MICROBIOLOGY LABORATORY GUIDEBOOK

UNITED STATES DEPARTMENT OF AGRICULTURE
FOOD SAFETY AND INSPECTION SERVICE
OFFICE OF PUBLIC HEALTH AND SCIENCE
MICROBIOLOGY DIVISION

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3RD EDITION, 1998
VOLUMES 1 & 2

FOREWORD

The 1993 *Escherichia coli* O157:H7 outbreak in the Pacific Northwest focused national attention on food safety. Since then, the number of requests for reprints on analytical methods used by the Microbiology Division, Office of Public Health and Science, Food Safety and Inspection Service, United States Department of Agriculture, has increased dramatically. Scientists within the Division have responded to these requests by completely revising and updating our Microbiology Laboratory Guidebook (MLG) for publication.

This MLG is our laboratory guidebook for the microbiological analysis of meat, poultry, and egg products that fall under the jurisdiction of USDA. It contains methods that FSIS prefers to use for the analysis of these foods. Since USDA does not endorse or approve methods for use by the food industry, inclusion of a particular method in the MLG should not be construed in this manner. Similarly, the mention of specific brand or trade names for a product, medium, chemical or reagent associated with methods contained herein does not constitute endorsement or selectivity by the authors or USDA over similar products that might also be suitable.

The use of the MLG comes with several caveats. This guidebook was written for microbiologists, and its interpretation and use should only be undertaken by trained microbiologists. FSIS assumes no responsibility for any economic, personal injury or other damage that may occur to individuals or organizations because of the use of methods contained in this guidebook. Users should note and pay particular attention to the safety caution symbol (†) and written warnings associated with certain hazardous chemicals or dangerous biological materials used in some of the methods. Users must act in a responsible manner at all times to protect themselves and the environment during performance of these methods. This guidebook must be supplemented with quality assurance and quality control programs as well as chemical, biological, and employee safety hazards management programs in order to operate a microbiology laboratory. These programs are beyond the scope of this guidebook and are the sole responsibility of the user to develop and implement.

This guidebook contains protocols for analytical tests that are required by FSIS regulatory activities. Some protocols, such as the Bioassay procedure for antibiotic residue detection and quantitation, may not be of value to commercial laboratories nor do we expect others to try to commercialize them. They are included here primarily as informational material since they are part of our current analytical methods.

The 1998, 3rd edition MLG publication consists of two separate volumes with a newly revised format utilizing a loose-leaf binder. This format should make the updating of chapters easier by allowing the substitution of a single chapter or page versus reprinting of the entire MLG. Because we anticipate the addition of new materials, the chapter numbers between volumes are not continuous in order to accommodate all changes.

Publishing this new 3rd edition MLG replaces all previous MLG versions and supersedes all Laboratory Communications, which should be discarded.

Finally, to produce a work of this magnitude requires a team of dedicated scientists and support staff. I would like to thank the following people for their efforts: Larry H. Dillard, Joseph Y. Chiu and James G. Eye for coordinating the FSIS Technical Support Laboratory reviews of the manual; Microbiology Division staff members Bhabani P. Dey, Stanley S. Green, Charles P. Lattuada, Bonnie E. Rose, Richard P. Mageau, and Gerri M. Ransom for composing, editing and proofreading many chapters; and Julie M. Hall for providing secretarial support in typing most of the chapters under trying conditions and meeting the demands of a diverse group of scientists.

Ann Marie McNamara, Sc.D. January 1998
Director
Microbiology Division
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In order to maximize the achievement of successful results when using the various methods in this MLG, it should be clearly understood that all methods and procedures should be performed at all times in a manner as close as possible to the prescribed directions. Particular attention should be paid to all details provided in a given analytical procedure. Changes or shortcuts should not be attempted in a method simply to accommodate factors, for example, such as processing a large number of similar samples through the method at the same time.

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Weight and volume measures: ± 1%
pH: ± 0.2 units
Time: hours ± 1 hour; minutes ± 1%
Temperature: ± 1.0°C

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Weight and volume measures: ± 1%
pH: ± 0.2 units
Time: hours ± 1 hour; minutes ± 1%
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CHAPTER 1. SAMPLE PREPARATION FOR MEAT, POULTRY AND PASTEURIZED EGG PRODUCTS

Charles P. Lattuada and B. P. Dey

1.1 Introduction

The purpose for the microbiological examinations of meat and poultry products is to obtain information. This information gathering may follow a qualitative or quantitative analytical format. The format followed is called the sampling plan. Many microorganisms are present in very low numbers and require one or more enrichment steps. If cell injury is anticipated, a non-selective enrichment frequently is used to resuscitate cells, followed by a more selective enrichment.

The analyst must study all records and correspondence before examining the sample. Care must be exercised in maintaining and handling the sample to insure that it is the same one that was collected, that it has not been tampered with, and that its condition is the same as it was at collection. The reserve sample must be stored properly to maintain its integrity in case additional analyses are required.

An analyst must be keenly aware that during all steps of the analysis, it is important to minimize the growth of non-critical microorganisms and to prevent entrance of environmental contaminants. The organism(s) isolated must come from the test sample and not from an outside source. These facts cannot be over-emphasized and can be accomplished only if strict attention is paid to the following rules:

The sampling operation must be well organized, with all supplies and equipment properly positioned before starting.

Ideally, sampling should be done in an area free of air currents following good aseptic procedures.

All work surfaces must be clean and sanitized.

Implements used for sampling must be sterile before use and protected from outside contamination during use.

The outside of the immediate container must be thoroughly sanitized.

Any laboratory person processing samples must be very familiar with aseptic techniques and the principles of sterilization, sanitization and disinfection. The person assigned to the sampling task should know the sampling protocol to be used and have a

reference copy at hand in case questions arise.

1.2 Sanitizing the Work Area

The work area must be clean and free from dust; detergent sanitizers are satisfactory for cleaning. Before work begins, the work area should be cleaned and a sanitizer/disinfectant applied liberally and given time to act. Quaternary ammonium compounds, sodium hypochlorite and phenolic compounds all are suitable for this purpose. The manufacturer's instructions regarding the concentration needed and the time required for the compound to act should be followed.

1.3 Sterilization of Instruments

a. All instruments and containers to be used in the sample analysis must be sterile. Any sterilization procedure may be used that is compatible with the material to be sterilized. Sterilization implies the total destruction of all viable organisms as measured by an appropriate culturing method.

b. An exception can be made, if necessary, when the number of instruments is limited (ie. chisels) and the testing protocol does not include sporeforming microorganisms. In which case, the instruments first are washed with soap and water, rinsed and inspected to be sure there is no organic matter in crevices or hinges, then they may be steamed for 30 minutes in an instrument sterilizer or placed in boiling water for two minutes.

c. Do not dip instruments into alcohol and flame them as a substitute for heat sterilization. It is not a substitution for the methods given above.

1.4 Disinfection of Outer Surface of the Immediate Container

a. The outside covering of the intact immediate container must be decontaminated to the greatest extent possible and particularly in the area where an opening will be made to expose the contents.

b. Hydrogen peroxide, tincture of iodine or 2500 ppm sodium hypochlorite solution may be used for this purpose. Allow time for the disinfectant to act before opening the container. Aseptically remove any residual disinfectant to prevent its entering the container when an opening is made.

1.5 Cutting and Weighing Samples

a. The sample should never be touched with bare hands. During the process of sanitizing the immediate container, the analyst should put on a pair of sterile gloves for handling samples.

b. Sterile instruments should be used for cutting, removing and manipulating all samples.

c. The sample must be taken aseptically according to the sampling protocol and placed in the proper sterile container for the next processing step. The remainder of the sample must be secured with an appropriate sterile closure that will preserve the sterility and integrity of the sample reserve. The sample reserve must be held according to the sampling protocol.

d. If the sample is to be weighed, the balance on which samples are weighed must be placed in an area that is clean and free of strong air currents.

e. If at all possible, the product should be weighed directly into the sterile container that will be used for dilution, mixing, blending and/or stomaching.

f. When weighing is complete, clean and disinfect the area with the same product used initially for disinfecting the work area. All instruments, containers, gloves and other materials that may have been in contact with the product must be incinerated or terminally sterilized before cleaning or disposal.

1.6 Selected References

Block, S. S. (ed.). 1984. Disinfection, Sterilization and Preservation, 3rd Edition. Lea & Febiger, Philadelphia, PA.

CHAPTER 2. PHYSICAL EXAMINATION OF MEAT AND POULTRY PRODUCTS

Charles P. Lattuada and B. P. Dey

2.1 Introduction

Microorganisms associated with meat and poultry products can be placed in three categories, beneficial, spoilage and pathogenic. Each product has a characteristic microbial profile called its "normal flora". Frequently information on changes in the "normal flora" can be obtained rapidly by simple observations. These observations can be grouped into a category called organoleptic observations. The term "organoleptic" refers to the use of the senses in determining the acceptability of a product. This would also include a direct microscopic examination.

Organoleptic analyses are of particular importance during investigations of certain food production problems such as detecting deleterious pre- or post-processing changes of canned products. Changes brought about by abusive handling and storage also may be detected by organoleptic observation.

In order to make a valid judgment, based upon one or more organoleptic observations, the analyst must know the physical characteristics of a "normal" product. This knowledge can be gained by experience and specialized training. Each laboratory should have Standard Operating Procedures (SOPs) describing the organoleptic standards for the acceptance or rejection of samples.

When judging a product to be abnormal, if possible, the decision should be based on a comparison of the suspect product with one that is normal, if readily available. This minimizes the subjectivity of the decision that a product has an "off odor", "off color", or other sensory abnormality. Tasting products as part of a microbiological examination is a dangerous practice and should be avoided. When the question to be answered is related to spoilage, odor is of primary importance; chemical and/or bacteriological results are corroborative and substantiating.

2.2 Examination

The following guideline establishes a standardized inter-laboratory procedure for characterizing samples.

a. Appearance: Changes in color; degradation of fat; presence of foreign materials such as metal, hair, feathers, sand, charcoal, etc.

b. Texture: Change in consistency; development of slime; breakdown of structure (proteolysis), etc.

c. Odor: Examples of words used to describe off-odors are: sour (acidic), moldy, musty, fishy, rancid, fruity, yeasty (beer-like) and putrid. However, if the analyst cannot decide how to classify an odor it is acceptable and appropriate to say simply: "off-odor" or "taint". Notations as to whether the off-odor is strong or slight are also in order.

2.21 Odor Examination By a Panel

In some cases results of odor examinations are equivocal and an odor detection panel, consisting of at least three members must be formed. The purpose of this panel is to evaluate aroma only, and its judgement must not be swayed by appearances. Only people with a good sense of smell can be assigned to it. The coordinator, who is not a panel member, will prepare the samples and ensure that the following procedures are followed:

a. The test must be conducted in a well-ventilated area free of strong odors.

b. At least 15 - 20% of the samples in the test group should be normal, wholesome, product-counterparts of the samples being examined. The normal controls should be as similar to the test product as possible with respect to ingredients, processing, packaging, size, age and handling procedures.

c. All samples should be presented to the smell panel in sequentially coded glass jars or polyethylene bags of the same size and shape, similar in weight and at the same temperature (usually 35°C). Both the normal and questionable products should be presented in a random order with a rest between samples. Do not decontaminate cans by flaming since heating and/or burning the contents could alter or mask any other odors that might be present.

d. Before beginning the examination, the panel members should smell and discuss the characteristic aroma of a normal product. They should be made aware that it is for general reference only, since normal products may vary slightly in odor and intensity. They then should rest until the samples are presented to allow recovery of the sense of smell which tires easily.

e. During the actual sample analysis, each panel member should remove the jar lid or open the bag, sniff the contents without glancing at them, replace the lid/close the bag and return the container to the panel coordinator. The panelist's sensory perceptions should

be entered on a score pad containing a list of appropriate terms with notations about whether the odor was strong or weak.

f. During the examination the panel members must not comment, exclaim or use body language that conveys their impression of the odors to other members of the panel.

Caution: It is not to be assumed that a smell panel composed of laboratory personnel will have the degree of skill attained by professional odor analysts. The purpose of a panel of laboratory personnel is to detect the odors of decomposition or product contamination with an odorous compound.

2.3 Determination of pH in Meat and Poultry Products

Potentiometric measurements should be used to determine the pH of a food product. The accuracy of most pH meters is approximately 0.1 pH units and reproducibility should be approximately ± 0.005 pH units. Both the glass and reference electrode are usually housed in a single tube, called the combination electrode. To obtain accurate results the same temperature should be used for standardization with the buffers and the sample. Measurements should be taken within the temperature range of 20 to 30°C.

2.31 Equipment and Reagents

a. Blender
b. Beaker, 100 ml
c. Separatory funnel
d. pH meter, suitable for reading pH from 0 to 14 in 0.1 unit increments. A rugged, designated combination electrode should be used for pH measurement of meats and poultry. A flat combination electrode works well for determining the surface pH of canned foods.
e. Distilled water
f. Certified buffer solutions of pH 7.00, and either pH 4.00 or 10.00. The buffers chosen should bracket the desired pH.

2.32 Procedure

a. Calibrate the pH meter, according to manufacturer's instructions, using certified buffers pH 7.00 and either pH 4.00 or 10.00.

b. Most products will be solid and require blending. A 1:5 or 1:10 dilution should be made with distilled water in a clean blender jar. Blend to a thin uniform consistency and perform the pH measurement. If fat or oil causes fouling of the electrode, transfer a portion

of the homogenate to a separatory funnel and draw off a portion of the aqueous phase. On certain products centrifugation may be required in order to recover a measurable aqueous phase.

c. Adjust the temperature control on the pH meter to that of the sample (ideally 25°C) and immerse the pH electrode into the liquid phase.

d. A surface electrode may be used with certain low fat products that present a flat, solid core surface. If a surface measurement is taken, ensure that the electrode has good contact with the product surface.

e. Record pH to the nearest 0.1 unit.

2.4 Determination of Water Activity (A_w) of Meat and Poultry Products

The free moisture level in food is called water activity (a_w). This is the water available to support microbiological growth in the food. It can be lowered by dehydration or by the addition of binding agents such as salt or sugar. The growth of different types and genera of microorganisms is controlled by the water activity level in a specific product. Much information exists on the water activity limits of growth for microorganisms. For example, the limit of growth for *Clostridium botulinum* occurs between an a_w of 0.935 and 0.945. Canned foods with an a_w of $\leq$0.85 are exempt by the FDA from the canned food regulations and cured meats without nitrates must have an a_w of $\leq$0.92. It is important, therefore, that the a_w in foods be measured very accurately. A detailed list of growth limiting a_w values can be found in Chapter 8 of the Compendium of Methods for the Microbiological Examination of Foods.

Measurement of the a_w in a food sample is affected by both time and temperature. It is dependent upon allowing enough time for the water vapor of the sample to reach equilibrium with the air space in a closed container, such as a closed jar, at a constant temperature. When incubation is required for equilibration, it is absolutely necessary to maintain accurate temperature control of the food samples inside the incubator used for a_w. It is equally important to allow ample time for the humidity of the air space above the sample to reach equilibrium with the food sample.

2.41 Decagon

The Decagon CX-2 will measure a_w in less than 5 minutes. The instrument has rapid vapor equilibration, does not require temperature equilibration and requires only a small sample (approximately 5 grams of food). The instrument does not have to be calibrated, but quality control samples, consisting of deionized water and various salt slushes, must be included in an analysis. When a very wet sample and a very dry one follow one another, two interim readings should be taken of the second sample before collecting data with the third reading. When a reading is completed,the instrument will "beep" continuously. The only reported material to interfere with a Decagon reading is propylene glycol. Foods containing propylene glycol should not be analyzed by this method.

2.42 Equipment and Materials

a. Decagon, Model CX-2 manufactured by Decagon Devices, Inc., Pullman, WA 99163-0835.
b. Blender and blending jars
c. Transfer pipettes

2.43 Procedure

a. In order to obtain a representative sample, approximately 100-200 grams of food should be blended.

b. Remove at least two samples, approximately 5 grams each, for a_w determination; the cup should never be filled above the fill level line molded into the side of the plastic cup.

c. Follow the manufacturer's directions contained in the Decagon Manual very carefully when performing this analysis.

d. Saturated salt solutions should be used for reference controls. The following saturated salt mixes and their expected a_w at 25°C normally are used:

NaCl ---------0.755
KBr ----------0.811
KCl ----------0.845
$(NH_4)H_2PO_4$-----0.934

Note: Never leave a sample in the instrument after a reading has been taken.

2.44 American Instrument Electronic Hydrometer

Another method for determining a_w is the American Instrument Electronic Hydrometer. Reportedly, it is an accurate instrument for measurement of the a_w in food products, provided the manufacturer's directions are followed carefully. The instrument measures the changes in electrical resistance of specially coated lithium chloride sensors. The electronic part of the instrument is very rugged and needs no special care. The sensors, like pH electrodes, are very sensitive and can be affected permanently by water condensation, desiccation, corrosive chemicals such as mercury vapor, unstable hydrocarbons such as ketones; halogen gases; and sulfur compounds such as hydrogen sulfide and sulfur dioxide. Sensors can be affected reversibly by polar vapors such as ammonia, amines, alcohols, glycols and glycerols. The response of sensors will return to normal, from slightly higher readings, if the polar vapors are removed by aeration.

2.45 Equipment and Materials

a. American Instrument Electronic Hydrometer (Model No. 30-87 or equivalent) manufactured by Newport Scientific, Inc., 8246E Sandy Court, Jessup, MD 20794.

b. Sensors, Color Code-Gray, (Cat-No. 4822W) for the above instrument, available from the same manufacturer. The Company makes different types of sensors for different ranges of humidities. This sensor is the one most commonly used in meat and poultry product analyses. They have an a_w range of about 0.81 to 0.99. Each sensor is unique and comes with its own factory calibration curve. When purchasing gray sensors specify that the a_w readings between 0.90 - 0.94 be inside the linear portion of the calibration curve. Also request that the correction factor of each sensor at 30°C (86°F) be incorporated into each calibration curve.

c. Sensor lids and 8-gang switch box. These socket type lids normally fit into the rims of standard pint size canning jars. The 8-gang switch box allows measurement of eight samples at a time. The sensor connectors should be labeled 1 to 8 to correspond to the switch position.

d. A forced-air incubator should be used to hold the samples at 30 ± 0.5°C. If necessary, cut a 1.5" diameter hole in the incubator to introduce the electrical leads for the eight sensors into the incubator. Be sure to fill the hole with sealant.

e. Clean and dry standard pint-size glass canning jars, without chips or cracks on the rims, for the samples.

f. Pipettes

g. Preparation of a saturated ammonium phosphate, monobasic, $[(NH_4)H_2PO_4]$ slush

$(NH_4)H_2PO_4$, reagent grade	200 g
Merthiolate	25 mg
Glass distilled water	

Place the ammonium phosphate and merthiolate in a new or clean pint-size jar, slowly add glass-distilled water (approximately 2-3 ml at a time), and stir vigorously with a spoon until approximately one half of the crystals are dissolved. Care must be taken to avoid splashing the salts onto the sides and rims of the jar.

Incubate the salt slushes at 30°C for 2-3 days to establish equilibrium.

h. Preparation of saturated potassium dichromate (K_2CrO_4) slush

Use the same procedure as above. Omit the merthiolate.

i. Store the salt slushes indefinitely in a 30°C incubator at all times except to install or remove sensors.

j. The a_w of the salt slushes should be (measured with a calibrated gray sensor):

$(NH_4)H_2PO_4$ slush	0.929 at 30°C
K_2CrO_4 slush	0.865 at 30°C

2.46 Procedure

a. Follow the manufacturer's directions very carefully when using this method.

b. Test each sensor first in $(NH_4)H_2PO_4$ and then in K_2CrO_4 salt slush and record the results on the analysis sheet. The sample test results will be recorded on the same sheet. Do not use sensors that differ from the expected value of the salt slush by more than a_w 0.01 unit.

c. If the a_w is going to be measured in other than the range specified for the grey sensor, be sure to use the appropriate sensor and prepare salt slushes appropriate for the expected range. A table of other salt slushes can be found in Chapter 8, "Measurement of water activity (a_w) and acidity", in the Compendium of Methods for the Microbiological Examination of Foods.

2.5 Selected References

Greenspan, L. 1977. Humidity fixed points of binary saturated aqueous solutions. J. Res. Nat. Bur. Stand. 81A:89-96.

Prior, B. A. 1979. Measurement of water activity in foods: A review. J. Food Prot. 42:668-674.

Troller, J. A., and V. N Scott. 1992. Measurement of water activity (a_w) and acidity, p. 135-151. *In* C. Vanderzant and D. F. Splittstoesser (ed.), Compendium of Methods for the Microbiological Examination of Foods. 3rd Edition. Amer. Publ. Hlth. Assoc. Washington, D.C.

CHAPTER 3. EXAMINATION OF FRESH, REFRIGERATED AND FROZEN PREPARED MEAT, POULTRY AND PASTEURIZED EGG PRODUCTS

Charles P. Lattuada, Larry H. Dillard and
Bonnie E. Rose

3.1 Introduction

The laboratory methods contained in this section of the Guidebook are used to detect and, when desired, quantitate selected microorganisms in samples collected in federally inspected meat, poultry and egg processing establishments. They generally follow the Compendium of Methods for the Microbiological Examination of Foods and AOAC International's Official Methods of Analysis. The methods presented in this section may be used to analyze samples of:

a. fresh, frozen, smoked, cured or dehydrated meat and poultry products;

b. prepared/ready-to-eat products such as pot pies, luncheon meats, dinners, battered or breaded meat and poultry products;

c. refrigerated meat or poultry salads;

d. dehydrated soups and sauces containing the requisite amount of meat or poultry;

e. meat snacks, hors d'oeuvres, pizza and specialty items;

f. various ingredients incorporated with meat and poultry products such as spices, vegetables, breading material, milk powder, dried egg, vegetable proteins;

g. pasteurized egg products;

h. environmental samples from areas in which any of the above are processed or manufactured.

The quantity and types of mesophilic microorganisms present in or on any of these products offer a means of evaluating the degree of sanitation used during the process. If the results obtained for coliforms, *Escherichia coli*, and *Staphylococcus aureus* are unusually high, they might result in some type of official follow-up action. Any such follow-up analysis will use the appropriate Final Action Method found in the latest edition of Official Methods of Analysis of AOAC International or any of its supplements. Pertinent sections in the 16th Edition are:

- **Aerobic Plate Count (APC): 966.23**
- **Coliform Group and *E. coli*: 966.24**
- ***S. aureus*: 987.09**

3.11 Comparison With the AOAC Method

The procedures in the following sections of this Chapter are either the same as those published by the AOAC or generally follow an AOAC method. The following is a listing of deviations:

a. The procedure for determining numbers of coliform and *E. coli* differ from the AOAC procedure as follows:

i. Use a single tube of laurel sulfate tryptose broth (LST) per dilution, rather than three tubes per dilution.
ii. Incubate inoculated LST and EC broths for 24 ± 2 h.
iii. Consider the presence of gas in LST and EC broths as positive for coliform and *E. coli* respectively, with no further testing required.

b. The procedure for the enumeration of *S. aureus* differs from the AOAC procedure in that only one tube, instead of three, per dilution is used to determine the estimated count.

3.12 General Guidelines for Testing Fresh or Prepared Foods

a. Do not combine the components of composite items such as frozen dinners into a single sample. To the greatest extent possible, examine as separate samples the vegetable or non-meat portion(s) and the meat portion.

b. The quantity, condition and suitability of the sample are very important.

i. The quantity should be sufficient to perform the analysis and have a reasonable amount in reserve for repeat testing.
ii. The condition of receipt should be in keeping with good microbiological practices for the analysis(es) requested.
iii. The sample should be, to the greatest extent possible, representative of the whole of the original product at the time the sample was taken.
iv. When appropriate and if possible, samples should be received at the laboratory in their original unopened package(s) (intact sample).

3.13 Tests Covered in This Section

a. Aerobic plate count
b. Coliform and *E. coli* quantitative estimates
c. *S. aureus*

3.2 Equipment and Materials

a. Balance, capacity ≥2 kg, sensitivity ± 0.1 g
b. Blender and sterile blender jars
c. Stomacher™ and sterile stomacher bags
d. Incubators at 35 ± 1.0°C, and 20 ± 1.0°C
e. Water bath at 45.5 ± 0.05°C
f. Water bath at 37 ± 1.0°C
g. Manual or Automatic colony counter and tally register
h. Sterile, disposable/reusable dishes, pans or trays for sample cutting
i. Sterile forceps, spoon, knife, scissors and other sterile sampling equipment
j. Sterile 1, 5 and 10 ml pipettes
k. Sterile 100 x 15 mm petri dishes
l. Transfer loop, 3 mm
m. Microscope and clean slides
n. Refrigerated centrifuge
o. Refrigerator
p. pH meter

3.21 Media

a. Plate count agar (PCA) in containers suitable for making pour plates
b. Laurel sulfate tryptose (LST) broth with fermentation tubes
c. EC broth with fermentation tubes
d. Surface dried Baird-Parker plates (egg tellurite glycine pyruvate agar, ETGPA)
e. Brain heart infusion (BHI) broth
f. Trypticase soy broth with 10% sodium chloride and 1% sodium pyruvate (PTSBS)
g. Toluidine blue DNA agar

3.22 Reagents

a. Butterfield's phosphate diluent
b. Gram stain reagents
c. Desiccated rabbit plasma (coagulase) EDTA
d. Tris Buffer
e. Ammonium sulfate [$(NH_4)_2SO_4$], reagent grade
f. Triton X-100
g. 3M trichloroacetic acid solution
h. 1N HCl solution

3.3 Preparation and Dilution of Samples

See Section 1.3 - 1.5 (Sterilization of Instruments, Disinfection of Containers, and Cutting and Weighing Samples)

3.31 Food Homogenates

a. Using sterile spoons, forceps, scissors, etc., aseptically weigh 50 ± 0.1 g of the sample into a sterile blender jar or stomacher bag.

b. If the sample is frozen, remove portions, whenever possible, without thawing the larger sample and weigh 50 ± 0.1 g of the sample into a sterile blender jar or stomacher bag. It is well known that freeze/thaw cycles are damaging to bacteria. This is particularly important when a re-examination of the product may be necessary. Otherwise, partially thaw the sample at 2-5°C for about 18 h, or by placing the sample in a watertight container and immersing it in cold water for 1-2 h.

c. Add 450 ml sterile Butterfield's phosphate diluent and stomach for 2 minutes, or blend at high speed for two minutes. The total volume in the blender jar must completely cover the blades. This becomes the 1:10 dilution.

d. Permit the foam to settle; then pipet 10 ml of the blended 1:10 dilution into a 90 ml dilution blank to make the 1:100 dilution. Repeat this procedure to prepare serial dilutions of 10^{-3}, 10^{-4}, etc. Shake all dilutions 25 times in a one foot arc. Use a separate 10 ml pipette to prepare each dilution. Pipettes must deliver accurately the required volumes. Do not deliver less than 10% of a pipette's volume. For example, to deliver one ml, do not use a pipette of more than 10 ml volume.

e. The analyst should strive to minimize the time from when the sample is stomached or blended until all the dilutions have been placed in or on the appropriate medium; ideally this time should not exceed 15 minutes whenever possible.

f. If the sample consists of less than 50 g, weigh about half the sample, and add the amount of diluent required to make a 1:10 dilution (nine times the weight of the portion of sample used) and proceed as above.

g. Hold reserves of each sample at or below -15°C (5°F), unless the product is stored normally at ambient

temperature or unless a specific protocol specifies otherwise. Samples should be held until a determination is made that a repeat test is not necessary or for the length of time designated by the testing protocol.

3.32 Whole Bird Rinse

a. Since there are differences between sample types and sizes (eg. chicken vs. turkey carcasses), be sure to check the specific program protocol before using this procedure.

b. Aseptically transfer the carcass to a sterile Stomacher 3500 bag (or equivalent), draining as much excess fluid as possible during the transfer.

Note: Larger (24 x 30-36 in.) bags will have to be used with turkeys.

c. Add 400 ml (chickens) or 600 ml (turkeys) of Butterfield's Phosphate Diluent (BPD) to the carcass in the bag. Pour approximately one half the volume into the interior cavity of the bird and the other half over the skin. Note: If *Salmonella* is the ONLY target analyte, Buffered Peptone Water (BPW) may be substituted for the BPD.

d. Rinse the bird, inside and out, with a rocking motion for 1 min at a rate of approximately 35 forward and back swings per minute. This is done by grasping the carcass in the bag with one hand and the closed top of the bag with the other. Rock with a reciprocal motion in an 18-24 inch arc, assuring that all surfaces (interior and exterior) are rinsed.

e. Aseptically remove the carcass from the bag, draining excess rinsed liquid into the bag, dispose of the carcass, and culture the bird rinse liquid according to protocol directions.

3.33 Egg Products

a. Liquid eggs must be held at 4.4°C (40°F) or below for valid analysis.

b. Frozen samples must be thawed as rapidly as possible in a water bath at 45°C.

c. Exposed or leaking samples should not be analyzed.

d. Mix the sample with a sterile spoon, spatula, or by

shaking.

e. Aseptically weigh a minimum of 100 g of egg sample into a sterile blender jar or sealable bag containing 900 ml of the appropriate enrichment or buffer. If a specific protocol requires a sample size greater than 100 g, the 1:10 ratio must be maintained in the same enrichment or buffer.

f. Mix the 1:10 sample enrichment/buffer well by shaking, stomaching, or blending.

g. Dried egg samples should be rehydrated slowly by gradually adding the enrichment/diluent to the sample. This is done by adding a small portion of liquid to the sample and mixing aseptically to obtain a homogeneous suspension. Repeat this procedure three times and then add the remainder of the liquid. Mix until a lump-free suspension is obtained.

h. Incubate or transfer to the appropriate enrichment medium and incubate according to the protocol(s) being used.

3.4 Aerobic Plate Count (APC)

a. Pour Plates (Reference AOAC 966.23 C)

i. Using the dilutions prepared in section 3.3, pipet 1 ml from the 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} etc. dilutions into each of four petri dishes, two for each incubation temperature. Plate additional dilutions when expecting higher bacterial levels.

ii. Use separate sterile pipettes for each dilution.

iii. Add molten Plate Count Agar cooled in a water bath to 45 ± 1°C. Uniformly mix the agar and the inoculum by gently swirling or tilting each plate, taking care not to generate bubbles.

iv. Allow the agar to harden and then place one series of duplicate plates in a 35 ± 1°C incubator for 48 h. Incubate the other series at 20 ± 1°C for four or five days.

v. Use a colony counter and count colonies on the duplicate plates in a suitable range (30-300 colonies per plate). If plates do not contain

30-300 colonies, record the dilution counted and the number of colonies found. Average the counts obtained from duplicate plates, multiply by the dilution factor and report this number as the aerobic plate count per gram or milliliter at the incubation temperature used.

b. Alternate Methods - AOAC

i. Aerobic Plate Count in Foods: Hydrophobic Grid Membrane Filter Method* (AOAC 986.32)

ii. Dry Rehydratable Film (Petrifilm Aerobic Plate™) Method* (AOAC 990.12)

iii. Spiral Plate Method* (AOAC 977.27)

*Since these methods are available commercially, the manufacturer's directions should be followed.

3.5 Coliform Group and *Escherichia coli*

a. Estimated Count Procedure (Reference AOAC 966.24)

i. Using the dilutions prepared in section 3.3, pipet 1 ml from the 10^{-1}, 10^{-2}, 10^{-3} etc. dilutions into LST broth, one tube per dilution. Inoculate additional dilutions when expecting higher bacterial levels. The highest dilution of sample must be sufficiently high to yield a negative end point.

ii. Use separate sterile pipettes for each dilution.

iii. Incubate the tubes of LST broth at 35°C for 24 ± 2 h.

iv. Examine each tube for gas formation as evidenced by displacement of fluid in the inverted tubes or by effervescence when tubes are shaken gently.

v. Consider any tube of LST broth displaying gas as coliform positive, and report the number of coliform per gram in accordance with the highest dilution with gas. When a "skip" occurs, report by using the missing estimate (for example: If the 10^{-1}, 10^{-2}, and 10^{-4} dilutions produce gas but the 10^{-3} dilution tube is non-gassing, report "1,000 coliforms per gram.")

b. Fecal Coliform (*E. coli*) Estimated Count Procedure (Reference AOAC 966.24)

i. Use a 3 mm calibrated loop to transfer one loopful

from every gas-positive LST broth tube to a correspondingly marked tube of EC broth.

ii. Incubate the EC tubes in a 45.5 ± 0.05°C covered water bath for 24 ± 2 h. Submerge the EC tubes in the bath so that the water level is above the level of medium in the tubes.

iii. Record every tube producing gas, as evidenced by displacement of liquid in the inverted tube or by effervescence when tubes are shaken gently.

iv. Report the number of *E. coli* per gram in accordance with the highest dilution displaying gas. When a "skip" occurs, report by using the missing estimate (for example: If the 10^{-1}, 10^{-2}, and 10^{-4} dilutions produce gas but the 10^{-3} dilution tube is non-gassing, report "1,000 *E. coli* per gram.")

c. Alternate Methods - AOAC

i. Coliform and *Escherichia coli* Counts in Foods: Hydrophobic Grid Membrane Filter/MUG Method*
ii. Coliform and *Escherichia coli* Counts in Foods: Dry Rehydratable Film*

*Since these methods are available commercially, the manufacturers's directions should be followed.

3.6 *Staphylococcus aureus*

a. Estimated Count Procedure (Reference AOAC 987.09)

i. Using the dilutions prepared in section 3.3, pipet 1 ml from the 10^{-1}, 10^{-2}, 10^{-3} etc. dilutions into tubes containing 10 ml of Trypticase (tryptic) Soy Broth with 10% sodium chloride and 1% sodium pyruvate (PTSBS), one tube per dilution. Inoculate additional dilutions when expecting higher bacterial levels. The highest dilution of sample must be sufficiently high to yield a negative end point.

ii. Use separate sterile pipettes for each dilution.

iii. Incubate the PTSBS tubes at 35°C for 48 h.
iv. Using a 3 mm calibrated loop, transfer a loopful from each growth-positive tube as well as from the tube of the next highest dilution to previously prepared plates of Baird-Parker agar. Streak in a manner to produce well-isolated colonies.

v. Incubate the Baird-Parker plates at 35°C for 48 h.

vi. Typical *S. aureus* colonies appear as circular, convex, smooth, grey-black to jet-black colonies on uncrowded plates and frequently have an off-white margin surrounded by a zone of precipitation (turbidity) followed by a clear zone. The colonies usually have a buttery to gummy consistency.

vii. Test two or more isolates, from each useable plate meeting the above description (3.6,vi), for coagulase as in Section 3.6 (c).

b. Direct Plating

i. If *S. aureus* counts of 100 cfu per gram or more are expected, direct plating can be done using Baird-Parker agar.

ii. Pipet 0.1 ml from each dilution on previously prepared and dried Baird-Parker agar plates. Use separate accurate pipettes for each dilution.

iii Distribute the inoculum evenly over the surface of the plates using separate, sterile, fire polished, bent-glass rods ("hockey sticks") for each plate. Mark plates according to the dilution used.

iv. Invert plates and incubate at 35°C for 48 h.

v. Select plates containing approximately 20 or more well-isolated typical *S. aureus* colonies. Count plates containing 20-200 colonies. Typical colonies are circular, convex, smooth, grey-black to jet-black and frequently have an off-white margin surrounded by a zone of precipitation (turbidity) followed by a clear zone. The colonies usually have a buttery to gummy consistency.

vi. Select 10 colonies from those counted and inoculate each into separate 13 x 100 millimeter tubes containing 0.2 ml of BHI broth for coagulase testing. Test for coagulase as in 3.6 (c).

vii. Calculate the total number of colonies represented by coagulase positive cultures and multiply by the appropriate sample dilution factor to record the number of coagulase positive staphylococci per gram.

c. Coagulase Test for *Staphylococcus aureus*

i. Use an inoculating needle to obtain a small amount of growth from each suspect colony and place it into 13 X 100 mm tubes containing 0.2 ml of BHI Broth.

ii. A known coagulase positive and a known negative culture should be inoculated into BHI broth at the same time as the samples.

iii. Incubate each tube at 35°C for 18-24 h.

iv. Add 0.5 ml of rabbit plasma with EDTA, reconstituted according to the manufacturer's directions, to the BHI cultures.

v. Mix thoroughly and place the tubes in a 35-37°C. water bath.

vi. Examine these tubes each hour, from one through six hours, for clot formation. Any degree of clotting should be interpreted as a positive reaction.

3.61 Special Sampling Procedure for Fermented Sausage Products

a. Introduction

During the early stages of sausage fermentation, staphylococci can grow extensively if the starter culture is not added or fermentation fails with no concomitant production of lactic acid and drop in pH. Failure can be caused by poor quality starter cultures or the improper use of starter cultures or "back inoculation". *S. aureus* growth is aerobic and usually confined to the outer 1/8 inch of the sausage. Enterotoxin may be formed as a result of this growth.

Coagulase-positive staphylococcal counts on large sticks of salami have been noted to vary widely. On large sticks, some areas may have very few staphylococci while other areas may have levels in excess of 10^6/g. Whenever possible, obtain 1-2 pounds of the suspect sausage. In order to obtain a representative sample, portions should be taken from several different areas and composited for testing.

b. Procedure

i. If the sausage is moldy, wipe the mold off the sausage casing with a piece of sterile tissue paper and proceed.

ii. To collect a sample, use a sterile, sharp knife and cut several thick slices from the sausage near the ends as well as in the middle. Aseptically trim and save the outer 1/8 to 1/4 inch portion of the sausage and label it "shell portion". Even if the amount of sample is limited, do not cut deeper than 1/4 inch.

iii. Working aseptically, blend 25-50 g of the shell portion for enterotoxin testing; the same blended sample can be used to test for viable coagulase-positive *S. aureus* as described in section 3.6.

iv. Analyze the sample by either of the following procedures.

3.62 The (Presumptive) Staphylococcal Enterotoxin Reverse Passive Latex Agglutination Test

The procedure for this test is given in (15.20) and usually is the method of choice.

3.63 Thermonuclease Assay

a. Introduction

This procedure is based on the detection of a heat stable DNase which is produced by most strains of *S. aureus,* including 98.3% of the enterotoxigenic strains. This heat stable DNase is produced in detectable amounts under all conditions which permit the growth of *S. aureus* and the production of enterotoxin. The DNase is able to survive processing conditions which would destroy viable *S. aureus.*

This method can be used to screen large sausages or a large number of samples to identify "hot spots".
It has been shown (Tatini, 1981) that the detection of DNase with this procedure is indicative of *S. aureus* populations of $\geq 10^5$ per gram.

b. Procedure:

i. Blend 20 g of shell, 10 g $(NH_4)_2SO_4$, and 2 ml Triton X-100 in 40 ml of distilled water.

ii. Adjust the pH of this slurry to 4.5-4.8 with 1N HCl.

iii. Centrifuge under refrigeration at 7-10,000 RPM for 15 min.

iv. Decant and discard the supernatant and add 0.05 ml cold 3M trichloroacetic acid for each ml of the original slurry, mix and centrifuge a second time as above.

v. Decant and discard the supernatant. Re-suspend the precipitate in 1 ml of Tris buffer, adjusted to pH 8.5, and then adjust the volume to 2 ml with Tris buffer.

vi. Boil the solution for $\geq$15 but $\leq$90 min, cool and store under refrigeration until needed.

vii. Cut 2 mm diameter wells into air dried Toluidine Blue DNA Agar.

viii. Dispense the food extract into one or more wells using a Pasteur pipette. Do not overfill the well.

ix. Incubate these plates, agar side down, at 37°C for 4 to 24 h.

x. Any pink halo, extending 1 mm beyond the well is considered positive for thermonuclease.

3.7 Selected References

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CHAPTER 6. ISOLATION, IDENTIFICATION, AND ENUMERATION OF *CAMPYLOBACTER JEJUNI/COLI* FROM MEAT AND POULTRY PRODUCTS

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6.1 Introduction

Procedures for the recovery of *Campylobacter* spp. from foods are evolving and no single method can be recommended for testing a wide variety of foods. Isolation of *Campylobacter jejuni* and *Campylobacter coli* is achieved both with and without selective broth enrichment. The procedures outlined below are among the most promising for the isolation and enumeration of these bacteria from raw/cooked meat and poultry products.

Campylobacters are sensitive to freezing and die off at room temperature. Samples intended for *Campylobacter* examination should be transported and held at 4°C. Sample analysis should begin as soon as possible since campylobacters can be overgrown by contaminating psychrotrophic bacteria. If freezing of samples cannot be avoided, cryoprotective agents should be used. Stern and Kotula, 1982, reported improved recovery of *C. jejuni* from ground beef stored frozen in 10% dimethyl sulfoxide or glycerol. Blankenship et al., 1983, found that brucella broth supplemented with 10% polyvinyl pyrrolidine was suitable for transporting frozen swab samples (from freshly processed poultry carcasses) to a central laboratory for analysis.

Campylobacters are microaerophilic and certain environmental stresses such as exposure to air, drying, low pH, and prolonged storage can be detrimental to their survival. Use of oxygen-quenching agents, a microaerobic atmosphere, and antibiotics that suppress competitors, significantly improve *Campylobacter* recovery.

6.2 Equipment, Reagents, and Media

6.21 Equipment

a. Phase-contrast microscope with 100X oil immersion objective
b. Agitating incubator(s)/water bath(s) at 37 ± 1.0°C and 42 ± 1.0°C
c. 42 ± 1.0°C incubator (static)
d. Balance, sensitivity of 0.1 g
e. Quart-size Qwik Seal® bags (Reynolds Metals Co., Richmond, VA; # RS78)
f. Anaerobic jars (vented or non-vented)

g. CampyPak Plus™ (BBL 71045)
or
Gas Generating Kits for *Campylobacter* (Oxoid BR56 for 3.0-3.5 liter jars, or BR60 for 2.5-3.0 liter jars)
h. Vacuum pump and gauge with appropriate tubing and connectors for evacuation of vented anaerobic jars
i. Gas cylinder containing a mixture of 5% O_2, 10% CO_2, and 85% N_2 with appropriate tubing and connectors for gassing vented anaerobic jars and Qwik Seal® bags
j. Regulator for gas cylinder compatible with Compressed Gas Association (CGA) connection on cylinder
k. Filter paper (for glycerol humectant and oxidase test)
l. Petri dishes (100 x 15 mm disposable)
m. Platinum or sterile plastic inoculating loops and needles
n. Microscope slides, cover slips, and immersion oil
o. 0.2 μm sterile membrane filters
p. 16 x 150 mm and 16 x 125 mm screw-cap test tubes
q. 250-ml screw-cap bottles
r. Sterile swabs or bent glass rods ("hockey sticks")
s. Sterile forceps and scissors
t. Sterile pipettes
u. Large sterile plastic bags
v. Stomacher™ 400, and Stamacher™ 400 bags
w. Centrifuge, rotor, and 250-ml sterile centrifuge bottles
x. Sterile cheesecloth-lined funnels

6.22 Reagents

a. Glycerol
b. 3% Hydrogen peroxide solution
c. Cephalothin antibiotic susceptibility discs (30 μg)
d. Nalidixic acid antibiotic susceptibility discs (30 μg)
e. Oxidase reagent (1% Tetramethyl-p-phenylenediamine dihydrochloride solution)
f. *Campylobacter* latex test kit (optional presumptive identification)

6.23 Media

a. Hunt Enrichment Broth (HEB)
b. 0.1% peptone water
c. Modified *Campylobacter* Charcoal Differential Agar (MCCDA)
d. Brucella-FBP (BFBP) Broth
e. Semisolid Brucella Glucose Medium
f. Brucella-FBP (BFBP) Agar
g. Enriched Semisolid Brucella Medium (optional)

6.3 Isolation and Enumeration

a. Place 25 g meat or swab samples into 100 ml of HEB in a Reynolds quart-size Qwik Seal® bag. Place the Qwik Seal® bag inside a Stomacher™ 400 bag for reinforcement and stomach for 2 minutes. Flatten the Qwik Seal® bag against the lab bench edge to remove as much air as possible without spilling the contents, then seal the bag, leaving a 1/2 inch opening at one end. Aseptically insert the tip-end of a sterile 10 ml pipette (or equivalent) into the bag through this opening. Be sure that the mouth-end of the pipette contains a sterile cotton filter. Connect the mouth-end of the pipette to the microaerobic Campy gas mixture (5% O_2, 10% CO_2, and 85% N_2) with sterile rubber tubing equipped with a sterile filter (a sterile filter can be made out of an autoclaved, shortened 25 ml volumetric pipette stuffed with glass wool). Slowly inflate the bag to capacity with the Campy gas mixture and continue to fill until excess gas flows from the bag. Then allow a small amount of gas to escape to provide for expansion, before securing the remainder of the seal. Proceed to step d.

b. Place a raw whole chicken carcass or meat pieces (up to 3 lb) in a large sterile plastic bag such as a Stomacher™ 3500 bag, and add 200 ml 0.1% peptone water. Twist bag to seal and shake contents for 2 minutes. Tilt the bag and hold back the meat pieces, allowing the rinse liquid to flow to one corner. Sanitize bag corner with 1000 ppm hypochlorite solution or 70% ethanol, then rinse in sterile distilled water. Aseptically cut the corner of the bag and pour the rinse through a sterile cheesecloth-lined funnel into a sterile 250 ml centrifuge bottle. Centrifuge at 16,000 x g for 15 minutes. Discard the supernatant and suspend the pellet in 10 ml 0.1% peptone water. For detection, inoculate 1 ml of rinse concentrate into 100 ml HEB in a Qwik Seal® bag. Then follow gassing steps as outlined, beginning with the third sentence of step a. above.

c. If enumeration is desired, prepare a three tube MPN series using HEB. Choose test dilutions and HEB volumes based on the expected numbers of campylobacters in the meat species being tested. For example, for poultry rinse samples (prior to centrifuging) begin by adding three 10 ml portions of the rinse to three 90 ml bottles of HEB. (Alternatively, Qwik Seal® bags may be used here [see step a. above]). Then add 1 ml portions of the rinse to each of three 9 ml tubes of HEB. Prepare serial dilutions of the rinse in 0.1% peptone water. Prepare subsequent MPN tubes by transferring 1 ml portions of the

decimal dilutions into 9 ml tubes of HEB in triplicate. Place all bottles and tubes in anaerobic jars. See step g. for jar gassing methods. Follow incubation steps beginning with step d. below. Use tubes or bottles found to contain confirmed *Campylobacter* to calculate MPN (refer to appropriate tables).

d. Incubate gassed Qwik Seal® bags or anaerobic jars containing bottles or tubes at 37 ± 1.0°C, shaking at 100 rpm for 4 h.

e. After the 4 h incubation at 37 ± 1.0°C, aseptically add additional sterile cefoperazone solution to bring the final concentration in each enrichment vessel to 30 mg/L. Reestablish the microaerobic atmosphere and increase the temperature to 42 ± 1.0°C. Continue the incubation for 20 h shaking at 100 rpm.

f. Swab/streak enrichments directly and at a 1:100 dilution onto MCCDA plates (for cooked products, a 1:50 dilution may be plated). Prepare the dilution by swirling a swab in the broth and twisting it against the side of the vessel to remove excess liquid. Break off the swab tip into a tube containing 9.9 ml of 0.1% peptone water and vortex. Inoculate the plates by placing a swab into the enrichment or dilution and removing excess liquid as above. Swab approximately 40% of the MCCDA plate, then streak from the swabbed area to yield isolated colonies. Alternatively, 0.1 ml portions of the enrichments or dilutions may be plated by spreading with a sterile bent glass rod. This plating technique may be used provided isolated colonies result.

g. Incubate the MCCDA plates at 42 ± 1.0°C for 24 h in an anaerobic jar under microaerobic conditions. Add about 4 drops of a humectant such as glycerol to a filter paper and place it in the jar to diminish typical confluent and swarming growth of *Campylobacter*. If no growth is achieved after 24 h, reincubate the plates for an additional 24 to 48 h to attempt recovery. The microaerobic conditions can be achieved in the jar by either of the following methods:

   i. Evacuate the air from a vented anaerobic jar to a partial vacuum of 20 inches of Hg and fill the jar with a gas mixture of 5% O_2, 10% CO_2, and 85% N_2. Repeat the evacuation-replacement procedure a total of three times to assure proper atmospheric conditions.

ii. CampyPak Plus™ (BBL) or Gas Generating Kits for *Campylobacter* (Oxoid). Follow the manufacturer's instructions on use and disposal of the kit materials. Keep jars away from flames when opening.

NOTE: Gas generator envelopes should be used if non-vented anaerobic jars are the only type available. Evacuation-replacement gassing of vented anaerobic jars is very economical.

To facilitate lid removal from a vented anaerobic jar, first release pressure by opening clamped tubing on port or by depressing the valve stem.

6.4 Identification of *Campylobacter*

Campylobacter colonies on MCCDA are smooth, shiny, and convex with a defined edge, or flat, transparent or translucent, and spreading with an irregular edge; colorless to grayish or light cream; and usually 1 to 2 mm in diameter but may be pinpoint to several mm in diameter. Plates of *Campylobacter* colonies may be stored up to 48 h refrigerated under microaerobic conditions if isolates cannot be picked immediately.

Use a platinum or plastic needle to pick three suspect *Campylobacter* colonies for each sample from the MCCDA plates and transfer each to 10 ml of brucella-FBP (BFBP) broth. Since campylobacters can vary greatly in colonial morphology, it is advisable to similarly culture at least one or all colony types present on the plates to assure the target is not overlooked. Alternatively, direct screening of colonies by phase-contrast microscopy can be done prior to picking isolates. To culture isolates, incubate the BFBP tubes with caps loosened for 24 to 48 h at 42 ± 1.0°C in an atmosphere of 5% O_2, 10% CO_2, and 85% N_2. Do not vortex culture tubes of *Campylobacter*, this will introduce oxygen into the media.

Perform the following identification tests on each BFBP broth culture:

a. Examine a wet-mount preparation of the BFBP broth culture with a phase-contrast microscope using a 100X oil immersion objective. Young cells of *Campylobacter* appear as narrow curved rods (0.2 to 0.8 μm wide by 1.5 to 5 μm long). The organisms show rapid movement with darting or corkscrew-like motility. Pairs of cells can resemble the silhouette of a gull's wing span or the letter S. Longer chains can appear helically curved, and multispiralled filamentous elongated forms may exist. Cells grown for more than 72 h may become non-culturable and coccoid. Campylobacters are Gram negative, but Gram staining may

be omitted since cell morphology and motility are more significant in the identification of these organisms. (Carbol fuchsin [0.5%] is used instead of safranin as a counter stain to improve Gram stain results.) Continue confirmation of those BFBP cultures that exhibit typical *Campylobacter* morphology.

b. Inoculate the top 10 mm layer of a tube of semisolid brucella glucose medium with several drops of the above BFBP broth culture. Incubate tubes with caps loosened in an anaerobic jar under microaerobic conditions at 42 ± 1.0°C for 1 to 3 days.

   i. Glucose fermentation test: Campylobacters are nonfermentative, so the color of the medium will remain red-orange. A positive reaction shows a yellow color (acid with phenol red indicator) in the semisolid brucella glucose medium.

   ii. Catalase test: After reading the results of the glucose fermentation test, add 1 ml of 3% hydrogen peroxide to the semisolid brucella glucose medium culture, let sit for two to three minutes, then gently invert the tube to distribute the reagent. Examine after 1 to 10 minutes for formation of bubbles, indicating a positive reaction. *C. jejuni* and *C. coli* are catalase positive.

c. Add about six drops of the BFBP broth culture to a BFBP agar plate, and spread the inoculum over the surface with a sterile swab or a bent glass rod. Aseptically place a disc of nalidixic acid (30 μg) and a disc of cephalothin (30 μg) on each plate. Press each disc with sterile forceps to adhere it to the agar surface. Incubate the plates in an anaerobic jar at 42 ± 1.0°C for 1 to 3 days in a microaerobic atmosphere.

   i. Susceptibility to nalidixic acid and cephalothin: Observe the growth patterns surrounding the antibiotic impregnated discs. *C. jejuni* and *C. coli* are sensitive to nalidixic acid, and a clear zone of inhibition will exist around the disc. A zone of any size indicates sensitivity. The organisms are both resistant to cephalothin, so growth will be present right up to the disc. Lawns of *Campylobacter* growth may be very light and can be difficult to see, so it is helpful to tilt the plate at an angle under a light for viewing.

   ii. Oxidase test: Place a 2 cm square piece of filter

paper in an empty petri dish and add 1 to 2 drops of oxidase reagent to the paper. Heavily smear cells from the above BFBP agar plate onto the reagent-impregnated paper in a spot 3 to 5 mm in diameter using a platinum or plastic loop. The test is positive if the cell mass turns dark purple within 30 seconds. Alternatively, the Difco DrySlide™ oxidase test may be used. Campylobacters are oxidase positive.

d. Optional tests

Other biochemical tests useful for differentiation of catalase-positive campylobacters include nitrate and nitrite reduction, H_2S production, growth in 1% glycine, growth in 3.5% NaCl, and growth at 25, 30.5, 37, and 42°C. *C. jejuni/coli* grow well at 42°C and are curved or S-shaped with darting, corkscrew-like motility. Biochemically, they are catalase positive, oxidase positive, nonfermentative, nalidixic acid sensitive, and cephalothin resistant. Distinguishing between *C. jejuni* and *C. coli* is usually not necessary in a food microbiology laboratory since both are causes of human campylobacteriosis. The few existing tests to separate these species are not dependable. Hippurate hydrolysis appears to be the most reliable and useful test for this purpose. A convenient rapid disk method is available (Cacho et al., 1989). *C. jejuni* is positive for this test, while *C. coli* yields a negative reaction.

6.5 Multiple Start Days

Analysis should begin on a Monday, Tuesday, Wednesday, or Thursday to avoid weekend work. Samples received on a Friday should be analyzed immediately or begun on Saturday; starting either day will require weekend work. Follow the table below according to the day analysis is to begin.

	Analysis To Be Done On Days				
Starting Date	Enrichment	Plating	Pick Colonies	Inoculate Biochemicals	Read/ Perform Tests
MON	MON	TUE	WED	THU	FRI
TUE	TUE	WED	THU	FRI	MON
WED	WED	THU	FRI	MON	WED
THU	THU	FRI	MON	TUE	THU
FRI	FRI	SAT	MON	TUE	THU
SAT	SAT	SUN	MON	TUE	THU

6.6 Storage and Transport of Stock Cultures

Inoculate overnight BFBP broth cultures into tubes of Brucella broth with 0.15% agar. Loosen the screw-caps and incubate for 24 to 48 h at 42 ± 1.0°C in an atmosphere of 5% O_2, 10% CO_2, and 85% N_2. Store refrigerated under this atmosphere for up to a month without serial passage. Cultures in this medium can be transported by mail. Seal tightened caps with adhesive tape to prevent leakage during shipment.

Cultures grown in enriched semisolid brucella medium may be stored under atmospheric conditions at room temperature with caps tightened, for at least three weeks. This medium is also suitable for transporting cultures by mail.

Cultures may also be preserved frozen. To prepare these stocks, swab 6 drops of a 24 h BFBP broth culture onto a BFBP agar plate and incubate microaerobically at 42 ± 1.0°C for 24 to 48 h. Then remove the plate growth with a swab and suspend the cells in 4 ml of Brucella broth with 15% sterile glycerol. The suspension can be stored frozen at -70°C in 1 ml portions for 6 months or longer. Thawing and refreezing these stocks will usually result in loss of viability.

6.7 Media Quality Control

Pay strict attention when preparing all media to assure proper supplement additions. Ingredients, reagents, and media that are past expiration date should be discarded. It is important to discard all unused liquid media more than one month old and all plating media more than two weeks old, since absorbed oxygen will generate peroxides which can be detrimental to campylobacters. Store all media refrigerated, tightly sealed, and shielded from light.

Inoculated media controls should be incubated with each batch of tests to assure proper media formulation and atmospheric conditions. When enriching, include a Qwik Seal® bag of HEB inoculated with an actively growing BFBP broth culture of *C. jejuni* as a control. Similarly, in each anaerobic jar, include an appropriate agar plate or broth inoculated with a known *C. jejuni* strain. Use of positive and negative controls for all biochemical tests is also recommended. An uninoculated control of all test media should also be included to allow assessment of sterility and any changes that may occur in the medium.

Listed below are some recommended controls for the *Campylobacter* biochemical tests:

a. Glucose fermentation test:
   Inoculate a semisolid brucella glucose tube with an *Escherichia coli* strain and incubate aerobically to generate a positive reaction. Inoculate a *C. jejuni* strain and incubate microaerobically to yield a negative reaction.

b. Catalase test:
   Use a *C. jejuni* strain as a positive control and a *Streptococcus* spp. as a negative control.

c. Susceptibility to nalidixic acid and cephalothin:
   Use a *C. jejuni* strain to demonstrate the desired sensitive/resistant pattern.

d. Oxidase Test:
   Use a *C. jejuni* strain as a positive control and an *E. coli* strain as a negative control.

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CHAPTER 7. ISOLATION AND IDENTIFICATION OF *AEROMONAS* SPECIES FROM MEAT AND POULTRY PRODUCTS

Bonnie E. Rose and Anita J. G. Okrend

7.1 Introduction

Members of the genus *Aeromonas* typically are aquatic bacteria and sometime pathogens of fish and cold-blooded vertebrates that inhabit wet environments. Nevertheless, aeromonads are isolated (often in considerable numbers) from various foods of animal origin. These include seafood, raw milk, beef, pork, lamb, and poultry. They grow readily at refrigeration temperatures. Production of enterotoxins can be demonstrated using various laboratory assays, and indirect epidemiological evidence suggests that members of the genus *Aeromonas* have been involved in sporadic human gastroenteritis outbreaks involving seafood. However, no fully confirmed foodborne outbreak has been described in the scientific literature.

The method presented describes procedures for isolation and identification of species of the *Aeromonas hydrophila* group which consists of *A. hydrophila*, *A. sobria* and *A. caviae*. A procedure for detection of hemolysin(s) is also provided. Burke et al., 1983, reported a 97% correlation between hemolysin production and enterotoxin production among *Aeromonas* species.

7.2 Equipment, Reagents and Media

7.21 Equipment

(isolation/identification)

a. Incubator, static 28 ± 1°C
b. Osterizer-type blender with sterilized cutting assemblies and adapters for use with Mason jars, or Stomacher™ (Tekmar) with sterile Stomacher™ bags
c. Sterile bent glass rods ("hockey sticks")

(hemolysin test)

d. Incubator, static 37°C
e. Microtiter plate reader equipped to read at 540 nm
f. Centrifuge capable of 12,000 RPM

g. Shaker incubator (30°C; 210 RPM)
h. Screw-cap Erlenmeyer flasks, 125 ml
i. Sterile screw-cap centrifuge tubes: 15 ml conical and 50 ml round bottom
j. 96-well microtiter plates
k. Membrane filters, 0.2 µm
l. Bench top clinical centrifuge

7.22 Reagents

(isolation/identification)

a. Butterfield's phosphate diluent (BPD)
b. Mineral oil, sterile
c. N,N-dimethyl-p-phenylenediamine monohydrochloride (1% aqueous solution)

(hemolysin test)

d. Rabbit blood, defibrinated
e. Phosphate buffered saline (PBS)
f. Distilled water, sterile

7.23 Media

(isolation/identification)

a. Tryptic soy broth plus 10 µg/ml ampicillin (TSBA)
b. Starch-ampicillin (SA) agar
c. Triple sugar iron (TSI) agar
d. Nutrient agar
e. Mannitol fermentation broth with Andrade's indicator
f. Arginine decarboxylase broth (Moeller)
g. Ornithine decarboxylase broth (Moeller)
h. Decarboxylase broth base (Moeller)
i. Glucose fermentation broth with Andrade's indicator
j. Bile esculin agar

(hemolysin test)

k. Brain heart infusion (BHI) broth

7.3 Isolation Procedure

Serial dilutions of meat samples may be surface-spread directly on SA agar. However better recovery of *Aeromonas* will be achieved by

using enrichment procedures, particularly when the aeromonads have been freeze-injured or are low in number.

a. Blend 25 g of meat in 225 ml TSBA with a blender or Stomacher™ for 2 minutes. Incubate at 28°C for 18 to 24 h.

b. After incubation prepare serial dilutions of the enrichment cultures in BPD. Transfer 0.1 ml of the 10^{-4} to 10^{-6} dilutions onto the surface of SA plates. Evenly spread the inoculum with sterile bent glass rods. The plates must be free of surface moisture if single colonies are to be obtained. Incubate the plates at 28°C for 18 to 24 h.

c. Pick three typical colonies per sample from the SA agar plates to TSI agar and nutrient agar slants. Incubate overnight at 28°C. *Aeromonas* colonies are typically 3 to 5 mm in diameter and appear yellow to honey-colored on SA agar.

7.4 Identification

a. Read the TSI reactions. *Aeromonas* reactions on TSI are as follows: acid butt, acid or alkaline slant, H_2S negative, positive or negative gas production.

b. Perform the oxidase test on the nutrient agar slants. Add a few drops of a N,N-dimethyl-p-phenylenediamine monohydrochloride solution (prepared fresh daily) to the growth on the nutrient agar slant. Oxidase positive cultures develop a pink color which successively becomes maroon, dark red, and black in 10 to 30 min. All aeromonads are oxidase-positive and fermentative.

c. Transfer all oxidase-positive fermenters from the TSI agar slants to the following media for biochemical confirmation: mannitol fermentation broth, arginine decarboxylase broth, ornithine decarboxylase broth, glucose fermentation broth, and bile esculin agar. After inoculation, layer the decarboxylase media with sterile mineral oil and incubate at 28°C for 48 h. Incubate the remainder of the confirmation media at 28°C for 24 h.

d. Record the biochemical characteristics of each isolate. All aeromonads produce acid from mannitol and are arginine positive, ornithine negative. Species of the *A. hydrophila* group can be differentiated according to the biochemical characteristics shown below:

Test (Substrate)	*A. hydrophila*	*A. sobria*	*A. caviae*
Gas from Glucose	+	+	-
Esculin hydrolysis	+	-	+

NOTE: Esculin hydrolysis imparts a dark brown color to the medium.

e. Transfer isolates of suspected *Aeromonas* that are to be tested for hemolysin production from TSI agar to nutrient agar slants and incubate overnight at 28°C.

7.5 Hemolysin Test

The hemolysin test described below is based on that of Burke et al., 1983 and 1984.

7.51 Preparation of Culture Filtrate

a. Transfer growth from the nutrient agar slant to BHI broth (25 ml broth in a 125 ml Erlenmeyer flask). Incubate overnight at 30°C on a shaker incubator at 210 RPM.

b. Centrifuge the broth culture at 11,950 RPM (SS-34 Dupont-Sorvall rotor) for 30 minutes. Decant and save the supernatant liquid; discard the cell pellet.

c. Filter sterilize the supernatant through a sterile disposable membrane filter (0.2 µm).

d. Hold the sterile culture filtrate at 4°C until needed, and test it for hemolysin activity within 24 h of preparation.

7.52 Preparation of Rabbit Erythrocyte Suspensions

a. Centrifuge 10 ml of defibrinated rabbit blood in a 15-ml conical centrifuge tube at 2400 RPM in a bench top clinical centrifuge for 5 minutes.

b. Remove the supernatant and white blood cell layer by suction and discard.

c. Add 10 ml of cold PBS to the packed erythrocytes, mix gently, and centrifuge as described above. Discard supernatant.

d. Wash the erythrocytes in PBS two more times, as described above.

e. After the final wash, note the volume of packed erythrocytes in the centrifuge tube. Prepare a 10% and a 1% erythrocyte suspension in PBS. Hold the two suspensions at 4°C until needed (use within 24 h).

7.53 Preparation of Hemoglobin Standard Curve

a. Transfer 1 ml of the 10% erythrocyte suspension into 8 ml of sterile distilled water. Shake the mixture until all cells are lysed. Add 1 ml of 10X PBS to obtain a 1% hemoglobin solution.

b. Add 1% hemoglobin solution and 1% erythrocyte suspension to conical centrifuge tubes in the following volumes:

Volume (ml)	% hemoglobin										
	0	10	20	30	40	50	60	70	80	90	100
Hemoglo -bin	0	.1	.2	.3	.4	.5	.6	.7	.8	.9	1.0
Erythro -cytes	1.0	.9	.8	.7	.6	.5	.4	.3	.2	.1	0

c. Centrifuge tubes at 2400 RPM for 5 minutes in a clinical centrifuge. Transfer 0.5 ml of supernatant from each tube into wells of a 96-well microtiter plate. Hold the plate for the hemolysin test (Section 7.54).

7.54 Hemolysin Test

a. Add 1 ml of sterile culture filtrate (Section 7.51) to 1 ml of the 1% erythrocyte suspension (Section 7.52) in a conical centrifuge tube and mix gently.

b. Incubate at 37°C for 1 h, then incubate for an additional 1 h at 4-5°C.

c. Centrifuge at 2400 RPM for five minutes.

d. Transfer 0.5 ml of supernatant to the 96-well plate containing the standards (Section 7.53).

e. Read the plate on a microtiter plate reader at 540 nm.

f. A positive hemolysin test is defined as the production of an O.D. reading $\geq$ the O.D. of the 20% hemoglobin standard in the standard curve prepared above in Section 7.53.

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CHAPTER 9. ISOLATION & IDENTIFICATION OF PATHOGENIC *YERSINIA ENTEROCOLITICA* FROM MEAT AND POULTRY PRODUCTS

Jennifer L. Johnson

9.1 Introduction

Yersinia enterocolitica and other *Yersinia* species such as *Y. frederiksenii* and *Y. kristensenii* are ubiquitous in the natural environment, and may be recovered from water, soil, animals, and food. There is considerable variation within the species *Y. enterocolitica*, and member organisms range from the so-called "*Y. enterocolitica*-like" organisms and "environmental" strains of *Y. enterocolitica* to strains capable of causing serious disease in humans. Hogs have been shown to be a reservoir for certain types of pathogenic *Y. enterocolitica* and pork products have been implicated in human disease. The presence of pathogenic *Y. enterocolitica* on food products is a special concern since those organisms are capable of growth at refrigerator temperatures.

Pathogenic *Y. enterocolitica* organisms are significant causes of human disease in many parts of the developed world. Epidemiological evidence from Belgium, Norway, Denmark, The Netherlands, Japan, Canada, and elsewhere strongly implicates consumption of pork products in human disease. In fact, disease due to *Y. enterocolitica* in the United States may be on the rise, and more information on contamination of meat (especially pork) and poultry is needed.

The term "pathogenic serotype", when used in reference to *Y. enterocolitica*, typically refers to one of 11 O-antigen groups in the *Y. enterocolitica* serotyping scheme. Some strains belonging to these serotypes have been implicated in human disease and have demonstrated pathogenicity in animal models or tissue culture cell invasiveness tests. Until recently, serotypes O:4,32; O:8; O:13a,13b; O:18; O:20; and O:21 have accounted for the majority of pathogenic serotypes recovered in the U.S. Only recently have serotype O:3 organisms been identified as a common cause of yersiniosis in the United States of America. In a recent American survey of hospitalized gastroenteritis patients, 92% of the *Y. enterocolitica* isolates were serotype O:3 while 5% were serotype O:5,27. Serotypes O:3, O:9, and O:5,27 are well-established human pathogens in other areas of the world. The so-called "North American serotypes" of *Y. enterocolitica* (serotypes

O:8, O:13, and O:21) represent a genetically distinct lineage from that of the other pathogenic serotypes.

While the term "pathogenic serotype" is in common usage, several authors have stated that terms such as "pathogenic phenotype", "pathogenic bio-serotype", and "pathogenic bio-serogroup" are more descriptive since they differentiate between pathogenic and nonpathogenic members of a generally pathogenic serotype. Biogrouping, the phenotypic characterization of *Y. enterocolitica*, can serve as a useful indication of the likely pathogenicity of a given strain. Testing for markers of pathogenicity like calcium dependence, crystal violet dye binding, auto-agglutination, and pyrazinamidase activity provide additional information. Markers are not perfectly correlated with pathogenicity but provide useful information under conditions where animal testing is undesirable or impractical.

Virulence in *Y. enterocolitica* is mediated by both chromosomal and plasmid-borne genes. While chromosomal determinants are stable, plasmids containing virulence genes may be lost during culture and confirmational procedures. Temperatures above 30°C are known to cause the loss of virulence plasmids in pathogenic *Y. enterocolitica*, but plasmid loss may also occur under other, less well-defined, circumstances.

Numerous enrichment schemes have been described for the recovery of *Yersinia enterocolitica* from meat samples. These enrichment procedures include cold enrichment for up to a month, direct selective enrichment, or two-step pre-enrichment/selective enrichment procedures. It appears that some enrichment procedures are better suited for the recovery of pathogenic *Y. enterocolitica* than others, though recovery may be influenced by the type of meat product. Even when using an enrichment and plating scheme reported to give good recovery from a particular meat product, considerable variation in recovery may be observed. Methods reported to provide good recovery of pathogenic *Y. enterocolitica* in one part of the world may not work so well in another geographical area, possibly due to differences in levels of *Y. enterocolitica* and competing flora.

Recovery of pathogenic *Y. enterocolitica* is contingent upon a number of factors including: the level of background flora on the product; the amount of background flora coming through enrichment and plating; the level of pathogenic *Y. enterocolitica* present on the sample; the numbers of non-pathogenic *Y. enterocolitica* and non-pathogenic *Yersinia* spp. present on the product; and loss of

virulence factors during enrichment and plating. Furthermore, a recovery method which gives good recovery of one serotype of pathogenic *Y. enterocolitica* may not be suited to other serotypes. In order to recover any of the important pathogenic serotypes of *Y. enterocolitica* which might be present, multiple enrichment broths and plating media are usually recommended for the recovery of the organism from naturally-contaminated foods.

As there is no "universal" enrichment scheme capable of reliably isolating all important pathogenic serotypes of *Y. enterocolitica*, recovering serotypes O:3, O:8, and O:5,27 necessitates the use of parallel procedures. This protocol specifies the use of three separate enrichment procedures in combination with two selective/differential agars. Even with the use of multiple cultural enrichment schemes, however, shortcomings of conventional cultural procedures for the recovery of pathogenic *Y. enterocolitica* undoubtedly result in an under-estimation of the prevalence of this organism in foods and in clinical specimens. A study reported that while 18% of raw pork products were found to contain *Y. enterocolitica* serotype O:3 by two cultural procedures, use of a genetic probe on plated enrichments gave a detection rate of 60%. One of the main difficulties encountered during conventional cultural isolation of pathogenic *Y. enterocolitica* appeared to be overgrowth of small numbers of pathogenic *Y. enterocolitica* by nonpathogenic yersiniae and other microorganisms. The use of conventional cultural procedures for the detection and recovery of pathogenic *Y. enterocolitica* by FSIS sets the stage for a move towards use of genetically-based detection methods.

A great deal of effort must be expended in the recovery and characterization of presumptively-pathogenic *Y. enterocolitica*. Sequential levels of characterization tests include: identification of presumptive *Yersinia*, speciation to *Y. enterocolitica*, biogrouping the *Y. enterocolitica*, followed by testing for pathogenicity markers. *Y. enterocolitica* is more active biochemically at 25°C than at 35-37°C, meaning that disparate results for a given test may be obtained depending on incubation temperature. This characteristic, coupled with the known temperature-sensitivity of the *Y. enterocolitica* virulence plasmid, makes strict adherence to temperature and time requirements a necessity. A word to the reader: although the extensive characterization protocol appears intimidating, the vast majority of non-*Y. enterocolitica* are effectively eliminated with minimal work by the first tier of testing.

The enrichment and characterization procedures described in this protocol are well-documented in the literature. The inclusion of these procedures in the latest edition of the "Compendium of Methods for the Microbiological examination of Foods" is further evidence of their acceptance by the scientific community.

9.2 Equipment, Reagents and Media

9.21 Equipment

a. Sterile scissors, forceps, knives, pipettes, hockey sticks, and other supplies
b. Balance (sensitivity of ± 0.1 g)
c. Inoculating needles and loops
d. Vortex mixer
e. Stomacher™ and sterile stomacher bags
f. Freezer (-70°C)
g. Stereomicroscope and oblique lighting (optional)
h. Incubators capable of holding temperatures at 4 ± 1°C, 25 ± 1°C, 28 ± 1°C, 30 ± 1°C, 32 ± 1°C, 35 ± 1°C and 37 ± 1°C.

9.22 Reagents

a. 0.25% KOH in 0.5% NaCl aqueous solution
b. Crystal violet (85 µg/ml aqueous solution)
c. Sterile mineral oil
d. 1% Ferrous ammonium sulfate (prepare fresh on day of use)
e. Kovacs' reagent
f. Voges-Proskauer (VP) test reagents
g. Oxidase reagent or reagent-impregnated disc/strip
h. Glycerol (sterile)
i. 1 N HCl solution

9.23 Media

a. Irgasan-Ticarcillin-Cholate (ITC) broth
b. Trypticase Soy Broth (TSB)
c. Bile-Oxalate-Sorbose (BOS) broth
d. 0.01 M Phosphate Buffered Saline (PBS, pH 7.6)
e. Cefsulodin-irgasan-novobiocin (CIN) agar (MUST BE MADE ACCORDING TO FORMULATION IN APPENDIX)
f. Salmonella Shigella Deoxycholate Calcium (SSDC) agar
g. Kligler's Iron agar (KIA) slants

h. Simmon's Citrate agar slants
i. Christensen's urea agar slants
j. Lysine decarboxylase medium (0.5% lysine)
k. Ornithine decarboxylase medium (0.5% ornithine)
l. CR-MOX (Congo Red Magnesium Oxalate) agar
m. Methyl Red-Voges Proskauer (MR-VP) broth
n. β-D-Glucosidase test medium
o. Purple broth with 1% filter-sterilized salicin
p. Purple broth with 1% filter-sterilized xylose
q. Purple broth with 1% filter-sterilized sucrose
r. Purple broth with 1% filter-sterilized trehalose
s. Purple broth with 1% filter-sterilized rhamnose
t. Esculin agar slants
u. Sterile Saline (0.85% NaCl)
v. Tween 80 agar (lipase test agar)
w. DNase test agar
x. Tryptophan broth (indole test medium)
y. Pyrazinamide agar slants
z. Veal infusion broth
aa. Trypticase Soy agar or Brain Heart Infusion agar plates

NOTE: Formulations for all the very specialized media and reagents used for the isolation and identification of *Yersinia* are presented at the end of this chapter.

9.3 Isolation Procedures

9.31 Preparation of Sample Homogenate

a. For meat samples other than surface samples: Add 25 g of sample to 100 ml of 0.01 M Phosphate Buffered Saline (PBS: pH 7.6). Homogenize for 2 minutes in a Stomacher™. Allow homogenate to stand undisturbed at room temperature for 10 minutes to allow settling of large meat particles.

b. For carcass surface samples: Add PBS to surface sample so as to prepare a 2:1 ratio of volume to surface area (e.g. add 100 ml PBS to a 50 cm^2 sample). Homogenize for 2 minutes in a Stomacher™. Allow homogenate to stand undisturbed at room temperature for 10 minutes.

9.32 Enrichment & Plating Procedures

In order to improve the chances of recovering pathogenic *Y. enterocolitica*, three enrichment procedures (ITC, TSB/BOS, and PBS) should be used. Although this will increase a laboratory's work-load, it is the best way to insure that any serotype of pathogenic *Y. enterocolitica* present in the product will be recovered. ITC broth provides good recovery of serotype O:3 and probably serotype O:9 *Y. enterocolitica*. TSB/BOS permits recovery of serotype O:8. PBS-cold enrichment has been shown to recover serotype O:5,27. KOH treatment of *Y. enterocolitica* enrichment cultures decreases background flora. Two selective plating media, SSDC and CIN agars, are recommended for the isolation of pathogenic *Y. enterocolitica*. Figure 1 illustrates the enrichment procedures which are included in this protocol.

a. ITC broth: Transfer 2 ml of sample homogenate supernatant into 100 ml ITC broth contained in an Erlenmeyer flask. Incubate at 25°C for 2 days. Spread-plate 0.1 ml onto SSDC agar and incubate the plates at 30°C for 24 h. Spread-plate 0.1 ml onto CIN agar, and incubate the plates at 32°C for 18 h. Also, remove 0.5 ml of the ITC enrichment, treat it with KOH, then streak onto CIN. Reincubate the ITC enrichment at 25°C for another 24 h. After the plate incubation is complete, examine the plates as described below. If colonies having typical *Y. enterocolitica* morphology are not visible on the plates, the ITC culture should be plated out as before.

b. TSB/BOS: Transfer 20 ml of sample homogenate supernatant into 80 ml TSB. Incubate at 25°C for 24 h. Transfer 0.1 ml of the TSB culture into 10 ml BOS. Incubate at 25°C for 3 days. Spread-plate 0.1 ml onto SSDC agar and incubate the plates at 30°C for 24 h. Spread-plate 0.1 ml onto CIN agar, and incubate the plates at 32°C for 18 h. Also, remove 0.5 ml of the BOS enrichment, treat it with KOH, then streak onto CIN. Reincubate the BOS enrichment culture at 25°C for 2 additional days, then plate as before.

c. PBS: Refrigerate the remainder of the PBS homogenate at 4°C for 14 days. Spread-plate 0.1 ml onto CIN agar, and incubate the plates at 32°C for 18 h. Also, remove 0.5

ml of the PBS enrichment, treat it with KOH, then streak onto CIN. Also, use KOH treatment with plating onto CIN.

d. KOH treatment: Add 0.5 ml of enrichment culture to 4.5 ml KOH/NaCl. Vortex briefly (3-4 sec) and IMMEDIATELY streak a loop-full of the KOH-treated broth onto CIN agar (Do NOT use KOH treatment in combination with SSDC agar).

9.33 Selection of Colonies from Plating Media

Due to the fact that SSDC and CIN agars are not completely inhibitory to non-yersiniae, a variety of non-*Yersinia* organisms may be recovered from these agars. Some of these organisms (e.g. strains of *Citrobacter* and *Enterobacter*) have a colonial morphology similar to that of *Y. enterocolitica*. Care must be exercised in the selection of suspect colonies from SSDC and CIN agars in order to minimize picking non-yersiniae. It may be helpful for the analyst to compare colonies growing on sample plates to colonies on the positive control plates. Colony appearance can change over time so strict adherence to time/temperature recommendations is necessary.

a. SSDC: On SSDC, *Y. enterocolitica* colonies are typically round, about 1 mm in diameter and opaque or colorless. When observing plates through a stereomicroscope with oblique transillumination, look for irregular colony edges with a finely granular colony center (never iridescent). Non-yersiniae present either an entire edge or a coarser pattern or both.

b. CIN: On CIN, typical *Y. enterocolitica* colonies have a red bulls-eye which is usually very dark and sharply delineated. The bulls-eye is surrounded by a transparent zone with varying radii, with the edge of the colony either entire or irregular; colony diameter is ca. 1-2 mm (larger colonies are usually not *Yersinia*). Again, the use of a stereomicroscope and oblique transillumination may facilitate examination of plates.

9.4 Identification and Confirmation Procedures

9.41 Identification of *Yersinia*

Select a colony on CIN or SSDC having morphology typical of *Y. enterocolitica* and emulsify colony in about 1 ml of sterile saline (0.85%). Use this to first inoculate a slant of Simmon's Citrate Agar, then inoculate Kligler's Iron Agar, and a tube of urea agar. Repeat until 5 colonies having morphology typical of *Y. enterocolitica* have been selected from each plate of selective agar. Table 1 presents the testing scheme to which isolates recovered from SSDC and CIN will be submitted.

a. Simmon's Citrate: Only Streak-inoculate the slant of a tube of Simmon's Citrate agar; do NOT stab the butt. Incubate at 28°C for 24 h. Presumptive *Y. enterocolitica* are citrate negative (-) and the citrate slant will remain the original green color (a positive (+) reaction is characterized by the agar turning a vivid blue color).

b. Kligler's Iron Agar: Stab-inoculate the butt and streak the slant. Incubate at 28°C for 18-24 h. Presumptive *Y. enterocolitica* should present an alkaline (red) slant and acid (yellow) butt, without gas or H_2S on KIA.

c. Christensen's urea agar: Streak the slant with a heavy inoculum load; do NOT stab the butt. Incubate at 28°C for 24-72 h. Presumptive *Y. enterocolitica* are (+) for urease and will turn the agar to an intense red-pink color.

9.42 Confirmation and Biogrouping of *Yersinia enterocolitica*

Any organism which is citrate negative (-), urease positive (+), and gives an alkaline slant/acid butt without gas or H_2S on KIA should be submitted to further testing. Inoculum for further testing may be obtained from the KIA slant; the KIA slant should then be refrigerated pending the test results. THE TESTS LISTED BELOW ARE ALL NECESSARY TO CONFIRM AND BIOGROUP POTENTIALLY-PATHOGENIC *Y. enterocolitica*. Do NOT attempt to biogroup any isolate until the results are available from ALL tests! Similarly, do NOT discard any culture until ALL tests have been completed. See Holt et al., 1994, for additional information on speciating *Yersinia*.

a. Oxidase test: Test colony growth from the KIA slant of any presumptive *Y. enterocolitica* isolates using oxidase reagent or commercially-available, reagent-impregnated test strips/discs. *Yersinia* are oxidase negative (-).

b. Lysine and ornithine decarboxylase: Inoculate one tube each of lysine decarboxylase medium and ornithine decarboxylase medium; overlay each inoculated tube with sterile mineral oil (4-5 mm deep layer). Incubate at 28°C for 4 days. *Y. enterocolitica* are LYS negative (-) and ORN positive (+).

c. Rhamnose, sucrose, xylose, and trehalose utilization: Inoculate one tube of each of these carbohydrate broths, and incubate at 25°C for 10 days, reading after 1,2,3,7, and 10 days. *Y. enterocolitica* are rhamnose negative (-) and sucrose positive (+). Xylose and trehalose reactions vary between biogroups.

d. Salicin utilization: Inoculate a tube of salicin broth, and incubate at 35°C, reading after 1,2,3, and 4 days. Salicin reactions vary between biogroups.

e. Esculin hydrolysis: Inoculate a tube of esculin agar. Incubate at 25°C for 10 days, reading after 1,2,3,7 and 10 days. Blackening indicates esculin hydrolysis. Esculin reactions vary between biogroups of *Y. enterocolitica*.

f. Indole test: Inoculate a tube of Tryptophan broth (indole test medium). Incubate (with loosened caps) at 28°C for 48 h. Add 0.5 ml of Kovacs' reagent, mix gently, then allow tubes to stand about 10 minutes. A dark red color developing below the solvent layer is evidence of a positive (+) test while the color will remain unchanged in a negative (-) test. Indole test results vary with biogroup of *Y. enterocolitica*.

g. VP test: Inoculate a tube of MR-VP broth, and incubate at 25°C for 24 h. After incubation, add 0.6 ml α-naphthol to the tube, and shake well. Add 0.2 ml 40% KOH solution with 0.3% creatine and shake. Read results after 15 minutes and 1 hour. Development of a pink to ruby red color is a positive test. Results vary with biogroup.

h. β-D-Glucosidase test: Emulsify culture in saline to McFarland 3 turbidity. Add 0.75 ml of culture suspension to 0.25 ml of β-D-glucosidase test medium. Incubate at 30°C overnight (16-20 h). A distinct yellow color indicates a positive reaction. Results vary with biogroup.

i. Lipase test: Inoculate *Y. enterocolitica* isolate onto a plate of Tween 80 agar (more than one isolate may be tested per plate). Incubate at 28°C, and examine after 2 and 5 days. Lipase activity is evidenced by an opaque halo surrounding the streak, and varies with biogroup.

j. Deoxyribonuclease (DNase) test: Inoculate *Y. enterocolitica* strain onto a plate of DNase test agar by streaking the medium in a band (about 3/4 inch length streak). Four or more strains may be tested per plate. Incubate plates at 28°C for 18-24 h. Following incubation, examine plates as follows. For DNase test agar, flood plate with 1 N HCl. A zone of clearing around a colony indicates a positive test. Observe for clear zones surrounding the streak (no clearing or a uniformly opaque agar indicates a negative reaction). DNase test agars containing toluidine blue or methyl green may also be used; follow manufacturer's instructions for interpreting results.

k. Pyrazinamidase test: Inoculate strains over entire slant of pyrazinamide agar and incubate at 25°C for 48 h. Flood slant surface with 1 ml of freshly prepared 1% (w/v) aqueous solution of Fe^{+2} ammonium sulfate. Read after 15 minutes; a pink to brown color indicates PYR positive (+); (presence of pyrazinoic acid) while no color development is observed with PYR negative (-) strains. Pathogenic strains are PYR negative (-).

9.43 Testing for Pathogenicity Markers

Presumptive pathogenic *Y. enterocolitica* are LYS negative (-), ORN positive (+), sucrose positive (+), salicin negative (-) and esculin negative (-). Once the results from all the biogrouping tests are available, Table 2 should be consulted for information on biogroup designation. *Y. enterocolitica* isolates belonging to Biogroups 1B, 2, 3, 4, or 5 should be subjected to further testing for pathogenicity markers.

a. Auto-agglutination in MR-VP broth: Inoculate 2 tubes of MR-VP broth; incubate one at 25°C for 24 h, and the other at 35°C for 24 h. After incubation, the tube incubated at the lower temperature should exhibit turbidity from cell growth. The tube which had been incubated at 35°C should show agglutination (clumping) of bacteria along the walls and/or bottom of tube and clear supernatant fluid. Test is plasmid-dependent.

b. Congo red binding/crystal violet binding: Grow isolates in TSB at 25°C for 16-18 h, then dilute in saline to obtain about 10^4 cfu/ml and dilute to 10^{-5}. Spread-plate 10 µl of diluted suspension on CR-MOX plates. Incubate plates at 37°C for 24 h. A predominance of tiny red colonies is indicative of a positive response for both congo red binding and calcium dependency (some large colorless colonies [CR-MOX negative] may be present due to loss of the virulence plasmid). Perform crystal violet binding on the same agar by flooding each plate with about 8 ml of crystal violet (85 µg/ml), allowing this to stand for 2 minutes, then decanting off the dye. If desired, plates may be observed with a stereo dissecting microscope at 40X magnification. Examine colonies as soon as possible as color tends to fade with time; positive isolates display small, intensely purple colonies. CR-MOX permits demonstration of calcium dependency, Congo red binding, and crystal violet dye binding. Test is plasmid-dependent.

9.5 Method Quality Control Procedures

Due to the variety of bio-serogroups of *Y. enterocolitica* which can be found on meat and poultry, a cocktail of control cultures (including serotypes O:3 and O:8) should be used as a positive control. In addition, an uninoculated media control should be utilized for each of the different enrichment media.

Inoculate control strains into separate tubes of TSB. Incubate at 25°C for 18-24 h. In order to provide ca. 30-300 cfu/ml, make a 10^{-7} dilution of each culture in sterile saline. Add 1 ml of the 10^{-7} dilution of each culture to a single bottle containing 50 ml PBS. Mix well. From this point forward, treat the PBS/*Y. enterocolitica* positive-control cocktail as a sample, following the instructions given above in Section 9.32. Confirm

at least one isolate (of each morphological type present on each of the agars) recovered from the positive-control sample.

9.6 Storage of Isolates

9.61 Maintenance of *Y. enterocolitica* Control Strains

Because of the possibility of plasmid loss in virulent *Y. enterocolitica*, it is recommended that control strains of *Y. enterocolitica* be immediately subcultured upon receipt (incubating at temperatures below 30°C), then preserved in a frozen state.

Inoculate a tube of veal infusion broth with each control strain. Incubate for 48 h at 25°C. Add sterile glycerol to a final concentration of 10% (e.g. 0.3 ml in 3 ml veal infusion broth), dispense into several sterile vials, and freeze immediately at -70°C. Preparation of a batch of vials for each strain is recommended so that one vial can be held in reserve to serve as a source of inoculum for preparation of a new batch of frozen stocks.

When a fresh culture of a control strain is needed, a small portion of frozen suspension may be removed aseptically and transferred to a tube of TSB. Incubation should be at 25°C for 24 h, followed by streaking onto a non-selective agar such as TSA or BHI agar with incubation at 25°C for 24 h.

Strains may be kept on TSA or BHI slants at 4°C for short periods of time, but it is not recommended that such strains be transferred due to the possibility of plasmid loss.

Periodically, control cultures should be tested for pathogenicity markers as described above. Cultures which have lost the virulence plasmid should be destroyed, and replaced by a fresh subculture from the frozen stock preparation.

9.62 Maintenance of Isolates During Confirmation

Due to the possibility of plasmid loss during extensive subculturing (even at temperatures below 30°C), it is recommended that presumptive *Y. enterocolitica* isolates be frozen following *Y. enterocolitica* confirmation testing.

From the KIA slant of a presumptive *Y. enterocolitica* isolate, inoculate a tube of veal infusion broth.

Incubate for 48 h at 25°C. Add sterile glycerol to a final concentration of 10%, and freeze immediately at -70°C.

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Figure 1. Enrichment schemes used for the recovery of pathogenic *Y. enterocolitica* from meat or poultry samples.

Homogenize Sample in PBS

2 ml into 100 ml ITC broth	20 ml into 80 ml TSB	remainder of homogenate
2 days 25°C	1 day 25°C	14 days 4°C
--Onto SSDC 24 h 30°C	--0.1 ml TSB culture + 10 ml BOS 25°C 3 days	--Onto CIN --KOH Onto CIN
--Onto CIN 18 h 32°C	--Onto SSDC --Onto CIN	
--KOH treatment Onto CIN	-KOH treatment Onto CIN	
After 1 additional day[a] of broth incubation	After 2 additional days of broth incubation	
--Onto SSDC	--Onto SSDC	
--Onto CIN	--Onto CIN	
--KOH treatment	--KOH treatment	

Onto CIN Onto CIN

[a] Plating should only be done if colonies having typical *Y. enterocolitica* morphology are not present on plates inoculated on previous day.

Table 1. Sequence of Confirmation, Biogrouping, and Pathogenicity-marker Tests used for *Y. enterocolitica*

Yersinia Confirmation Tests	Simmons' Citrate slant	Kligler's Iron Agar slant & butt	Christensen's urea agar
	28°C, 24-72 h	28°C, 18-24 h	28°C, 18-72 h
	Citrate (-) (green) little/no gas	Alk/Acid no H_2S	Urea (+) (pink)
Y. enterocolitica Confirmation Tests	Oxidase Lysine decarboxylase Ornithine decarboxylase Rhamnose utilization Sucrose utilization		
Y. enterocolitica Biogrouping Tests	Lipase DNase Indole Xylose VP β-D-Glucosidase		

	Pyrazinamidase	
	Salicin;	Esculin
	Trehalose;	Nitrate Reduction
Pathogenicity-Marker Tests	Autoagglutination in MR-VP broth	
	Congo Red Binding	
	Crystal Violet Binding	

Table 2. Biogrouping Scheme for *Yersinia enterocolitica* [a]

	Biogroups[b]					
	1A	1B[c]	2[c]	3[c]	4[c]	5[c]
Lipase (Tween-esterase)	+	+	-	-	-	-
Esculin/salicin 24 h[d]	+,-	-	-	-	-	-
Indole	+	+	(+)[e]	-	-	-
Xylose	+	+	+	+	-	V[f]
Trehalose/NO_3[g]	+	+	+	+	+	-
Pyrazinamidase	+	-	-	-	-	-
β-D-Glucosidase	+	-	-	-	-	-
Voges-Proskauer	+	+	+	+[h]	+	(+)
DNase	-	-	-	-	+	+

[a] Modified from Wauters et al., 1987.

[b] Reactions from tests incubated at 25-28°C, with the exception of β-D-Glucosidase which was incubated at 30°C and salicin which was incubated at 35°C. Incubation at other temperatures may result in different results and biogroupings.

[c] Biogroup contains pathogenic strains.

[d] Esculin and salicin reactions for a given strain of *Y. enterocolitica* are nearly always identical so they are listed together in this table.

[e] Indicates a delayed positive reaction.

[f] Indicates variable reactions.

[g] **Trehalose and nitrate reduction reactions for a given strain of *Y. enterocolitica* are nearly always identical so they are listed together in this table.**

[h] **Rarely, a serotype O:3 strain may be negative for VP.**

ADDENDUM

Formulations for Media and Reagents for *Yersinia enterocolitica* Isolation and Identification

β-D-Glucosidase test

Add 0.1 g 4-nitrophenyl-β-D-glucopyranoside to 100 ml 0.666 M NaH_2PO_4 (pH 6.0), dissolve, then filter-sterilize.

BOS broth

$Na_2HPO_4*7H_2O$	17.25 g
Na oxalate	5.0 g
Bile salts No. 3 (Difco)	2.0 g
NaCl	1.0 g
0.1% solution of $MgSO_4*7H_2O$	10.0 ml
Distilled deionized H_2O	639.0 ml

Combine ingredients and mix until dissolved, adjust pH to 7.6 with 5 N HCl, then autoclave at 121°C for 15 minutes.

Add the following filter-sterilized solutions:

100 ml of 10% sorbose
100 ml of 1.0% asparagine
100 ml of 1.0% methionine
10 ml of 2.5 mg/ml metanil yellow
10 ml of 2.5 mg/ml yeast extract
10 ml of 0.5% Na pyruvate
1 ml of 0.4% solution of Irgasan DP300 (2,4,4'-trichloro-2'-hydroxydi

Adjust pH to 7.6 with either 5 N NaOH or HCl as required. Store at 4°C for up to 7 days.

On day of use, add 10 ml of 1.0 mg/ml Na furadantin (from stock solution stored at -70°C) to the above complete base. Aseptically dispense 10 ml portions into sterile tubes.

CIN agar

MUST CONTAIN Cefsulodin at 4 mg/L:

This formulation is commercially available from Difco; premixes available from other manufacturers contain different levels of cefsulodin.

Oxoid special peptone	20.0 g
Yeast extract	2.0 g
Mannitol	20.0 g
Na pyruvate	2.0 g
NaCl	1.0 g
0.1% aqueous stock solution of $MgSO_4*7H_2O$	10.0 ml
Na deoxycholate	0.5 g
Oxoid No. 4 (L11) agar	12.0 g
Distilled deionized H_2O	748.0 ml

Bring to a boil in order to dissolve agar completely (do NOT autoclave). Cool to around 80-85°C.

Add 10 ml of Irgasan DP300 (2,4,4'-trichloro-2'-hydroxydiphenyl ether, Ciba Geigy) solution (0.04% in 95% ETOH). Shake vigorously to disperse ethanol. Cool in a water bath to ca. 50-55°C.

Add 1 ml of 5 N NaOH, then 10 ml of each of the following aqueous, filter sterilized (0.22 µm pore size) stock solutions:

neutral red (3 mg/ml)
crystal violet (0.1 mg/ml)
cefsulodin (0.4 mg/ml)
novobiocin (0.25 mg/ml).

[Stock antibiotic solutions are stored at -70°C and thawed at room temperature just before use]

Adjust final pH to 7.4 with 5 N NaOH. Store prepared plates at around 20-25°C for up to 9 days.

CR-MOX agar

Tryptic soy agar	40.0 g
Distilled deionized H_2O	825.0 ml

Mix and autoclave at 121°C for 15 minutes. Cool basal medium to 55°C.

Add the following solutions:

a) 80 ml of 0.25 M sodium oxalate (Sigma) solution (sterilized by autoclaving at 121°C for 15 minutes)
b) 80 ml of 0.25 M magnesium chloride solution (sterilized by autoclaving at 121°C for 15 minutes)
c) 10 ml of 20% D-galactose solution (sterilized by autoclaving at 115°C for 10 minutes)
d) 5 ml of 1% Congo red solution (sterilized by autoclaving at 121°C for 15 minutes)

Mix well and dispense into 15 X 100 mm petri dishes. Store prepared media in plastic bags at 4°C for up to 3 months.

DNase test Agar

Tryptose	20.0 g
Deoxyribonucleic acid	2.0 g
Sodium chloride	5.0 g
Agar	15.0 g
Distilled water	1.0 L

Suspend all ingredients and heat to boiling to dissolve completely. Sterilize in the autoclave at 121°C for 15 minutes, final pH = 7.3. Dispense into sterile Petri dishes.

Esculin agar

Polypeptone (Oxoid)	10.0 g
Esculin	1.0 g
Ferric ammonium citrate	1.0 g
Agar	5.0 g
Distilled deionized H_2O	1.0 L

Mix well. Dispense into tubes, and autoclave at 121°C for 15 minutes.

Indole test medium

Prepare a 1% solution of Bacto Peptone (Difco) OR 1% Trypticase peptone (BBL) OR use Tryptone Water (Oxoid). Dispense 5 ml quantities into tubes. Sterilize by autoclaving at 121°C for 15 minutes.

ITC broth

Tryptone	10.0 g
Yeast extract	1.0 g
$MgCl_2*6H_2O$	60.0 g
NaCl	5.0 g
0.2% (w/v) malachite green solution (aqueous)	5.0 ml
$KClO_3$	1.0 g
Distilled deionized H_2O	1.0 L

Mix above ingredients, autoclave at 121°C for 15 minutes, cool. Then add,

a) 1 ml of Ticarcillin solution (1 mg/ml in H_2O; filter-sterilized) (Ticarcillin available from Sigma)
b) 1 ml of Irgasan DP300 (1 mg/ml in 95% ethanol); AKA 2,4,4'-trichloro-2'-hydroxydiphenyl ether (CIBA-Geigy, Basel)
c) Mix well. Dispense 100 ml into sterile 100 ml Erlenmeyer flasks (it is important to minimize the surface area:volume ratio). Store at 4°C for up to 1 month.

Kligler's iron agar (KIA) slants

Polypeptone peptone	20.0 g
Lactose	20.0 g
Dextrose	1.0 g
NaCl	5.0 g
Ferric ammonium citrate	0.5 g
Sodium thiosulfate	0.5 g
Agar	15.0 g
Phenol red	0.025 g
Distilled water	1.0 L

Heat with agitation to dissolve completely. Dispense into 13 X 100 mm screw-cap tubes and autoclave for 15 minutes at 121°C. Cool and slant to form deep butts. Final pH = 7.4.

KOH solution

NaCl	5.0 g
KOH	2.5 g
Distilled deionized H_2O	1.0 L

Dispense 4.5 ml amounts in small screw-cap tubes, and sterilize at 121°C for 15 minutes. Tighten caps when cool. Make only a small number of tubes at a time since pH decreases with storage time; store at 4°C for no more than 7 days.

Pyrazinamide agar

Tryptic soy agar (Difco)	30.0 g
Pyrazine-carboxamide (Merck)	1.0 g
0.2 M Tris-maleate buffer (pH 6)	1.0 L

Mix well, dispense 5 ml amounts in tubes (160 X 16 mm). Autoclave at 121°C for 15 minutes. Slant for cooling.

SSDC agar

SS agar (quantity per liter as stated by a particular Manufacturer)

Yeast extract	5.0 g
Na deoxycholate	10.0 g
$CaCl_2$	1.0 g
Distilled deionized H_20	1.0 L

Adjust pH to 7.2 to 7.3 Bring agar almost to a boil on a hot plate (Do NOT autoclave). Temper agar to 55-60°C, mix and pour while still warm, making thick plates. Store prepared plates for 7 days at 20-25°C in the dark. Do NOT store at 4°C.

Tween 80 agar (Lipase test agar)

Peptone	10.0 g
NaCl	5.0 g
$CaCl_2*H_2O$	0.1 g
Agar	15.0 g
Distilled deionized H_2O	1.0 L

Sterilize agar base by autoclaving at 121°C for 15 minutes. Temper to 45-50°C.

Sterilize Tween 80 by autoclaving at 121°C for 20 minutes.

Add sterile Tween 80 to tempered agar base to give a final concentration of 1% (v/v). Mix well. Dispense into Petri dishes, and allow to solidify.

Veal infusion broth

Veal, infusion from	500.0 g
Proteose peptone # 3	10.0 g
NaCl	5.0 g
Distilled water	1.0 L

Heat with agitation to dissolve all ingredients. Dispense 7 ml portions into 16 X 150 mm tubes and autoclave at 121°C for 15 minutes. Final pH = 7.4.

CHAPTER 10. EXAMINATION OF HEAT PROCESSED, HERMETICALLY SEALED (CANNED) MEAT AND POULTRY PRODUCTS

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10.1 Introduction

Thermally processed meat and poultry products in hermetically sealed containers include both shelf stable products as well as those that must be kept refrigerated (i.e. perishable product). There are a wide variety of packages designed to totally exclude air. These include traditional rigid containers, such as metal cans and glass jars; semi-rigid containers such as plastic cans, bowls and trays; and flexible containers such as retortable pouches and bags. The microbiological examination of these food products requires knowledge and a thorough understanding of food microbiology, food science, and packaging technology and engineering. Many books and scientific articles are available on the processing and the laboratory testing of these products. Individuals who perform these analyses should be familiar with the current procedures and methods. Some of these references are listed in section 10.6.

10.2 Important Terms and Concepts

a. Shelf Stability (commercial sterility):

The term "shelf stability" traditionally has been used by the Agency and is synonymous with the terms "commercial sterility" or commercially sterile". Shelf stability is defined in CFR title 9, part 318, Subpart G, 318.300 (u) of the Food Safety and Inspection Service (meat and poultry) USDA regulations. Shelf stability (commercial sterility) means "the condition achieved by application of heat, sufficient, alone or in combination with other ingredients and/or treatments, to render the product free of microorganisms capable of growing in the product at non-refrigerated conditions (over 50°F, 10°C) at which the product is intended to be held during distribution and storage". Such a product may contain viable thermophilic spores, but no mesophilic spores or vegetative cells. These products usually are stable for years unless stored at temperatures of 115-130°F (46-55°C) which may allow swelling or flat sour spoilage to

occur because of germination and growth of the thermophilic spores. Many low acid canned meat/poultry products contain low numbers of thermophilic spores. For this reason, samples of canned foods are not routinely incubated at 55°C because the results usually will be confusing and provide no sound information. Canned food lots that are to be held in hot vending machines or are destined for tropical countries are exceptions to this rule.

b. Hermetically Sealed Container:

A container that is totally sealed to prevent the entry or escape of air and therefore secure the product against the entry of microorganisms.

c. Adventitious contamination:

Adventitious contamination may be defined as the accidental addition of environmental microorganisms to the contents of a container during analysis. This can occur if the microbiologist has not sterilized the puncture site on the container surface or the opening device adequately, or is careless in manipulating equipment or cultures. Strict attention to proper procedures is required to avoid this type of contamination.

d. Cured Meat/Poultry Products:

Many canned meat/poultry products contain curing salts such as mixtures of sodium chloride and sodium nitrite. When included in a canned meat/poultry product formulation, sodium chloride and sodium nitrite inhibit the outgrowth of bacterial spores, particularly clostridial spores. Lowering the pH and increasing the sodium chloride concentration enhance the inhibitory action of sodium nitrite. Thus, most canned, cured meat/poultry products are minimally heat processed and are rendered shelf stable by the interrelationship of heat, pH, sodium chloride, sodium nitrite and a low level of indigenous spores. Spoilage in canned cured meat/poultry products attributed to underprocessing is rare. When it occurs, it is usually the result of improper curing rather than inadequate heating. The heat processes used for canned, cured, shelf stable meat/poultry products are unique in that they usually

are not designed to destroy mesophilic bacterial spores but merely to inhibit their outgrowth.

e. Uncured Meat/Poultry Products:

Canned uncured meat/poultry products are given a much more severe heat treatment than canned cured products. The treatment given to canned uncured meat/poultry products is commonly referred to as a "full retort cook".

10.21 Classification of Containers

a. Metal and plastic cans with metal double sealed end(s):

Cans must be at room temperature for classification. Cans are classified as NORMAL if both ends are flat or slightly concave; FLIPPER when one end of a normal-appearing can is struck sharply on a flat surface, the opposite end "flips out" (bulges) but returns to its original appearance with mild thumb pressure; SPRINGER if one end is slightly convex and when pressed in will cause the opposite end to become slightly convex; SOFT SWELL if both ends are slightly convex but can be pressed inward with moderate thumb pressure only to return to the convex state when thumb pressure is released; HARD SWELL if both ends are convex, rigid and do not respond to medium hard thumb pressure. A can with a hard swell will usually "buckle" before it bursts. Hard swollen cans must be handled carefully because they can explode. They should be chilled before opening except when aerobic thermophiles are suspected. Never flame a can with a hard swell, use only chemical sanitization.

b. Glass jars:

Classify glass jars by the condition of the lid (closure) only. Do not strike a glass jar against a surface as you would a can. Instead shake the jar abruptly to cause the contents to exert force against the lid; doing so occasionally reveals a flipper. Scrutinize the contents through the glass prior to opening. Compare the contents of the abnormal/questionable jar with the contents of a normal jar (e.g., color, turbidity, and presence of gas bubbles), and record observations.

c. Flexible containers (pouches):

Pouches usually are fabricated from laminates consisting of two or more layers (plies) of material. Retortable pouches are the most common type of flexible container used for canned, shelf-stable products. Most pouches are 3-ply: an outer ply of polyester film, a middle ply of aluminum foil, and an inner ply of polypropylene. The polyester functions as the heat resistant, tough protective layer; the aluminum foil as a moisture, gas and light barrier; and the polypropylene functions as the food contact surface and the film for heat sealing. The polypropylene also provides added strength, and protects the aluminum film against corrosion by the food product. Not all retortable pouches contain an aluminum foil ply. Pouches and paperboard containers used for non-retorted, shelf stable products (e.g. pH-controlled and hot-filled product) or aseptically filled containers may be quite different from retortable pouches in construction. Pouches and other flexible containers are either factory-formed and supplied ready for filling, or are formed by the processor from roll stock.

10.22 Container Abnormalities

To determine the cause of product abnormalities, both normal and abnormal containers from the same production lot should be examined. All observed microbiological results should be correlated with any existing product abnormalities (Section 10.46 a) such as atypical pH, odor, color, gross appearance, direct microscopic examination, etc. as well as the container evaluation findings (Section 10.46, b,c). Non-microbial swells (such as hydrogen swells) are usually diagnosed by considering all product attributes because culture results are negative or insignificant.

a. Metal cans, plastic containers and glass jars:

Conditions such as "swells" are defined in Section 10.21 (a). The defects and abnormalities associated with these containers have been extensively detailed by others. Rather than include extensive descriptions for each of them in this section, the analyst is referred to several excellent references presented in Section 10.6. These references provide detailed information on the numerous defects and abnormalities that can occur with these containers. The analyst should be familiar with these conditions before beginning any analysis of a

defective or abnormal container. The effect of processing failures, such as overfilling, closure at low temperature or high altitude; container damage; and storage temperature changes, must be taken into consideration as the analyst evaluates possible causes for the defect or abnormality. For quick reference, a Glossary of Terms is provided in Appendices I and II.

b. Pouches:

A Glossary of Terms for these containers can be found in Appendix III. It is imperative to follow uniform procedures (Section 10.46,c) when examining defective or abnormal pouches. The APHA, 1966 reference (Section 10.6) provides detailed information on the analysis of pouch defects.

10.3 Analysis of Containers

The number of containers available for analysis will vary. However, it is important that the number be large enough to provide valid results. Unless the cause of spoilage is clear cut, at least 12 containers should be examined. With a clear cut cause, one half this number may be adequate. If abnormal containers have been reported, but are not available for analysis, incubation of like-coded containers may reproduce the abnormality. The "normal" cans should be incubated at 35°C for 10 days prior to examination. Incubation temperatures in excess of 35°C should not be used unless thermophilic spoilage is suspected. This incubation may reproduce the abnormality, and thereby document progressive microbiological changes in the product. Examine the incubated cans daily. Remove any swells from the incubator as they develop and culture them along with a normal control. After the 10 day incubation period, cool the cans to room temperature and reclassify. Swollen, buckled and blown containers should NOT be incubated but analyzed immediately along with a normal control. All steps in the analysis should be conducted in sequence according to protocol.

10.31 Physical Examination of Metal and Plastic Containers

a. Before opening, visually examine the double end seam(s) and side seam (if present) for structural defects, flaws and physical damage; record pertinent observations.

b. Run thumb and forefinger around the inside and outside of the double seams for evidence of roughness, unevenness, or sharpness.

c. Using a felt marker, make three slash marks at irregular intervals across the label and the code-end seam. Remove the label and copy any label code-numbers to the side of the container along with a mark indicating the code end of the can. Correlate any stains on the label with suspicious areas on the side panel (can body) by returning the label to its exact position relative to the slash marks.

d. Examine all non-seam areas of the can and ends for any evidence of physical damage. If the code is embossed, carefully examine it for any evidence of puncturing. Circle any suspect and/or defective areas with an indelible pen and record this information on the work sheet. For an illustration of these defects see the APHA, 1966 reference (Section 10.6).

10.32 Physical Examination of Glass Jars

a. Before opening, remove the label and, using a good light source such as a microscope light, examine the container for apparent or suspected defects. Microorganisms may enter jars through small cracks in the glass. Make note of any residue observed on the outer surface and the location.

b. Test the closure gently to determine its tightness. After sampling has been completed, examine the lid (closure) and the glass rim (sealing surface) of the jar. Look for flaws in the sealing ring or compound inside the closure; for food particles lodged between the glass and the lid; and for chips or uneven areas in the glass rim.

10.33 Physical Examination of Pouches

a. Pouches should be examined using an illuminated 5X magnifier.

b. Hold the pouch in one hand, examine it for abnormalities, such as swelling, leakage, overfilling, and defects such as delamination and severe distortion. Record any pertinent observations.

c. Hold the pouch at both ends and examine both sides for noticeable cuts, cracks, scratches, food residues, punctures, missing labels, foreign materials or other abnormalities.

d. Carefully examine all seal areas for incomplete fusion. Pay attention to such defects as entrapped product, wrinkles, moisture and foreign material in the seal. Particular attention should be given to the final or closing seal.

e. All actual and suspected defects should be circled with an indelible marking pen for more detailed examination after all sampling is complete.

10.4 Analysis of the Contents

Processing errors occur infrequently with canned products, but may result in the improper processing of large quantities of product. Swollen cans, for instance, may signal a microbial spoilage problem. Each abnormality in a "canned" product must be investigated thoroughly and correctly. The following procedures should be followed carefully.

10.41 Equipment and Material

a. Incubators 20°, 35° & 55 ± 1°C
b. Vertical laminar flow hood
c. Microscope, microscope slides & cover slips
d. pH meter equipped with a flat electrode
e. Felt-tip indelible marker
f. Illuminated 5X magnifier
g. Sterile Bacti-disc cutter or other suitable opening device
h. Large, sterile plastic or metal funnel
i. Large autoclavable holding pans
j. Sterile towels
k. Clean laboratory coat and hair covering(s)
l. Sterile wide bore pipettes or 8 mm glass tubing with cotton plugs
m. Sterile serological pipettes with cotton plugs
n. Safety aspiration device for pipetting (e.g. pro-pipette)
o. Sterile petri dishes, beakers, and large test tubes
p. Sterile triers, cork borers, scissors, knives and 8" forceps. Triers can be made from the tail piece of

chrome finish sink drain pipe, 1 1/2" in diameter, flanged on one end and sharpened on the other end.

q. Sterile cotton swabs with wooden handles in glass test tubes, one per tube, or commercially sterilized swabs in paper sleeves
r. Sterile gloves
s. Small wire basket to hold pouches in an upright position
t. Seam analysis tools (micrometer, calipers, saw, countersink meter, metal plate scissors, nippers).
u. Vacuum gauge
v. Light source such as a microscope light
w. Sonic cleaning apparatus
x. Transparent acrylic plate with a hole and tubing to a vacuum source
y. Bituminous compound in strips (tar type strips usually available in hardware stores) stored in the 35°C incubator
z. Seamtest Type U (Concentrate), Winston Products Co., Inc Box 3332, Charlotte, N.C., Dilute 1:300 with distilled water for use.
aa. Wooden dowels, 1/2" diameter
bb. Gas cylinder clamp
cc. Abrasive chlorinated cleaner or a scouring pad

10.42 Media and Reagents

a. Modified Cooked Meat Medium (MCMM) STEAM JUST BEFORE USE
b. Brom Cresol Purple Broth (BCPB) or Dextrose Tryptone Broth
c. Plate Count Agar
d. APT Agar
e. KF Broth
f. Strong's Sporulation Medium
g. Gram stain reagents
h. Spore stain
i. Dishwashing detergent
j. Chlorine solution, (Commercial Bleach with approximately 5% available chlorine diluted 1:100 with 0.5 M phosphate buffer, pH 6.2)

10.43 Preparation

a. The Analyst

i. The analyst must wear a clean full length laboratory coat.

ii. Hair must be completely covered with a clean, disposable operating room type hair cover. A surgical face mask should be worn; if the analyst has facial hair such as beards and sideburns, the mask must completely cover it.

iii. Hands, forearms and face should be washed with germicidal soap and water.

iv. The analyst should wear safety glasses or goggles, preferably in combination with some type of face shield when opening swollen cans or cans suspected of being contaminated with ***Clostridium spp***.

b. Preparing the Environment

i. If possible, the analysis should be done in a vertical laminar flow hood. If a hood is not available, the area used must be clean and draft-free.

ii. Flat cans should be opened in the laminar flow hood.

iii. Swells may explode or spew, therefore they should be opened outside the hood and the container transferred to the hood only after it is opened and all gas released.

iv. Disinfect the work surface before beginning any work.

c. Preparing Metal Cans Prior to Opening

i. Scrub the non-coded end of the metal can with abrasive cleaner or a scouring pad. This removes bacteria-laden oil and protein residues. Rinse well with tap water. Cans with an "easy open" end usually are coded on the bottom. Record the code exactly and prepare the code end as described above.

ii. Sanitize the cleaned end with chlorine solution (Section 10.42 j) either by placing clean tissues over the end and saturating it with chlorine solution or by immersing the end in a shallow pan containing the solution. Allow a 15-minute contact

time; wipe dry with sterile towels or tissue. (An alternative sanitization procedure which can be used on Normal-appearing cans ONLY is to heat the entire can surface using a laboratory burner or a propane torch until the metal becomes slightly discolored from the heat.) Proceed as outlined in Section 10.44.

d. Preparing Jars Prior to Opening

i. Scrub the surface of the jar closure with abrasive cleaner or scouring pads. Rinse well with tap water.

ii. Sanitize the jar closure with chlorine (Section 10.42 j) either by placing clean tissues over the closure and saturating it with chlorine solution or immersing the closure in a shallow pan containing the solution. Allow a 15-minute contact time; wipe dry with sterile towels or tissue.

e. Preparing Plastic Containers Prior to Opening

i. Scrub the bottom surface of the container with abrasive cleaner or scouring pads. Rinse well with tap water.

ii. Sanitize the bottom with chlorine solution (Section 10.42 j) by placing clean tissues over the bottom and saturating it with chlorine or immersing the bottom of the container in a shallow pan containing the solution. Allow a 15-minute contact time; then wipe dry with sterile towels or tissue.

f. Preparing Normal and Abnormal-Appearing Flexible Retortable Pouches Prior to Opening

i. Clean the outside of the pouch with a sanitizer and rinse well.

ii. Sanitize the entire pouch in a suitably sized pan with chlorine solution (Section 10.42 j). Allow a 15-minute contact time; then wipe dry with sterile towels or tissue.

g. Preparing Swollen Cans Prior to Opening

i. Scrub the non-coded end of the chilled metal can with an abrasive cleaner or a scouring pad. This removes bacteria-laden oil and protein residues. Rinse well with tap water.

ii. Sanitize the cleaned end with chlorine solution (Section 10.42 j) either by placing clean tissues over the end and saturating it with chlorine solution or immersing the end in a shallow pan containing the solution. Allow a 15-minute contact time; then wipe dry with sterile towels or tissue.

h. Opening Devices

i. The preferred type of opening device is the adjustable Bacti-disc cutter (available from the Wilkens-Anderson Company, 4525 W. Division Street, Chicago, IL.; a similar device is available from the American National Can Co., 1301 Dugdale Rd., Waukegan, IL. Order Number WT2437). The opener should be pre-sterilized or heated in a flame to redness. If this type of device is not available, individually packaged and heat sterilized regular, all metal, kitchen-type can openers may be used. The advantage of the Bacti-disc type opener is that it causes no damage to the double seam (simplifying later examination) and the size of the opening can be adjusted.

ii. Sometimes a large can (e.g. a #10 size can) may be difficult to open. The analyst could be exposed to pathogens or their toxins if the can is not properly secured. The container can be held tightly with a gas cylinder clamp secured in an inverted position in a shallow metal drawer or tray lined with a large disposable poly bag or an autoclavable tray to contain any overflow. Place the #10 container against the clamp and secure the strap. Rotate the can and continue cutting until the opening is completed. The metal tray and liner may be removed for cleaning and the clamp is autoclavable.

10.44 Sampling

a. Normal-Appearing Metal Cans and Jars with Metal Closures

i. Prepare the area and can or jar closure as described in section 10.43.

ii. Shake the container to distribute the contents.

iii. Use a sterilized opening device to cut the desired size entry hole. Transfer samples immediately to the selected media with a sterile pipette or swab and proceed as outlined in Section 10.45.

iv. Aseptically transfer a representative amount of the product to a sterile test tube or other sterile container as a working reserve. Use a pipet or sterile spoon to accomplish this.

v. Caution: The contents from overfilled cans may flow out of the hole onto the surrounding lid surface at the time of opening. This material can then drain back into the can when the opening device is removed. Should this occur, terminate the analysis.

b. Normal and Abnormal-Appearing Plastic Containers

i. Immediately after removing the container from the chlorine solution and wiping the excess liquid, use a very hot, sterilized opening device to cut the desired size entry hole. Transfer samples immediately to the selected media with a sterile pipette or swab and proceed as outlined in Section 10.45.

ii. Aseptically transfer a representative amount of the product to a sterile test tube or other sterile container as a working reserve. Use a pipet or sterile spoon to accomplish this.

c. Normal and Abnormal Appearing Flexible Retortable Pouches

i. Place the disinfected pouch upright in a sterile beaker and cut a two inch strip about one quarter

of an inch under the seam edge using a sterile scissors. If possible, use a pipette to remove some of the pouch contents, otherwise use a swab. Transfer the samples immediately to the selected media with a sterile pipet or swab, proceed as in section 10.45.

ii. Aseptically transfer a representative amount of the product to a sterile test tube or other sterile container as a working reserve. Fold the edge of the opened pouch over against itself several times and secure with tape until the microbiological analysis is complete.

d. Swollen Cans

i. Cans displaying a hard swell should be chilled before opening. Most foods spoiled by ***Bacillus stearothermophilus*** will not produce gas (flat sour spoilage). However, if nitrate or nitrite is present in the meat/poultry product, gas may be produced by this microorganism. Cold usually will kill ***B. stearothermophilus*** resulting in no growth in Bromcresol Purple Broth. If possible, save one or two cans and store without refrigeration.

ii. NEVER FLAME A SWOLLEN CONTAINER - IT MAY BURST. Place the container to be opened in a large, shallow, autoclavable pan. The side seam, if present, should be facing away from the analyst. A container with a hard swell may forcefully spray out some its contents, posing a possible hazard to the analyst if the contents are toxic. Therefore, these cans should be considered a biohazard and precautions must be taken to protect the analyst. Protective gloves should be worn and the lab coat should be tucked inside the cuffs of the gloves or at least secured around the wrist. Some type of facial shield is also recommended.

iii. Place the sanitized container into a biohazard bag and cover with a sterile towel or invert a sterile funnel with a cotton filter in the stem over the can. Place the point of the sterile opening device in the middle of the container closure. Make a small hole in the center of the sterilized end/closure. Try to maintain pressure over the

hole. Release the instrument slowly to allow gas to escape into the towel or funnel.

iv. After the gas pressure has been released, enlarge the opening to the desired size to permit sampling and aseptically remove some of the container contents. Sample as outlined in (a) above.

10.45 Culturing

a. Inoculation of Culture Media

i. The sampling and transfer processes must be conducted aseptically; care must be taken to prevent contamination during the various manipulations.

ii. Transfer the sample at once to the selected media, inoculating each tube at the bottom. Whenever possible, use a pipet and pro-pipette to remove 1-2 ml of product for inoculating each tube of medium. When the nature of the meat/poultry product makes it impossible to use a pipet, use a sampling swab (holding it by the very end of the shaft) to transfer 1-2 g of the product to each tube. This is accomplished by plunging the swab into the product, then inserting the swab as far as possible into the appropriate tube of medium and breaking off the portion of the shaft that was handled. Use one swab for each tube of medium. When inoculating MCMM, force the broken swab to the bottom of the tube by using the tip of another sterile swab.

iii. For each sample, inoculate 2 tubes of MCMM which were steamed (or boiled) for 10 minutes and cooled just before use and 2 tubes of Bromcresol Purple Broth. If a tube of KF medium is inoculated at the same time, the presence of enterococci can be determined rapidly.

iv. As a process control, place uninoculated swabs into each of two tubes of MCMM and BCP and one swab into KF broth (if used). Additionally, label two uninoculated tubes of each medium to serve as controls. If multiple samples are cultured at the same time, only one set of control tubes are needed for each medium and each temperature.

v. After all tubes have been inoculated with a sample, aseptically transfer approximately 30 ml or a 30 g portion of the container contents to a sterile tube, Whirl-Pak® or jar for retention as a working reserve sample. Appropriately label the container and store it in a refrigerator at approximately 4°C.

vi. Finally, transfer a portion of the container contents to a sterile Petri plate, clean jar or beaker for pH, microscopic, organoleptic and other relevant analyses (10.46).

vii. Cover the hole made in the container with several layers of sterile aluminum foil, secure the foil with tape and then store the container in a refrigerator at approximately 4°C. This serves as the primary reserve. Re-enter it only as a last resort. If the sample is a regulatory sample, chain of custody records must be maintained on it.

b. Incubation of Culture Media

i. Incubate one tube each of MCMM and BCP at 35°C and one tube each at 55°C. If used, incubate the tube of KF medium at 35°C. For the MCMM and BCP controls, incubate one tube at 35° and one at 55°C.

ii. Observe all tubes at 24 and 48 h. Tubes incubated at 35°C that show no growth should be incubated for 5 days before discarding. Tubes incubated at 55°C should be incubated for 3 days before discarding. Subculture any questionable tubes, especially if the product under examination contributes turbidity.

c. Identification of Organisms

i. Use conventional bacteriological procedures to characterize the type(s) of microbial flora found in the contents of the container.

ii. Use descriptive terms such as: mixed culture or pure culture, anaerobic or aerobic growth, spore former or non-sporeformer, mesophile or thermophile, cocci or rods.

iii. Cultures should be examined using a Gram stain. Gram stains should be done only on 18-24 h cultures. Record the morphological types observed and their Gram reaction. If the container contents are examined microscopically using a methylene blue stain, record those observations as well. If endospores are present, the spore stain can be used for better definition of spore type and placement.

iv. Record all biochemical test results in addition to any characteristic growth patterns on differential and/or selective media.

v. MCMM tubes showing a bright yellow color with visible gas bubbles, and containing gram positive or gram variable rods should be suspected of containing gas-forming anaerobes. If ***Clostridium botulinum*** is suspected, sub-cultures should be made and incubated for 4-5 days. The original tube should be reincubated to check for spores. After 4 - 5 days incubation, test the cultures for toxin by the mouse bioassay (see Chapter 14).

10.46 Supportive Determinations

a. Examination of Container Contents

i. Determine the pH of the sample (10.45, a, vii) using a flat electrode. Disinfect the electrode after taking this measurement.

ii. If applicable, determine the water activity of the sample (Section 2.4).

iii. Examine the sample microscopically by making a simple methylene blue or crystal violet stain. A Gram stain is of no value since the age of the cells is not known and Gram-stain reactions may not be dependable in the case of old cells. Prepare a spore stain if the contents of a swollen container show signs of digestion and few bacterial cells.

iv. Note abnormalities observed in the container contents such as off-odors, off-color, changes in consistency and texture when compared with normal product. DO NOT TASTE!

b. Examination of Metal and Plastic Cans

NOTE: Whenever possible a "normal" companion can should be examined along with the abnormal one.

i. After a reserve sample has been taken and all examinations are complete, discard any remaining product into an autoclavable bag and terminally sterilize.

ii. Disinfect the inside of the container with a phenolic disinfectant and carefully clean it with a stiff brush or use an ultra sonic bath. Do not autoclave the container since this may destroy any defects.

iii. Examine the interior lining of metal containers for blackening, detinning and pitting.

iv. The container code should have been recorded prior to analysis; if it was not, do so now. Sometimes embossed codes are poorly impressed and can be revealed by rubbing a pencil on a paper held over the code. If this does not work, place a thin smooth piece of paper over the code, hold securely and rub the paper with a clean finger in order to impress the paper. Rerub the paper with a finger coated with graphite. This is superior to using a pencil to rub the code. If that fails, rub the code with carbon paper. Place transparent adhesive tape over the code and rub the tape with the back of a fingernail. Lift the tape and transfer it to any document requiring the can code. The latter two techniques allow a record to be kept of any partial numbers or symbols. It is also possible to wait until the can is emptied, then view the reverse of the code from the inside. If needed, the code can be viewed in a mirror.

v. When leakage from double seams or side seams is suspected, remove excess metal from the opened end, leaving a 0.5 - 1 cm flange. Dry thoroughly,

preferably overnight, in the 55°C incubator. Add leak detection liquid (10.41z) to the can to a depth of 2-4 cm. Place a microleak detector on the open end of the container. The leak detector consists of a transparent acrylic plate with a vacuum gauge and connector for a vacuum source. Place a gasket (cut pieces of an automobile tire inner tube will do) between the apparatus and the can. If the fit is not tight (e.g., end seam is bent), use modeling clay to fill in the gaps. Large cans without beading or thin metal cans having a wider diameter than height may collapse when vacuum is applied. To prevent this from happening, use 1/2" wooden dowels cut to the appropriate length to support the can sides. Bituminous compound on the dowel ends will hold them in place. Generally, 4 dowels are sufficient for a #10 can. Apply the gasket and any bituminous compound, to the open can end and fit the leak detector plate in place. Connect the vacuum and apply 10 inches vacuum to the can. Swirl the liquid to dissipate bubbles formed by gases dissolved in the liquid. Examine seams by covering them with the diluted Seamtest. Leaks are identified by a steady stream of bubbles or a steadily increasing bubble size. After carefully examining all seams for leaks, increase the vacuum to 20 inches vacuum and re-examine the seams. Leave the can under vacuum until a leak appears or for a maximum of 2 h, and examine at half-hour intervals. Mark the location of leaks on the can's exterior using a marking pen. When reporting, note which seam, and the distance from the side seam or some other appropriate reference point. If no leaks were found, note test conditions (time and amount of vacuum drawn).

vi. Perform a tear-down examination of the double seams. The following references in Section 10.6 will guide you through this process: APHA, 1966; Food Processors Institute, 1988; Double Seam Manual; Evaluating a Double Seam, FDA Bacteriological Analytical Manual, 1992.

vii. The tightness of double seams formed by plastic cans and metal can ends may be evaluated by comparing the actual seam thickness to the

calculated thickness of the plastic flange, neck, or metal end. This would include three thicknesses of plastic and two of metal. Also, assess tightness by inspecting the pressure ridge, since it reflects the compression of the plastic body wall. The pressure ridge should be visible and continuous. Each packer may have different specifications for the finished seams; if necessary, the analyst must call the in-plant inspector and ask for specifications for the container of interest.

c. Examination of Pouches

i. The best way to determine if a pouch has leaked is by the type of microorganisms recovered.

ii. The pouch should be examined microscopically looking for points of light coming through the film. These are potential leakage sites.

10.47 Interpretation of Results

Use Tables 2, 3 and 4 to arrive at possible causes of spoilage based on all laboratory results. Caution: The tables are based on a single cause of spoilage. If there are multiple causes, the tables may not help.

10.5 Examination of Canned, Perishable Meat/Poultry Products

Perishable meat and poultry products, such as hams, luncheon meats, and loaves are packaged in hermetically-sealed containers and then heat-processed to internal temperatures of not less than 150°F (65.5°C) and usually not greater than 160°F (71°C). "Perishable, Keep Refrigerated" must appear on the label of these products. Although they are not shelf stable, good commercial processing usually will destroy vegetative bacterial cells. The combined effects of sodium nitrite, salt, refrigeration, and low oxygen tension retard the outgrowth of the few vegetative cells and/or spores that may survive the process. Such products can retain their acceptable quality for 1 to 3 years when properly processed and refrigerated.

10.51 Analysis of Containers

See Sections 10.3 - 10.33

10.52 Analysis of the Contents

a. Equipment and Material

See Section 10.41

b. Media and Reagents

See Section 10.42

c. Preparation

See Section 10.43

d. Sampling

i. Using procedures already described (Section 10.44) remove approximately 50 g of sample with a sterilized trier, large cork borers, scissors, knife or forceps.

ii. Place the sample into a sterile blender jar or Stomacher bag, add 450 ml of sterile Butterfield's Phosphate Diluent and homogenize for 2 minutes. This is a 1:10 dilution; make additional dilutions through at least 10^{-4}. Proceed with the culturing steps given in Section 10.52 (e, f & g).

iii. After sampling, cover the container opening with sterile aluminum foil several layers thick and secure with tape. Place the opened sample unit in the freezer until the analysis is complete.

e. Aerobic Plate Counts

i. Pipet 1 ml of each dilution prepared in 10.52 (d) into each of two sets of duplicate pour plates according to the instructions given in Section 3.4.

ii. Prepare one dilution set with Plate Count Agar. Incubate this set at 35°C for 48 h.

iii. Substitute APT agar for the Plate Count Agar in the other set of plates. Incubate this set at 20°C for 96 h.

iv. Count and record the results from both sets as described in Section 3.4.

f. Gas-Forming Anaerobes (GFAs)

i. Steam tubes of MCMM for 10 minutes and cool just prior to use.

ii. Inoculate each tube with l ml of each dilution prepared in 10.52 (d). Begin with the 1:10 dilution and continue with subsequent dilutions. Use a separate pipet for each dilution. Dilutions must be sufficiently high to yield a negative endpoint. Be sure that the inoculum is deposited near the bottom of the tube.

iii. Incubate these tubes for 48 h at 35°C, but read daily.

iv. Consider any MCMM tubes showing a bright yellow color, containing visible gas bubbles, and containing gram positive or gram variable rods as positive for GFAs.

v. Based upon the highest dilution showing these organisms, report the approximate number of gas-forming anaerobes per gram, calculated as the reciprocal of the highest positive dilution. If skips occur, disregard the final actual dilution and calculate the end point at the dilution where the skip occurred. This is only an approximation of the gas forming anaerobe count. A minimum of three tubes per dilution and an MPN table must be used for a more accurate determination.

vi. If *Clostridium botulinum* is suspected, representative tubes that have not been opened should be reincubated for a total of 4 - 5 days and then tested for botulinum toxin using the mouse bioassay (Chapter 14).

g. Enterococci

i. Transfer 1 ml of each dilution prepared in 10.52(d) to individual tubes of KF broth. Use a separate pipette for each dilution. Begin with the 1:10 dilution and continue with each subsequent

dilution. Dilutions must be sufficiently high to yield a negative end point.

ii. Incubate these tubes at 35°C for 48 h. Tubes showing a yellow color, turbidity and buttoning of growth are presumptive positives.

iii. Confirm all presumptive positives microscopically. Either wet mounts examined under low light or gram stained preparations are suitable for these microscopic determinations. Microscopic determinations yielding cells with ovoid streptococcal morphology shall be considered confirmed positive.

iv. Report the approximate number of enterococci per gram, calculated as the reciprocal of the highest positive confirmed dilution. If skips occur, disregard the final actual dilution and calculate the end point at the dilution where the skip occurred. This is only an approximation of the number of enterococci. A minimum of three tubes per dilution and an MPN table must be used for a more accurate determination of organisms as described in 10.43-10.45 and Tables 2, 3 and 4.

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Appendix I

Glossary of Metal/Plastic Can Seam Terminology for Container Components and Defects

The same terms that are used to describe an all-metal seam apply equally well to the metal end/plastic body seam.

Base Plate: Part of a closing machine which supports cans during seaming operation.

Beaded Can: A can which is re-enforced by having ring indentations around the body. The bead tends to keep the can cylindrical and helps to eliminate paneling of the can body.

Body: Principal part of a container - usually the largest part in one piece containing the sides (thus sidewall or body wall).

Body Hook: Can body portion of double seam. Prior to seaming, this portion was the flange of the can.

Bottom Seam: Factory end seam. The double seam of the can end put on by the can manufacturer.

Buckling: A distortion in a can end.

Can Size: Two systems are commonly used to denote can size:

i. An Arbitrary system (1, 2, etc.) with no relation to finished dimension.

ii. A system indicating the nominal finished dimensions of a can; e.g. "307 x 512." In this example, the first group of digits ("307") refers to the can's diameter and the second set ("512"), the can's height. The first digit in each set represents inches, and the next two digits represent sixteenths of an inch. Hence, the example can has a diameter of 3-7/16 and a height of 5-12/16 (or 5-3/4) inches.

Chuck: Part of a closing machine which fits inside the countersink and in the chuck wall of the end during seaming.

Closing Machine: Also known as a double seamer. Machine which double seams the lid onto the can bodies.

Compound: Rubber or other material applied inside the end curl to aid in forming a hermetic seal when the end is double seamed on the can body.

Contamination in Weld Area: Any visible burn at one or more points along the side seam of a welded can. This is a major defect.

Countersink: On a seamed end, the perpendicular distance from the outermost end panel to the top seam.

Cover: Can end placed on can by packer. Also known as top, lid, packer's end, canner's end.

Cover Hook: That part of double seam formed from the curl of the can end.

Cross Over: The portion of a double seam at the lap.

Cross Section: Referring to a double seam, a section through the double seam.

Curl: The semi-circular edge of a finished end prior to double seaming. The curl forms the cover hook of the double seam.

Cut Code: A break in the metal of a can due to improper embossing-marker equipment.

Cut-Over: During certain abnormal double seaming conditions, the seaming panel becomes flattened and metal is forced over the seaming chuck forming a sharp lip at the chuck wall. In extreme cases the metal may split in a cut-over.

Dead-Head: An incompletely rolled finished seam. Also known as a skip, skid or spinner.

Double Seam: The joint between the end and the can body formed by rolling the curl under the flange (1st operation) and then pressing the metal together (2nd operation).

Droop: A smooth projection of double seam below the bottom of a normal seam. While droops may occur at any point of the seam, they usually are evident at the side seam lap. A

slight droop at the lap may be considered normal because of additional plate thickness incorporated into the seam structure.

Excessive Slivers: One or more slivers which are 1/32" or longer. This is a minor defect of welded cans.

Factory End: Bottom or can manufacturer's end.

False Seam: A seam fault where the end and body hook are not over-lapped (engaged), although they give the appearance of a properly formed seam. Also see Knockdown Flange.

Feather: Beginnings of a cut-over. See Sharp Edge.

First Operation: The first operation in double seaming. In this operation, the curl of the end is tucked under the flange of the can body which is bent down to form cover and body hook, respectively.

Flange: The flared portion of the can body which facilitates double seaming.

Flange Crack: Any crack at the flange or immediately adjacent to the weld of welded cans. This is a major defect.

Headspace: The free space above the contents of a can and the can lid.

Heavy Lap: A lap containing excess solder. Also called a thick lap.

Hook: (i). The bent over edges of a body blank, which form the side seam lock (ii). The body and cover hooks in a double seam.

Internal Enamel: A coating applied to the inside of the can to protect the can from chemical action by the contents or to prevent discoloration. A lacquer is usually clear; an enamel is pigmented and opaque.

Jumped Seam: A double seam which is not rolled tight enough adjacent to the crossover caused by jumping of the seaming rolls at the lap.

Knockdown Flange: A seam defect in which the flange is bent against the body of the can. The cover hook is not tucked

inside the body hook, but lies outside of it. False seams, knockdown flanges and soft crabs are degrees of the same effect. In order to distinguish the degree of the defect, the following terminology is suggested:

False Seam: The cover hook and body hook are not tucked for a distance of less than an inch. Thus it may not be possible to detect a false seam until the can is torn down.

Knockdown Flange: As above, but more than an inch in length. Body hook and cover hook in contact, but not tucked.

Soft Crab: A defect in which the body of the can is broken down and does not contact the double seam. Thus, there is a wide open hole in the can below the double seam where the body was not incorporated into the seam.

Lap: The soldered but not locked portions of a side seam at the ends of the can body before seaming and removing the can from the chuck at completion of the operation.

Lid: See Cover.

Lip, Spurs or Vees: Irregularities in the double seam due to insufficient or sometimes absent overlap of the cover hook with the body hook, usually in small areas of the seam. The cover hook metal protrudes below the seam at the bottom of the cover hook in one or more "V" shapes.

Loss of Overlap: Any observable loss of overlap along the side seam of a welded can. This is a critical defect.

Loose Tin: A metal can which does not appear swollen, but slight pressure reveals a looseness.

Mislock: A poor or partial side seam lock, due to improper forming of the side seam hooks.

Neck: The thickness of the top of the sidewall (body wall) of a plastic tub, one tenth of an inch below the junction of the flange and the sidewall.

Notch: A small cut-away portion at the corners of the body blank. This reduces droop when double seaming.

Oozier: An imperfect can which allows the escape of the contents through the seam.

Open Lap: A lap failed due to various strains set up during manufacturing operations. Also caused by improper cooling of the solder (See Weak Lap). A lap which is not properly soldered so the two halves are not properly joined.

Over Lap: The distance the cover hook laps over the body hook.

Paneling: A flattening of the can side. Also used to define concentric (expansion) rings in can ends.

Peaking: Permanent deformation of the expansion rings on the can ends due to rapid reduction of steam pressure at the conclusion of processing. Such cans have no positive internal pressure and the ends can be forced back more or less to their normal position.

Perforation: Holes in the metal of a can resulting from the action of acid in food on metal. Perforation may come from inside due to product in the can or from outside due to material spilled on the cans.

Pleat: A fold in the cover hook which extends from the edge downward toward the bottom of the cover hook and sometimes results in a sharp droop, vee or spur.

Pressure Ridge: A ridge formed on the inside of the can body directly opposite the double seam, as a result of the pressure applied by the seaming rolls during seam formation.

Pucker: A condition which is intermediate between a wrinkle and a pleat in which the cover hook is locally distorted downward without actual folding. Puckers may be graded the same way as wrinkles.

Sanitary Can: Can with one end attached, the other end put on by the packer after the can is filled. Also known as packer's can or open top can.

Sawtooth: Partial separation of the side seam overlap at one or more points along the side seam after performing the pull test on a welded side seam. This is a critical defect.

Seam Arrowing: A readily visible narrowing of the weld at either end of the can body. This is a major defect.

Seam Width: The maximum dimensions of a seam measured parallel to folds of the seam. Also referred to as the seam length or height.

Seam Thickness: The maximum dimension measured across or perpendicular to the layers of the seam.

Second Operation: The finishing operation in double seaming. The hooks formed in the first operation are rolled tight against each other in the second operation.

Sharp Edge: A sharp edge at the top of the inside portion of the double seam due to the end metal being forced over the seaming chuck.

Side Seam: The seam joining the two edges of a blank to form a body.

Skipper / Spinner: See Deadhead.

Uneven Hook: A body or cover hook which is not uniform in length.

Vee: See Lip.

Weak Lap: The lap is soldered and both parts are together. However, strain on this lap (e.g. by twisting with the fingers) will cause the solderbond to break.

Weld Crack: Any observable crack in a welded side seam. This is a critical defect.

Worm Holes: Voids in solder usually at the end of the side seam. May extend completely through the width of the side seam.

Wrinkle: The small ripples in the cover hook of a can. A measure of tightness of a seam.

Appendix II

Glossary of Glass Container Parts

From a manufacturing standpoint, there are three basic parts to a glass container based on the three parts of glass container molds in which they are made. These are the finish, the body and the bottom.

Finish: The finish is that part of the jar that holds the cap or closure. It is the glass surrounding the opening in the container. In the manufacturing process, it is made in the neck ring or the finish ring. It is so named since, in early hand glass manufacturing, it was the last part of the glass container to be fabricated, hence "the finish". The finish of glass containers has several specific areas as follows:

Continuous Thread: A continuous spiral projecting glass ridge on the finish of a container intended to mesh with the thread of a screw-type closure.

Glass lug: One of several horizontal tapering protruding ridges of glass around the periphery of the finish that permit specially designed edges or lugs on the closure to slide between these protrusions and fasten the number of lugs on the closure and their precise configuration is established by the closure manufacture.

Neck Ring Parting Line: A horizontal mark on the glass surface at the bottom of the neck ring or finish ring resulting from the matching of the neck ring parts with the body mold parts.

Sealing Surface: That portion of the finish which makes contact with the sealing gasket or liner. The sealing surface may be on the top of the finish, or may be a combination of both top and side seal.

Vertical Neck Ring Seam: A mark on the glass finish resulting from the joint of matching the two parts of the neck ring. NOTE: Some finishes are made in a one-piece ring and do not have this seam.

Body: The body of the container is that portion which is made in the "body-mold" in manufacturing. It is the largest part of the container and lies between the finish and the bottom.

The characteristic parts of the body of a glass container are:

Heel: The heel is the curved portion between the bottom and the beginning of the straight side wall.

Mold Seam: A vertical mark on the glass surface in the body area resulting from matching the two parts of the body mold.

Shoulder: That portion of a glass container in which the maximum cross-section or body area decreases to join the neck or finish area. Most glass containers for processed foods have very little neck. The neck would be a straight area between the shoulder and the bottom of the bead or, with beadless finishes, the neck ring parting line.

Side Wall: The remainder of the body area between the shoulder and the heel.

Bottom: The bottom of the container is made in the "bottom plate" part of the glass container mold. The designated parts of the bottom normally are:

Bearing Surface: That portion of the container on which it rests. The bearing surface may have a special configuration known as the "stacking feature" which is designed to provide some interlocking of the bottom of the jar with the closure of another jar on which it might be stacked for display purposes.

Bottom Plate Parting Line: A horizontal mark on the glass surface resulting from the matching of the body mold parts with the bottom plate.

Appendix III

Glossary of terms - Flexible Retortable Pouches.

Adhesive: A substance applied to ply surfaces to cement the layers together in a laminated film: (a). Polyurethane adhesive for the outer layer (b). Maleic anhydride adduct of polypropylene for the inner layer.

Blisters: Bubbles/gaseous inclusions/particulate material, may be present between layers of laminate, usually are found in the seal area.

Bottom of Closing Seal: Portion of closing (packer) seal adjustment to the pouch contents.

Bottom Seal: A seal applied by heat and pressure to the bottom of a flexible pouch.

Cosmetic Seal: Area above the primary seal designed to close the edges of the pouch thus preventing the accumulation of extraneous material.

Cuts, Punctures, Scratches: Mechanical defects that penetrate one or more layers of the pouch.

Delamination: Any separation of plies through adhesive failure. This may result in questionable integrity of the package and safety of the product.

Dirty: Smeared with product or product trapped in top edges (where there are no cosmetic seals).

Disintegrated Container: Evidence of delamination or degradation after retorting.

Final Seal: A seal formed by heat and pressure by the packer after pouch filling and prior to retorting.

Foil Flex Cracks/Foil Roll Holes: Visible cracks in the aluminum foil layer caused by flexing of the pouch or pin holes (roll holes) in the foil caused through manufacture of the aluminum ply.

Foreign Materials: Any material (solid food, condensate, grease, voids, blemishes) that may be entrapped between the plies but usually found in the seal area.

Fusion Seal: A seal formed by joining two opposing surfaces by the application of heat and pressure.

Hard Swell or Blown: Distention or rupture due to internal gas formation.

Inner Ply: Polypropylene coating bonded to the food surface side of the aluminum foil.

Laminate: Two or more layers of material held together by adhesive(s).

Leaker: Product leaking through any area of the pouch.

Outer Ply: The polyester film bonded to the exterior surface of the aluminum foil.

Over Carton: A separate container (usually cardboard) in which the flexible pouch is packaged for additional protection.

Package Dimensions: The measurements of retortable flexible pouches stated as length, the longest dimension (LGT), width the second longest dimension (W), and thickness, the shortest dimension (HGT). All are given as internal measurements.

Pin Holes, Roll Holes: Holes in the aluminum foil layer only, originating during manufacturing; usually do not leak.

Preformed Seals: Seals formed by heat and pressure, by the manufacturer of the pouches, along the sides and at the bottom of the pouches.

Primary Seal: A fusion seal formed by the food processor by applying heat and pressure immediately after filling.

Seal: A continuous joint of two surfaces made by fusion of the laminated materials.

Seal Width: The maximum dimension of the seal measured from the leading outside edge perpendicular to the inside edge of the same seal.

Severely Damaged: Punctures, cuts or ruptures which penetrate all layers of the pouch and expose the product to contamination.

Side Seals: Seals formed by applying heat and pressure to the sides of the pouch's laminates to form the "preformed pouch".

Tear Nicks or Notch: Notches near the final seal to aid the consumer in opening the pouch.

Wrinkle: A crease or pucker in the seal (Packer or Factory) areas.

Appendix IV

Table 1. Normal pH Values for a Few Representative Canned Meat/Poultry Products.

Kinds of Food	pH
Beans with Wieners	5.7
Beef Chili	5.6
Beef Paté	5.7
Beef Stew	5.4 - 5.9
Beef Taco Filling	5.8
Beef and Gravy	5.9 - 6.1
Chicken Noodle Soup	5.8 - 6.5
Chicken Soup with Rice	6.7 - 7.1
Chicken Broth	6.8 - 7.0
Chicken and Dumplings	6.4
Chicken Vegetable Soup	5.6
Chicken Stew	5.6
Chicken Vienna Sausage	6.1 - 7.0
Chorizos	5.2
Corned Beef	6.2
Corned Beef Hash	5.0 - 5.7
Egg Noodles & Chicken	6.5
Ham	6.0 - 6.5
Lamb, Strained Baby Food	6.4 - 6.5
Pork Cocktail Franks	6.2
Pork with Natural Juices	6.2 - 6.4
Pork Sausage	6.1 - 6.2
Roast Beef	5.9 - 6.0
Spaghetti and Meatballs	5.0
Spaghetti Sauce with Beef	4.2
Stuffed Cabbage	5.9
Sloppy Joe	4.4
Turkey, Boned in Bouillon	6.1 - 6.2
Turkey with Gravy	6.0 - 6.3
Vienna Sausage	6.2 - 6.5
Wieners, Franks	6.2

Appendix V

Table 2. KEY TO PROBABLE CAUSE OF SPOILAGE IN CANNED FOODS

Group 1.- Low-Acid Foods
pH Range 5.0 to 8.0

Condition of cans	Characteristics of Material in Cans						
	Odor	Appearance	Gas (CO_2 & H_2)	pH	Smear	Cultures	Diagnosis
Swells	Normal to "metallic"	Normal to frothy (Cans usually etched or corroded)	More than 20% H_2	Normal	Negative to occasional organisms	Negative	Hydrogen swells
	Sour	Frothy; possibly ropy brine	Mostly CO_2	Below Normal	Pure or mixed cultures of rods, cocci, yeasts or molds	Growth, aerobically and/or anaerobically at 35°C., and possibly at 55°C.	Leakage
	Sour	Frothy; possibly ropy brine, food particles firm with uncooked appearance	Mostly CO_2	Below Normal	Pure or mixed cultures of rods, coccoids, cocci and yeasts	Growth, aerobically and/or anaerobically at 35°C., and possibly at 55°C. (If product received high exhaust, only spore formers may be recovered)	No process given
	Normal to sour-cheesy	Frothy	H_2 and CO_2	Slightly to definitely below normal	Rods, med. Short to med. long, usually granular; spores seldom seen	Gas, anaerobically at 55°C., and possibly slowly at 35°C.	Post-processing temperature abuse Thermophilic anaerobes
	Cheesy to putrid	Usually frothy with disintegration of solid particles	Mostly CO_2; possibly some H_2	Slightly to definitely below normal	Rods; usually spores present	Gas anaerobically at 35°C.	Underprocessing - mesophilic anaerobes (possibility of Cl. botulinum)
	Slightly off – possibly ammoniacal	Normal to frothy		Slightly to definitely below normal	Rods; spores occasionally seen	Growth, aerobically and/or anaerobically with gas at 35°C and possibly at 55°C. Pellicle in aerobic broth tubes. Spores formed on agar and in pellicle.	Underprocessing - B. subtilis type

No vacuum and/or Cans buckled	Normal	Normal	No H_2	Normal to slightly below normal	Negative to moderate number of organisms	Negative	Insufficient vacuum, caused by: 1) Incipient spoilage, 2) Insufficient exhaust, 3) Insufficient blanch, 4) Improper retort cooling procedures, 5) Over fill
Flat cans (0 to normal vacuum)	Normal to sour	Normal to cloudy brine		Slightly to definitely below normal	Rods, generally granular in appearance; spores seldom seen	Growth without gas at 55°C. Spore formation on nutrient agar	Post-Processing temperature abuse Thermophilic flat sours.
	Normal to sour	Normal to cloudy brine; possibly moldy		Slightly to definitely below normal	Pure or mixed cultures of rods, coccoids, cocci or mold	Growth, aerobically and/or anaerobically at 35°C., and possibly at 55°C.	Leakage

Appendix VI

Table 3. KEY TO PROBABLE CAUSE OF SPOILAGE IN CANNED FOODS

Group 3. Semi-Acid Foods
pH Range 4.6 to 5.0

Condition of cans	Characteristics of Material in Cans						
	Odor	Appearance	Gas (CO_2 & H_2)	pH	Smear	Cultures	Diagnosis
Swells	Normal to "metallic"	Normal to frothy (Cans usually etched or corroded)	More than 20% H_2	Normal	Negative to occasional organisms	Negative	Hydrogen swells
	Sour	Frothy; possibly ropy brine	Mostly CO_2	Below Normal	Pure or mixed cultures of rods, coccoids, cocci, yeasts or molds	Growth, aerobically and/or anaerobically at 35°C., and possibly at 55°C.	Leakage
Note: Cans are Sometimes flat	Sour	Frothy; possibly ropy brine, food particles firm with uncooked appearance	Mostly CO_2	Below Normal	Pure or mixed cultures of rods, coccoids, cocci and yeasts	Growth, aerobically and/or anaerobically at 35°C., and possibly at 55°C. (If product received high exhaust, only spore formers may be recovered)	No process given
	Normal to sour-cheesy	Frothy	H_2 and CO_2	Slightly to definitely below normal	Rods - med. Short to med. long, usually granular; spores seldom seen	Gas, anaerobically at 55°C., and possibly slowly at 35°C.	Post-processing temperature abuse Thermophilic anaerobes
	Normal to cheesy to putrid	Normal to frothy with disintegration of solid particles	Mostly CO_2; possibly some H_2	Normal to slightly below normal	Rods; possibly spores present	Gas anaerobically at 35°C. Putrid odor	Underprocessing – mesophilic anaerobes (possibility of *Cl. Botulinum)*

	Slightly off - possibly ammoniacal	Normal to frothy		Slightly to definitely below normal	Rods; occasionally spores observed	Growth, aerobically and/or anaerobically with gas at 35°C and possibly at 55°C. Pellicle in aerobic broth tubes. Spores formed on agar and in pellicle.	Under-processing - *B. subtilis* type
	Butyric acid	Frothy, large volume gas	H_2 and CO_2	Definitely below normal	Rods - bipolar staining; possibly spores	Gas anaerobically at 35°C. Butyric acid odor	Under processing - butyric acid anaerobe
No vacuum and/or Cans buckled	Normal	Normal	No H_2	Normal to slightly below normal	Negative to moderate number of organisms	Negative	Insufficient vacuum, caused by: 1) Incipient spoilage, 2) Insufficient exhaust, 3) Insufficient blanch, 4) Improper retort cooling procedures, 5) Over fill
Flat cans (0 to normal vacuum)	Sour to "medicinal"	Normal to cloudy brine		Slightly to definitely below normal	Rods, possibly granular in appearance	Growth without gas at 55°C. and possibly at 35°C. Growth on thermoacidurans agar	Underprocessing *B. coagulans*
	Normal to sour	Normal to cloudy brine; possibly moldy		Slightly to definitely below normal	Pure or mixed cultures or rods, coccoid, cocci or mold	Growth, aerobically and/or anaerobically at 35°C., and possibly at 55°C.	Leakage

Appendix VII

Table 4. Characteristics of Normal and Abnormal Perishable Canned Meat/Poultry Products

Condition of Cans	Odor	Appearance	pH	Smear	Cultures	Probable Cause
Flat Cans (0 to Normal Vacuum)	Normal	Normal	Normal	Negative to occasional organisms	0 to low # APC, APT agar count	Normal product
0 to degrees of swelling	Sour to off odor	Normal to mushy, possible gel liquification	Slightly to definitely below normal	Mixed culture of rods & enterococci	Low # mesophiles, high # psychrophilic non-spore formers (enterococci, lactobacilli	1. Prolonged storage at low temperatures 2. Abnormal high levels in raw materials 3. Substandard process
Swell	Sour or off odor, possibly putrid	Normal to mushy, possible gel liquification	Slightly to definitely below normal	Mixed culture of rods, cocci	High # mesophilic spore formers and non-sporeformers	Product held without refrigeration
Swell	Normal to sour	Normal	Below normal	Cocci, rods or both	Enterococci, rods or both	Leakage if shell higher than core. Underprocessing if core higher than shell
Swell	Off odor	Normal to off color	Below normal	Rods	Psychrotrophic clostridia (rarely occurs in U.S.).	Low brine levels
Swell	Normal to putrid, depending on length of storage.	Ranges from uncooked appearance to digested	Normal to low, depending on length of storage.	Vary	Vary	Missed processing cycle. Most of these are detected soon after distribution.

CHAPTER 11. TESTS FOR ENZYMES IN MEAT AND MEAT PRODUCTS

Charles P. Lattuada, James G. Eye, John M. Damare and B. P. Dey

11.1 Catalase Test

11.11 Introduction

Tests for catalase in meat are limited to products that have been given a heat treatment since the enzyme normally is present in all raw meat. It is particularly useful for roast beef. This procedure will detect under-processing when the product is scheduled to be heated to 145°F (62.8°C) or higher internal temperature. Tests for catalase in cooked beef are indicative of the presence of somatic catalase. Somatic catalase is destroyed at approximately 145°F and the test indicates whether or not temperatures higher than 145°F were reached.

Detection of catalase in a canned meat product could be indicative of flat sour spoilage. At canning temperatures all somatic catalase should be destroyed, and the presence of the enzyme in a freshly opened can is indicative of bacterial catalase produced by growth.

11.12 Equipment and Supplies

a. Clean plastic teaspoon
b. Clean paper towels
c. Felt-tip marking pen
d. Adhesive tape or paper labels
e. Whirl-Pak® clear plastic bags (3" x 4")
f. Clear plastic Zip-Loc® bags (12" x 12")
g. Clean and sanitized slicing knife
h. Clean and sanitized large spoon or spatula
i. 3% Hydrogen Peroxide - 1 pint
j. Baby Shampoo
k. Active dry baker's yeast

11.13 Procedure

a. Preparation of the Peroxide Reagent

    i. Remove the caps from both the peroxide and the shampoo bottles.

ii. Add one teaspoonful of the shampoo to the pint of hydrogen peroxide (peroxide reagent).

iii. Replace the caps securely on each bottle.

iv. Slowly invert the peroxide reagent bottle 3-4 times to mix the contents.

v. Label the reagent bottle "Prepared Reagent" followed by the date of preparation.

vi. Store the peroxide reagent in a refrigerator, the unused shampoo can be stored on a shelf with the chemicals.

b. Testing the Peroxide Reagent

i. Label a 3" x 4" Whirl-Pak® bag "Reagent Test".

ii. Carefully open the Whirl-Pak® bag and pour approximately 10 granules of the baker's yeast into the bag.

iii. Hold the Whirl-Pak® bag upright and pour approximately ½ inch of the peroxide reagent into the bag.

iv. Securely hold the top of the bag with the fingers of one hand and securely hold the bottom of the bag with the fingers of the other hand. Position the bag so that the fluid/foam level in the bag is aligned along the edge of the work surface. Keep the bag pressed against the edge of the work surface. Carefully pull the bag downward toward the open end to expel all excess air from the bag. Fold the top over several times and secure it with the built-in clips.

v. Securely replace the cap on the peroxide reagent bottle and then use it to support the upright "Reagent Test" bag.

vi. Record the time and then add 5 minutes to it for the "Read Time".

vii. At the read time note whether the bag has abundant foam and is somewhat inflated (Positive Test) or non-foamy and flat (Negative Test). Record this information in the appropriate Quality Control Log. If the peroxide reagent gives a positive test, proceed to the product test, if otherwise, prepare a fresh aliquot of the peroxide reagent first.

c. Roast Beef Cooking Temperature Test

i. Prepare the product for sampling and secure a clean sanitized (145°F + hot water) slicing knife. Dry the knife with a clean, preferably sterilized, paper towel.

ii. Wipe the knife and slicing surface with a 5% hypochlorite solution.

iii. Make a slice through the roast beef at the thickest part of the sample (maximum circumference). Examine the two halves to see if there are areas that appear to be more rare than others.

iv. Label a Whirl-Pak® bag with the sample identification number and then carefully open it.

v. Cut a ¼ inch thick slice from one of the surfaces, lay it down on a sterile surface and carve out a 1" square section from what appears to be the least cooked area of the slice. Using the knife blade, transfer this 1" square to the Whirl-Pak® bag.

vi. Shake the bag to transfer the piece to the bottom of the bag. Cover the piece with Peroxide Reagent and proceed according to steps b. iv through vi, with the exception that the reaction time between the reagent and the sample is extended to 15 minutes.

vii. Record the results on the form that accompanied the sample and proceed as you would with any other positive or negative official sample.

d. Canned Product

i. Label a 12" x 12" zip-lock® bag with the appropriate sample identification number. Do the same for a 3" x 4" Whirl-Pak® bag.

ii. Aseptically open the suspect can and transfer the contents to the large zip-lock® bag. It may be necessary to use a clean and sanitized large spoon or spatula to facilitate this transfer.

iii. Carefully close the zipper, expelling all air in the process.

iv. Carefully manipulate the contents of the zip-loc® bag in a manner to thoroughly mix the contents.

v. Carefully open the zip-loc® bag, and using a clean, sanitized teaspoon, remove a level spoonful of test material from the bag and transfer it to the Whirl-Pak® bag. Reseal the zip-lock bag and set it and the empty container to one side for possible future use.

vi. Add peroxide reagent to the Whirl-Pak® bag with the sub-sample to completely cover the sample and the peroxide reagent fills the bottom third of the bag. Use the teaspoon to evenly disperse the sub-sample throughout the reagent.

vii. Quickly fold the top of the bag four times the width of the tab tape and secure with the side tabs. Proceed according to steps b. iv through vi, with the exception that the reaction time between the reagent and the sample is one minute.

viii. Allow the sample test bag to stand undisturbed for an additional 15 minute period and then make a final reading.

ix. Record the results on the form that accompanied the sample and proceed as you would with any other positive or negative official sample.

11.2 Selected References

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CHAPTER 12. EXAMINATION OF MEAT AND POULTRY PRODUCTS FOR *BACILLUS CEREUS*

Charles P. Lattuada and Dennis McClain

12.1 Introduction

Bacillus cereus is one of the few sporeforming, aerobic bacteria recognized as a bacterial pathogen. It is widespread in soil, milk, the surfaces of meat and poultry, cereals, starches, herbs and spices. Its' role as a food-borne pathogen is relatively recent and somewhat uncommon in the United States. Two distinct types of illness have been attributed to the consumption of food contaminated with ***B. cereus***. The more common manifestation is a diarrheal illness with an incubation time of 8-16 h characterized by abdominal pain and diarrhea. The other is an emetic illness with an incubation time of 1-5 h and characterized by nausea and vomiting. While the emetic type is usually associated with cereal type products such as rice, the diarrheal type is more widely associated with many foods.

B. cereus typically is a very large, aerobic, Gram positive, sporeforming rod with peritrichous flagella. It grows over a wide temperature range (10 to 48°C) with an optimum range of 28 to 35°C. It will grow over a wide pH range (pH 4.9 - 9.3) and in sodium chloride concentrations approximating 7.5%. Microscopically it may be seen in chains. Macroscopically the colonies have a dull or frosted appearance on a nutrient agar plate. Its association with disease is usually related to counts $>10^5$ cfu/g in the suspect food. Since ***B. cereus*** does not ferment mannitol, does produce lecithinase and is resistant to polymyxin, a selective medium consisting of mannitol-yolk-polymyxin (MYP) is commonly used for its isolation. Colonies typically are pink in color and surrounded by a zone of precipitate. An ELISA test is available to detect the diarrheal toxin.

12.2 Equipment, Reagents, Media

12.21 Equipment

a. Balance capable of weighing to 0.1 g
b. Stomacher™ (model 400 by Tekmar, or comparable model), sterile plastic bags (with twist ties or self-sealing)

OR blade-type blender, sterile cutting assemblies and blender jars

c. Sterile supplies, spoons or spatulas, pipettes (1 ml), bent glass rods "hockey sticks", aluminum pie pans (or equivalent)
d. Incubator, 30 ± 1°C
e. Incubator, 35 ± 1°C
f. Light or Darkfield Microscope
g. Platinum inoculating loops, 3 mm diameter
h. Microscope slides and cover slips
i. Meeker/Bunsen burner with tripod, or hot plate
j. Pyrex beaker, 250-300 ml size

12.22 Reagents

a. Butterfield's Phosphate Diluent (BPD) for sample extraction
b. BPD dilution blanks, 9 ml volume
c. Basic fuchsin staining solution, 0.5% aqueous

12.23 Media

a. Plates of Mannitol Yolk Polymyxin (MYP) Agar
b. Nutrient Agar Slants
c. BC Motility Medium
d. Nutrient Agar Plates
e. Blood Agar Plates, 5% Sheep RBC

12.3 Sampling and Dilution Procedure

a. Aseptically composite a 25 g or 25 ml sample in sterile bag or blender jar.

b. Add 225 ml Butterfield's Phosphate Diluent (BPD) to each sample taken.

c. Stomach or blend for 2 minutes and then prepare serial dilutions of 10^{-2} to 10^{-6} in 9 ml BPD dilution blanks.

12.31 Plating and Examination of Colonies

a. Pipette 0.1 ml of the homogenate (10^{-1}) and spread it over the entire surface of duplicate, predried MYP plates with a "hockey stick". Repeat the procedure for each of the other dilutions through 10^{-6}. Use a

separate, sterile "hockey stick" for each dilution. Allow the inoculum to dry before incubating the plates.

b. Incubate all plates in an upright position for 20 to 24 h at 30°C.

c. After incubation, examine all plates for colonies that are surrounded by a zone of precipitate (lecithinase production) against an eosin pink to lavender agar background (non-fermentation of mannitol). If the areas of lecithinase production coalesce between colonies, look for plates with 10-100 colonies. Count all typical colonies and determine the presumptive count per gram. Remember that the count will be tenfold higher than the dilution, because only 0.1 ml was placed on a plate.

12.32 Confirmatory and Differential Procedures/Tests

a. Select 4-6 typical colonies for confirmation. Each of these colonies is subcultured on a predried Nutrient Agar Plate and incubated at 30°C for 24 - 48 h. Note the presence or absence of rhizoid growth on the nutrient agar plate.

b. At the same time inoculate a tryptic soy sheep blood agar plate that has been divided into 4 - 6 segments. A 2 mm loop should be used to deposit the inoculum in the center of the segment. Note the size of the hemolytic zone (and whether it is partial or complete).

c. Motility test - use BC motility medium method by making a center line stab inoculation with a 3 mm loop and incubating the tube at 30°C for 18-24 h. Observe for diffuse growth into the medium away from the stab as an indication of a motile organism.

Alternatively a microscopic motility test may be used. The slide motility test is done by adding 0.2 ml of sterile water to a nutrient agar slant and then inoculating the aqueous phase with a 3 mm loopful of a 24 h slant culture. Incubate for 6-8 h at 30°C. Place a loopful of the liquid culture on a glass slide and overlay with a cover slip. *B. cereus* and *B. thuringiensis* are actively motile while *B. anthracis* and the rhizoid strains of *B. cereus* are non-motile.

d. Rhizoid growth - to test for rhizoid growth, inoculate several well isolated areas of a predried Nutrient Agar Plate. Use a 3 mm inoculating loop to make a point of contact inoculation. Incubate the plate in an upright position at 30°C for 24-48 h. If hair-like projections (rhizoids) develop outward from these colonies, the isolate is *B. cereus* var. *mycoides* and not considered to be a human pathogen.

e. Protein toxin crystal stain - Make a smear on a microscope slide with sterile water from a 2-3 day old nutrient agar plate or slant. Allow the slide to air dry and then gently heat fix it. After cooling, flood the slide with methanol, wait 30 seconds and pour it off. Then flood the slide with 0.5% aqueous solution of basic fuchsin. Gently heat the slide until steam is observed, remove the heat, wait 1-2 minutes and repeat the procedure. Let the slide cool and rinse well with water. Examine under oil immersion for free spores and darkly stained, diamond shaped, toxin crystals. Toxin crystals should be present if the cells have lysed and free spores are observed. The presence of toxin crystals is strongly indicative that the organism is *B. thuringiensis*.

f. Other Tests - If further biochemical testing is warranted, consult either Bergey's Manual of Systematic Bacteriology or the Compendium of Methods for the Microbiological Examination of Foods.

12.33 Interpretation of Test Results

a. *B. cereus* usually is: lecithinase positive, strongly hemolytic on sheep blood agar, actively motile, does not produce rhizoid colonies and does not produce protein toxin crystals (diamond shaped).

b. Other lecithinase positive or weakly positive cultures may be *B. cereus* var. *mycoides*, *B. thuringiensis*, or *B. anthracis*. Caution: non-motile, non-hemolytic colonies could be *B. anthracis* and should be handled with special care.

12.4 Method Quality Control Procedures

A minimum of three method control cultures is recommended for use whenever a new batch of medium is made or acquired as well as each time that an analysis is performed. These controls should consist of at least one strain each of *B. cereus*, *B. cereus* var. *mycoides*, and *B. thuringiensis*. This also will assist the analyst in becoming more familiar with the morphological and cultural differences of these *B. cereus* variants.

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CHAPTER 13. EXAMINATION OF MEAT AND POULTRY PRODUCTS FOR *CLOSTRIDIUM PERFRINGENS*

Ann Marie McNamara and Charles P. Lattuada

13.1 Introduction

Clostridium perfringens is a spore-forming, anaerobic bacterium that is widespread in soil, water, foods, spices, and the intestinal tract of humans and animals. Viable, sporulating strains that produce typical foodborne illness belong to Type A and produce an enterotoxin that causes typical symptoms of acute abdominal pain and diarrhea. Symptoms of nausea, vomiting and fever are rare. Symptoms usually appear 8-12 (range 6-24) hours after ingestion of a contaminated food, usually cooked meat or poultry. The infectious dose for humans is high, generally considered to be 10^6 - 10^7 cells/g. In foodborne disease outbreaks, findings of hundreds of thousands or more organisms per gram of food supports a diagnosis of ***C. perfringens*** foodborne illness when appropriate clinical and epidemiological evidence exists. There are four other types of ***C. perfringens***: types B, C, D and E. Some strains of type C produce an enterotoxin that causes a rare form of necrotic enteritis that is often fatal and rarely seen outside of New Guinea.

This method for isolating and identifying ***C. perfringens*** in foods is a modification of the ***C. perfringens*** method found in the Compendium of Methods for the Microbiological Examination of Foods, 3rd Edition (Labbe & Harmon, 1992).

For use in the FSIS Nationwide Microbiological Baseline Data Collection Programs and product surveys, the following "presumptive" isolation and enumeration method will suffice. This method is considered to be a "presumptive" method because other species of ***Clostridia*** besides ***perfringens*** can reduce sulfite and produce black colonies which are egg-yolk positive in TSC and EY-free TSC agar (Labbe and Harmon, 1992). Additionally, some strains of ***C. perfringens*** may not produce a halo surrounding their black colonies, so all black colonies should be counted whether a halo is present or not (Labbe and Harmon, 1992). For outbreak investigations or investigation of epidemiologically-linked cases, the more lengthy and time-consuming confirmation method should be used.

All samples should be shipped as refrigerated samples (0 - 10°C); this is particularly important with outbreak samples. Samples should be analyzed promptly upon laboratory receipt (Labbe and Harmon, 1992). *C. perfringens* in foods stored for prolonged periods of time or frozen many lose viability. If frozen samples must be shipped, food samples should be treated with buffered glycerol salt solution to give a 10% final concentration of glycerol. Samples should be shipped on dry ice and be stored frozen at -55°C to -60°C until the samples are analyzed.

13.2 Equipment, Reagents and Media

13.21 Equipment

a. Incubator at 35 ± 1°C
b. Anaerobic containers
c. Anaerobic gas mixture consisting of 90% N_2 + 10% CO_2
d. Colony counter with a piece of white tissue paper over the counting background area to facilitate counting black colonies
e. Stomacher™ 400 and sterile stomacher bags or Blender and sterile blender jars
f. Vortex mixer
g. Water bath 46 ± 1°C
h. Sterile, bent, glass rods ("hockey sticks")

13.22 Reagents

a. Nitrate reduction reagents (Method 1)
b. 0.1% peptone water diluent
c. Phosphate-buffered saline (PBS)
d. Physiological saline (0.85% sodium chloride)
e. Butterfield's Phosphate Diluent
f. Buffered Glycerol Salt Solution (for frozen samples)

13.23 Media

a. Tryptose Sulfite Cycloserine (TSC) agar
b. EY-free TSC agar
c. Trypticase Peptone Glucose Yeast Extract Broth (buffered)
d. Fluid Thioglycollate Medium
e. Motility-Nitrate Medium (buffered)
f. Lactose Gelatin Medium

g. Spray's Fermentation Medium (1% salicin, or 1% raffinose)

13.3 Presumptive Test

13.31 Sample Preparation

a. Meat Samples:

i. Label a sterile stomacher bag so that it corresponds to the label on the sample bag.

ii. Aseptically remove portions of the sample at random to obtain 25 grams. Place these portions in the sterile stomacher bag.

iii. Add 225 ml Butterfield's Phosphate Diluent (BPD) to the stomacher bag of each sample taken.

iv. Stomach for 2 minutes. Prepare serial dilutions of 10^{-2} to 10^{-6}.

b. Poultry Samples:

i. Prepare serial dilutions of 10^{-1} to 10^{-3} of the whole bird rinse.

13.32 Enrichment and Plating

a. Make duplicate spread plates on thin (6-7 ml) TSC with egg yolk agar base, using 0.1 ml/plate of undiluted sample rinse/extract as well as each dilution.

b. Equally distribute the inoculum using sterile "hockey sticks". Use a new sterile "hockey stick" for each dilution.

c. After the inoculum has dried slightly, overlay the surface with approximately 10 ml or more of egg yolk free TSC agar. Allow the plates to solidify before placing them, lid side up, in an anaerobic jar. Flush jar 3 or 4 times with 90% N_2 + 10% CO_2 leaving this atmosphere in after the last flush, or alternatively use a system which catalytically removes oxygen.

d. Incubate all plates for 24 h at 35°C.

13.33 Examination of Plates

a. After incubation, count the number of presumptive *C. perfringens* colonies. These colonies will be black and usually surrounded by a 2-4 mm opaque zone (halo).

b. Multiply the number of colonies counted by 10 (since only 0.1 ml used) and then multiply by the appropriate dilution factor to obtain your total count.

13.4 Confirmatory Procedure (for epidemiologically linked cases)

13.41 Colony Selection

a. Select 10 representative black colonies from each TSC agar plate counted and inoculate each into a freshly boiled (deaerated) and cooled tube of fluid thioglycollate broth.

b. Incubate for 4 h in a water bath at 46°C or overnight at 35°C. After incubation prepare a Gram stain from each tube and examine microscopically. *C. perfringens* organisms are short, fat Gram positive rods. Endospores are rarely produced in fluid thioglycollate medium.

c. If contaminants are observed, re-streak the contaminated culture onto the surface of a TSC (with egg yolk) agar plate (do not overlay) and incubate anaerobically before proceeding with any confirmatory tests. Surface colonies will appear as yellowish-grey colonies measuring approximately 2 mm in diameter. If re-streaking was done, it is necessary to repeat a. and b. of Section 13.41 (above).

13.42 Confirmatory Tests

a. Motility - nitrate reduction test

i. Stab inoculate each tube of motility-nitrate medium with two, 2 mm loopfuls of the fluid thioglycollate medium culture.

ii. The medium contains 0.5% each of glycerol and galactose to improve the consistency of the nitrate

reduction reaction with different strains of the organism.

iii. Incubate the inoculated medium at 35°C for 24 h and check motility. Since *C. perfringens* is non-motile, growth should occur only along the line of inoculum and not diffuse from the stab line.

iv. Test for reduction of nitrate to nitrite. A red or orange color indicates reduction of nitrate to nitrite. If no color develops, test fluid thioglycollate for residual nitrate by addition of powdered zinc.

b. Lactose gelatin medium

i. Stab inoculate each tube of lactose gelatin medium with two, 2 mm loopfuls of the fluid thioglycollate medium culture.

ii. Incubate at 35°C for 24 to 48 h. Lactose fermentation is indicated by gas bubbles and a change in color of the medium from red to yellow. Gelatin usually is liquefied by *C. perfringens* within 24 to 48 h.

c. Carbohydrate fermentation

i. Inoculate 0.15 ml of the fluid thioglycollate broth culture into 1 tube of freshly deaerated Spray's fermentation medium containing 1% salicin, 1 tube containing 1% raffinose, and 1 tube of medium without carbohydrate for each isolate.

ii. Incubate these three media at 35°C for 24 h and then check for production of acid. To test for acid, transfer 1 ml of culture to a test tube or spot plate and add 2 drops of 0.04% bromthymol blue. A yellow color indicates that acid has been produced.

iii. Reincubate negative raffinose tubes for an additional 48 h and retest for the production of acid.

iv. Salicin is rapidly fermented with the production of acid by culturally similar species such as *C. paraperfringens*, *C. baratii*, *C. sardiniense*, *C. absonum*, and *C. celatum*, but usually not by *C. perfringens*.

v. Acid is produced from raffinose within 3 days by *C. perfringens* but is not produced by culturally similar species.

13.43 Quantitation of *C. perfringens* Populations Based on Confirmed Anaerobic Plate Counts

a. Cultures obtained from presumptive *C. perfringens* black colonies on selective, differential TSC or EY-free TSC medium are confirmed as *C. perfringens* if they are:

i. nonmotile
ii. reduce nitrate
iii. ferment lactose
iv. liquefy gelatin within 48 h
v. produce acid from raffinose.

b. Calculate the number of confirmed *C. perfringens* per gram of food sample as follows:

i. Average the paired plates counted, then adjust the average presumptive plate count to 1.0 ml by multiplying by 10.

ii. Multiply the adjusted presumptive plate count by the reciprocal of the dilution plated to arrive at the total of presumptive *C. perfringens* colonies.

iii. The confirmed colony count is then determined by using the ratio of the colonies confirmed as *C. perfringens* to the total colonies tested.

13.5 Quality Control

a. The following authentic, reference cultures can be used as control organisms in the above procedures:

C. perfringens ATCC 13124
C. absonum ATCC 27555

b. The expected reactions produced by these control organisms are as shown in the following table:

Organism	Motility	H_2S	Gelatin liq.	Nitrate reduct.	Lactose ferm.	Salicin ferm.	Raffinose ferm.
C. perfringens ATCC 13124	-	+	+	±	+	-	d
C. absonum ATCC 27555	±*	+	d	+	+	+w	-

*** usually + in young cultures; d = delayed; w = weak**

3.6 Selected References

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CHAPTER 15. IMMUNOASSAYS FOR THE DETECTION AND QUANTITATION OF STAPHYLOCOCCAL ENTEROTOXINS FROM MEAT AND POULTRY PRODUCTS AND/OR BROTH CULTURE FLUIDS

Richard P. Mageau

15.1 Introduction

Some strains of coagulase positive *Staphylococcus aureus* are endowed with the genetic capacity to produce certain extracellular proteins which, when ingested, cause a severe gastrointestinal disturbance. These proteins are known as staphylococcal enterotoxins. There are five distinct, major, serological types of enterotoxins currently recognized as significant and they are designated as serotypes A, B, C (C_1, C_2, C_3), D and E. In 1995 a new serotype, SEH, was identified and reported in the literature, however, it's significance to foodborne illness is still undetermined. When an enterotoxigenic strain of *Staphylococcus aureus* becomes established in a food product, environmental growth conditions may become optimum to allow for high proliferation of the organism and resulting production of the enterotoxin. Ingestion of this food usually results in a foodborne illness. For regulatory and epidemiological purposes in investigating foodborne illnesses it is important to be able to recognize the presence and serotype of staphylococcal enterotoxins in a suspect food product.

Recent advances and refinements in the development of immunoassays and immunological reagents, specifically with regard to the staphylococcal enterotoxins, have allowed the completion and implementation of assays for quantitative detection of these toxins. These new assays provide advantages of increased sensitivity, reduced analysis time, and a capability for greater sample number analyses due to the reduction of high labor intensive operations associated with procedures previously employed. The following provides a detailed description of two immunoassay procedures which are to be used by the Field Service Laboratories for the determination of the major staphylococcal enterotoxins in various meat and poultry product samples and/or broth culture fluids. The procedure described in PART A is to be used only as a presumptive, qualitative screen test. The procedure described in PART B is to be used as the confirmative test which will provide quantitative and qualitative information.

PART A

15.2 (Presumptive) Staphylococcal Enterotoxin Reverse Passive Latex Agglutination Test

15.21 Introduction and Principles

A Staphylococcal Enterotoxin - Reverse Passive Latex Agglutination (SET-RPLA) test for the qualitative determination of enterotoxin serotypes A, B, C and D is commercially available. This test system is available as a complete, stable kit form. The test kit was evaluated by the Immunology Section of the Microbiology Division and was found to be suitable for use as a presumptive, qualitative screen test on meat sample extracts or broth culture filtrates. The SET-RPLA test was found to be specific and capable of detecting each homologous enterotoxin down to at least 1 ng/ml of sample extract fluid.

A latex agglutination test employed for presumptive screen testing of meat and poultry food samples for staphylococcal enterotoxins should meet or exceed the following performance characteristics:

Sensitivity	≥99% *
Specificity	≥99%
False Negative Rate	≤ 1%
False Positive Rate	≤ 1%
Efficiency	≥99%

* All at a toxin concentration level of ≥1 ng/ml of sample extract fluid and/or Protein A concentration level of <50 ng/ml of sample extract fluid.

The test functions on the principle of using individual suspensions of red latex particles which are each sensitized with specific antibody against a particular enterotoxin serotype. The presence of homologous enterotoxin will then cause visible agglutination of the specific antibody sensitized latex particles after an appropriate incubation period. The absence of toxins or the presence of heterologous toxin serotypes will not cause agglutination of the latex particles. The presence or absence of visible agglutination is discerned by observing the characteristic settling pattern of the red latex particles on the bottom of the reaction well.

The following details provide all the necessary instructional information to perform the SET-RPLA. These instructions are to be used in place of the instruction sheet supplied with the test kit.

15.22 Equipment and Supplies

a. **Rainin Pipetman, Model P-200 adjustable digital microliter pipette and RC-20 disposable microliter pipette tips. (Rainin Instrument Co., Woburn, MA.)**
b. **Minishaker for microtiter plates, Cat. #002-963-0900 (Dynatech Laboratories, Inc., Alexandria, VA).**
c. **Microtiter Test Reading Mirror, Cat. #001-010-4900 (Dynatech).**
d. **Microtiter plates, 96 well, "V" bottom, polystyrene, Cat.#001-010-2602 and lids for above plate, Cat. #001-010-5550 (Dynatech).**
e. **Eppendorf Repeater Pipette, Cat. #G20551 with accessory of 1.25 ml capacity Combitips, Cat. #G20552B (Daigger Scientific Co.).**
f. **Waring blender and appropriate blending vessel.**
g. **Centrifuge, refrigerated, capable of operation at 32,000 X G and appropriate centrifuge tubes resistant to chloroform.**
h. **Kimwipes®.**
i. **Glass separatory funnels, with stopper, 125 ml size.**

15.23 Chemicals and Reagents

a. **NaCl (Fisher, S-271).**
b. **Chloroform† (Fisher, C-298).**
c. **SET-RPLA test kit consisting of the following items:**

    i. **Vials of antibody sensitized latex suspensions of Anti A, Anti B, Anti C, Anti D, and Control latex (unsensitized).**

    ii. **Vials of enterotoxin† reference standards of A, B, C, and D serotypes.**

    iii. **Vials of buffered diluent.**

NOTE: Store entire kit at 4°C when not in use. DO NOT FREEZE.

15.24 Preparation of Stock Reagent Solutions

0.2 M Sodium chloride solution at pH 7.5.

Add 11.69 grams of NaCl to 1 liter of distilled water. Dissolve the salt completely and adjust pH to 7.5 with use of 0.1 N NaOH solution.

15.25 Sample Preparation for Enterotoxin Analysis

a. Meat Food Products

i. Blend 20 grams of meat sample together with 40 ml of 0.2 M NaCl solution, pH 7.5, at high speed in a Waring blender for 3 minutes.

ii. Centrifuge the resulting slurry at 32,000 X G for 15 minutes in a refrigerated centrifuge.

iii. Pour off the supernatant fluid and adjust the pH to 7.5 with 1 or 0.1 N NaOH solution.

iv. In a separatory funnel, in a chemical fume hood with the exhaust on, extract the supernatant fluid with a 1/3 volume (about 10 ml) of cold chloroform by shaking vigorously and letting stand for 15-30 minutes.

v. Pour the supernatant - chloroform mixture into chloroform resistant centrifuge tubes and centrifuge the mixture at 32,000 X G for 15 minutes in a refrigerated centrifuge.

vi. Pour both the supernatant layers through a double layer of kimwipes® back into a clean separatory funnel (make sure solid particles are retained by the Kimwipes®) and <u>without</u> further shaking allow the two layers to settle and clearly separate.

vii. Discard the chloroform (lower layer), and collect the clear meat extract (upper layer) free of any chloroform into a clean tube and use in the immunoassay. Keep the extract refrigerated until actually used in the performance of the assay.

b. Culture Fluids

i. Occasionally it may be of interest to determine if an isolated, coagulase positive, culture of *S. aureus* is capable of producing one or more enterotoxins (enterotoxigenic). This can be accomplished by first growing the pure culture for 24 h at 37°C in a medium such as Brain Heart Infusion Broth on a shaker at 150 RPM.

ii. Centrifuge the 24 h broth culture at 15,000 X G for 15 minutes and obtain the cell free culture fluid.

iii. Make a 1:100 dilution of the culture fluid in the buffered diluent supplied in the SET-RPLA kit. Use this diluted culture fluid directly in the assay to determine the presence of enterotoxins.

15.26 Performance of the SET-RPLA Test

a. Obtain the SET-RPLA test kit from the refrigerator, allow to equilibrate to room temperature and see that all the necessary kit components are present.

b. For the first time that the kit is used, rehydrate each of the lyophilized enterotoxin standards (A, B, C, and D) with the appropriate volume (given on kit instruction sheet or vial label) of buffered diluent and mix well. They can now be used without any further modifications in all subsequent assay performances.

c. Obtain the meat sample extracts previously prepared and make a 1:2 dilution of each in the buffered diluent in separate tubes. Culture fluids, if any are to be assayed, can be used directly as previously prepared.

d. Obtain a 96 well, "V" bottom, Dynatech microtiter plate and cover from stock supplies.

e. Place 25 µl of buffered diluent in each well of column 1 in rows A, B, C, D, and E using the Pipetman and a disposable tip.

f. Place 25 µl of reference enterotoxin A, B, C, and D into the wells of column 2 in rows A, B, C, and D respectively.

g. Place 25 µl of any one of the reference enterotoxin standards (your choice) in the well of column 2 in row E.

h. Place 25 µl of each test sample extract in each well of a single, respective column in rows A, B, C, D, and E, beginning with column 3.

i. Obtain the individual vials of latex Anti A, Anti B, Anti C, Anti D, and Control latex suspensions and mix each thoroughly but gently to produce uniform latex suspensions.

j. Using an Eppendorf Repeater Pipette and individual 1.25 ml capacity combitips, dispense 25 µl of latex Anti A, Anti B, Anti C, Anti D, and Control latex into each occupied well of rows A, B, C, D, and E respectively.

k. Mount the plate on the carrier of the Minishaker and carefully shake the plate at a "medium" dial setting for 15 seconds to thoroughly mix, but not spill, the contents of each well.

l. Allow the covered plate to remain undisturbed at normal room temperature for 24 h before the final reading is made.

15.27 Test Reading and Sample Interpretation

a. After the appropriate period of time, remove the cover from the plate, mount it on the Microtiter Test Reading Mirror and observe from the bottom of the plate the pattern of settled red latex particles in each well.

b. The pattern of settled red latex particles determines whether or not agglutination has taken place. Nonagglutination is determined by observing that all of the latex particles have settled into a distinct pile at the bottom of the "V" in a particular well; usually referred to as a "button". Agglutination is determined by observing that all the latex particles in a given well are uniformly spread out over the entire surface of the "V" bottom without any distinct pile or "button". The agglutination patterns illustrated on the SET-RPLA kit instruction sheet may be helpful in regard to understanding this.

c. Observe each well and record whether or not agglutination has taken place.

d. To insure that the test is working properly, the following results should be obtained with regard to the controls employed. All wells of column 1 should be negative (no agglutination) as these are negative controls. All wells of column 2 of rows A, B, C, and D should be positive (agglutination) as these serve as positive homologous controls. The single well in column 2 of row E should be negative.

e. If all of the above controls have reacted properly, proceed to the interpretation of sample results. If any controls did not react properly, the test must be considered invalid and the procedure must be repeated and technical assistance should be sought to determine the nature of the problem.

f. Each sample can be interpreted with regards to the presence or absence of enterotoxins by observing the reactions of that sample column with respect to rows A, B, C, D, and E, which, of course, correspond to Anti A, B, C, D, and Control latex respectively. A positive reaction in any well of Anti A, B, C, or D identifies the presence of that particular toxin serotype. The control latex well (row E) should never show agglutination. If the sample column contains no positive wells, then the sample may be considered to be free of enterotoxins A-D and can be reported out as such.

g. If a sample contains enterotoxin it will usually be of only one serotype. The presence of more than one serotype in a food sample or culture fluid is possible but is rather unusual and one should not normally expect to find this.

h. If a sample should produce a positive reaction in Anti A, B, and C wells simultaneously (but not for the Anti D or Control latex wells) this is usually indicative of the presence of Protein A and the sample must be further treated, as described below, before it can be accurately assessed with regards to the presence of enterotoxins.

i. Add normal rabbit serum to a total concentration of 5% (v/v) to a sample extract suspected of containing significant concentrations of Protein A. Allow the sample to incubate for 30 minutes at 37°C. Centrifuge the sample at 10,000 X G for 15 minutes. Obtain the sample supernatant and perform the SET-RPLA test again to determine the presence of enterotoxins. The normal rabbit serum treatment should effectively neutralize the interfering reactivity of the Protein A.

j. All SET-RPLA positive samples or those with questionable results are to be confirmed by the procedure outlined in PART B.

15.28 Quality Control Procedures

a. Store and maintain the SET-RPLA kit components at refrigerator temperature (4 - 8°C) when not in use. DO NOT ALLOW THEM TO FREEZE.

b. Observe the kit manufacturer's expiration date for all test kit components. Kits should not be used beyond their expiration date.

c. Use only "V" bottom microtiter plates to perform the assay.

d. Allow all test components to equilibrate to room temperature prior to performing an analysis.

e. Thoroughly but gently resuspend the settled latex particle reagents in their vials to produce uniform latex suspensions immediately prior to dispensing this reagent in the test.

f. Always run negative and positive enterotoxin controls and control latex (unsensitized) when performing the analysis.

g. All negative and positive controls must give expected correct results before correct interpretation of test sample results can be made.

h. Do not allow the plate to be disturbed once all reagents have been added and properly mixed. Disturbing the plate may cause the settling pattern of agglutinated or

nonagglutinated latex to form abnormally and thus produce erroneous results.

† Safety Caution: Do not dispose of hazardous (chloroform) or biohazardous (enterotoxin) fluids by pouring down the sink drains.

Collect these liquid wastes in separate containers and dispose of according to standard waste management procedures for your laboratory.

Do not allow human exposure to chloroform vapors.

15.29 Selected References

Bergdoll, M. S. 1980. Staphylococcal food poisoning, p. 108-119. *In* H. D. Graham (ed.), The Safety of Foods, 2nd Edition, AVI Publishing Company, Inc., Westport, CT.

Parks, C. E., and R. Szabo. 1986. Evaluation of reversed passive latex agglutination (RPLA) test kits for detection of staphylococcal enterotoxins A, B, C and D in foods. Can. J. Microbiol. 32:723-726.

Sanjeev, S., and P. K. Surendran. 1992. Evaluation of reversed passive agglutination test kits for the detection of staphylococcal enterotoxins A, B, C and D in fishery products. J. Food Sci. Technol. 29:311-312.

PART B

15.3 (Confirmative) Biotin-streptavidin Enzyme Linked Immunosorbent Assay for Staphylococcal Enterotoxins

15.31 Introduction and Principles

Enzyme Immunoassay (EIA) provides an alternative approach to the immunological detection of staphylococcal enterotoxins. EIA offers the major advantages of being more reliable in their reactions than latex agglutination and they can also be used for quantitation of the material under analysis. The Immunology Section of the Microbiology Division developed a Biotin-streptavidin Enzyme Linked Immunosorbent Assay (ELISA) for the quantitative detection of staphylococcal enterotoxin serotypes A, B, C, D, and E. This developed assay makes use of a biotin-streptavidin amplification reaction for the indicator portion of the assay.

The biotin-streptavidin ELISA described in this procedure is one of a solid phase, double antibody, "sandwich" type with a final biotinylated antibody-streptavidin peroxidase reaction to provide visual evidence of the degree of reaction upon substrate addition. The brief functional principles of this assay are as follows. Specific antibody (capture) against a particular enterotoxin serotype is bound to the walls of a microtiter plate (solid phase) and is allowed to react with test material which may contain enterotoxin(s). Only the homologous enterotoxin will react and bind to the wall bound antibody. A second antibody (probe) is introduced into the system with the same specificity as the first wall bound antibody and can now react with previously bound homologous enterotoxin. This second antibody is one which has had biotin chemically introduced into the molecule and is referred to as biotinylated antibody. Five "sets" of specific antibody pairs are simultaneously but individually employed in the assay corresponding to each of the five enterotoxin serotypes in question. A commercial preparation of streptavidin-peroxidase conjugate is next generally introduced into the assay system. This reaction makes use of the natural, very high, chemical binding affinity of biotin and streptavidin. Amplification is achieved by the fact that each molecule of streptavidin can bind four molecules of biotin. The streptavidin-peroxidase introduced into the assay will therefore bind to any biotinylated antibody present. With the final addition of the substrate to the system, the visible evidence of a positive reaction is produced from

conversion of the substrate to a colored end product by the enzyme peroxidase. If homologous toxins are not present, biotinylated antibody does not bind and subsequent reactions cannot take place, which therefore results in no colored change in the added substrate.

The following details provide all the necessary information for the performance of the Biotin-streptavidin ELISA for the quantitative determination of staphylococcal enterotoxin serotypes A, B, C, D, and E from meat and poultry products or broth culture fluids. All samples giving positive or questionable results in the SET-RPLA analysis (PART A) must be subjected to this confirmative Biotin-streptavidin ELISA for a final quantitative determination of enterotoxin presence before the final analytical results are reported.

15.32 Equipment and Supplies

a. Flow (ICN) Laboratories Titertek Multiskan MC Plate Reader, Cat. #78-530-00.
b. Flow (ICN) Laboratories Titertek Microplate Washer, Cat. #78-431-00.
c. Flow (ICN) Vacuum Pump for above washer, Cat. #78-426-00.
d. Flow (ICN) Titertek Multichannel Pipette, 8 channel, adjustable 50-200 µl volume, Cat. #77-859-00.
e. Eppendorf Repeater Pipette (Daigger Scientific Co., Cat. # G-20551) with accessory of 2.5 ml capacity Combitips (Daigger, Cat. #G-20552C) and 5.0 ml capacity Combitips (Daigger, Cat. #G-20552D).
f. Dynatech Laboratories Microelisa Plates, Immulon I, flat bottom, 96 wells, Cat. #11-010-3350 and covers.
g. Incubator, 37°C (any properly operating brand).
h. Centrifuge, refrigerated, capable of operation at 32,000 X G and appropriate centrifuge tubes resistant to chloroform.
i. Microtest Manifold, Wheaton, straight, 8 place with Luer Lock connection (Daigger, Cat. #G-20560A).
j. Kimwipes®.
k. Glass separatory funnels, with stopper, 125 ml size.
l. Waring Blender and appropriate blending vessel.
m. Rainin Pipetman, Model P-200 adjustable digital microliter pipette and RC-20 disposable microliter pipette tips. (Rainin Instrument Co., Woburn, MA.)

15.33 Chemicals and Reagents

a. Na_2HPO_4 (Fisher, Cat. #S-374).
b. NaH_2PO_4 (Fisher, Cat. #S-369).
c. NaCl (Fisher, Cat. #S-271).
d. Citric acid, anhydrous (Fisher, Cat. #A-940).
e. Hydrogen peroxide, 30% reagent grade (Fisher, Cat. #H-323).
f. Tween 80 (Fisher, Cat. #T-164).
g. Sodium azide† (NaN_3), purified (Fisher, Cat. #S-227).
h. Bovine Serum Albumin, powder, fraction V (Sigma, Cat. #A-4503), store in refrigerator.
i. Chloroform† (Fisher, Cat. #C-298).
j. ABTS substrate indicator; 2,2' azino-di-(3-ethyl Benzthiazoline Sulfonic acid), (Sigma, Cat. #A-1888).
k. Streptavidin-peroxidase conjugate, Cat. #43-4323 (Zymed Laboratories, Inc., San Francisco, CA), store in refrigerator.

15.34 Staphylococcal Biochemical Reagents

a. Anti-staphylococcal enterotoxin A, B, C, D, and E antibody stock solutions.
b. Biotinylated anti-staphylococcal enterotoxin A, B, C, D, and E antibody stock solutions.
c. Staphylococcal enterotoxin† A, B, C, D, E standard reference stock solutions.

NOTE: The above 3 sets of items must be stored in the frozen state at all times to maintain stability.

15.35 Preparation of Stock Reagent Solutions

a. 0.15 M Phosphate Buffered Saline at pH 7.2 (PBS).

Add 10.35 grams of NaH_2PO_4 and 4.38 grams of NaCl to 1 liter of distilled water and dissolve completely to prepare the "acid" solution. Add 10.65 grams of Na_2HPO_4 and 4.38 grams of NaCl to 1 liter of distilled water and dissolve completely to prepare the "base" solution. While mixing with a magnetic stirrer and monitoring the pH on a pH meter, add a sufficient quantity of the "acid" solution to the "base" solution to achieve a final, stabilized pH of 7.2. Dispense into glass containers, autoclave at 121°C for 15 minutes and store

at room temperature. It is most convenient to make up this buffer in 5 liter quantities at a time.

b. Phosphate Buffered Saline containing 0.5% Tween 80 (PBS-Tween).

To 1 liter of prepared 0.15 M phosphate buffered saline at pH 7.2 add 0.5 ml of Tween-80 and mix (not on magnetic stirrer) for several hours at room temperature until completely dissolved. Store this prepared solution in the refrigerator (4°C).

c. Phosphate Buffered Saline containing 0.5% Bovine Serum Albumin (PBS-BSA).

To 1 liter of prepared 0.15 M phosphate buffered saline at pH 7.2, add 5 grams of powdered bovine serum albumin and 1 gram of sodium azide (NaN_3) and mix (not on magnetic stirrer) at room temperature until completely dissolved. Store this prepared solution in the refrigerator (4°C).

d. ABTS - H_2O_2 Substrate Buffered Solution.

Prepare a 0.1 M citric acid stock solution by dissolving 1.92 grams of anhydrous citric acid in 100 ml of distilled water. Prepare a 0.1 M dibasic sodium phosphate stock solution by dissolving 1.42 grams of Na_2HPO_4 in 100 ml distilled water. Add sufficient quantities of these two stock solutions together while mixing with a magnetic stirrer and monitoring the pH on a pH meter to prepare 100 ml of a 0.1 M citrate-phosphate buffer at a final stabilized pH of 4.0.

To 100 ml of the above prepared 0.1 M citrate-phosphate buffer add 22 mg of ABTS [2,2' azino-di-(3-ethyl Benzthiazoline Sulfonic acid)] and 15 µl of stock 30% hydrogen peroxide, mix gently by hand (no magnetic stirrer) until completely dissolved. Pass this substrate solution through a 0.45 µm Millex® filter, place in a sterile glass container, and store in the dark at room temperature until needed. This substrate solution should be prepared 24 h in advance of need and may be used as long as it retains its original light green color. A solution which has deteriorated to the

point where it cannot be used is evidenced by a dark azure-green color formation.

e. 0.2 M Sodium Chloride Solution at pH 7.5.

Add 11.69 grams of NaCl to 1 liter of distilled water. Dissolve the salt completely and adjust pH to 7.5 with use of 0.1 N NaOH solution.

15.36 Sample Preparation for Enterotoxin Analysis

Sample extracts for enterotoxin analysis from meat and poultry products or culture fluids are prepared exactly as described under the similar section (15.25 a. or b.) of PART A for SET-RPLA. These should be prepared in advance of the actual ELISA performance and kept refrigerated until needed.

15.37 Performance of the Biotin-streptavidin ELISA

a. Obtain a flat bottom, 96 well Dynatech Immulon I microtiter plate and cover from stock supplies.

b. Dilute the anti-staphylococcal enterotoxin antibody stock solutions in PBS in individual tubes to contain the following amounts of antibody protein as shown below for each respective serotype.

Anti-SEA antibody = 5 µg/ml
Anti-SEB antibody = 5 µg/ml
Anti-SEC antibody = 1 µg/ml
Anti-SED antibody = 5 µg/ml
Anti-SEE antibody = 5 µg/ml

c. Sensitize wells of the Immulon I microtiter plate with antibody for enterotoxin serotypes A, B, C, D, and E by placing 200 µl of the above concentrations of each antibody protein solution (PBS) in the wells of rows A, B, C, D, and E respectively. Leave all wells of column 2 empty.

d. Incubate the covered plate for 3 h at 37°C.

e. Remove the plate from the incubator, remove the cover and mount on the carrier of a Flow Titertek Microplate Washer which has been primed with PBS-Tween and set to deliver 300 µl fluid to each well.

f. Remove the solution from the wells by aspiration with the washer and wash the wells once with 300 µl fluid to each well.

g. Remove the plate from the washer, invert over a sink, hold the plate tightly in one hand and flick several times to remove any remaining excess liquid from the wells.

h. Tap the plate in an inverted position several times on a soft paper towel (Sorgs Laboratory towels) placed on the surface of the lab bench and allow the plate to remain inverted for 1-2 minutes to complete the draining process. Place the plate right-side up and cover until next reagent addition.

i. Block unwanted reactive sites on the plastic wells by filling all wells (including those in column 2) with 250 µl of PBS-BSA per well, dispensed from an 8 place microtest manifold attached to a Cornwall syringe.

j. Replace the cover on the plate and let stand undisturbed overnight at normal room temperature.

k. Wash the wells once by repeating steps (e thru h).

l. With a Pipetman microliter pipette place 200 µl of PBS-BSA to all wells of column 1 and 2 to serve as negative controls.

m. Obtain previously prepared standard reference enterotoxin solutions of serotypes A, B, C, D, and E at concentrations of 1, 5, 10, 25, and 50 ng/ml in PBS-BSA.

n. Place 200 µl of each of the above concentrations of toxins A, B, C, D, and E to the homologous antibody sensitized wells of rows A, B, C, D, and E respectively, beginning with column 3 wells at the lowest concentration.

o. Place 200 µl of each previously prepared sample extract in each well of a single, respective column in rows A, B, C, D, and E, beginning with column 8.

p. Incubate the covered plate for 2 h at 37°C.

q. Wash the wells twice by repeating steps (e thru h).

r. Prepare the following dilutions of biotinylated anti-staphylococcal enterotoxin antibody stock solutions in PBS-Tween in individual tubes as shown below for each respective serotype.

Biotinylated Anti-SEA antibody = 1:5000
Biotinylated Anti-SEB antibody = 1:5000
Biotinylated Anti-SEC antibody = 1:2500
Biotinylated Anti-SED antibody = 1:5000
Biotinylated Anti-SEE antibody = 1:1500

s. Place 200 µl of the above dilutions (PBS-Tween) of each biotinylated antibody serotype to all wells in a respective row of homologous, primary antibody sensitized wells (i.e., Anti-A in row A, Anti-B in row B, etc.).

t. Incubate the covered plate for 2 h at 37°C.

u. Wash the wells three times by repeating steps (e thru h).

v. Prepare a 1:5000 dilution of the commercial Streptavidin-peroxidase conjugate in PBS-Tween in a separate tube.

w. Add 200 µl of the 1:5000 dilution (PBS-Tween) of Streptavidin-peroxidase conjugate to all wells of the plate with the use of an Eppendorf Repeater pipette and a 5 ml capacity combitip.

x. Incubate the covered plate for 30 minutes at 37°C.

y. Wash the wells three times by repeating steps (e thru h).

z. With the use of the Flow 8 channel pipette, add 200 µl of ABTS-H_2O_2 substrate buffered solution to all wells.

aa. Place the cover on the plate and incubate for 30 minutes at 37°C.

bb. Twenty minutes prior to the end of the above incubation period turn on the power to the Flow Titertek Multiskan MC plate reader and allow it to warm up.

cc. After the 30 minutes incubation period of step (aa) is complete, remove the plate from the incubator, remove the cover, and place the plate on the carrier of the Multiskan MC plate reader.

dd. Program the reader for the current date, Mode 1 (single wavelength absorbance), Wavelength Filter #2 (414 nm), push the carrier and plate into the measuring head and blank the instrument (zero O.D. point set) on column 1.

ee. Press the START button and obtain a printed paper strip of the Optical Density (O.D.) values for all of the reaction wells on the plate.

ff. Remove the plate from the reader and visually examine the plate to see that the obvious colored reaction intensities generally correspond to the numerical values on the printed data sheet to assure that the plate has been properly read in the instrument.

gg. Turn off the power to the Multiskan MC plate reader and discard the plate (save the cover for reuse) after completion of the Data Analysis Plotting and Sample Interpretation Section described below.

15.38 Data Analysis, Plotting, and Sample Interpretation

a. All wells in column 1, which serve as the zero-blank negative control, should have no color reaction, indicating a proper lack of non-specific attachment of biotinylated antibody or Streptavidin-peroxidase to the antibody sensitized wells. Under these conditions these wells are excellent controls to blank in (zero point set) the O.D. reading instrument.

b. All wells in column 2 serve as BSA negative controls to assess non-specific attachment of biotinylated antibody and also Streptavidin-peroxidase. Since the wells originally were never sensitized with anti-enterotoxin antibodies but only blocked with BSA, no positive reactions (high O.D. values) should ever be observed.

c. Wells in columns 3, 4, 5, 6 and 7 of rows A, B, C, D and E represent the standard quantitative dose response values of the reaction with regard to enterotoxin serotypes A, B, C, D, and E respectively. The response (O.D.) observed in this ELISA should be one of a direct linear relationship to increased dose concentration of enterotoxin.

d. The remaining wells of individual columns 8-12 for rows A, B, C, D, and E represent reaction values for individual test sample extracts with regards to the presence or absence of enterotoxins A-E respectively.

e. Obtain a piece of 4 cycle semi-logarithmic graph paper. Label the ordinate (10 division to the inch) with O.D. values from 0-2.0 in increments of 0.05. Label the abscissa (4 cycle logs) with enterotoxin concentrations of 0, 1, 5, 10, 25, and 50 ng/ml.

f. Plot the O.D. values against standard enterotoxin concentrations for each individual serotype together on the same piece of graph paper. Draw straight lines from point to point for each homologous set of enterotoxin concentrations. You will now have 5 individual standard curves for enterotoxin serotypes A-E respectively, which will have similar appearances to each other but still be distinctly different. The curves should illustrate the direct linear dose-response relationship in regards to increasing toxin concentration for each serotype.

g. To determine if a test sample contains enterotoxin and its' quantity if present, proceed as follows:

   i. Obtain the O.D. values of individual sample column wells with regards to rows A, B, C, D, and E (which correspond to Anti A, B, C, D, and E antibodies respectively) and determine if any sample O.D. values exceed the 1 ng enterotoxin standard O.D. value for each individual serotype.

   ii. Any sample O.D. value exceeding the 1 ng enterotoxin standard of a given serotype is to be considered as a positive identification reaction for the presence of that enterotoxin serotype in the sample.

iii. Determine the quantitative amount of an enterotoxin which is present by interpolating the O.D. value with regards to concentration from the standard curve for that particular serotype identified and multiply by 3 (food sample) or 100 (culture fluid).

iv. If the sample O.D. value does not fall within the more linear portion (1-25 ng/ml) of the standard curve of a given serotype, then the sample analysis should be repeated using standard dilutions of the original extract in PBS. The dilution factor which produces readable results would then need to be included in the final quantitative calculations.

v. If sample O.D. values are less than those of the 1 ng standards of each serotype, the sample should be considered free of enterotoxins A-E and reported out as such.

h. If a sample is found to contain an enterotoxin, it will usually be of only one serotype. The presence of more than one serotype toxin in a given sample is possible but rather unusual.

i. If a sample is found to produce a strong positive reaction in all the serotype wells, except Anti-D, this usually indicates that the sample contains a significant amount of Protein A and the sample must be treated as described in PART A, 15.27 step i, before a repeat ELISA analysis can be performed to accurately determine the presence of enterotoxins.

j. All enterotoxin positive samples should be reported out by using a statement such as the following. "This food sample was found to contain Staphylococcal enterotoxin serotype _____, at a concentration of _____ ng/g as confirmed by an ELISA procedure." The serotype and quantitative values would, of course, be filled in from your analytical data.

15.39 Quality Control Procedures

a. The assay reagents have been standardized for use only with Dynatech Immulon I microtiter plates. No other plates should be used.

b. All stock reagent solutions must be properly prepared and maintained free of contamination or chemical breakdown.

c. The stock ABTS-H_2O_2 substrate buffered solution should not be used if it has turned to a significantly darker shade of green from that of the original preparation.

d. Be sure the stock, commercial Streptavidin-peroxidase reagent has not deteriorated to the point of producing abnormally low final O.D. readings. Use only an unexpired lot of this reagent.

e. All standard negative and positive enterotoxin control values must be in the correct range before proper interpretation of test sample results can be reliably made.

f. The standard curves generated from the standard enterotoxin concentrations for each serotype should always be of the same general shape and value from run to run. Drastic changes in the shape of these curves usually indicate critical reagent deterioration (or misuse).

g. Standardized reference enterotoxin concentrations must always be carefully and properly prepared from higher concentrated stock solutions to assure reliability of the generated standard curves.

† Safety Caution: Do not dispose of hazardous (chloroform, sodium azide) or biohazardous fluids (enterotoxin) by pouring down sink drains.

Accumulation of sodium azide in lead drains may result in an explosion.

Collect these liquid wastes in separate containers and dispose of according to standard waste management procedures for your laboratory.

Do not allow human exposure to chloroform vapors.

15.4 Selected References

Freed, R. C., M. L. Evenson, R. F. Reiser, and M. S. Bergdoll. 1982. Enzyme-linked immunosorbent assay for detection of staphylococcal enterotoxins in foods. Appl. Environ. Microbiol. 44:1349-1355.

CHAPTER 16. AGAROSE THIN-LAYER ISOELECTRIC FOCUSING (TLIEF) FOR SPECIES DETERMINATION OF RAW MUSCLE TISSUES

Richard P. Mageau

16.1 Introduction

Improvements in the developed biochemical technique of isoelectric focusing have allowed the application of this technique to be used for species determination of raw muscle tissue. This method provides for the relatively rapid species determination of a large number of samples in a definitive, less subjective manner, in a single analytical run without the use of anti-species sera. The principle of this technique involves the separation and focusing of proteins under an electrical field in a stable pH gradient dependent upon differences in the isoelectric points of the various proteins present. Since various species tissues contain multiple proteins of different isoelectric points, an aqueous extract of a particular species tissue when subjected to TLIEF will produce a stained protein band pattern unique and distinct for that species. By using the method described below, a total of 24 samples (48 if sample filter papers are cut in half along their long axis) may be analyzed in a single determination in one day as to their correct species. The use of this established method is intended to aid in the rapid species analysis of a large influx of raw tissue samples resulting from particular meat species problems which may be encountered in the Agency's inspection system.

16.2 Materials and Equipment

a. Multiphor for high Performance Analytical Electrofocusing in Agarose; to include 2117-301 Multiphor Basic Unit, 2117-107 Analytical Electrofocusing Lid, 2117-701 Capillary Gel Casting Kit, and 1850-100 Agarose-EF Accessory Kit. (LKB Instruments.)
b. 2197-001 D.C. Power Supply for Electrofocusing and Electrophoresis. (LKB Instruments.)
c. 185-101 Multiphor Gelbond film, 124 x 258 mm. (LKB Instruments.)
d. 2030-710 Bayonet female plastic tubing connector and 2030-702 Bayonet male plastic tubing connector. (LKB Instruments.)
e. 2117-109 Multiphor Staining Kit. (LKB Instruments.)
f. 1403 Coomassie Brilliant Blue R-250 dye (Fisher).
g. S-460 D-sorbitol powder, reagent grade (Fisher).

h. A-322 Trichloroacetic acid, reagent grade (Fisher).
i. A-297 5-sulfosalicylic acid, crystal, reagent grade (Fisher).
j. 14-198-5A High pressure hose clamps, 1/4" to 5/8" size (Fisher).
k. K-10 Kerosene (Fisher).
l. 17-0468-01 Agarose IEF (Pharmacia Fine Chemicals).
m. 17-0453-01 Pharmalyte Carrier Ampholyte, pH 5-8 range (Pharmacia).
n. Schleicher and Schuell #470 filter paper, 12.5 x 26 cm size and Schleicher and Schuell #577 filter paper, 12.5 x 26 cm size (PGC Scientific Corp.).
o. W 3237-10 Lauda Brinkman, Model K-4/RD Circulating water bath. (American Scientific Products.)
p. B-1206-2 Whirl-Pak® bags, 3" x 5". (American Scientific Products.)
q. R5316-8 Tygon tubing, formula S-50-HL, 5/16" x 1/16". (American Scientific Products.)
r. Hair dryer (hot and cold).
s. Rubber print roller, 6" wide.
t. Silicone gasket, 0.75 mm thick, overall dimensions of 12.5 x 26 cm, 3 sided of 5 mm width. (Potomac Rubber Co., Inc., Washington, DC.)
u. Water bath and incubator/oven capable of maintaining 65°C.
v. Centrifuge capable of 9,000 x G maximum.
w. Stomacher®

16.3 Procedure

a. Initial Reagent Preparations

i. Fixing solution:

Dissolve 25 g sulfosalicylic acid and 50 g of trichloroacetic acid in distilled water and dilute to a final volume of 500 ml.

ii. Destaining solution:

Mix 700 ml of ethyl alcohol and 200 ml glacial acetic acid together and dilute to a final volume of 2,000 ml with distilled water.

iii. Staining solution:

Completely dissolve 1 g Coomassie Brilliant Blue R250 dye in 500 ml of destaining solution.

iv. Cathode solution: (1 M NaOH, 100 ml)

v. Anode solution: (0.05 M H_2SO_4, 100 ml)

b. Sample Preparation

i. Obtain 1 g of diced, raw, muscle tissue and place in a small whirl-pak® bag together with 9 ml of distilled water.

ii. Thoroughly macerate the tissue by stomaching for 1-2 minutes and then leave overnight at 4°C.

iii. Centrifuge the resulting solution at 9000 x g for 10 minutes at room temperature and apply to sample filter papers when ready to electrofocus.

c. Apparatus Assembly

i. Set up and align the Lauda K-4/RD circulating water bath, LKB 2117 Multiphor Basic unit, and LKB 2197 D.C. Power supply on a laboratory bench such that the water bath is adjacent and convenient to the Multiphor unit and the power supply is on the adjacent side of the Multiphor unit.

ii. When placed on the same table or workbench, the LK-4/RD circulating waterbath causes a vibration problem that may cause the bands on the final agarose gel plate to be irregular. This problem can be corrected by isolating the waterbath, either by moving the waterbath to a separate table or to the floor. In cases where the lab has a raised or suspended floor, the addition of vibration damping elements (Fisher 01-914045) may be necessary to further isolate the vibration.

iii. Install the cooling plate in the Multiphor unit according to LKB instruction manual and attach appropriate, insulated, circulation hoses to the water bath and secure to make leak-proof.

iv. Adjust and calibrate the water bath temperature to assure an adequate supply of water is circulating through the cooling plate at 4°C.

v. Turn on the circulating, calibrated water bath at least 30 minutes prior to the preparation of a gel plate on the day that an analytical run is to be performed.

d. Agarose Gel-plastic Film Preparation

i. Mix 0.3 g Agarose-IEF (Pharmacia) and 3.6 g sorbitol in a conical flask with 27 ml distilled water and heat with stirring in a boiling water bath until all solids are dissolved.

ii. Place the flask containing the dissolved ingredients in a 65°C water bath and allow the solution to cool and equilibrate to 65°C.

iii. Add 1.9 ml of Pharmalyte, pH 5-8 range, ampholyte solution (Pharmacia) with needle and syringe, while gently swirling the 65°C tempered, liquid agarose solution. The final agarose solution is 30 ml total volume with an ampholine concentration of about 2.5% and agarose concentration of 1%. Leave the liquified agarose solution in the 65°C water bath until needed, after completing step (viii).

iv. Obtain a glass plate 125 x 260 mm (LKB 2117-701) Capillary Gel Casting Kit) that has been previously treated with the surface wetting agent Prosil-28 according to product instructions and place a small amount of distilled water on the glass surface.

v. Obtain a sheet of gel-bond film and place it on the wet glass plate such that the hydrophobic side of the sheet is down and in contact with the water and the hydrophilic side is up. Properly align the edges of the film sheet with the edges of the glass plate and remove excess water and air bubbles by rolling the surface of the film sheet with a rubber roller. Carefully remove excess water with absorbent towels.

vi. Place the three-sided, orange, silicone gasket on the film sheet and align the gasket edges with the edges of the film sheet.

vii. Place a 125 x 260 mm Prosil-28 treated glass plate on top of the orange gasket and align the leading edges with the gasket. Place five clamps around the three gasket-glass edges (2 each on long sides and 1 on the short end). When properly set up you will have a glass-film sheet sandwich arrangement which is leak proof on three sides where the gasket is and one open end with a space of about 0.75 mm (equal to gasket thickness) between the bottom of the top glass plate and the top of the gel-bond film sheet.

viii. Place this glass-film sheet sandwich arrangement in a 60-65°C oven for 10 minutes to warm up along with a 50 cc syringe and 21 gauge needle.

ix. Remove the warm glass-film sheet sandwich from the oven and set-up on a rack near the water bath containing the previously prepared liquid agarose solution at 65°C. Quickly fill a 50 cc syringe fitted with a 1 inch 21 gauge needle with the liquid agarose solution. Insert the needle in the space between the gel bond film sheet and bottom of the top glass plate. Rapidly but evenly inject the liquid agarose solution to fill this space without air bubbles before the agarose solution starts to gel.

x. Allow the agarose filled sandwich to set undisturbed until the agarose has solidified and then place in a refrigerator for 30 minutes to completely solidify the agarose.

xi. Carefully remove the five clamps and the top glass plate from the sandwich and obtain the agarose coated gel-bond film sheet from the bottom glass plate. When properly executed you will have a gel-bond film sheet containing a uniform, bubble free solidified agarose-ampholine layer of approximately 0.75 mm thickness.

xii. Several agarose gel-bond film plates may be prepared at the same time in order to reduce preparation time for future runs. The prepared plates must be preserved until needed by storage in the LKB Humidity Chamber (LKB-2117-110). These chambers are stackable and come in a kit holding up to three gel plates. Plates stored refrigerated for as long as 6 weeks in the humidity chamber show no loss in performance.

NOTE: Do not perform step (xi) above until just prior to starting step (iii) of section (e) below.

e. Isoelectric Focusing of Samples and References

i. Smear a small amount of reagent grade kerosene (Fisher) on the top of the cooling plate (which has 4°C water circulating through it) of the Multiphor unit.

ii. Place an LKB sample position template on top of the kerosene covered cooling plate, position in proper alignment with the cooling plate and smooth out so that no air bubbles are present under the template. Blot excess kerosene from edges of the template with absorbent towels.

iii. Smear a small amount of kerosene on top of the template and place the previously prepared agarose film sheet on top of the kerosene covered template, align edges with the cooling plate, remove any trapped air bubbles and blot excess kerosene from the edges.

iv. Soak filter paper strips (10 x 5 mm) in sample or reference tissue extracts and apply to the surface of the agarose gel near the anode using the visible template under the agarose-film sheet as a guide. A maximum of 24 samples total (including desired reference extracts) may be placed on the agarose surface. Be sure that the sample paper strip is in complete contact with the agarose surface and rinse off the tweezers between the handling of each sample strip with distilled water.

An alternative approach to sample application is to first place 24 blank paper strips in the proper position on the agarose surface and then with the use of a micropipetting device place a standard amount (25 µl) of sample extract on each respective strip. If it is desirable to employ small paper strips (10 x 2.5 mm) to accommodate a larger number of samples (48) for analysis, these strips should have only 10-15 µl sample extract applied to them and care must be taken to not cause overloading and mixing of adjacent samples.

v. Soak electrode filter paper strips with appropriate solutions for cathode (1 M NaOH) and anode (0.05 M H_2SO_4), blot excess off on paper toweling and guided by the visible template apply the wet electrode strips to the surface of the agarose in the proper anode and cathode positions and cut to the proper size of the agar.

vi. Place the LKB electrofocusing lid on the Multiphor unit over the cooling plate in the proper alignment such that the platinum electrode wires are centered and make good firm, complete contact with the respective soaked anode and cathode filter paper strips.

vii. Connect the electrical cables of the electrofocusing lid to the small pins on the front of the Multiphor unit.

viii. Mount the cover by first introducing the hooks on the cover into the rectangular holes on the rear side of the Multiphor unit, lower the cover and press the large electrode pins into the holes on the cover.

ix. Connect the electrical leads from the cover to the proper terminals (check for like charge) on the LKB 2197 D.C. power supply.

x. Turn on the power supply and adjust to provide the following conditions: 10 watts constant power, 700 V constant voltage and current unlimited (wide open) for a period of 45 minutes.

xi. After this period of time, change power to the following conditions: 10 watts constant power, 1000 V constant voltage and current unlimited for a period of 60 minutes.

xii. Turn off power after this period of time, remove the cover and electrofocusing lid and proceed to section (f) below.

f. Fixing, Staining, and Destaining

i. After completing the isoelectric phase of separation in Section 16.3 e, remove the agarose-film sheet, discard the electrode filter paper strips and sample filter paper strips. Place the agarose-film sheet in the LKB staining tray and immerse in fixing solution for 30 minutes with occasional gentle agitation. Perform this and all subsequent steps in a chemical fume hood with the exhaust turned on.

ii. Remove the agarose sheet from the first tray and place in a second tray containing destaining solution. Wash for a 30 minute period changing the fluid once.

iii. Remove the agarose sheet from the destaining solution and place on a glass plate. Place one sheet of Schleicher and Schuell #577 filter paper (12.5 x 26 cm) over the agarose surface so that no air pockets are trapped under the paper. Then place 2 sheets of Schleicher and Schuell #470 (12.5 x 26 cm) on top of the #577 filter paper, followed by a second glass plate and 1 kg weight. Allow sheets to remain in this manner for 15 minutes to effect an initial drying of the agarose gel.

iv. Remove the weight, glass plate, and filter papers (discard). Complete the thorough drying of the agarose gel with a draught of hot air from a hand held hair dryer. The agarose must be completely dry and adhering to the gel-bond sheet as a thin film of its' own before proceeding to the next step.

v. Place the dried agarose-film sheet in the staining solution for 10 minutes.

vi. Remove, drain, and place in destaining solution until background is sufficiently clear.

vii. Remove, drain, and dry to a final state with the hair dryer.

viii. Examine and compare the isoelectric focused protein patterns of the unknown samples to those of the reference tissue extracts used to identify the samples in question. The final dry preparation may be kept without further modifications as a permanent record of sample analysis.

16.4 Quality Control of Key Reagents or Procedures

In order to assure the integrity and reproducibility of the previously outlined TLIEF procedure, special attention should be given to the considerations cited below.

a. Agarose Gel-plastic Film Preparation.

Be sure to maintain the sterility of the stock ampholyte solution by using aseptic techniques and a new sterile needle and syringe to withdraw the necessary volume of ampholyte needed to prepare the liquified agarose solution. Ampholytes are susceptible to microbial contamination and this would destroy their intended function.

b. Do not allow air bubbles to form during the injection of the liquid agarose solution into the glass sandwich. Air bubbles at this stage will produce a void in that area on the solidified agarose sheet. The presence of air bubbles during electrofocusing will cause a discontinuous electrical resistance between the electrodes. This may ultimately result in improper band migration for the applied sample at that point.

c. Isoelectric Focusing of Samples and References.

Extracts from reference tissues should be prepared from relatively fresh tissues. Old tissues stored in the freezer for a period of time beyond 6-12 months begin to demonstrate fewer bands. Reference tissue extracts (controls) should be applied to each agarose sheet used for an analytical determination of unknown samples. Do

not rely on the use of previously prepared, dried, stained sheets of reference tissues for comparative purposes.

d. Fixing, Staining, and Destaining.

Proper staining contrast of the dried agarose sheet and protein bands depends upon complete removal of ampholytes and total drying of the agarose gel prior to staining.

Care should be given to wash well after the fixing step (step i; Section 16.3 f) and not to reuse the same quantity of fixing solution too many times as this will cause a build-up of ampholytes in it. Complete drying must be accomplished in step iv (Section 16.3 f) by careful use of the hot air dryer prior to staining (step v; Section 16.3 f). Destaining (step v; Section 16.3 f) must be accomplished carefully and empirically by frequent examination of the sheet to insure that under or over destaining is not allowed to occur such that all protein bands are optimumly stained and appear readily visible.

16.5 Selected References

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Chapter 18. SPECIES IDENTIFICATION FIELD TESTS (SIFT)

Mark E. Cutrufelli and Richard P. Mageau

18.1 Introduction

A series of individual, serological screen tests has been developed for rapid species verification of raw whole/ground meat tissue or emulsified meat products in field environments. They are collectively referred to as the Species Identification Field Tests (SIFT). The individual tests which comprise SIFT are as follows: ORBIT (Overnight Rapid Bovine Identification Test), PROFIT (Poultry Rapid Overnight Field Identification Test), PRIME (Porcine Rapid Identification Method), SOFT (Serological Ovine Field Test), REST (Rapid Equine Serological Test), and DRIFT (Deer Rapid Identification Field Test).

The basis of these tests is that of an agar-gel immunodiffusion technique using stabilized reference antigen and antibody reagent impregnated paper discs and prepared agar-gel plates that have a printed template for correct placement of test components. Identification of a species tissue is demonstrated by a reaction of complete fusion between sample and reference antigen immunoprecipitin bands which become plainly visible after overnight incubation of the immunodiffusion plate at room temperature. Key components are stable for at least one year when stored under refrigerator conditions. Each test has been shown to have adequate sensitivity and specificity for its intended purpose of the particular species in question. These tests are reliable, practical, economical, and very easy to perform and interpret in any work environment. Individual species tests for beef, pork, poultry and sheep are commercially available as a complete test kit. As a result of an Association of Official Analytical Chemists (AOAC) collaborative study, the method of these tests is an official AOAC first action method.

18.2 Materials and Methods

All materials necessary for the performance of SIFT for beef, pork, poultry and sheep species may be commercially purchased as individual test kits. The method of performing SIFT for beef species detection using an ORBIT test kit is described below. Performance of SIFT for other species, using the other SIFT kits available, would be conducted in an identical manner except for the substitution of the appropriate dye colored - template marked agar-

gel plates and species reference antigen and antibody reagent discs relative to the species being tested. Specific formulations for preparation of the agar-gel plates and the reference antigen and antibody reagent discs for each species SIFT kit are detailed in the individual references cited at the end of this protocol.

18.21 ORBIT Kit Composition is as Follows:

a. ORBIT agar-filled plates with pink dye; pattern for disc placement silk screened on plate bottom.
b. Vial of Anti-Beef Antibody Discs-A-.
c. Vial of Beef Reference Antigen Discs-B-.
d. Vial of Blank Discs-S-.
e. One piece flat black construction paper.
f. Three pieces of white paper.
g. One felt-tip marking pen.
h. Polyethylene sample bags.
i. Three forceps.
j. Hyperion viewer (optional accessory).

18.22 Ground Meat Accessory Kit Composition is as Follows:

a. Wooden applicator sticks - six inches long.
b. Sample cups - silk screen printed with two permanent measurement lines on outside.
c. Forceps.

18.3 Procedure

a. Remove prepared ORBIT agar-gel immunodiffusion plates and reagent discs from the refrigerator and allow equilibration to room temperature.

b. Using the forceps carefully place one anti-beef antibody disc, flat on the agar surface, such that the A lettered circle of the template is completely and evenly covered by the disc.

c. In an identical manner place one beef reference antigen disc over the B lettered circle of the same plate.

d. Sample discs may be prepared from either thawed whole muscle tissue or from ground/formulated meat products:

i. If the sample is whole tissue, make a vertical slice about 38 mm deep in an area which is free of fat or connective tissue. With clean forceps place one blank sample disc halfway into the depth of the slit and gently squeeze the slit closed such that both sides of the disc are in contact with the tissue. Let the disc remain in this position 10 - 30 seconds to absorb tissue fluids and appear obviously wet.

ii. If the sample is of a ground/formulated type, place about 1 gram well packed into the sample cup such that it is filled level with the bottom black measuring line. Add sufficient quantity of cold tap water to fill the beaker level to the top black measuring line. Mix sample and water with a clean wooden applicator stick such that a uniform emulsion results. Tilt the cup 45° and with clean forceps immerse a blank sample disc in the emulsion to a depth necessary for complete saturation. Excess fluid and meat particles are removed from the disc by wiping it on a cup rim during removal.

e. The sample disc, from either type of sample is placed over one of the S lettered circles of the ORBIT plate containing the reference discs.

f. Treat a second sample in an identical fashion and place that disc over the remaining unoccupied S lettered circle of the same plate.

g. Tightly seal the lid on the plate and leave undisturbed overnight (15 - 24 h) at room temperature.

h. The plates are then examined with an indirect white light source against a flat black background. This may be done with a Hyperion viewer or by using black paper taped to and suspended vertically from the rear part of a desk lamp's housing.

i. Examine the plate for the formation of characteristic immunoprecipitin lines in the agar among the four discs to determine which sample contain beef.

18.4 Results

Immunodiffusion reactions for the ORBIT test are interpreted as are those for other SIFT plate reactions. A reference band should always be visible between the reference antigen-B- and reference antibody-A- discs. Complete fusion of this line with a band formed between the antibody-A-disc and the sample-S-discs is indicative of a positive reaction for that sample. Absence of a band between the sample and the antibody disc is read as negative. Any lines formed near the sample disc that are not extensions of the reference band are also negative reactions.

18.5 Quality Control Procedures

a. Maintain storage of unused prepared plates and reagent discs at refrigeration conditions (4°C) in order to assure adequate shelf life and proper reactivity. DO NOT FREEZE.

b. Do not use any kit components beyond their expiration date.

c. Use separate, clean forceps for each individual disc placement to prevent reagent or tissue fluid carry over and cross contamination.

d. Proper disc placement and positioning is critical to obtaining expected reactions.

e. An immunoprecipitin band must always be produced between the reference antigen and antibody discs, as this serves as the positive control and assures the proper reactivity of the test system. If a reference band is not produced, the test system is invalid, samples should not be interpreted and the cause of the failure to produce the reference band must be determined and corrected before subsequent testing can proceed.

f. Do not attempt to read any immunodiffusion plates that have reacted for more than 24 h.

g. The normal room temperature for proper incubation of immunodiffusion plates is considered to be in the range of 70 - 78°F (21.1 - 25.6°C).

18.6 Selected References

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CHAPTER 19. COMPETITIVE ENZYME-LINKED IMMUNOASSAY (CELIA) FOR THE DETECTION AND QUANTITATION OF CHLORAMPHENICOL

Richard P. Mageau

19.1 Introduction and Principles

Enzyme Immunoassays (EIA) have become increasingly popular to detect and quantitate a wide range of biological molecules of interest. The excellent specificity and sensitivity afforded by EIA are two major factors contributing to the development and use of this technique for quantitative detection of low molecular weight haptenic molecules such as antibiotics. The Immunology Section of the Microbiology Division developed and published an original EIA procedure to detect and quantitate the antibiotic chloramphenicol (CA).

The specific type of EIA developed was an indirect Competitive Enzyme-linked Immunoassay (CELIA) system. The principles of this assay are as follows. The binding of the limiting number of specific rabbit CA antibody molecules in liquid phase to solid phase bound CA antigen is competitively inhibited by free liquid phase CA in the sample under assay. Bound antibody (not displaced) is indicated by using an enzyme linked anti-rabbit antibody preparation which is subsequently reacted with an appropriate substrate. Enzyme activity, measured spectrophotometrically, is inversely proportional to the concentration of CA in the sample.

The CELIA procedure for CA when performed on bovine muscle tissue extracts or phosphate buffered saline CA standards has the following characteristics: sensitivity of 1 ng/ml ($P<0.05$), linear quantitative displacement over the range of 1-100 ng/ml, a mean 50% displacement end point of 15 ng/ml and excellent specificity with respect to other antibiotics and related chemicals.

The specific procedure subsequently described provides the complete information necessary to perform the CELIA for CA. This procedure represents a modified version of the originally developed and published manual method. This modified version is automated and employs 96 well microtiter plates and Flow (ICN) automatic plate washing and optical density reading equipment. This automated version affords the potential opportunity for high volume sample analysis and effective cost savings by the reduced use of extremely expensive developmental biochemical reagents.

19.2 Equipment and Supplies

a. Flow (ICN) Laboratories Titertek Multiskan MC plate reader; #78-530-00.
b. Flow (ICN) Laboratories Titertek Microplate Washer; #78-431-00.
c. Flow (ICN) Vacuum pump for above washer; #78-426-00.
d. Flow (ICN) Titertek Multichannel pipette; 8 channel, adjustable 50-200 ul volume; #77-859-00.
e. Eppendorf Repeater Pipette (Daigger Scientific Co.#G20551) with accessory of 2.5 ml capacity Combitips (Daigger #G20552C) and 5.0 ml capacity Combitips (Daigger #G20552D).
f. Dynatech Laboratories Microelisa plates, Immulon I, flat bottom, 96 wells, #11-010-3350 and covers.
g. Incubator, 37°C (any properly operating brand).
h. Stomacher®, Model 80 (Tekmar Co., Cincinnati, OH).
i. Whirl-pak® bags; 75 x 180 cm size.
j. Centrifuge, capable of operation at 15,600 x g (Eppendorf, Model 5412; Brinkman Instruments, Inc.), and appropriate centrifuge tubes.
k. Refrigerator (4°C).
l. Microtest Manifold, Wheaton, straight, 8 place with Luer Lock connection (Daigger #G20560A).

19.21 Chemicals and Reagents

a. Na_2HPO_4 (Fisher, S-374).
b. NaH_2PO_4 (Fisher, S-369).
c. NaCl (Fisher, S-271).
d. Citric acid, anhydrous (Fisher, A-940).
e. Hydrogen peroxide, 30% reagent grade (Fisher, H-323).
f. Tween 80 (Fisher, T-164).
g. Sodium azide†; NaN_3, purified (Fisher, S-227).
h. Bovine Serum Albumin, powder, fraction V (Sigma, A-4503), store in refrigerator.
i. Chloramphenicol, crystalline (Sigma, C-0378), store in refrigerator.
j. ABTS substrate indicator; 2,2' azino-di-(3-ethyl Benzthiazoline Sulfonic acid), (Sigma, A-1888).

19.22 Biochemical Reagents and Supplies

a. Anti-chloramphenicol serum (undilute).
b. Chloramphenicol-BSA conjugated antigen (50 µg/ml stock).

c. Goat anti-rabbit immunoglobulin G horseradish peroxidase (GARP) conjugate; Miles-Yeda, Israel, (undilute).
d. Chloramphenicol negative beef tissue (initial supply only; used to set up tissue-CA standards).
e. Normal Rabbit Serum (undilute).

NOTE: The above 5 items must be stored in the frozen state at all times to maintain stability.

19.23 Preparation of Stock Reagent Solutions

a. 0.15 M Phosphate Buffered Saline at pH 7.2 (PBS)

Add 10.35 grams of NaH_2PO_4 and 4.38 grams of NaCl to 1 liter of distilled water and dissolve completely to prepare the "acid" solution. Add 10.65 grams of Na_2HPO_4 and 4.38 grams of NaCl to 1 liter of distilled water and dissolve completely to prepare the "base" solution. While mixing with a magnetic stirrer and monitoring the pH on a pH meter, add a sufficient quantity of the "acid" solution to the "base" solution to achieve a final, stabilized pH of 7.2. Dispense into glass containers, autoclave at 121°C for 15 minutes and store at room temperature. It is most convenient to make up this buffer in 5 liter quantities at a time.

b. Phosphate Buffered Saline Containing 0.05% Tween 80 (PBS-Tween)

To 1 liter of prepared 0.15 M phosphate buffered saline at pH 7.2 add 0.5 ml of Tween-80 and mix (not on magnetic stirrer) for several hours at room temperature until completely dissolved. Store this prepared solution in the refrigerator (4°C).

c. Phosphate Buffered Saline Containing 0.5% Bovine Serum Albumin (PBS-BSA)

To 1 liter of prepared 0.15 M phosphate buffered saline at pH 7.2, add 5 grams of powdered bovine serum albumin and 1 gram of sodium azide (NaN_3) and mix (not on magnetic stirrer) at room temperature until completely dissolved. Store this prepared solution in the refrigerator (4°C).

d. ABTS - H_2O_2 Substrate Buffered Solution

Prepare a 0.1 M citric acid solution by dissolving 1.92 grams of anhydrous citric acid in 100 ml of distilled water. Prepare a 0.1 M dibasic sodium phosphate stock solution by dissolving 1.42 grams of Na_2HPO_4 in 100 ml distilled water. Add sufficient quantities of these two stock solutions together while mixing with a magnetic stirrer and monitoring the pH on a pH meter to prepare 100 ml of a 0.1 M citrate-phosphate buffer at a final stabilized pH of 4.0.

To 100 ml of the above prepared 0.1 M citrate-phosphate buffer add 22 mg of ABTS [2,2' azino-di-(3-ethyl Benzthiazoline Sulfonic acid)] and 15 µl of stock 30% hydrogen peroxide, mix gently by hand (no magnetic stirrer) until completely dissolved. Pass this substrate solution through a 0.45 µm Millex® filter, place in a sterile glass container, and store in the dark at room temperature until needed. This substrate solution should be prepared 24 h in advance of need and may be used as long as it retains its original light green color. A solution which has deteriorated to the point where it cannot be used is evidenced by a dark azure-green color formation.

e. PBS Chloramphenicol (CA) Standards

Prepare a stock 1 mg/ml chloramphenicol (CA) solution by weighing out 10 mg powdered, pure CA on an analytical balance and placing in 10 ml PBS. Allow the CA to dissolve thoroughly into solution by occasional mixing over a period of 24-48 h, or longer if necessary, due to limited solubility of the CA. From this stock 1 mg/ml CA solution make serial ten-fold dilutions in PBS (10 ml quantities) to produce CA standards at concentrations of 10,000, 1,000, 100, 10, and 1 ng/ml respectively. Store these standards in the refrigerator (4°C) until used in the assay.

f. Tissue Extract CA Standards

Prepare tissue extract from known CA free, raw, bovine muscle tissue by the following manner:

i. Place 5 grams of diced tissue in a 75 x 180 cm Whirl-pak® bag.

ii. Add 10 ml PBS.

iii. Place bag in Model 80 Stomacher® and stomach for 30 seconds.

iv. Remove bag from Stomacher® and leave undisturbed for 1 h at room temperature.

v. Pour off the liquid contents from the extraction bag into a centrifuge tube.

vi. Centrifuge at 15,600 x g for 15 minutes.

vii. Collect the clear supernatant tissue extract. If necessary, filter to remove all debris and lipid particulates, and place in a sterile glass container.

Using the PBS-CA standards prepared in (e) above, make ten-fold dilutions of each needed 10X higher concentration standard into the freshly prepared beef tissue extract to produce CA standards at concentrations of 1,000, 100, 10, and 1 ng/ml respectively. These tissue extract CA standards should be made fresh each time a standard curve is to be run in the CELIA. The tissue extract originally prepared, without CA, should be stored in the refrigerator and may be used for subsequent CA standards preparation as long as the extract shows no evidence of microbial contamination or protein precipitation. Tissue extracts should always be prepared from tissues similar to those being analyzed for the presence of CA with respect to species and organ or tissue type.

19.3 Performance of CELIA for CA

a. Obtain a flat bottom, 96 well Dynatech Immulon I microelisa plate and cover from stock supplies.

b. Prepare a sufficient quantity of the Chloramphenicol-Bovine Serum Albumin (CA-BSA) conjugated antigen for plate well sensitization. Make a small volume dilution

of the stock 50 µg/ml CA-BSA antigen solution in PBS such that a final concentration of 50 ng/ml CA is obtained.

c. By using the 8 channel pipette, place 200 µl of the 50 ng/ml CA-BSA (in PBS) sensitizing antigen solution into all wells except those of column 2. Leave these wells empty for the present time.

d. Place a cover on the plate and allow the CA-BSA antigen to passively absorb to the wells by incubating the plate for 3 h at 37°C.

e. Test sample extractions should now be concurrently started at this stage in the following manner:

   i. Place 5 grams of diced tissue in a 75 x 180 cm Whirl-pak® bag.

   ii. Add 10 ml of PBS.

   iii. Place bag in Model 80 Stomacher® and stomach for 30 seconds.

   iv. Remove bag from Stomacher® and leave undisturbed for 1 h at room temperature.

   v. Pour off liquid contents from the extraction bag into a centrifuge tube.

   vi. Centrifuge at 15,600 x g for 15 minutes. (Eppendorf Model #5412 centrifuge using 1.5 ml volume centrifuge tubes is very convenient for this).

   vii. Place the clear, test sample supernatant extracts in the refrigerator (4°C) until called for in step (p) of this assay procedure.

f. Remove the plate from the incubator [continued from step (d)], remove the cover and mount on the carrier of a Titertek Microplate Washer which has been primed with PBS-Tween and set to deliver 300 µl fluid to each well.

g. Remove the CA-BSA sensitizing antigen solution from the wells by aspiration with the washer and wash the wells <u>once</u> with 300 µl of PBS-Tween per well.

h. Remove the plate from the washer, invert over a sink, hold the plate tightly in one hand and flick several times to remove any remaining excess liquid from the wells.

i. Tap the plate in an inverted position several times on a soft paper towel (Sorg Laboratory Towels) placed on the surface of the lab bench and allow the plate to remain inverted for 1-2 minutes to complete the draining process. Place the plate right-side up and cover until next reagent addition.

j. Block unwanted reactive sites on the plastic wells by filling all wells (including those in column 2) with 250 µl of PBS-BSA per well, dispensed by using a 8 place microtest manifold attached to a Cornwall syringe.

k. Replace the cover on the plate and incubate for 2 h at 37°C.

l. Remove the plate from the incubator, place on the carrier of the washer, aspirate the PBS-BSA blocking solution out of each well and wash the wells twice with 300 µl of PBS-Tween per well.

m. Repeat steps (h) and (i).

n. With an appropriate pipetting device place 150 µl of PBS in the wells of column 1, 2, 3, and 4 of row A and B. Place 150 µl of CA free tissue extract in the wells of column 1, 2, 3, and 4 of row C and D and the wells of column 1 and 2 of row E, F, G, and H. These wells all serve as negative reagent controls (column 1 and 2) or 0 level controls (column 3 and 4).

o. Place 150 µl of PBS CA standards at concentrations of 1, 10, 100, and 1000 ng/ml in wells of column 5 and 6, 7 and 8, 9 and 10, 11 and 12 respectively of rows A and B. Place 150 µl of tissue extract CA standards at concentrations of 1, 10, 100, and 1000 ng/ml in wells of column 5 and 6, 7 and 8, 9 and 10, 11 and 12 respectively of rows C and D. These wells serve to produce the standard CA inhibition curves in PBS (rows A and B) and tissue extract (rows C and D).

p. Place 150 µl of each test sample extract [from step (e)] in 2 adjacent wells (duplicates) of an individual row. All wells of column 3-12 of row E-H are available for use for duplicate analysis of individual test sample extracts (20 test sample capacity/plate). Record in some appropriate fashion the location of each test sample extract within the available wells for sample analysis for future reference.

q. With the use of an Eppendorf Repeater pipette and a 2.5 ml Eppendorf combitip attached, add 50 µl of normal rabbit serum diluted 1:700 in PBS to all wells of column 1. The wells of this column serve as zero blank normal rabbit serum controls, producing no visible reactions and are used to blank in the reader making spectrophotometric measurements of the reactions in all subsequent wells in each row.

r. With the use of the repeater pipette and a new 2.5 ml combitip attached, add 50 µl of anti-chloramphenicol serum diluted 1:700 in PBS to all remaining wells.

s. Carefully mix and distribute the contents in each well by gently rocking the plate and tapping the ends against your fingers. DO NOT allow the contents of any well to spill out as this will invalidate this result.

t. Place the cover on the plate and incubate overnight (16-18 h) in the refrigerator at 4°C.

u. Remove the plate from the refrigerator, allow equilibration to room temperature, place on the carrier of the washer, aspirate the contents out of each well and wash the wells twice with 300 µl PBS-Tween per well.

v. Repeat steps (h) and (i).

w. By using the 8 channel pipette, add 200 µl of goat anti-rabbit immunoglobulin G horseradish peroxidase (GARP) conjugate diluted 1:5000 in PBS-Tween to all wells.

x. Place the cover on the plate and incubate for 2 h at 37°C.

y. Remove the plate from the incubator, place on the carrier of the washer, aspirate the contents out of each well and wash the wells three times with 300 µl of PBS-Tween per well.

z. Repeat steps (h) and (i).

aa. With the use of the 8 channel pipette, add 200 µl of ABTS-H_20_2 substrate buffered solution to all wells.

bb. Place the cover on the plate and incubate for 90 minutes at 37°C.

cc. Twenty minutes prior to the end of the above incubation period, turn on the power to the Titertek Multiskan MC plate reader and allow it to warm up.

dd. After the 90 minute incubation period of step (bb) is complete, remove the plate from the incubator, remove the cover and place the plate on the carrier of the Multiskan MC plate reader.

ee. Program the reader for the current date, Mode 1 (single wavelength absorbance), Wavelength filter #2 (414 nm), push the carrier and plate into the measuring head and blank the instrument (zero O.D. point set) on column 1.

ff. Press the START button and obtain a printed paper strip of the Optical Density (O.D.) values for all of the reaction wells on the plate.

gg. Remove the plate from the reader and visually examine the plate to see that the obvious colored reaction intensities generally correspond to the numerical values on the printed data sheet to assure that the instrument properly read the plate.

hh. Turn off the power to the Multiskan MC plate reader and discard the plate (save the cover for reuse) after completion of the Data Analysis, Plotting, and Sample Interpretation Section described below.

19.4 Data Analysis, Plotting, and Sample Interpretation

a. All wells in column 1, which serve as the zero-blank normal rabbit serum control, should have no color

reaction. This indicates a proper lack of non-specific attachment of rabbit serum or GARP conjugate to the bound CA antigen in the wells. Under these conditions these wells are excellent controls to blank in (zero point set) the O.D. reading instrument.

b. All wells in column 2 serve as BSA negative controls to assess non-specific attachment of anti-CA antibody (and also GARP). Since these wells were never sensitized with CA antigen and only blocked with BSA, no positive reactions (high O.D. values) should be observed. These controls may also be considered as a check on the other half of the primary antigen-antibody component of the assay system initiated in column 1.

c. Wells in columns 3 and 4 of rows A, B, C, and D should demonstrate maximum binding of anti-CA antibody (zero inhibition) and have the highest O.D. values. These represent the zero controls for the standard inhibition curves produced by subsequently increasing concentrations of CA.

d. The remaining wells of rows A and B represent the standard inhibition curve for PBS CA standards and those of rows C and D represent the standard inhibition curve for tissue extract CA standards. The O.D. values in both of these series of wells should decrease with increasing concentrations of CA due to inhibition of binding of anti-CA antibody.

e. The remaining wells of the plate (columns 3-12 of rows E-H) represent reaction values for test sample extracts relative to the presence or absence of CA in the original samples.

f. For each pair or set of wells containing exactly the same test materials, calculate the average O.D. value.

g. Obtain a piece of 5 cycle semi-logarithmic graph paper containing 100 numerical scale divisions. Label the ordinate (100 numerical scale divisions) with O.D. values from 0 to 2.0 in increments of 0.2 units. Label the abscissa (5 cycle logs) with CA concentrations of 0, 1, 10, 100, and 1000 ng/ml.

h. Plot the average O.D. values generated for the PBS CA standards and tissue extract CA standards from 0 to 1000 ng/ml respectively on the graph paper. Draw straight lines from point to point. You will now have two inhibition (displacement) curves for increasing concentrations of CA in PBS or tissue extracts.

i. Examine the two inhibition curves and compare the slopes and overall O.D. values. The PBS CA standard displacement curve represents the basic reaction level of the primary antigen-antibody system influenced only by pure CA. The tissue extract CA standard displacement curve represents the influence of CA and interaction of various proteinaceous materials extracted from the test sample. If the tissue extract CA inhibition curve is significantly different from the PBS CA inhibition curve (which it usually is) use the former for determining positive CA concentration levels in test samples.

j. Calculate the 50% displacement end point for both standard inhibition curves (50% of the 0 standard O.D.). Values in the range of 5 to 20 ng/ml with a mean value of around 15 ng/ml should be obtained as an indication of properly operating displacement systems.

k. To determine if a test sample contains CA and to quantitate the amount, if it is present, proceed as follows:

   i. Obtain the O.D. value for the test sample and determine the relationship to the tissue extract CA standard curve.

   ii. If this value is between 0 and 1 ng/ml (i.e. O.D. greater than the 1 ng/ml standard), the sample is considered to be free of CA.

   iii. If the value falls within the linear portion of the standard curve, from 1-100 ng/ml, the sample is considered to contain CA. To determine the amount of CA present per gram of tissue, interpolate from the curve the ng/ml CA value on the abscissa relative to the particular O.D. obtained for that sample and multiply it by 2. This assumes that all of the CA from the original 5 gram of tissue is extracted into the 10 ml PBS volume and the

resulting dilution therefore is 1:2 rather than the usual 1:3.

iv. If the O.D. value falls beyond the linear portion of the standard curve (ie. O.D. less than the 100 ng/ml standard), the sample is also considered to contain CA but accurate quantitation is not possible from this particular analytical run. More accurate quantitation in this case would be achieved by taking this sample extract, making serial ten-fold dilutions of it in PBS (10^1 -10^6), repeating the CELIA analysis a second time on these dilutions and determining which dilution produced an O.D. value within the linear portion (1-100 ng/ml) of the PBS CA standard curve.

Calculations for this sample would then be reduced to: interpolated CA value of ng/ml from the PBS CA standard curve abscissa x ten-fold dilution factor x 2 = ng CA/gram of tissue.

19.5 Quality Control Procedures

a. The assay reagents have been evaluated for use only with Dynatech Immulon I microtiter plates. No other plates should be used.

b. All stock reagent solutions must be properly prepared and maintained free of contamination or chemical breakdown.

c. All stock immunochemical reagents must be stored in the frozen state at all times to maintain stability.

d. The stock ABTS-H_2O_2 substrate buffered solution should not be used if it has turned to a significantly darker shade of green from that of the original preparation.

e. Be sure the stock, commercial preparation of Goat anti-rabbit immunoglobulin G horseradish peroxidase (GARP) conjugate reagent has not deteriorated to the point of producing improper final O.D. readings. Use only an unexpired lot of this reagent.

f. To insure validity of the quantitative aspects of this assay, extreme care must be exercised to accurately

prepare the standard CA concentrations in PBS and CA free tissue extracts from stock sources of the pure CA drug.

g. The CA free tissue used to prepare extracts for subsequent preparation of the CA tissue extract standards should be initially validated as being free of CA by a reliable procedure.

h. Standard curves for CA in PBS and CA in tissue extracts must always be run in an analytical determination for the presence of CA in test samples.

i. The tissue source used to prepare the CA tissue extract standard curve must be of the same species and organ type as that of the test sample to be quantified.

j. The standard CA inhibition curves should always be quite similar from run to run and the 50% displacement end point should always be in the same general range. Drastic deviations in the above indicates an improperly operating displacement system due to critical reagent deterioration or technical error in the assay set-up and must therefore be corrected.

k. A valid test run can only be assured by the demonstration of proper CA standard inhibition curves for each particular analytical determination.

† Safety Caution: Do not dispose of spent sodium azide PBS-BSA solution by pouring down sink drains.

Collect in separate liquid waste container and dispose of as hazardous waste according to standard waste management procedures for your laboratory.

Accumulation of sodium azide in lead sink drains may result in an explosion.

19.6 Selected References

Campbell, G. S., R. P. Mageau, B. Schwab, and R. W. Johnston. 1984. Detection and quantitation of chloramphenicol by competitive enzyme-linked immunoassay. Antimicrob. Agents Chemother. 25:205-211.

Shekarchi, I. C., J. L. Sever, Y. J. Lee, G. Castellano, and D. L. Madden. 1984. Evaluation of various plastic microtiter plates with measles, toxoplasma, and gamma globulin antigens in enzyme-linked immunosorbent assays. J. Clin. Microbiol. 19:89-96.

CHAPTER 20. QUALITY ASSURANCE PROGRAM TO ENSURE CORRECT PERFORMANCE OF THE FLOW (ICN) TITERTEK MULTISKAN MC PLATE READER

Richard P. Mageau

20.1 Introduction

Due to the increased use of enzyme immunoassay procedures for the analysis of important residues, it is important to assure that the instrument used to measure the data produced in these assays is operating properly. This is especially important with regard to assays that have defined optical density values for positive, negative, and control parameters. Many of the current enzyme immunoassays implemented in the Field Service Laboratories employ the ABTS/H_2O_2 substrate and the Flow (ICN) Multiskan MC Plate Reader to obtain data. This substrate when acted upon by the enzyme peroxidase produces a product which has a maximum absorbance at the 414 nm wavelength (filter #2). There is no way to be certain that the daily optical density readings obtained by the instrument during the performance of an enzyme immunoassay are correct, except perhaps by complacent trust. The easy procedure described in this chapter is an attempt to ensure that the readings generated by the Flow (ICN) Multiskan MC Reader at the 414 nm wavelength filter (#2) are indeed correct and that the instrument is operating properly.

20.2 Procedure

a. Prepare 200 ml of a stock 15% (w/v) solution of nickel sulfate (nickelous sulfate, 6-hydrate, crystal, Baker 2808-1) in distilled water in a volumetric flask. Store this stock solution in an air-tight glass container to prevent evaporation and ensure that deterioration does not occur due to contamination or chemical decomposition.

b. Obtain a Dynatech Immulon I, 96 well microtiter plate.

c. Leave all wells of column 1 empty. Accurately place 200 µl of the stock 15% nickel sulfate solution into all wells of columns 2 and 3 (16 wells total).

d. Turn on the Flow (ICN) reader, allow it to warm up and program it for Mode 1 (singe absorbance) and Filter #2 (414 nm wavelength).

e. Push the plate containing the nickel sulfate wells into the reader, blank the instrument on column 1 and obtain optical density readings for the wells of columns 2 and 3.

f. Calculate the mean O.D. value for the 16 wells of columns 2 and 3.

g. Perform the exact same procedure each month and keep a log book of the monthly mean O.D. values for the 16 nickel sulfate wells.

h. If the instrument is performing correctly there should be no significant change in the monthly mean O.D. values. A significant change (most likely a decrease) in these values indicates a problem with the instrument, probably with regard to the light source (lamp), the 414 nm wavelength filter (#2), or the internal electronics of the instrument itself. A systematic check out of these areas in that order is recommended.

NOTE: Spare lamps, replacement filters, electronic repair/instrument check out, or technical assistance may be obtained from:

ICN Biomedical Instruments
330 Wynn Drive
Huntsville, Alabama 35805
Tele: 1-800-426-8869

Prior to returning the instrument for repair, you must first obtain a Return Goods Authorization (RGA) number by calling the above and making the necessary arrangements.

Chapter 21. ANIMAL SPECIES DETERMINATION, IMMUNOLOGICAL

Richard P. Mageau

PART A

21.1 (Presumptive) Tube Ring Precipitin Test

21.11 Introduction

The accurate identification of animal meat species at a significant level of sensitivity in raw meat and poultry products is an important aspect of the Agency's ability to meet the legislative mandate providing for the assurance of a safe, wholesome, unadulterated and accurately labeled meat and poultry supply to consumers. Raw meat species identification can generally be accomplished by physicochemical procedures such as electrophoresis, isoelectric focusing (see Chapter 16) or high performance liquid chromatography and by immunological procedures such as immunoprecipitin (immunodiffusion) reactions (see Chapter 18) or enzyme-linked immunosorbent assay (ELISA), (see Chapter 17).

The immunological methods described in Parts A, B, and C of this chapter of the Microbiology Laboratory Guidebook concerning raw meat species identification have been selected, adapted and implemented for use in the FSIS Technical Support Laboratories because of their suitability as scientifically sound methods, defendable in a court of law when litigation arises from violative results and their practical working use in high volume, routine sample analysis in regulatory laboratories. The methods in Parts A and B are to be used only as presumptive screen tests and all positive, violative results are to be further subjected to a final confirmation by the procedure described in Part C.

The analytical screen test formerly used by the Technical Support Laboratories for determination of the species of animal tissue in raw meat and poultry products was the Ring Precipitin test. Although this immunoassay was successfully used for many years, it was subject to certain limitations or requirements. These consisted of such factors as unremarkable and variable sensitivity levels for species adulterants in different base meat tissues; the availability of significant quantities of expensive, specific anti-species sera; the exact titration of these antisera against standardized reference 30,000 total protein solutions in a specific timed reaction interval; preparation of crystal clear sample

extracts for test reactivity against the titered, specific antisera in the timed reaction interval; and the known observation that certain meat product ingredients such as spices or soy proteins may interfere with obtaining correct test results when certain samples containing such are analyzed by this standardized procedure. In short, this assay required several immunochemical reagents and much, exact standardization of reagents and test performance in order to insure reliable test results. Although this assay has been replaced for routine use by a commercial Immunostick ELISA screen test procedure described in Part B, the Ring Precipitin test procedure is presented below in detail to provide information as an alternative acceptable method if the need should arise.

21.12 Equipment and Materials

a. Culture tubes, glass, 6 x 50 mm, disposable.
b. Pipettes, Pasteur type, 9" (22.8 cm) and 5-3/4" (14.6 cm), disposable, sterile.
c. Pipettes, calibrated, assorted sizes, sterile.
d. Serum vials, rubber stoppered, 15 and 30 ml size, sterile.
e. Racks for holding 6 x 50 mm culture tubes.
f. Culture tubes, glass, 20 x 150 mm or larger.
g. Filter paper, Whatman #42, 11 cm diameter.
h. Millipore Millex® disposable membrane filter units, Luer-lock fitting, 0.45 or 0.22 µm porosity.
i. Syringes, disposable, assorted sizes.
j. Hypodermic needles, disposable, 20 and 22 gauge by 1" (2.5 cm) long; 19 gauge by 1-1/2" (3.8 cm) long.
k. Centrifuge, preferably refrigerated.
l. Centrifuge tubes, plastic, autoclavable, 50 ml capacity.
m. Spectrophotometer, Bausch & Lomb Spectronic 20.
n. Calworth Stomacher®, Model 80.
o. Whirl-Pak® polyethylene bags, 22.8 x 11.4 cm size.
p. Mechanical Shaker.
q. New Zealand albino rabbits, 2.3 kg.

Precaution: All non-disposable glassware must be thoroughly cleaned in detergent, followed by final distilled water rinse and heating in a drying oven for at least 2 h at 200°C to prevent foreign protein contamination.

21.121 Reagents

a. **Normal Saline (0.85% sodium chloride solution):**

Dissolve 8.5 g NaCl in 1000 ml distilled water.

b. **2X Saline (1.7% sodium chloride solution):**

Dissolve 17 g NaCl in 1000 ml distilled water. Add merthiolate to a final concentration of 1:10,000.

c. **2X Saline Containing 10% Normal Rabbit Serum:**

Add 10 ml of normal rabbit serum to 90 ml of 2X saline (above) and mix thoroughly.

d. **Merthiolated Saline:**

To normal saline add sufficient powdered merthiolate to produce a final concentration of 1:10,000.

e. **Normal Sera:**

Obtain authentic normal horse, beef, pork, sheep, chicken, and turkey sera from a reputable commercial source or by directly bleeding the appropriate animal.

f. **10% Solution of Aluminum Potassium Sulfate in Distilled Water**

g. **Specific Antisera to Animal Species:**

Obtain anti-horse, beef, pork, sheep, chicken, and turkey sera following rabbit immunizations.

h. **Biuret Solution**

21.13 Preparation of Proom's Alum Precipitated (PAP) Antigens for Rabbit Immunizations

The preparation of alum precipitated antigens from the normal serum of various animal species is as follows by the method of Proom (Proom, 1943).

a. **Obtain 25 ml of authentic normal serum of the particular species required and thaw completely from the preserved frozen state.**

b. **To this 25 ml of normal serum add 80 ml of sterile distilled water and 90 ml of 10% aluminum potassium sulfate solution and mix thoroughly.**

c. **Using a pH meter, adjust the pH of the resulting solution to 6.35 very carefully with 5 N NaOH.**

d. **Pour the adjusted solution into centrifuge tubes, centrifuge in the cold at 3,000 RPM for 20 minutes and discard the supernatant fluid.**

e. **To the packed precipitate add 100 ml of merthiolated saline, thoroughly resuspend the precipitate and pour into a large plastic bottle.**

f. **Place this bottle and solution on a mechanical shaker and shake vigorously at room temperature for 25 minutes.**

g. **Pour the solution back into centrifuge tubes and centrifuge as described in Step (d). (Or centrifuge in large bottles.)**

h. **Repeat steps (e thru g) for a total of 4 times.**

i. **After the final centrifugation and liquid discard, add merthiolated saline to the fluffy white precipitate for a final volume of 100 ml and thoroughly resuspend.**

j. **Place 25 ml aliquots of this alum precipitated antigen into sterile serum vials and label the appropriate species represented.**

k. **Store this antigen in the refrigerator until needed for rabbit immunization. DO NOT FREEZE.**

l. **Prepare alum precipitated antigens, as outlined above, for each species of animal to which specific antiserum is required.**

21.14 Antiserum Production

Prepare specific antisera against each species required by the following method:

a. Obtain a healthy 2.3 kg New Zealand albino rabbit and using a syringe fitted with a 20 gauge, 2.5 cm long needle obtain 5 ml of blood from the medial artery of the ear.

b. Separate the serum and test this preimmune serum against the prepared test antigens by the tube ring precipitin test to assure that the rabbit is free of existing antibodies.

c. Using a syringe fitted with a 22 gauge, 2.5 cm long needle inject 0.5 ml of thoroughly mixed, previously prepared alum precipitated antigen of the desired species, intramuscularly into each hind leg of the rabbit (1.0 ml total) as the primary injection.

d. On Day 21 post primary injection, inject 0.5 ml antigen into each leg, as the initial booster.

e. On Day 28 post primary injection, trial bleed the rabbit from the medial artery of the ear, obtain the serum and perform a titration to determine the relative antibody content as described under Section 21.16, Antiserum Titration and Specificity Tests. If the immune serum has a titre of 1:10 or greater, proceed to obtain a large bleeding from the rabbit.

f. If the serum titer of the above trial bleeding is considerably lower than 1:10, proceed to give a second booster injection of antigen as in step (d) on Day 36 post primary injection.

g. After 14 days from this second booster injection of antigen obtain a large bleeding from the rabbit.

h. Large bleedings may be obtained by using a large syringe fitted with a 20 gauge, 2.5 cm needle and bleeding carefully through the medial artery of the ear or by placing the rabbit ventral side up in an appropriate restraining device and performing intracardiac bleeding with a 100 ml disposable syringe fitted with a 19 gauge,

3.8 cm long needle. If the rabbit is to be kept for subsequent bleeding or reimmunizations, DO NOT bleed for more than 35 ml at any one time. Remove the needle and gently aspirate the blood from the syringe into a sterile container.

i. The serum is obtained by allowing the blood to clot at room temperature for 2-4 h, ring the clot from the walls of the container and place in the refrigerator overnight. Decant the serum, centrifuge at 3,000 RPM to remove all RBC's and filter sterilize through a 0.22 µm Millex® membrane filter unit directly into a sterile, rubber stoppered serum vial. Merthiolate may be added to a final concentration of 1:10,000 as a preservative, but only as a last resort in lieu of strict aseptic handling of the serum at all times. More information relative to Steps (h) and (i) may be found in "Methods in Immunology", 1977.

j. Label the vial as to the specific anti-species serum represented and keep refrigerated until further use in the tube ring precipitin test. DO NOT FREEZE.

21.15 Preparation of Normal Serum Antigens for Controls in The Ring Precipitin Test

Antigens to be used for controls and antisera titering in ring precipitin tests are prepared from authentic normal sera obtained from various animal species. Maintain these normal sera in a frozen sterile condition prior to dilution and use.

Since the protein content of sera varies from animal to animal within a species, as well as among species, it is necessary to determine and adjust the amount of antigen used for controls. This is done on the basis of the total protein (TP) content of each normal sera.

The TP content of each sera is determined by the biuret method (Section 21.19). Prepare a 1:500 working dilution of TP using the following formula:

(5 x % TP) - 1 = D_v 500. In which % TP = % TP in serum, and D_v 500 = Volume of normal saline to be added to one volume of serum to attain a 1:500 dilution.

Examples:

Serum A = 7% TP	Serum B = 6.5% TP
(5 x 7) - 1 = 34	(5 x 6.5) - 1 = 31.5
1 ml Serum A + 34 ml	1 ml Serum B + 31.5 ml
Normal Saline = 1:500 TP	Normal Saline = 1:500 TP

From this 1:500 working dilution of TP prepare the following TP dilutions in normal saline: 1:1,500 TP; 1:3,000 TP; and 1:30,000 TP. The 30,000 TP serum antigen will serve as the homologous test antigen, while the 3,000 TP and 1,500 TP serum antigens will serve as heterologous test antigens in the procedures that follow.

Filter these diluted serum antigens through a Millex® filter (0.45 µm) into sterile vials or screw cap tubes. Store these diluted antigens at 4-6°C. DO NOT FREEZE. Discard after 8 weeks, or if cloudy or precipitated.

21.16 Antiserum Titration and Specificity Tests

Since the specific antibody content varies within different lots of a particular prepared anti-species serum, it is necessary to quantitate and standardize this antibody level for use in routine sample analysis by the ring precipitin test. It is also necessary to verify the specificity of the reactivity of an anti-species serum towards its homologous antigen at this time.

a. Using 2X saline containing 10% normal rabbit serum, prepare the following dilution series of the anti-species serum to be titered: undilute; 1:2, 1:3, 1:4, 1:5, 1:6, 1:7, 1:9, 1:10, 1:15, 1:20, and higher if deemed necessary. (Large volumes are not necessary.)

b. Test each of the above dilutions against 30,000 TP homologous serum antigen and 1,500 TP heterologous sera antigens previously prepared using the described Ring Precipitin Test. NOTE EXCEPTIONS: To test anti-bovine and anti-ovine sera with their respective heterologous sheep and beef antigens, use 3,000 TP instead of 1,500 TP. Make the same exception for anti-turkey and anti-chicken sera.

c. Choose as the working dilution of antiserum for subsequent use in routine ring precipitin testing on unknowns the highest dilution of antiserum that gives a positive test with the 30,000 TP homologous antigen

within 6 minutes, and fails to give a positive test with the 1,500 TP heterologous antigen (Note Above Exceptions) within 10 minutes. This establishes the antiserum titer and confirms specificity.

An additional test on specificity may be performed by the agar gel immunodiffusion test using undiluted antiserum and saline extracts of tissues from authentic heterologous and homologous animal species.

d. Prepare a 5-6 ml volume of working dilution of each anti-species sera required in 2X saline containing 10% normal rabbit serum and filter sterilize through Millex® filters (0.22 µm) into sterile 15 ml screw cap vials. Refrigerate at 4-6°C until needed. DO NOT FREEZE. Reconfirm the titer and specificity of the working dilution of antisera against appropriate TP antigens each week and discard the sera upon loss of titer or specificity, or development of autoprecipitation or microbial contamination.

21.17 Sample Extraction

a. Fresh Tissue

Weigh 25 g of fresh tissue, using the inner portion of the piece if possible. Dice the tissue and place into an appropriate receptacle (polyethylene bag or beaker) and add 100 ml normal saline. Allow to stand for 1-1/2 to 2 h at room temperature. Filter 5-6 ml of the extract through three-fold filter paper (Whatman #42) into 20 x 150 mm tubes. The filtrate must be crystal clear, but may be colored from straw to dark red. If the filtrate is not crystal clear, subject it to centrifugation and/or filtration through a Millex® syringe filter unit (0.45 or 0.22 µm pore size). Run the test as soon as possible, before the filtrate becomes cloudy.

b. Partially Cooked or Cured Tissue

When a tissue has been heated above 165-175°F, the proteins become insoluble and cannot be extracted. Frequently, however, an interior section may not have reached the denaturing temperature and will release enough soluble proteins for a test. The same applies to cured products. For cooked, uncured tissues, extract as

for fresh tissue and let stand in the refrigerator at least 18 h, then test aliquots at intervals for 5 days. If no reaction occurs after 5 days' extraction, report sample as not giving an antigenic response. If possible, perform the ELISA cooked meat species procedure (see Chapter 17) to identify and differentiate these non-reactive samples. Use the same procedure for cured tissue, but extract with distilled water instead of saline.

c. Chopped, Ground or Emulsified Tissue

Proceed as for fresh tissue.

d. Alternative Extraction Method

Place 12.5 g of tissue and 50 ml normal saline in a 22.8 X 11.4 cm Whirl-Pak® polyethylene bag. (Do not deviate from above amounts.) Place the bag and contents in a Calworth Stomacher®, model 80, and stomach for the following times found to be optimum for the various types of sample products listed (Table 1):

Table 1. Stomaching Time for Samples

Sample Types	Stomaching Time/seconds
Raw ground meats, emulsions and sausage formulations	0 (manually knead bag and contents)
Raw muscular tissue, diced	5-10 (maximum)
Cooked and cured samples, hard processed meats (salami, bologna, frankfurters, etc)	15-30 (maximum)

After stomaching, allow the bag and contents to sit at room temperature for 15-20 minutes. Proceed to prepare a crystal clear filtrate of this extract in the usual manner outlined for fresh tissue extraction.

21.18 Ring Precipitin Tube Test

In an appropriately marked rack, place one 6 X 50 mm tube for each species for which the sample is to be tested (e.g., horse, beef, pork, sheep, chicken, turkey). Place in each tube about 0.2 ml of the working dilution of respective anti-species serum using individual, sterile Pasteur pipettes. Fill another Pasteur pipette with the unknown tissue extract to be tested. Tilt the tube at a 45^{o} angle and slide the pipette down the side of the tube just above the antiserum. Then allow the extract of the unknown to flow gently over the surface of the antiserum, while withdrawing the pipette, keeping it ahead of the advancing interface. Do not allow the pipette to touch the antiserum, or to disturb the interface. Clean the surface of the tube with moist toweling, then wipe it dry. After 3 to 5 minutes, and again up to 10 minutes, read the tube by indirect light against a black background.

A cloudy white ring at the interface is a positive test. Also test heterologous TP dilutions, and read up to 10 minutes as a test of acceptability of antisera. If the heterologous TP dilution for one species gives a positive test against the serum of another species within 10 minutes, check for possible contamination of the antiserum. (Note: Quality Control Section, 21.110)

Retest the antiserum for specificity and retest the sample, extracting at least two times. If more than one piece of tissue was used, then retest each piece separately using, if possible, the innermost portions of the pieces. If the sample is ground or chopped, retest another extraction of the sample; repeat two times if the reaction indicates possible violation. Record the reaction times.

21.19 Total Protein by Biuret Method

21.191 Biuret Solution

In a one liter volumetric flask place 1.5 g cupric sulfate, and 6.0 g fine crystals of potassium sodium tartrate. Add sufficient distilled water to dissolve. Add slowly with agitation of the flask, 300 ml 2.5 N sodium hydroxide and mix. Add 1 g potassium iodide and shake until dissolved. Dilute to one liter total volume. Discard when black or reddish precipitate forms.

21.192 Method

a. Place 9.5 ml 0.85% NaCl in a test tube. Add 0.5 ml of sample. Rinse out pipette by drawing in and expelling some of the mixture.

b. Into one of 2 test tubes place 2 ml of the diluted sample, above; in the other, 2 ml 0.85% NaCl solution (blank).

c. Add 8 ml biuret reagent (above) to each tube, and mix.

d. Set 100% transmission with "blank" at wavelength 540 nm.

e. Immediately after adding biuret reagent read transmission of sample and obtain concentration from the following (Table 2).

Table 2. Percent protein, as determined by percent transmission of Biuret reaction in Bausch and Lomb Spectronic 20. (Note: Quality Control Section).

% TR* (540 nm)	0	1	2	3	4	5	6	7	8	9
	Percent Protein									
0										
10										
20										
30								13.8	13.4	13.0
40	12.7	12.4	12.0	11.7	11.4	11.1	10.8	10.5	10.2	9.9
50	9.6	9.3	9.0	8.8	8.6	8.3	8.0	7.8	7.6	7.3
60	7.1	6.9	6.6	6.4	6.2	6.0	5.8	5.6	5.4	5.2
70	5.0	4.8	4.6	4.4	4.2	4.0	3.8	3.7	3.5	3.3
80	3.1	2.9	2.8	2.6	2.4	2.3	2.1	2.0	1.8	1.6
90	1.5	1.4	1.2	1.0						

* TR (Transmission)

Example: % transmission = 47. Concentration of protein = 10.5%

21.110 Quality Control Procedures

In order to assure the integrity and reproducibility of the procedures previously outlined, special attention should be given to the following considerations cited for each section listed below:

a. **Normal serum of authentic species: It is absolutely essential that the species authenticity of the normal sera be initially established since these sera serve as the starting material for anti-species sera production and standardized test antigens. This can be accomplished by directly bleeding the live animal species required and preparing the serum from the blood. If a commercial source of normal serum of a particular species is used, it should be verified in a known, correctly functioning, serological test system.**

b. **Total Protein Determinations and TP Dilutions: Care and attention should be given to the correct test performance, data interpretation and calculations to arrive at the total protein content of each normal sera. Caution must also be exercised in the mechanical preparation of the correct TP dilutions of heterologous and homologous sera antigens. Improperly prepared or calculated values for the above will lead to erroneous anti-species sera titration or specificity data. This in turn might render the antisera dilution finally chosen for use, totally ineffective for reacting with an adulterant tissue in an unknown sample.**

c. **Antiserum Titration and Specificity Checks: The most important component of the ring precipitin test system which ultimately is responsible for the successful detection of an adulterant tissue is the standardized anti-species serum. It cannot be stressed too strongly that periodic checks on the performance characteristics of these diluted antisera must be made with the 1,500 TP and 30,000 TP normal serum antigens to assure that the antisera are reacting in the expected manner. Previously titered antisera can on occasion, with age, produce a change in the titration endpoint. Appropriate adjustments in the working dilutions of these antisera would therefore need to be made in order to compensate for this fact.**

d. Biuret Protein Determination Table: It should be noted that the convenient table provided for the determination of protein by the Biuret reaction is valid only if the exact test procedure is followed and the percent transmission values are obtained using a Bausch and Lomb Spectronic 20 spectrophotometer with the standard, round, tube shaped cuvettes. If a different protein determination test or spectrophotometer is to be employed, then a new standard table must first be prepared with the use of known protein standards.

e. Sample Extracts and Anti-species Sera Working Dilutions: Reagents must be crystal clear following Millex® filtrations just prior to performing the ring precipitin test. Any degree of cloudiness will make it more difficult to visualize any reacting immunoprecipitin line at the interface.

f. Overlaying the Working Dilution of Each Respective Anti-species Serum with the Sample Extracts: Overlayering must be done in a careful, gentle manner so as to not create a mixture of the two reagents at the interface. A mixture at the interface will tend to create a broad, diffuse immunoprecipitin band and cause difficulty in visualizing a positive reaction within the specified time period, rather than the usually expected sharp band.

21.111 Selected References

Garvey, J. S., N. E. Cremer, and D. H. Sussdorf. 1977. Methods in Immunology: A Laboratory Text for Instruction and Research, p. 7-38. 3rd Edition. W. A. Benjamin Inc. Reading, MA.

Kenny, F. 1985. A practical species testing programme, p. 155-159. *In* R. L. S. Patterson (ed.), Biochemical Identification of Meat Species. Elsevier Science Publishing Co., Inc. New York, NY.

Proom, H. 1943. The preparation of precipitating sera for the identification of animal species. J. Path. Bact. 55:419-426.

PART B

21.2 (Presumptive) Commercial ELISA Immunostick Screen Test Kit.

21.21 Introduction

Modern developments in immunoassay technology have made available alternative procedures which have the advantage of eliminating or greatly reducing the limitations previously cited for the Ring Precipitin test. One such procedure is the Enzyme-Linked Immunosorbent Assay (ELISA) method, which is now available in a commercialized kit form capable of rapid, specific species identification of raw meat and poultry tissue products, inclusive of all current species of interest to our National Testing Program. The original ELISA raw species test kit was developed and manufactured as a solid phase microwell plate system. The system was subsequently modified slightly by incorporation of NUNC dip-stick paddles (immunosticks) as the solid phase and the use of pre-dispensed, standardized reagents in color coded tubes. It is currently marketed and distributed in the U.S. in a complete (25 test) kit form and is referred to as a commercial ELISA Immunostick Raw Meat Species Screening Test Kit.

This raw meat species screen test is a double antibody "sandwich" ELISA procedure with antibody specificity directed against the various species albumins which are contained in meat tissues. Specific antibody sensitized immunosticks are allowed to capture homologous species albumin from sample tissue extracts, then reacted with the second peroxidase labeled antibody of the same specificity, followed by a final reaction step in ABTS/H_2O_2 chromogen/substrate solution. A short incubation period and a brief tap water rinse is performed between each of the first two steps. A positive reaction, indicating the presence of the test species tissue in the sample, is evidenced by a distinct green color formation in the last reagent tube. Each species kit contains all necessary reagents, controls and accessories to perform the test in an extremely easy fashion with the production of very accurate results.

The Immunology Section of BCB, MD at Beltsville conducted an evaluation of the ELISA Immunostick Screen test kits for all available species. They were found to be very specific, reliable, easy to use and capable of detecting an adulterant tissue at the 1% sensitivity level. It is with the above considerations in mind and the aim of technical improvement over the previous screen test procedure that these commercial Immunostick Screen Tests were

implemented in all FSIS, Technical Support Laboratories for raw meat species determinations. The Immunostick Screen Test is now used in place of the standard Ring Precipitin test procedure. All positive Screen Test results which represent sample violations are to be confirmed in the usual manner by the standard agar-gel immunodiffusion procedure described in Part C.

A commercial ELISA Immunostick Screen Test employed for presumptive identification of species composition of raw meat and poultry tissues should meet or exceed the following performance characteristics:

Sensitivity - produce positive reactions down to the 1% level (W/W) of adulterant or contaminant tissue in a base meat tissue mixture such that a 0% False Negative Rate is observed.

Specificity - produces no positive cross reactions with any heterologous species tissues such that a 0% False Positive Rate is observed.

21.22 Reagents and Equipment

a. Commercial ELISA Immunostick Raw Meat Species Screen Test Kits. Color codes for individual species kits are as follows (Table 1):

Table 1. Color Codes for Commercial ELISA Immunostick Screen Test Kits.

Color Code	Species	Color Code	Species
Red	Beef	Orange	Horse
Yellow	Pork	Lilac	Rabbit
Blue	Poultry*	Grey	Kangaroo
Pink	Chicken*	Brown	Turkey*
Green	Sheep	Various	Mixed

* The ELISA immunostick Poultry screen test does not differentiate between chicken or turkey. If it should become necessary to do

so, this can be accomplished by performing the traditional agar-gel immunodiffusion procedure (Part C), or by using the ELISA immunostick chicken or turkey screen tests. Since these latter two screen tests have less than the required sensitivity, their use should be limited to whole meat or poultry tissues or a mixed meat/poultry emulsion where the poultry component is known to constitute over 5% of the final meat block.

Each individual species kit contains the following items:

i. Twenty-five - color coded, white plastic immunosticks sensitized with specific anti-species capture antibody in tubes of preservative buffer solution.
ii. Twenty-five - color coded tubes containing species specific antibody-enzyme conjugate reagent.
iii. Twenty-five - tubes (non-color coded) containing color development buffer reagent.
iv. One vial of concentrated ABTS color reagent.
v. One vial of aqueous sodium fluoride stop solution.
vi. One vial of positive control solution (homologous species albumin).
vii. Disposable polypropylene pasteur pipettes - NOT TO BE USED.
viii. Product insert test kit instruction pamphlet.

b. Rainin Gilson Pipetman® (P-200) adjustable pipette and appropriate disposable pipette tips.
c. Calworth Stomacher®, Model 80.
d. Whirl-Pak® polyethylene bag, 6 oz size (7.5 x 17 cm).

21.23 Raw Sample Preparation

All types of raw meat and poultry product samples are prepared as follows:

a. Weigh out 1 gram of thawed, diced, raw sample product which is a homogeneous, representative portion of the whole sample.

b. Place in a 6 oz Whirl-Pak® bag.

c. Add 9 ml of distilled water.

d. Place the bag and it's contents in a Model 80 Calworth Stomacher® and stomach for a period of 60 seconds.

e. Allow the extract to settle for 2-3 minutes until a particle free liquid layer is formed in the top portion of the bag's contents. Use this upper liquid layer as the sample extract in the following test procedure.

21.24 Test Procedure

The following procedure is to be used, which represents minor modifications from the original product insert test kit instruction pamphlet. These procedural modifications are designed to improve the accuracy, precision and reproducibility of test results. The subsequent instructions represent the testing of 1 sample through 1 species test procedure. Obviously multiple samples and/or species tests may be performed simultaneously, as long as one is careful to keep track of reaction times, washing steps, various reagent steps, etc. relative to each given test sample.

a. Remove the appropriate color coded species Immunostick tube, antibody-enzyme conjugate reagent tube, and color development buffer tube (a set of 3) from refrigerated storage and allow to equilibrate to room temperature.

b. Label Immunostick caps and all tubes with appropriate sample identification codes.

c. Prepare the color development buffer reagent tube (non-color coded) for later use by adding 40 µl of ABTS concentrate to this tube, replace cap and mix in a gentle but complete manner.

d. Obtain the first color coded Immunostick tube, unscrew the cap and remove the immunostick-paddle, add 200 µl of prepared sample extract to the liquid in the tube, replace the immunostick-paddle in the tube and mix contents by rotating the cap rapidly 4-6 times and tighten the cap. DO NOT INVERT tubes to accomplish mixing at any stage in this procedure. Handle the paddle at all times only by it's attached cap, DO NOT TOUCH paddle with fingers.

e. Allow this tube to stand for 10 minutes at room temperature.

f. Remove the immunostick-paddle and wash the paddle and entire cap completely by placing it under a gentle stream of cold tap water for 10 seconds, then shake to remove excess water.

Note: Water dispensed from a squeeze bottle can also be used to carefully perform this wash step.

g. Place the washed immunostick-paddle into the second color coded tube of antibody-enzyme conjugate reagent, mix contents by rotating the cap rapidly 4-6 times and tighten the cap.

h. Allow this antibody-enzyme reagent tube to stand for 10 minutes at room temperature.

i. Remove the immunostick-paddle and wash the paddle and entire cap completely by placing it under a gentle stream of cold tap water for 30 seconds, then shake to remove excess water.

Note in step (f) above also applies here.

j. Place the washed immunostick-paddle into the final, non-color coded, tube of ABTS prepared (step c) color development buffer reagent, mix contents by rotating the cap rapidly 4-6 times and tighten the cap.

k. Allow the color development reagent tube to stand for 10 minutes at room temperature.

l. Add 200 µl of sodium fluoride stop solution to this color development tube, leave the paddle in, and mix well to stop the reaction.

m. Observe the above tube with the white paddle in it for the presence of any discernable green color in the solution or on the paddle surface. A green color indicates a positive test and the presence of the test species in the original meat sample. A colorless solution around the white paddle indicates a negative test and the absence of the test species in the sample.

All ELISA Immunostick positive species results which represent sample violations are to be confirmed by the traditional agar-gel immunodiffusion procedure as described in Part C.

21.241 Test Controls

The occasional use of positive and negative controls in performing this species screen test will ensure proper quality control and reliable test performance of this method. This should ALWAYS be done initially upon opening and placing into use a brand new kit package.

Each species test kit is supplied with a positive control vial (homologous species albumin solution) for this purpose. The negative control for any one particular species test kit may be obtained by using the positive control solution from any of the other heterologous species test kits: (eg. horse albumin solution should always give negative results in all other species kits except horse).

Control testing may be performed in the following manner:

a. Remove the cap and Immunostick from a tube of an individual test series to be used for control testing.

b. Add 200 µl of negative or positive control solution to the liquid in the tube.

c. Replace the immunostick-paddle in the tube, mix contents by rotating the cap rapidly 4-6 times and tighten the cap.

d. Proceed with the remainder of the test procedure exactly as described above by continuing and completing steps e-m (Section 21.24; Test Procedure). Be sure to initially prepare an ABTS color development buffer reagent tube in the usual manner when you start your control tests.

21.25 Quality Control Procedures

a. Store all kit components at refrigerator temperature (4-8^{o} C) when not in use to preserve and maintain reactivity of immunoreagents.

b. Perform positive and negative control testing of an initially opened kit package and occasionally thereafter to insure proper test performance.

c. Observe the manufacturer's one year expiration date of all test kit components. Kits should not be used beyond the expiration date.

d. The concentrated ABTS color reagent solution tube should be observed over the kit shelf life. If this ABTS concentrate should start to turn a much darker shade of green than when it was originally received, this indicates decomposition, and a new tube of ABTS concentrate should be requested from the vendor.

e. All volumetric additions of sample extracts or reagents to the test procedure should be made only with the Rainin Gilson Pipetman® pipette instrument.

f. Kit components should be allowed to equilibrate to room temperature before commencing test procedure.

g. The 1 gram test sample used for extraction must be representative of the entire original sample in order to insure that test results accurately reflect the true composition of the original sample.

h. Preparation of the color development buffer reagent tube by the addition of ABTS concentrate (step c of Section 21.24, Test Procedure) should only be accomplished just prior to commencing the test procedure. Preparation of this reagent tube should not be done in advance (hours/days) because of the inherent chemical instability of ABTS in buffered substrate for extended time periods.

i. Accurate timings of washing and reaction steps should be performed.

j. Assure that all surfaces of the white immunostick-paddle and cap are adequately washed during the two timed wash steps.

k. Do not use hot or warm water for immunostick-paddle washing, only cold.

l. Since all reactions of this solid phase immunoassay occur on the surfaces of the white immunostick-paddle, it is very important not to touch the paddle surface with fingers or any other physical objects which might interfere with the immunoreaction.

m. When performing different species tests simultaneously on the same sample, be sure to maintain the proper continuity of color coded reagent tubes for each respective test species as you complete the test procedure. (eg. An anti-beef species immunostick (red color code) that has reacted with a beef sample extract if improperly placed in an anti-pork enzyme conjugate reagent tube (yellow color code), will produce a false negative result).

21.26 Technical Assistance

If any problems should arise during the performance of this species screen test or technical assistance is required on any aspect of the procedure, contact the following:

Dr. Richard P. Mageau
Microbiology Staff Officer
USDA, FSIS, OPHS, MD, EMIB
Washington, DC 20250
Telephone (202) 501-7600

21.27 Selected References

Anonymous. 1991. Commercial Immunostick Raw Meat Species Screening Kits; product insert instruction pamphlet.

Fukal, L. 1991. Review Article. Modern immunoassays in meat-product analysis. Die Nahrung 35(5):431-448.

PART C

21.3 (Confirmation) Agar Gel Immunodiffusion Test

21.31 Introduction

The final determination (confirmation) of an adulterant species of animal tissue in raw meat and poultry products is based upon the results of sample analysis by the agar-gel double immunodiffusion procedure presented in Part C. All presumptive positive violative results from the analytical methods in Part A or B are subjected to confirmation by agar-gel immunodiffusion before definitive compliance or legal actions are undertaken.

The agar-gel immunodiffusion procedure described in this section is based upon fundamental principles established previously by Ouchterlony, 1968, and modified for specific application and Agency use by Fugate and Penn, 1971. Agar-gel immunodiffusion is notable for it's qualitative ability to demonstrate similarities and resolve differences in related proteins based upon the formation of specific immunoprecipitin lines resulting from the diffusion of specific antigens and antibodies from wells or troughs cut into an agar matrix after they have reached their optimum proportions. As such, this procedure is ideally suited for meat species protein identification. In addition to being relatively easy to perform and providing results within a 24 hour period, the procedure also has the advantage of generally not being affected by the same factors which tend to produce false positive reactions in other immunoassays such as the Ring Precipitin test. If any false or "non specific" reactions should occur in a double immunodiffusion assay, it is possible to distinguish them from true positive reactions by carefully observing the immunoprecipitin pattern formed and it's relationship to known antigen extracts. The three basic types of reactions usually observed in double immunodiffusion assays are lines of identity, lines of partial identity and lines of non-identity. With a little practice and experience these types of reactions can be easily distinguished and their interpretation in relation to resolving the identity and/or relationships of similar proteins can be made in a definitive and reliable manner.

Although several different patterns of wells or troughs may be generally used in an agar-gel to perform double immunodiffusion reactions, the pattern ultimately employed is usually dependent upon the intended, specific application of the assay. Hvass, 1985, used a relatively simple, common, 7 well , circular pattern to differentiate raw meat species, while Fugate and Penn, 1971, used a

more complicated pattern consisting of 3 antisera troughs and 24 antigen extract wells. The latter was designed with the intention of demonstrating relationships among more than one species on a single plate and also to provide several identical reaction areas on the same plate showing the identity or non-identity relationship of an unknown meat species sample with known reference species tissue extracts. The concept of demonstrating several areas of identical results using several positive and negative controls within the same single reaction system provides almost irrefutable evidence in a court of law when applying this already well recognized immunodiffusion procedure to establish identity of a meat species in a case of fraudulent adulteration.

21.32 Equipment and Materials

a. Dish, Petri, plastic, 15 X 100 mm disposable
b. Pipettes, disposable, capillary, Pasteur type
c. Box, plastic, humidity chamber, or other air tight container used to maintain high humidity.
d. Cutter, agar-gel, or template pattern
e. Flask, side arm
f. Tubing, rubber or neoprene, high vacuum type
g. Tubing, brass (Cork borer), 5/32 x 1-3/4 inch (3.95 x 44.5 mm)
h. Applicators, wooden, cotton tipped
i. Pipettes, graduated, serological, assorted sizes
j. Dishes, staining (only if agar is to be dried and stained)
k. Slides, microscope, 1 x 3 inch (2.54 x 7.62 cm);(only if agar is to be dried and stained)
l. Filter paper, Whatman No. 1 and No. 42
m. Pans, plastic, 6 x 12 x 6 inch (15.2 x 30.5 x 15.2 cm), or other suitable containers (used only if agar is to be air dried and stained).
n. Assorted laboratory flasks, beakers, tubes, etc.

Clean all glassware, rinse in distilled water and heat a minimum of two hours at 200°C in a dry heat oven to eliminate contamination from prior use.

21.33 Reagents

a. Normal saline, (0.85 percent sodium chloride solution):

Dissolve 8.5 g NaCl in 1000 ml distilled water.

b. Buffered saline (0.85 percent sodium chloride solution, pH 7.2 phosphate buffered):

To 1000 ml normal saline, add 1.25 ml stock phosphate buffer solution. Adjust pH to 7.2 if required.

c. Phosphate buffer stock solution - pH 7.2:

Dissolve 34 g monobasic potassium phosphate (KH_2PO_4) in 500 ml distilled water. Adjust pH to 7.2 with 1 normal sodium hydroxide (NaOH), (requires approximately 175 ml). Dilute to 1000 ml with distilled water. Store under refrigeration.

d. Agar, 1.0 percent (Oxoid Purified Agar, L28):

To 99 ml buffered saline, add 1.0 g purified agar. Heat with constant stirring until agar is melted. Filter hot agar through glass wool or several thicknesses of cheese cloth.

Dispense into screw cap flasks or tubes and sterilize by autoclaving for 20 minutes at 15 pounds pressure. Cool agar to 49-50°C and add 1.0 ml of stock merthiolate solution (1:100) per 100 ml melted agar, to give a final concentration of 1:10,000. Tighten caps (airtight) and store until needed. Remelt when needed in boiling water bath. (Agar can be stored for extended periods of time if caps are airtight and no desiccation or growth occurs).

e. Tissue extracts from known animal species:

Cut muscle tissue collected from animals (known species) into 10 g portions and freeze until needed. To 10 g of ground or finely diced tissue, add 30 ml normal saline and stomach for specified times as shown in Table 1 Section 21.17. Let stand a minimum of 90 minutes. Decant liquid and filter through Whatman No. 42 filter paper. Use immediately. (note Section Quality Control of key reagents or procedures).

f. Antisera:

Undiluted anti-horse, beef, pork, sheep, chicken and turkey species serum, or others as may be required.

g. Tissue extract-(unknown samples to be confirmed):

Extract unknown tissue(s) as in (e) above, using 25 g tissue and 75 ml normal saline.

h. Staining solution:

Dissolve 2 g acid fuchsin in 500 ml absolute methyl alcohol; add 400 ml distilled water and 100 ml glacial acetic acid.

i. Destaining solution:

To 500 ml absolute methyl alcohol, add 400 ml distilled water and 100 ml glacial acetic acid.

j. Acidified Distilled Water:

To 1000 ml distilled water, add 0.2 ml glacial acetic acid.

k. Mounting fluid:

A commercially available material for mounting cover slips permanently.

21.34 Preparation of Agar-Gel Immunodiffusion Plates

a. Agar Plate Preparation.

Remelt purified agar prepared above and dispense 18-20 ml into the 15 x 100 mm plastic petri dishes. Allow to solidify and refrigerate for a minimum of 30 minutes. Store no more than 2 weeks under refrigeration in a high humidity atmosphere. Do not use plates showing desiccation or microbial growth. (Note: Quality Control Section 21.35)

b. Cutting Pattern of Wells and Troughs.

Remove the plates from refrigeration and cut the desired pattern by one of the two methods described below:

i. Use a gel cutting tool which has the proper well and trough cutting tubes and knife edges permanently

embedded in a fixture such as plexiglas or other solid substance.

Figure 1 illustrates one such tool. Align the tool carefully on the agar surface to obtain a perpendicular cut, then press down firmly to cut the agar.

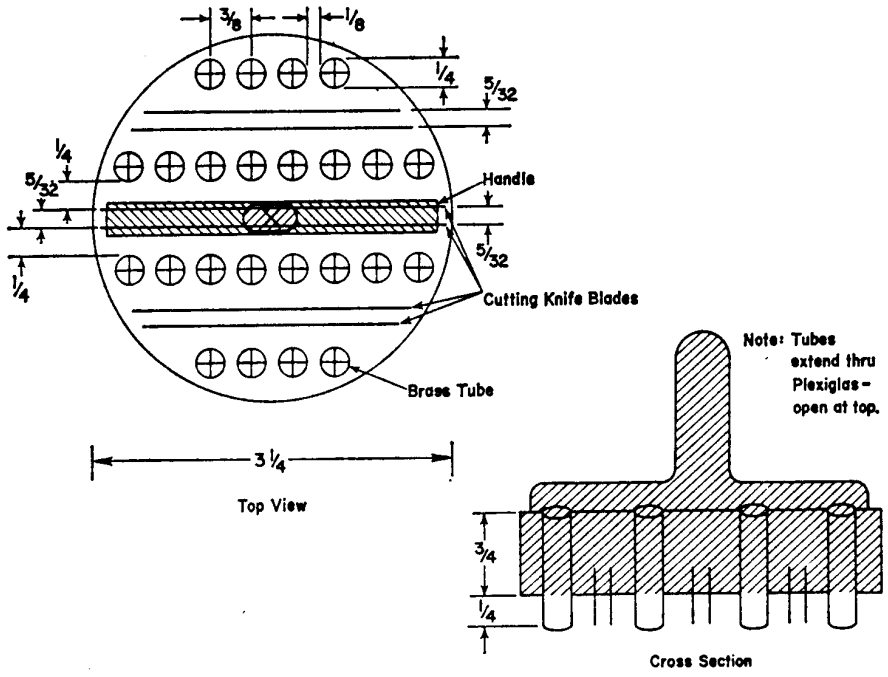

FIG. 1 - Cutting tool used to cut pattern of wells and troughs in agar-gel. (Fugate and Penn, 1971)

ii. Using a pattern of the desired arrangement drawn on graph paper, center the plate over the pattern, agar side up. Press a metal tube of acceptable diameter, connected to a vacuum source by a vacuum tube and side arm flask, through the agar at the indicated places on the pattern. Then cut the troughs with a razor blade or scalpel along the lines of the pattern; or use a tool fashioned with two blades or

knife edges the correct distance apart, and with a downward motion cut the agar.

Remove the agar plugs in the wells with a metal tube connected to a vacuum source. Experience will dictate how to avoid tearing the agar surrounding the wells. Remove the trough plugs with an applicator stick which has one end shaved to present a shovel edge. Gently push the applicator stick to the dish bottom and guide it along the cut, raising the strip of agar as a plow would.

Remove the remaining agar in the wells and troughs with a cotton tipped applicator very carefully so as to not tear the surrounding agar surface.

c. Sealing Wells and Troughs

Hold the plate at a 45^{o} angle and, with a Pasteur pipette, place a thin layer of agar on the floor of each well and trough, sealing the bottom edges of the cut agar to the plate. Do not add an excess of agar. Repair torn wells or troughs in a similar way; if necessary, refill the well or trough and recut it. Caution: An overfilled well will distort the agar and the reaction bands.

d. Preparation of Tissue Extracts: (Protein antigens)

Using the desired known animal species muscle tissue, prepare saline extracts as described in Reagents Section 21.33 e. and g. Do the same for unknown tissue that is to be analyzed. (Note: Quality Control, Section 21.35)

e. Charging the Wells

Mark the outside of the plate to identify the location and contents of each well and trough. Using a Pasteur capillary pipette, partially fill the wells with the known and unknown extracts, maintaining a concave meniscus. Overfilling to form a convex meniscus will interfere with diffusion and may cause wells to overflow. Always place the extract of the unknown between known antigens of two different species. Like antigens will form continuous reactant bands in the agar media, and unlike antigens will form discontinuous bands (See Figure 2).

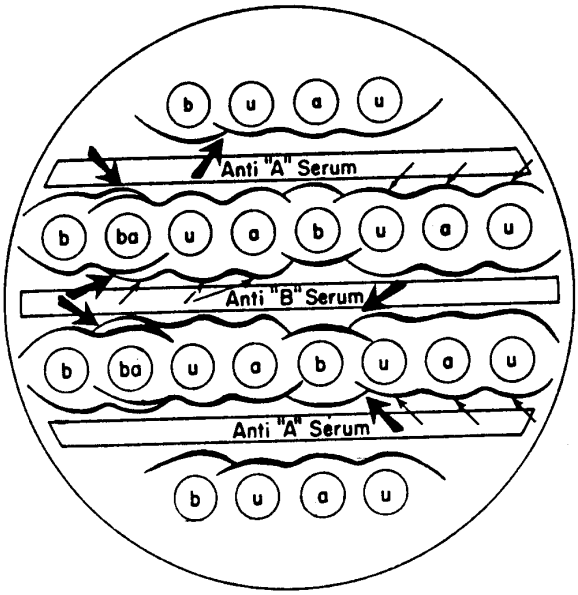

FIG. 2 - Precipitin pattern resulting from heterologous antigen-antisera reactions: a, antigens derived from species A; b, antigens derived from species B; u, antigens derived from unknown; ⇒ , lines of partial identity; → , lines of identity. Although atypical, the above pattern results when all antigens react with antisera used. The identification of unknown antigen u is accomplished by lines of identity formed with antigen a. Both a and u form lines of partial identity with lines formed by antigen b, which is indicated by a spur reaction. It can be concluded that antigen u is derived from species A and is similar but not identical to species B. (Fugate and Penn, 1971)

f. Charging of Troughs:

Fill troughs with the antisera. Use one plate to determine two species only. (e.g., beef and sheep, or beef and horse, etc.). Use the top and bottom troughs for one antiserum, and the center trough for the other. (See Figure 2).

g. Incubation and Observation:

Replace the plate covers and allow the plates to remain at room temperature for 1 1/2 to 2 h. Refill the wells and troughs with the appropriate antigens and antisera. Line the bottom of an airtight chamber with wet filter paper or cotton. Incubate the plates in this high humidity chamber at room temperature for 18 to 24 h. To read the plates, direct a light source parallel to the agar surface, i.e., from the side of the plate, and hold the plate over a dark black background. The reactant bands will appear white on a grey surface. If the bands are not fully developed, refill the wells and troughs, and continue incubation in the chamber for an additional 24 to 48 h under refrigeration.

Following incubation, remove the plates from the humidity chamber, discard the remaining reactants and gently wash the plates under a stream of distilled water. Use a soft cotton applicator to remove any film from the agar surface and precipitated matter from the wells and troughs. Dry the bottom of the petri dish with a soft laboratory tissue and observe the plate for reaction bands. Position the plate in alignment with the worksheet (Figure 3) and draw the reaction bands observed on the plate onto the worksheet.

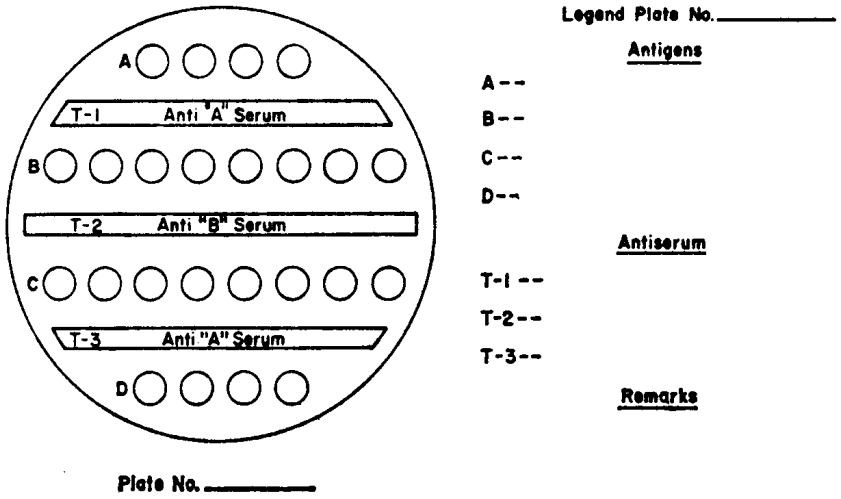

FIG. 3 - Worksheet showing well and trough arrangement and antigen-antisera placement. (Fugate and Penn, 1971)

h. Interpretation of Precipitin Reactions

Interpretation of results depends upon lines formed with known and unknown antigens. Figure 4 (A) illustrates an identity line, i.e., the precipitin line that forms when the antigens are identical. Figure 4 (B) shows partial identity lines, i.e., the lines that form when extracts contain similar but not identical proteins which react with the same antiserum. Figure 2 (page 21-26) illustrates a typical reaction with an unknown and 2 known antigens, showing lines of identity and partial identity. Since unknown antigen u forms a continuous wave pattern with known antigen a, lines of identity form. The lines formed by known antigen b appear as spurs of those formed by antigen a and u, and are typical lines of partial identity.

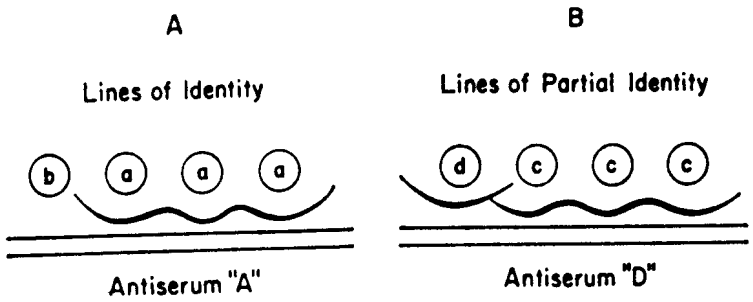

FIG. 4 - Precipitin lines of identity and partial identity. A, lines of identity formed with homologous antigen-antiserum only (antigen a vs. antiserum A); B. lines of partial identity formed when similar antigens react with the same antiserum. Note the typical spur formed, indicating lines of partial identity. (Antigen c and d are similar but not identical). (Fugate and Penn, 1971).

Figure 2 also illustrates the pattern of precipitin lines formed when the sample contains tissue antigens from 2 species (wells ba). In the majority of cases, the antisera will not react with heterologous antigens and lines of partial identity do not form. This occurs when the animal species are closely related (such as bovine and ovine).

Figure 5 illustrates areas containing identical antigen alignment. Four of the 6 areas have antigens reacting with antiserum A and 2 of the 4 areas are in position to react with antiserum B. The 2 remaining areas (2 and 4) are control as well as indicative sites. The mixtures of antigens a and b in wells marked ba are in position to react with both antisera and illustrate precipitin lines that occur when the sample contains tissues from both species.

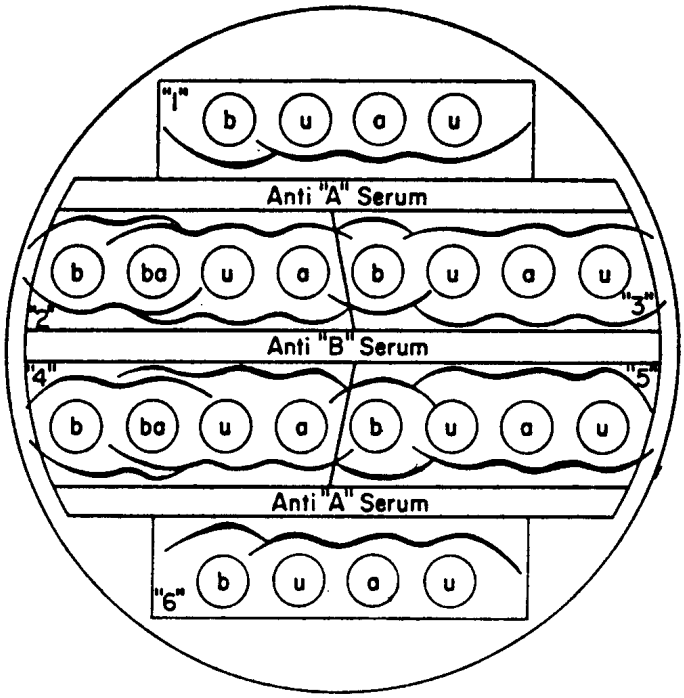

FIG. 5 - Position and reaction sites (6 areas) each consisting of 4 antigen wells. With the exception of areas 2 and 4, antigen placement is identical in each area. Areas 2 and 4 utilize one well each for a mixture of the 2 known antigens (ba), and illustrate precipitin reactions when sample consists of tissues from both species. All areas, except 1 and 6, are positioned to react with both antisera. Interpretation of results from areas 1, 3, 5, and 6 should correlate. Lines enclosing areas indicate portion of plate mounted on slides for preservation. (Fugate and Penn, 1971)

i. Staining Reaction Bands

To keep a permanent record, dialyze to remove free proteins and salts, then dry, stain, and prepare a mount under a cover slip, as follows:

Flood the plate with 500 to 1000 ml pH 7.2 buffered saline in a plastic pan. Replace with fresh buffer twice daily for three days, then once daily for two more days. Finally replace with acidified distilled water and let stand overnight.

Drain off the acidified distilled water, and cut a block of the reaction areas from the agar, and place it onto a 1 x 3 inch (2.54 x 7.62 cm) marked glass slide. Cover the block with a strip of filter paper, and dry in the incubator to a very thin film. Wash gently with a cotton applicator wetted with distilled water to remove adhering bits of the filter paper. Stain the films in acid fuchsin staining solution for 10 minutes. Remove the excess stain and rinse in destaining solution for a period of 15-20 minutes using 2-3 changes, until the agar is clear. Allow the slides to dry, then mount under cover slips with mounting fluid.

j. Photographic Recording of Reaction Bands

One of the easiest methods to obtain a permanent record of the immunodiffusion reaction is to photograph the entire unstained plate. Although there are many ways to achieve this, one of the easiest and quickest is to use a Cordis Immunodiffusion Camera. This is an instrument with preset optics, light source and Polaroid Camera which uses Polaroid Type 084 or 107 black and white film packs. The plate is placed in the instrument, the shutter is tripped, the film tab is pulled from the camera and within 25 seconds an excellent quality black and white print of the immunodiffusion reaction is produced.

21.35 Quality Control Procedures

a. Tissue Extracts from Known Species:

It is extremely important to establish the authenticity of these reference tissues before they are used, since

the basis for the types of immunodiffusion reactions obtained with unknown tissue extracts in the agar gel immunodiffusion test depends upon the use of known species tissue extracts.

b. Prepared Agar Gel Immunodiffusion Plates:

It is usually convenient to prepare a large number of plates at one time for future needs. Care must be taken to prevent deterioration of these plates during storage in the refrigerator. It has been found most useful to stack about 10 plates together in double or triple, air tight, tightly sealed plastic bags. Any plates showing microbial contamination, desiccation, or salt crystal formation should not be used as they will adversely effect the formation of immunoprecipitin lines.

c. The Specific Anti-species Sera: Sera used in the immunodiffusion procedure should always be initially checked for their proper reactivity against known, authentic reference tissues prior to their routine use as a diagnostic reagent.

21.36 Selected References

Fugate, H. G., and S. R. Penn. 1971. Immunodiffusion technique for the identification of animal species. J. Assoc. Off. Anal. Chem. 54:1152-1156.

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Ouchterlony, O. 1968. Handbook of Immunodiffusion and Immunoelectrophoresis. Ann Arbor Science Publishers, Inc., Ann Arbor, MI.

CHAPTER 32. DETECTION AND IDENTIFICATION OF EXTRANEOUS MATERIAL IN MEAT AND POULTRY PRODUCTS.

Mark M. Wheeler and James G. Eye

32.1 General Introduction

Food Safety and Inspection Service (FSIS) is responsible for insuring that the meat and poultry products offered to the consumer are safe, wholesome, unadulterated and truthfully labeled. In fulfilling this responsibility, the Agency's laboratories perform sanitation analyses of the meat and poultry products including investigations for extraneous or foreign materials. According to law, a meat or poultry product is adulterated if it consists in whole or in part of any filthy substance, is for any reason unsound or unwholesome, or if the product was prepared or packed under unsanitary conditions where it may have been contaminated [21 United States Code 601(m)(3)(4), 21 United States Code 453(g)(3)(4)].

Extraneous material is defined as any foreign material found in a food product and associated with objectionable conditions or practices in production, storage, or distribution. Examples of extraneous materials are: filth, metal, glass, sand, wood, paper or plastic. Filth is defined as any objectionable matter contributed by animal contamination of a product such as: rodent, insect or bird matter; or objectionable material contributed by unsanitary conditions. The presence of extraneous material in a food product is not only unappealing but represents a breakdown in good manufacturing practices and could pose a serious health hazard to the consumer. The isolation and identification of extraneous materials sometimes yields evidence that a product was stored or processed under unsanitary conditions and is unfit as human food.

The study of extraneous materials found in food is called Microanalytical Entomology. The U.S. Food and Drug Administration (FDA) and the Association of Official Analytical Chemists (AOAC) have published reference articles, books and methods on this subject. These publications discuss methods of analyses, contaminant identification, and contaminant significance.

This chapter contains the methods developed and used by FSIS Entomology and Extraneous Materials Laboratories (EEML) to isolate and to identify extraneous materials from meat and poultry products. These methods are intended for the stated product and

contaminant. Before using one of these methods on a different product or for a different contaminant, the method must be thoroughly evaluated for that purpose. Aside from the methods developed in our laboratories, FSIS EEMLs use many AOAC methods.

32.2 General Quality Control and Good Laboratory Practices for the Entomology and Extraneous Materials Laboratory

For extraneous materials analyses it is of the utmost importance to maintain a clean and contaminant-free laboratory. All possible action must be taken to prevent the contamination of the sample with insects or extraneous materials. Below are listed general practices and techniques which must be observed in the Entomology and Extraneous Materials Laboratories to insure a quality analysis.

32.21 Equipment and Reagents:

a. Sieves

i. Each analyst should be assigned a sieve. The analyst is responsible for maintaining his/her sieve. The sieve should be cleaned immediately after using it to prevent debris from drying on the sieve.

ii. As specified by the AOAC the #230 sieve should be a plain weave, not a twill weave.

iii. Before beginning an analysis, the sieve should be examined for rips and tears. Small tears can be mended with a drop of solder and will not affect the usefulness of the sieve. Sieves with tears and holes should not be used.

iv. The sieve should be backwashed by spraying water through the bottom of the sieve to remove any debris in the sieve.

v. Annual Cleaning - The sieves should be cleaned once a year (more often if needed) by the following procedures:

(1) Soaked in a 5% aqueous pancreatin suspension, at pH 8.2-8.5 for 4-5 h at 37-40^{o}C.

(2) Soaked in a 10% EDTA (tetrasodium ethylenediaminetetraacetic acid) for 2-3 h at 40-50°C.

(3) Soaked in a 10% NaOH (w/v) for 2-3 h at 80-90°C.

b. Magnetic Stirrers

i. Magnetic stirrers should be stored in a clean plastic container with lid. This should protect the stirrer from picking up metal fragments while not in use. The interior of this container should be kept clean.

ii. Magnetic stirring bars can be cleaned by removing the large particles with forceps and small filings by soaking in a "aqua regia"† solution (a 1:3 mixture of nitric acid and hydrochloric acids).

c. Filter Paper

Filter paper should be stored in a container that will protect it from extraneous materials contamination. A petri dish or a small plastic sandwich container with a tight fitting lid would be ideal. Of course, this precaution is worthless if the analyst does not replace the lid and leaves the filter paper container on the lab bench uncovered for extended periods of time. As with the container for the magnetic stirrers, the container for the filter paper must be kept clean.

d. Laboratory

The entomologists, technicians and aides will routinely:

i. Wipe the lab bench and the work area with a damp sponge before beginning an analysis.

ii. Clean the lab and the microscope room thoroughly at least once a month. This should include wiping down all benches, table tops and tops of any refrigerators or ovens, and cleaning or vacuuming all window sills. DO NOT clean up the laboratory while analyzing samples.

e. Glassware

i. Avoid use of plastic beakers, funnels, graduated cylinders, etc. because insect fragments and hairs adhere to plastics.

ii. After cleaning glassware allow it to dry in an inverted position. Store glassware inverted or cover the opening with aluminum foil. When it is not possible to store the glassware inverted or to cover it, the analyst should rinse the glassware with water prior to use.

f. Trap Rods

Clean the trap rod with soap and water after use.

g. Balances

i. All balances should be inspected and serviced by a trained service technician once a year.

ii. Every month the lab analyst should clean the exterior of the balance, level and check the accuracy of the balance with a 50 g calibration weight.

h. Microscopes

i. All microscopes should be inspected and serviced by a trained service technician on a yearly basis.

ii. Each analyst should be assigned a microscope and will be responsible for the daily maintenance of that instrument. The analyst will clean the exterior surface of the microscope, the eyepieces and the illuminators.

i. Reagents

i. Before mixing reagents, be sure to clean the top of the reagent bottles to prevent contaminants from falling into the solution.

ii. Rinse out carboys before preparing solutions.

iii. Label reagents with the "date prepared" and the "expiration date" (if the later is applicable).

iv. Request the "Certification of Analysis" for chemicals, such as paraffin oils.

j. Sample Handling Procedures

When opening the sample container maintain control of the closure mechanism. Remove rubber bands from bags. Do not cut or otherwise break rubber bands. Remove the staples from bags and paperwork. Do not pull open bags sealed with staples or rubber bands.

32.22 Laboratory Quality Control

a. Air Quality

A petri dish with filter paper wetted with glycerin should be left exposed for 24 h in the laboratory to detect any air borne contaminants. Place these petri dishes on the lab bench, in the fume hood, and near a window. Examine microscopically at 30X. Perform once a week. Record the results of this examination in a bound "Quality Control Notebook".

b. Water Quality

Sample the tap water (hot and cold) by running the water through a #230 sieve for 15 minutes. Wash the trappings from the sieve on to filter paper and examine microscopically at 30X. Perform this analysis once a week. Record the results in a bound "Quality Control Notebook".

c. Hairs and Fibers

The analyst should prepare microscope slide mounts of their head, arm, and eyebrow hair and be able to recognize their own hair from other hair. Include a slide mount with facial hair, if applicable. Analyst should prepare microscope slide mounts of fibers from personal clothes which have a loose knit and could fall into a product. All of these slides should be maintained in the lab as a record.

Part A

32.3 DETECTION OF LIGHT FILTH IN PREPARED INFANT FOODS CONTAINING MEAT AND POULTRY

Mark M. Wheeler and Barbara Bennett

32.31 Introduction

The presence of any objectionable animal material in a food product is defined as filth. Oleophilic filth is defined as light filth. Examples of light filth include insects, insect fragments, hairs, and feather barbules. These adulterants can be detected in a food product by separating them from the food in the oil phase of a oil/aqueous mixture.

The methods described here isolate insect fragments and rodent hairs from prepared baby food containing meat or poultry. Bovine hairs and feathers can also be recovered from the pure meat and poultry. The product is digested in a hydrochloric acid solution and the solubilized material is washed through a #230 sieve. In a pure meat/poultry product, the meat tissue is totally digested and can be washed through the sieve. The material remaining on the sieve can be transferred directly to filter paper. In the baby food dinners, meat products combined with cereals or vegetables, the plant material is not completely digested and thus does not pass through the sieve. In this case, a light filth flotation using paraffin oil is necessary to separate the filth material from the plant material. This flotation step will provide cleaner filter paper, thus easier and more accurate enumeration of the light filth.

32.32 Equipment:

a. Laboratory Balance,
b. Beaker, 2 L
c. Beaker, 600 ml
d. Wildman trap flask, 1 L
e. Hot plate, magnetic stirring
f. Sieve, stainless steel, U.S. Standard No. 230
g. Magnetic stirrer bar, teflon coated (1 X 5 cm)
h. Buchner funnel
i. Vacuum Pump
j. Watch Glass for a 2 L beaker
k. Petri Dish (2), 100 X 10 mm

l. Filter Paper, S&S #8 Ruled
m. Stereoscopic Microscope, 10 - 30X
n. Trap Rod
o. Aerator, local hardware store
p. 1 L graduated cylinder
q. 50 ml graduated cylinder
r. 25 ml graduated cylinder

32.33 Reagents

a. Igepal CO-730 (nonionic detergent, active at low pH) available through GAF Corporation, 140 W. 51st St. NY, NY 10020
b. Concentrated Hydrochloric acid (HCl) †
c. Tergitol #4, Sigma Chemical Co.
d. 40% isopropanol in filtered, distilled water.
e. Paraffin oil (Saybolt viscosity 125/135) Sargent - Welch
f. Glycerin/Ethanol mixture (vol:vol 1:1)
g. Sodium Bicarbonate

32.34 Procedure for Meat and Poultry

a. Preparation - Wash the exterior of the jar, particularly around the lid, to remove any contaminants which may be drawn into the jar upon opening.

b. Quantitatively transfer contents of one jar strained or one jar junior infant food to a two liter beaker with distilled water. Be sure to rinse the inside of the lid into the beaker.

c. Bring volume to around 800 ml with distilled water.

d. Add 5 ml Igepal CO-730 and 45 ml concentrated HCl with stirring. Cover with watch glass.

e. Bring to a boil and boil for 30 minutes.

f. Transfer the hot mixture to a 230 mesh sieve and wash with a forcible stream of hot aerated tap water until washings are clear and acid is removed. Wash the remaining material to one side of sieve. Retain the washings in a pan to neutralize at a later time with sodium bicarbonate.

g. Add 2 ml of "Tergitol #4" to contents of sieve and wash with forcible stream of hot aerated water, until foaming subsides. Wash all the remaining residue to one side of sieve.

h. Wash the contents of the sieve onto lined filter paper in a buchner funnel with isopropyl alcohol. Wash down the sides of the filter paper. Aspirate the paper to near dryness.

i. Add a small amount of glycerine/ethanol (32.33 f) to a petri dish. Using forceps, remove the filter paper from the buchner funnel and place in the petri dish.

j. Examine microscopically at 30X. (See Section 32.36)

32.35 Procedure for Baby Food Dinners

a. As an initial preparation, wash the exterior of the jar, particularly around the lid, to remove any contaminants which may be drawn into the jar upon opening.

b. Quantitatively transfer contents of one jar strained or one jar junior infant food to a two liter beaker with distilled water. Be sure to rinse the inside of the lid into the beaker.

c. Bring volume to around 800 ml with distilled water.

d. Add 5 ml of Igepal CO-730 and 45 ml of concentrated HCl with stirring. Cover with watch glass.

e. Bring to a boil and boil for 30 minutes.

f. Transfer the hot mixture to a 230 mesh sieve and wash with a forcible stream of hot aerated water until washings are clear and acid is removed. Wash the remaining material to one side of sieve. Retain the washings in a pan to neutralize at a later time.

g. Add 2 ml of Tergitol #4 to contents of sieve and wash with a forcible stream of hot water, until foaming subsides. Wash the remaining material to one side of sieve.

h. Transfer contents quantitatively to a 1 L Trap Flask with 40% isopropanol.

i. Bring volume of the liquid in the flask to 500 ml with 40% isopropanol.

j. Bring to a boil and continue simmering boil for 5 minutes with magnetic stirring.

k. Remove from heat and let stand for 1 minute. Insert trap rod into flask. With disc held just below liquid surface slowly add paraffin oil (29.33 e) by pouring it slowly down the trap rod.

l. Stir magnetically for 3 minutes at a speed sufficient to draw a vortex to the stirring bar without splashing and without introducing air into the liquid.

m. Allow the mixture to stand for 1 minute.

n. Fill the flask to the neck with 40% isopropanol by pouring slowly down the trap rod with the disc just below the oil layer.

o. Allow the mixture to stand for 20 minutes. Resuspend the material at the bottom of the flask by turning the flask in a clock-wise or counter clock-wise direction on the bench at 5 and 10 minutes to release any trapped oil, taking care not to disturb the oil layer.

p. Trap off the oil layer into a 600 ml beaker. With 40% isopropanol, rinse the neck of flask and stem of trap rod and pour rinsings into same beaker. Repeat rinsing procedure as necessary.

q. Pour the contents of the above beaker on to lined filter paper in a buchner funnel. Rinse the beaker with 100% isopropanol until all the oil is gone.

r. Wet a petri dish with a small amount of glycerine/ethanol and place the filter paper on this dish.

s. Examine microscopically at 30X. (See Section 32.36)

t. Add 25 ml of paraffin oil to the flask.

u. Slowly push the oil into the aqueous phase with the trap rod. Continue to slowly plunge up and down, about 1/2 the height of the flask, for 1 minute. Be careful not to introduce any air into the liquid.

v. Fill the flask up to the neck with 40% isopropanol and let stand for 15 minutes. Turn the flask at 5 and 10 minute intervals to release any trapped oil drops.

w. Trap off the oil layer into a 600 ml beaker. With 100% isopropanol, rinse the neck of flask and stem of trap rod and pour rinsings into the same beaker. Repeat rinsing procedure as necessary.

x. Continue as in Steps q & r. Examine at 30X. (See Section 32.36)

32.36 Results

The lined filter paper should be examined line by line at 30X magnification. Identify and count any hairs and insect fragments observed. Report the following:

- whole or equivalent insects (adults, pupae, maggots,
- larvae, cast skins)
- insect fragments, identified
- insect fragments, unidentified
- aphids, scale insects, mites, spiders, psocids, thrips,
- etc. and fragments of the above.
- rodent hairs (state the length of the hairs)

32.37 Quality Control

See Section 32.22

32.38 Safety Caution

† Do not dispose of hazardous waste by pouring down sink drains. Collect in separate containers and dispose of this waste according to standard waste management procedures for your laboratory.

Use caution when working with hydrochloric, other acids and strong bases. Wear goggles and gloves to protect eyes and skin when preparing the solution and when moving and wet

sieving the sample. Digest and wet sieve samples under a safety hood.

Part B

32.4 DETECTION AND IDENTIFICATION OF EXTRANEOUS MATERIALS IN NON-MEAT FOOD INGREDIENTS - MACROSCOPIC EXAMINATION

James G. Eye and Mark M. Wheeler

32.41 Introduction

A food is considered adulterated if "it consists in whole or in part" of any filth or decomposed substance or if the food is "otherwise unfit for human food." Extraneous materials detected in the ingredients indicate the product was prepared under unsanitary condition where it may have become contaminated. The presence of extraneous materials in the product ingredients would render the final product adulterated. The purpose of this procedure is to presumptively determine the presence of rodent excreta, insects, insect webbing, mold and other extraneous materials in the dry non-meat food ingredient. This method is intended as a screening procedure. A vast majority of samples analyzed by this procedure will be free of extraneous materials.

This method will allow for prompt examination of samples by all FSIS laboratories and insure that compliant samples are reported promptly to the operating inspectors. This procedure will also reserve the analytical time the analyst has for the smaller number of non-compliant samples that will require more time consuming analyses. This method is recommended only for screening; all positives or apparently violative samples are to be confirmed by AOAC or other accepted microscopic methods.

32.42 Terms and Concepts

The following terms are used in the macroscopic examination and reporting results:

Thrus: Any material going through the sieve.

Overs: Any material remaining on the sieve after sieving.

Animal Contaminated: Any material showing animal excreta or evidence of rodent or other animal chewing or gnawing.

Insect Infested: Any non-meat ingredient that contains live or

dead insects, webbing, excreta, or definite evidence of insect feeding.

Miscellaneous Extraneous Material: Includes stones, dirt, wire, string, non-toxic foreign seeds, etc.

Moldy: A product bearing any evidence of mold.

Rodent Excreta: Excretory pellets of the black rat (*Rattus rattus*), Norway rat (*Rattus norvegicus*) or the house mouse (*Mus musculus*), or pieces/fragments thereof, as determined by the presence of murine rodent hairs in the matrix of the fecal material.

Other Animal Excreta: Any excretory product, other than rodent, as identified by microscopic examination.

Whole Insects: Includes an adult insect, a pupa, a larva, or a major portion thereof.

32.43 Equipment:

a. Jones Riffle Sampler (8261-C10 Arthur H. Thomas)
b. Balance, Top-loading, 1 kg capacity
c. Balance, analytical, 500 g capacity
d. Sieves, U.S. Standard Series (4-881 Fisher Scientific Co.) 3 1/2 through 20
e. Magnifier-Lamp (L6039-2 Scientific Products) (LUXO-LFM2FE)
f. Trays, Cutting, (62686-363, VWR Scientific)

32.44 Procedure

32.441 Examination of Ground Spices

a. Mix sample received by passing through a riffle sampler 4 times, recombining separations before each pass.

b. Separate approximately 200 g of sample and weigh.

NOTE: Retain excess sample for use in confirmatory analysis, if needed.

c. Sift sample portion-wise through a #20 sieve, retain "thrus".

d. Transfer "overs" to a cutting tray and spread evenly so that all material can be observed.

e. If sufficient material is present to preclude spreading, place material on one side of tray and move portion-wise to middle.

f. Examine the middle portion at 3 to 5 magnifications with strong, even light. (A bench-top magnifier-lamp is suitable for this purpose).

g. Note and identify (if possible) all categories of extraneous material observed.

h. If confirmatory analysis is needed, place "thrus" and "overs" into a plastic bag along with the excess sample.

i. Send all portions for confirmatory analysis to an advanced reference laboratory, if available and necessary.

j. A written report of the extraneous material observed should accompany samples submitted for confirmation.

32.442 Examination of Whole Spices, Seeds, and Large Flake Leafy Spices

a. Mix sample received by passing through a riffle sampler 4 times, recombining separations before each pass.

b. Separate approximately (200 g for whole spices and seeds; 50 g for leafy spices and herbs).

c. Sift sample portion wise through a sieve of such size that more of the whole spices are retained as "overs".

NOTE: The sieve should never be of a smaller opening size than "Tyler Standard #8" or USA Standard 2.36 mm".

d. Transfer both "thrus" and "overs" separately on to a cutting tray and spread evenly so that all material can be observed.

e. Examine both portions at 3 to 5X magnification under strong, even light. (A bench-top magnifier lamp is suitable for this purpose).

NOTE: If sufficient material is present to preclude obtaining a single layer, place all material on one side of the tray and move portion-wise to middle and examine.

f. Note and identify (if possible) insects, insect damage, other filth and extraneous material observed.

g. If confirmatory analysis is needed place "thrus" and "overs" into a plastic bag along with the excess sample.

h. Send all sample portions for confirmatory analysis to an advanced reference laboratory, if available and necessary.

i. A written report of the extraneous material observed should accompany samples submitted for confirmation.

32.45 Reporting Results

a. Identify all categories of extraneous materials observed and record the quantity in each category.

b. To report the results as "percent extraneous material by weight", transfer the extraneous material to a tared dish and weigh.

Use the following formula to calculate the percentage:

$$\text{Percent}(\%) = \frac{\text{extraneous material (gm) in category X 100}}{\text{Sample Weight}}$$

32.46 Criteria for Confirmatory Analysis

These criteria are presented as internal guidelines to assist the analyst trained for macroscopic analysis in determining whether or not a sample should be subjected to a more extensive examination. Each type of contamination observed should be considered both on its own sanitation significance and in conjunction with other observations reported by the field or seen by the analyst.

Any sample exhibiting the following characteristics must be confirmed by analysts trained in more sensitive microscopic or chemical analytical techniques:

a. Any sample showing evidence of active or current infestation with insects and/or other animal contamination.

b. Any sample of whole seeds, herbs or other spice material that exhibits evidence of mold and/or insect damage.

c. Any sample that appears to contain in excess of 0.5% by weight of any non-hazardous extraneous material (stones, soil and non-toxic seeds).

d. Any sample appearing to contain animal excreta, including insect excreta identified during macroscopic examination.

32.47 Quality Control and Quality Assurance

See Section 32.22

Part C

32.5 DETECTION OF GLASS AND NON-ALUMINUM METALS IN MEAT AND POULTRY PRODUCTS

Mark M. Wheeler

32.51 Introduction

Meat and poultry products are exposed to a wide variety of materials during processing and packaging. Due to faulty processing, breakage in machinery or improper handling, pieces of the processing equipment or packaging material can be introduced into the finished product. The presence of extraneous materials in a finished product may pose a serious health risk to all consumers.

This method provides a fast, simple, and reliable means for isolating glass or metal contaminants from meat and poultry products. The sample is digested in an alkaline solution. The glass and non-aluminum metals are unaffected by the digestion. These contaminants are separated from other undigested material in a brine solution.

The laboratory equipment used in the analysis will depend on the type of contamination. When the suspected contaminant is glass, use of laboratory glassware in analysis must be avoided. Similarly, when the contaminant is suspected to be a metal, use of metal utensils and containers should be avoided. This will serve to protect the integrity of the sample during analysis.

32.52 Reagents and Material

32.521 Reagents

a. 7% Alcoholic Potassium Hydroxide (KOH) †

Dissolve 7 g of KOH in 100 ml of 95% Ethyl Alcohol

NOTE: KOH pellets can be used.

b. Sodium Chloride (NaCl) Solution

Prepare 2 L of NaCl solution at room temperature by adding 300 g of NaCl/L of distilled water.

c. Tergitol #4

d. Glycerol/Ethanol Mixture (vol:vol 1:1)

32.522 Materials

32.5221 For Metallic Contaminants

a. Heavyweight Plastic Picnic knives and forks or 40 lb.test Monofilament
b. Magnetic Stirring Hot Plate and Magnetic Bar (AOAC XIV 44.002 n)
c. #230 Sieve (AOAC XIV 44.002 r)
d. Filter Paper (AOAC XIV 44.002 i)
e. Hirsch Funnel with Screen (AOAC XIV 44.002 k)
f. 2 L Beaker, glass
g. 600 ml "tall" beaker, i.e. Pyrex #1060
h. 2 L Graduated cylinder
i. Watch Glass for a 2 L beaker

32.5222 For Glass Contaminants

The equipment is the same as above except do not use glassware in analysis and substitute with the following for glass beakers:

a. Stainless Steel Beaker with 2 L capacity
b. Reusable Plastic Beaker with 600 ml capacity (Nalgene Polypropylene #1201)
c. Polypropylene Graduated Cylinder
d. Plastic Basin to cover 2 L beaker, ie. Nalgene #69010040

32.53 Procedure

a. Cut sample to be digested into 1" x 1" pieces to facilitate the digestion process (Use plastic utensils or monofilament if examining for suspected metal contamination).

b. Weigh 225 g sample into a 2 L beaker.

c. Add 1.5 L 7% Alcoholic KOH. (see Section 32.56, Safety Caution)

d. Cover with a watch glass. Heat to a boil while stirring on a stirring hot plate until the sample is completely

digested. (Approximately 1 h). Initially, it will be difficult to stir the sample magnetically but after 10 min at medium to low heat the sample will be sufficiently digested to permit magnetic stirring.

e. Transfer sample to a No. 230 sieve. Apply a moderately forceful stream of hot water to push digested residues through sieve. Retain washings in a pan for hazardous chemical disposal.

f. If there is only a little residue present, transfer this directly to filter paper and examine microscopically.

g. If large amounts of undigested material remain, add 2 ml of Tergitol to help solubilize remaining residues. Repeat washing until suds subside. Transfer sample to a "tall" 600 ml beaker with distilled water.

h. Add 400 ml NaCl solution.

i. Wait 30 seconds, then pour off suspended material. Be careful not to disturb or pour off material on bottom.

j. Repeat steps h and i.

k. Wash the material remaining on bottom of beaker onto ruled filter paper with distilled water and examine microscopically.

NOTE: Check the magnetic stirring bar for metal contamination.

32.531 Procedure for Index Sample

a. If an index sample of the contaminant is available, put a portion of the index sample in the 7% Alcoholic KOH Solution. (see Section 32.56 Safety Caution)

b. Bring the solution to a boil and examine the index sample noting any chemical reaction it may have undergone.

c. Repeat the boiling and examine again. If the sample reacts with the solution, do not use an alkaline digestion. Use an acid or enzymatic digestion instead.

32.54 Result

The lined filter paper should be examined line by line at 30X magnification. Report the following:

a. Metal

- **Count the number of pieces of metal recovered.**
- **Record the size or the number of contaminants within a size range.**
- **Provide a general description of the contaminants recovered. Note the shape, thickness, color or discoloration, magnetism, and surface markings.**

b. Glass

- **Count the number of fragments recovered.**
- **Record the size or the number of contaminants within a size range.**
- **Provide a general description of the contaminants. Note the presence or absence of the following characteristics: very thin, cube shaped, mold markings, rounded edges, smooth curved surfaces, color.**
- **Examine suspected fragments under polarized light to determine if they are isotropic.**

32.55 Quality Control

See Section 32.22

a. In step 32.53 e, be sure to wash the heavy contaminants from the bottom of the beaker to the sieve. Heavy contaminants settle quickly to the bottom of the beaker and an ample stream of water is needed to wash them from the beaker. The beaker should be inverted over the sieve and the material in the beaker should be washed into the sieve with a gentle stream of water.

b. Check the magnetic stirring bar for small magnetic filings before beginning analysis.

32.56 Safety Caution

† Do not dispose of hazardous waste by pouring down sink drains. Collect in separate containers and dispose of as

hazardous waste as per standard waste management procedures for your laboratory.

Use caution when working with potassium hydroxide. Wear goggles and gloves to protect eyes and skin when preparing the solution and when moving and wet sieving the sample. Digest and wet sieve samples under a safety hood.

Part D

32.6 METHOD FOR THE ISOLATION OF GLASS FROM PREPARED MEAT AND POULTRY BABY FOODS

Mark M. Wheeler

32.61 Introduction

The recommended procedure for isolating glass from a food product is the heavy sediment procedure for that product. There is no heavy sediment procedure for meat/poultry baby food products. The method outlined below was developed in response to the need for a standard procedure for isolating glass from meat/poultry baby food products.

Bottled food can become contaminated with glass in a number of ways. The container may already be contaminated when it arrives at the food processors. The finished food product may become contaminated by glass breakage during processing. Containers can break during storage, shipping, retail, and consumer handling and fragments from broken containers can contaminate the exterior of other containers. If these exterior contaminants are in or around the jar opening, they could contaminate the product when the jar is opened.

This method is quick and easy. The sample is washed in a #60 sieve. The bulk of the sample is washed through the sieve. The remaining material is transferred to a beaker and mixed with a brine solution. In a brine solution the heavy contaminants, such as glass, settle to the bottom of the beaker. The brine solution and the suspended food material are poured off and discarded. These two steps, adding and pouring off the brine solution, are repeated three times. The heavy contaminants remaining on the bottom of the beaker are washed on to a filter paper which is examined microscopically. This isolation procedure takes less than 15 minutes. Suspect particles must be tested to confirm that they are in fact glass.

To protect the integrity of the sample, no glass should be used in any part of this method. After adding the brine solution to the plastic beaker containing the sample, any glass fragments will settle to the bottom within seconds. The longer the settling step, the more food material settles to the bottom which creates dirty

plates. Ten seconds is plenty of time for glass fragments to settle to the bottom.

32.62 Reagents

a. Sodium Chloride

32.63 Equipment

NOTE: DO NOT USE ANY GLASS APPARATUS DURING THE ANALYSIS.

a. Plastic Beaker w/ 600 ml capacity, ie. Nalgene #1201
b. Plastic Beaker w/ 2 L capacity, ie. Nalgene #1201
c. Plastic Basin to cover 2 L beaker, ie. Nalgene #69010040
d. Plastic Graduated Cylinder
e. #60 Sieve (AOAC 16.1.01(B)(r) 16th Ed.)
f. #230 Sieve (AOAC 16.1.01(B)(r) 16th Ed.)
g. Magnetic Stirrer and Bar (AOAC 16.1.01(B)(n) 16th Ed.)
h. Ruled Filter Paper (AOAC 16.1.01(B)(i) 16th Ed.)
i. Disposable Petri Dish (100 X 10 mm)
j. Lab Spatula
k. Hirsch Funnel
l. Side arm trap flask connected to vacuum pump
m. Laboratory Balance, 1 kg capacity
n. Compound Microscope with polarizer
o. Aerator, Water (AOAC 16.1.01(B)(a) 16th Ed.)

32.64 Reagent Preparation

a. Sodium Chloride Solution (300 gm/L)

Add 2 L of distilled water to 2 L plastic beaker. Add 600 g of NaCl while magnetically stirring. The above recommended plastic beaker will accommodate 2 L of salt solution. Cover beaker with plastic basin and continue stirring until NaCl is completely dissolved.

32.65 Procedure

32.651 Cleaning of Exterior of Sample Container

a. Thoroughly rinse exterior of jar and around lid on to a #230 sieve using hot water. Use the spatula to clean food residues from lid and jar threads. This step is included to be sure no glass is on exterior of the jar.

b. Wet filter paper with water and center it in Hirsch funnel. Turn on vacuum and fit filter paper to sides of funnel.

c. Transfer material on the sieve to filter paper with distilled water. Do not aspirate the paper to dryness otherwise the glass fragments will "pop" off the paper. If needed, wet the paper with a drop or two of water. The paper should be moist enough so that it adheres to the petri dish but it should not be soaked. If the paper is too wet, the water will hide small, flat pieces of glass.

d. Transfer filter paper to petri dish and examine paper microscopically for glass fragments.

e. Confirm any suspect particles using a compound microscope with polarized light.

f. Count, measure and describe all glass fragments found on the exterior of container. Report any particles of glass as contaminants found on the exterior of the sample container. Report number of fragments found within a size range. Fragments less than 1 mm can be reported as " Less than 1 mm." An excessive number of fragments can be reported as "Too Numerous To Count."

32.652 Sample Analysis

a. Quantitatively transfer contents of jar to #60 sieve. A spatula can be used to remove the bulk of the sample. Use water from a squirt bottle to thoroughly rinse interior of jar. Retain jar for further examination at step l.

b. Thoroughly wash sample in sieve with hot aerated water. When no more material passes thru sieve, wash remaining material to one side of sieve.

c. Quantitatively transfer contents of sieve to a plastic beaker w/ distilled water. Use no more than 200 ml of water.

d. Dilute to 400 ml with NaCl solution.

e. Let material settle 10 seconds, then pour off suspended material. More than 10 seconds is not needed. Glass will settle to bottom in 10 seconds. Waiting longer than 10 seconds allows more food material to settle to the bottom. Be careful not to disturb or pour off residues on bottom.

f. Repeat Steps d & e to remove excess plant material, usually twice more.

g. Wet filter paper with water and center it in Hirsch funnel. Turn on vacuum and fit filter paper to sides of funnel.

h. Wash residues remaining on bottom of beaker to ruled filter paper. Do not aspirate the paper to dryness otherwise the glass fragment will "pop" off the paper. If needed, wet the paper with a drop or two of water. The paper should be moist enough so that the paper adheres to the petri dish but the paper should not be soaked. If the paper is too wet, the water will hide small, flat pieces of glass.

i. Transfer paper to petri dish and examine microscopically.

j. Count, measure, and describe all glass fragments found in the food product. Report number of fragments found within a size range. Fragments less than 1 mm can be reported as " Less than 1 mm." An excessive number of fragments can be reported as "Too Numerous To Count."

k. Confirm any suspected particles using a compound microscope and polarized light. Glass is an isotropic compound and will not transmit crossed polar light. Sand or quartz are birefringent, thus will transmit crossed polar light.

l. Examine all retail sample jars for chips, fractures, or other defects if any glass fragments are found within product.

m. Maintain in reserve all glass fragments and all jars from which glass fragments were removed.

32.66 Characterization of Contaminants

If classification or comparison of glass contaminants is needed to identify a possible source, determine the refractive index of the glass contaminants.

32.67 Quality Control

See Section 32.22

a. Do not use any glassware in this analysis.

b. Before beginning an analysis, wipe down or wash the entire work area.

c. Rinse the beakers and graduated cylinder before using them.

d. Backwash the sieve by spraying water through the bottom to remove any debris in the sieve before using it.

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CHAPTER 33. DETECTION OF ANTIMICROBIAL RESIDUES IN MEAT AND POULTRY TISSUE BY SCREEN TESTS

B. P. Dey, Clarence A. White, Richard H. Reamer and Nitin H. Thaker

33.1 Introduction

Rapid microbiological screen tests are used in slaughter establishments to detect the presence of antimicrobial residues in food animal tissues. The Swab Test on Premises (STOP) is used for all red meat species except bob veal calves, where the Calf Antibiotic and Sulfa Test (CAST) is used. The Fast Antimicrobial Screen Test (FAST) developed recently and tested on bovine tissue, has been found to have greater sensitivity than STOP and CAST. The test is being conducted in bovine slaughter establishments on a limited basis. The FAST procedure is presently being tested in swine.

These microbial inhibition tests are simple to perform, cost effective and allow routine testing and release of large numbers of food animal carcasses in the shortest possible time. Use of these screen tests permit FSIS to analyze only those carcasses which were found to contain antimicrobial compounds by in-plant tests.

PART A

33.2 DETECTION OF ANTIMICROBIAL RESIDUES BY SWAB TEST ON PREMISES (STOP)

Clarence A. White, B. P. Dey and Richard H. Reamer

33.21 Background

The Swab Test on Premises (STOP) was developed for tentative detection of antimicrobial residues in carcasses. It is performed by inserting a sterile cotton swab into the kidney sample of a carcass. After 30 minutes, the tissue fluid soaked top, one-fourth portion of the swab is transferred to an agar plate seeded with *Bacillus subtilis* spores. After incubation for 16-18 h at 29°C, plates are examined for a zone of inhibition (ZI) around the swab. If no inhibition is seen, the carcass is free of antimicrobial residue at detectable levels. In case of inhibition, presence of antimicrobial residues is suspected and muscle, liver and kidney tissues from the suspect carcass are collected and submitted for confirmation and identification at FSIS laboratories.
In 1980, a modified version of the original test was introduced in slaughter establishments. The agar plates and vials of spores are

separately supplied. In the modified version, prior to performing the test, the plates are surface streaked with the spore suspension by sterile swabs. The rest of the procedure is similar to the original test. The supplies are now commercially available and are stable for 6 months when stored at either room or refrigerated temperatures.

Initially when the test was developed, it used tissues from kidney, liver, muscle, and injection site. However, at present kidney is the target tissue. The sensitivity of the STOP test for sulfonamide detection is unsuitable for regulatory purposes.

33.22 Equipment, Reagents and Supplies

33.221 Equipment

a. Laminar Flow Hood or equivalent clean room
b. Sorvall RC5C Refrigerated Centrifuge, Sorvall Rotor SS-34, and Sorvall Swinging Bucket Rotor HB-4 or equivalent. Centrifuge must be able to operate at 20,000 x G at a constant 5°C. It should also operate with a swinging bucket rotor at 1,500 x G at room temperature or equivalent.
c. Virtis homogenizer, Model 60K or equivalent
d. Sterile Virtis jars
e. Vortex mixer or equivalent
f. Incubators, one maintaining 37°C and the other 29°C
g. Precision water bath (48 ± 1°C) with cover (Model 183) or equivalent
h. Quebec Colony Counter or equivalent
i. Fisher-Lilly Antibiotic Zone Reader (Fisher Scientific, Cat. No. 07-906) or equivalent

33.222 Reagents

a. Distilled water:

The distilled water must be prepared using an all glass still (Corning Megapure 6L or equivalent) and stored in a glass or any acceptable reservoir which is not a part of the system. All spore lots must be prepared using glass distilled water.

* Do not use deionized water.

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* Resins of some systems produce quaternary ammonium compounds which interfere with the analysis.

b. Phosphate buffer (3 M, pH 7.1)

Dissolve 306.9 g of K_2HPO_4 and 168.6 g KH_2PO_4 per liter of

distilled water. If necessary, adjust pH by dropwise addition of either 0.1 N HCl or NaOH depending upon pH reading. Sterilize by autoclaving at 121°C for 15 minutes or filtering through a 0.2 μm filter.

c. Ethyl alcohol (USP grade, 200 proof)

Dehydrated Alcohol, USP, Ethyl Alcohol, 200 Proof Punctilious®, (Ethyl Alcohol [Ethanol] CAS #64-17-5, Warner-Graham Company, 160 Church Lane, Cockeysville, MD 21030). For a 50% solution, mix 1 part of ethyl alcohol with 1 part glass distilled water. Prior to use, filter sterilize through a 0.2 μm filter.

d. Polyethylene glycol, Mol. Wt. 4000 (Baker Chemicals) Sterilize in a covered beaker by autoclaving prior to use.

e. Butterfield's Phosphate Buffer, sterile

33.223 Supplies

a. Sterile Roux bottles
b. Sterile glass beads, 4 mm diameter
c. Sterile 100 ml graduated glass stoppered cylinders or volumetric flasks
d. Sterile centrifuge tubes, 40 ml (Nalgene 3118 or equivalent)
e. Sterile pipettes, 10 ml and 1 ml graduated to the tip
f. Sterile, clear glass vials 51 x 15 mm with deep seated screw caps
g. Pressure sensitive labels not to exceed 2" x 1/2"
h. Acetate shrink-wrap material for sealing 51 x 15 mm glass vials or equivalent closure material
i. Forceps
j. Permanent marking pen
k. Antimicrobial sensitivity discs containing 5 mcg of the antibiotic neomycin (N5)
l. Sterile cotton swabs on hollow plastic tubes
m. Sterile, plastic 60 mm diameter X 15 mm petri plates (Falcon Cat. # 1007 or equivalent)

33.23 Media

a. Brain Heart Infusion broth (BBL or equivalent); reconstitute according to manufacturer's directions, dispense 10 ml/tube and sterilize (121°C for 15 minutes)
b. Blood agar plates (Columbia Blood Agar Base, 5% HRBC)
c. Antibiotic Agar No. 5 (Streptomycin Assay Agar)
d. Mueller-Hinton Agar

e. A-K Sporulating Agar No. 2

i. Agar slants - reconstitute A-K Sporulating Agar No. 2 according to manufacturer's directions with an extra 0.5% Purified Agar (Difco or equivalent), sterilize by autoclaving at 121°C for 15 minutes and prepare as slants.

ii. Roux bottles - add 300 ml reconstituted A-K Sporulating Agar No. 2 with an extra 0.5% Purified Agar. Sterilize (121°C for 15 minutes) and allow medium to harden in Roux bottles placed in a horizontal position.

33.24 Test Organism

Bacillus subtilis ATCC 6633 (American Type Culture Collection, Rockville, MD)

33.241 Purity and Biochemical Properties of *Bacillus subtilis*

a. Reconstitute a lyophilized culture in Brain Heart Infusion broth and incubate at 37°C for 18 h. Streak blood agar plates with the broth culture, incubate at 37°C for 18 h and check for culture purity.

b. For isolation, streak the culture onto two Columbia Agar plates with 5% defibrinated horse blood. Incubate at 37°C for 18 h.

c. Prepare a Gram stain of three well isolated colonies. All cultures should be Gram positive.

d. Stain a drop of the spore suspension with malachite green and counterstain with carbol-fuchsin solution. The spores will appear green, whereas the vegetative cells will appear red or pink.

e. Use one Columbia Agar plate with 5% defibrinated horse blood from the culture to test for the presence of catalase. *Bacillus* sp. are catalase positive.

f. Use colonies from the other plate to check biochemical characteristics of the culture by inoculating O-F glucose, Voges-Proskauer, and mannitol broths. Incubate at 35°C for 18 h. The biochemical patterns of *B. subtilis* should agree with the following chart:

Catalase	Gram stain	Spore forming	O-F glucose	Voges-Proskauer	Mannitol
+	+	+	O	+	V

(+) = positive; (-) = negative; (F) = fermentative; (O) = oxidative; (A) = acid; (V) = variable.

g. If the organism does not meet all the above criteria, replace with a new ATCC culture of the organism.

33.242 Preparation of *B. subtilis* spores

a. After the culture meets all biochemical criteria, pick several well isolated colonies from the plates and streak A-K Sporulating Agar No. 2 slants (one per Roux bottle) and incubate the slants at 37°C for 18 h.

b. To each agar slant, add 4-6 sterile glass beads and 2-3 ml sterile distilled water and gently shake for 2 minutes to dislodge bacterial growth.

c. Aseptically transfer the slant suspensions to a Roux bottle containing A-K Sporulating Agar No. 2 and spread with the help of the glass beads. Multiple cultures may be prepared and pooled for transferring.

d. Incubate the Roux bottles horizontally for 18-24 h at 37°C and then at room temperature for the remainder of 1 week (6 days).

e. Harvest the growth from the Roux bottles by adding 20-30 sterile glass beads and approximately 25 ml of sterile distilled water per bottle. Gently agitate bottles to dislodge bacterial growth. (Care must be taken not to break the agar during harvesting).

f. Aseptically transfer the bacterial suspension into sterile centrifuge tubes (40 ml volume) and heat the tubes in boiling water (100°C) for 10 minutes.

g. Wash the heated suspension three times with sterile distilled water by centrifuging and decanting in the following manner:

   i. Centrifuge at 5°C for 20 minutes at 20,000 x G.
   ii. Pour off supernatant.
   iii. Resuspend the pellet in 20 ml sterile distilled water.
   iv. Repeat Steps i, ii and iii two more times.

h. Wash and coat a Virtis jar with a mixture of sterile phosphate buffer and sterile polyethylene glycol in the following manner:

   Mix 34.1 ml of sterile phosphate buffer and 11.8 g of sterile polyethylene glycol in a 100 ml sterile glass stoppered volumetric flask and shake vigorously. Bring to volume with sterile distilled water. Pour the mixture into a Virtis jar and place the jar on the homogenizer. Blend for 5 minutes at 5,000 RPM. Discard the mixture. Repeat the process.

i. Prepare a fresh solution of sterile buffered polyethylene glycol (34.1 ml of phosphate buffer and 11.8 g of polyethylene glycol) in a 100 ml glass stoppered sterile volumetric flask. Add 25 ml of the washed spore mixture and bring to volume with distilled water. Shake vigorously. Pour the mixture into a coated Virtis jar and homogenize for 5 minutes at 5,000 RPM.

j. Dispense the mixture equally into four sterile centrifuge tubes and centrifuge in a swinging bucket rotor at 1,500 x G (3,000 RPM in H-4 Rotor in Sorvall RC5C) for 2 minutes at room temperature.

k. A two-phase system with an interface will be formed in the centrifuge tube. Being careful not to disturb or disperse the interface layer, transfer the spore containing, upper phase using a 10 ml pipette to a second set of sterile centrifuge tubes.

l. Centrifuge the tubes at 20,000 x G for 20 minutes at 5°C. Pour off the supernatant. Resuspend the pellet in each tube with 20 ml sterile distilled water and pool the contents of all tubes into a sterile container.

m. Pipette 25 ml aliquots of spore suspension into each sterile centrifuge tube. Centrifuge tubes at 20,000 x G for 20 minutes at 5°C. Repeat the process five times after decanting the supernatant and re-suspending the

pellet in 20 ml of sterile distilled water.

n. After the last wash step, resuspend each spore pellet in 20 ml 50% ethyl alcohol. Pool all spore suspensions into a sterile bottle containing 15-20 sterile glass beads. Store the stock suspension at 35-40°F (2-4.4°C). (Properly preserved stock spore suspension may be used indefinitely).

33.243 Enumeration of *B. subtilis* Spores in Working Suspension

a. To determine the number of spores/ml in each new spore stock suspension, prepare tenfold serial dilutions (10^{-2}-10^{-10}) of the suspension using Butterfield's Phosphate Buffer. (Pipet 1.0 ml of well mixed spore stock suspension (use vortex mixer) into 9 ml buffer and then make serial dilutions up to 10^{-10}.).

b. Using separate pipettes, pipette 1.0 ml of each dilution into triplicate 100 x 15 mm plates.

c. Pipette 15 ml molten Plate Count Agar (cooled to 50 ± 1°C) into each plate. Mix by swirling or tilting plates to evenly disperse the inoculum throughout the medium. Incubate for 48 h at 37 ± 1°C.

d. Count colonies (30-300) in triplicate plates on a Quebec Colony Counter. Record and average the number of colonies/ml in each dilution. Determine the number of colony forming units (cfu)/ml of the stock solution.

e. To prepare the final spore suspension at a concentration of 1 x 10^6 cfu/ml in 50% ethyl alcohol from the stock spore suspension, use the following formula:

$$\text{Concentration of stock spore suspension (cfu/ml)} = \text{Dilution factor} \times \text{Desired concentration of working spore suspension (cfu/ml)}$$

Example:

Stock spore suspension = 1 x 10^9 spores/ml
Desired concentration of working spore suspension = 1 x 10^6 spores/ml:

$$(1 \times 10^9 \text{ cfu/ml}) = (x)\ (1 \times 10^6 \text{ cfu/ml})$$

$$\frac{(1 \times 10^9 \text{ cfu/ml})}{(1 \times 10^6 \text{ cfu/ml})} = x$$

x = 1000

In this example, the stock spore suspension must be diluted 1:1000 (1 part stock spore suspension plus 999 parts diluent) in 50% ethyl alcohol to prepare the 1 x 10^6 spore/ml concentration.

33.244 *Packaging of *B. subtilis* Spore Suspension (for Field Use)

a. Dispense 4.0 ml of the final (working) spore suspension (1 x 10^6 cfu/ml in 50% ethyl alcohol) into sterile 51 x 15 mm clear, glass vials with deep seated, leak-proof screw caps.

b. After securely capping spore vials, seal with shrink-seal, or equivalent closure material, to prevent leakage or dehydration.

c. Label the vials with the following information on a transparent mylar pressure sensitive label, or equivalent:

i. "STOP spores"
ii. *B. subtilis* ATCC 6633
iii. Lot Number
iv. Packaging Date

NOTE: *B. subtilis* spores (1 x 10^6 or 1 x 10^7 cfu/ml) can also be obtained from EDITEK, Burlington, NC, by special order.

33.25 *Preparation of STOP Plates (for Field Use)

a. Add 25.5 g of Antibiotic Agar No. 5 (Streptomycin Assay Agar) powder into 1 L of glass distilled water. Heat while stirring and bring to a boil. Sterilize at 121°C for 15 min. Cool and mix the medium thoroughly in a 48°C water bath. Continue mixing during cooling and dispensing.

b. Aseptically add 6.0 ml of the agar to each 60 x 15 mm plate and distribute evenly. Place plates on a flat level surface and allow agar to harden.

*NOTE: Under FSIS contract, STOP spores (1 x 10^6 cfu/ml) and plates are now produced commercially and are routinely available for use. After they meet all quality control specifications they are used in

slaughter plants.

c. Label the lid of each plate with the following information:

i. "STOP PLATE"
ii. Lot Number
iii. Expiration Date

d. Refrigerate plates in sealed double plastic bags to prevent moisture evaporation. These plates can be used for a period of 90 days.

33.251 Preparation of STOP Plates (Used in Laboratory)

a. Add 25.5 g of Antibiotic Agar No. 5 (Streptomycin Assay Agar) powder to 1 L of distilled water. Heat while stirring and bring to a boil. Sterilize at 121°C for 15 minutes. Cool and mix the medium thoroughly in a 48°C water bath.

b. Aseptically add 1 ml of 1 x 10^7 cfu/ml *B. subtilis* spore suspension per 100 ml of the agar. Mix thoroughly. Pipette 8 ml of the agar into each 100 x 15 mm plate and tilt plates to insure even distribution. Allow the plates to harden on a flat, level surface.

c. Label the lid of each plate with the following information:

i. "STOP PLATE"
ii. Date

d. Refrigerate plates in sealed double plastic bags to prevent moisture evaporation. These plates can be used for a period of 10 working days.

33.26 Performing the STOP Test

33.261 Sample Condition

a. Assure that the samples are cold, 4°C or below.

b. Identify samples according to standard operating procedures.

NOTE: Presently STOP is used only on kidney tissue of all classes of animals, i.e., bovine, swine, sheep/goat, and horses with the exception of bob veal calves.

33.262 Procedure

a. Allow frozen samples to thaw completely at room temperature for a sufficient period of time such that ice crystals are no longer present within the sample.

b. Open a sterile cotton swab pack, remove one swab, and insert the sharp end of the swab shaft about 1/2" to 3/4" into each kidney tissue.

c. Move the swab shaft back and forth several times to macerate the tissue, disrupting tissue cells and releasing tissue fluid. Remove the swab shaft.

d. Reverse the swab and insert the cotton tip into the tissue opening, twisting to make sure that the cotton tip is in good contact with the macerated tissue.

e. Allow swabs to remain in the tissue for a minimum of 30 minutes.

f. Allow refrigerated plates to warm to room temperature for about 10 minutes before streaking. Check each plate for absence of contamination, cracking of agar or dryness.

g. Lift the plate cover slightly and mark an "X" reference mark on the outer side wall of the plate. Place the covered plate bottom side down on the work place surface with the reference mark at the 12 o' clock position. With a fine-tip permanent marking pen, start at the "x" and draw a line across the bottom of the plate dividing it into two equal sections.

h. Check for seal integrity of vials containing spores.

i. Shake the ***B. subtilis*** spore vial (1×10^6 cfu/ml) and dip a sterile swab in the solution. Gently touch the swab to the side of the vial to remove excess fluid. Replace the screw cap on the vial.

j. Streak the surface of the agar plates with the swab from a point marked on the side of the plate moving up and down and from left to right. Turn the plate 1/4 turn and streak again.

k. Repeat this streaking process 2 more times. Finally turn the plate 1/2 turn and streak. (Use a separate swab for each plate)

NOTE: Above applies only for plates used in the plant. The plates used in laboratories are seeded at a different

concentration level and therefore should not be surface streaked.

l. Place a neomycin 5 μg disc on the agar surface near the vertical line on a plate.

m. Remove the swab from the tissue, break the shaft approximately two inches from the swab end.

NOTE: If the swabs appear dry, reinsert them in the tissue and squeeze the tissue around the swab to absorb tissue fluids. For small portions of dry muscle tissue, moisten swab with distilled water prior to inserting.

n. Gently place the swab on the surface of the plate with the broken end of the shaft near the neomycin 5 μg disc making sure not to break the agar surface. Make sure the swab has uniform contact with the agar.

NOTE: Swabs from two kidney tissues from two different carcasses can be placed on each plate provided they are properly identified on the plate.

If two tissue swabs are used per plate, place cotton tips in "rabbit ears" configuration (Fig. 1)

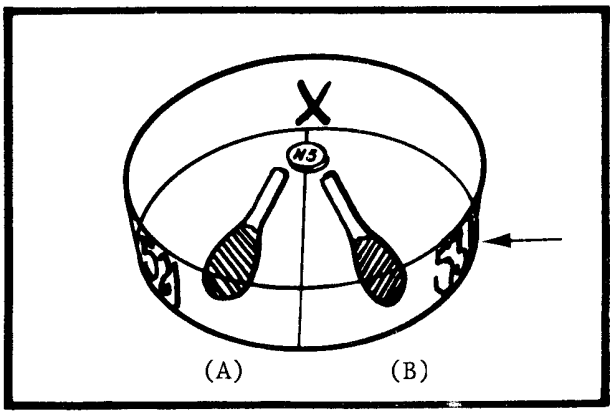

Figure 1. Swab placement on plate

o. Incubate the plates upright at 29 ± 1°C for 16-18 h.

p. Store samples under refrigeration until the test is completed.

33.27 Results and Interpretation

a. Remove the incubated plates from incubator and remove swabs.

b. Measure the ZI by the N5 disc with a mm ruler or with an antibiotic zone reader. The zone should be 20-26 mm wide. If the zone is not 20-26 mm in width, the test is inconclusive and should be repeated.

c. Observe the plates for inhibition of *B. subtilis* growth surrounding the swabs.

i. If a zone of inhibition is observed, the test is positive. Measure the length and the width of the zone and record results.

ii. If no zone of inhibition is observed, the test is negative. Record the result.

33.28 Selected References

Johnston, R. W., R. H. Reamer, E. W. Harris, H. G. Fugate, and B. Schwab. 1981. A new screening method for the detection of antibiotic residues in meat and poultry tissues. J. Food Prot. 44:828-831.

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Read, R. B., J. G. Bradshaw, A. A. Swatzentruber, and A. R. Brazis. 1971. Detection of sulfa drugs and antibiotics in milk. Appl. Microbiol. 21:806-808.

United States Department of Agriculture. 1982. The shelf stable swab test system for detecting antibiotic residues in tissues. Laboratory Communication No. 31. Food Safety and Inspection Service, S&T, Microbiology Division, Washington, D.C.

PART B

33.3 DETECTION OF ANTIMICROBIAL RESIDUE IN CALVES BY CALF ANTIBIOTIC AND SULFONAMIDE TEST (CAST)

Clarence A. White, B. P. Dey and Richard H. Reamer

33.31 Introduction

The Calf Antibiotic and Sulfa Test (CAST) is a modified form of the Sulfa Swab Technique (SST). Sulfonamides are frequently used in bob veal calves, a class of animals weighing under 150 pounds and less than three weeks old. This test is used to detect antibiotic and sulfonamide residues in bob veal calves at slaughter.

The inspectors performing the test at slaughter plants are supplied with agar plates and vials containing an alcohol suspension of spores. To perform the test, a sterile cotton tipped applicator (swab) is inserted into the kidney sample of a bob veal calf and left for 30 minutes to absorb tissue fluids. The agar plates are surface streaked by sterile swabs with the supplied *Bacillus megaterium* spore suspension. The swab is removed from the kidney, broken as close to the cotton tip as possible, and placed on to the agar plate streaked with spores. After 16-18 h incubation at 44°C, plates are examined for a zone of inhibition (ZI) around the swab. If no inhibition is seen, the carcass is free of antimicrobial residues at a detectable level. All carcasses presenting inhibition are subjected to laboratory confirmation.

33.32 Equipment, Reagents and supplies

33.321 Equipment

a. Laminar Flow Hood or equivalent clean room
b. Sorvall RC5C Refrigerated Centrifuge, Sorvall Rotor SS-34, and Sorvall Swinging Bucket Rotor HB-4 or equivalent. Centrifuge must be able to operate at 20,000 x G at a constant 5°C. It should also operate with a swinging bucket rotor at 1,500 x G at room temperature.
c. Virtis homogenizer, Model 60K or equivalent
d. Sterile Virtis jars
e. Vortex mixer or equivalent
f. Incubators 37°C and 44 + 1°C
g. Precision water bath (temperature 48 ± 1°C) with cover (Model 183) or equivalent
h. Quebec Colony Counter or equivalent
i. Fisher-Lilly Antibiotic Zone Reader (Fisher Scientific, Cat. No. 07-906) or equivalent

33.322 Reagents

a. **Distilled water:**

The distilled water must be prepared using an all glass still (Corning Megapure 6L or equivalent) and stored in a glass or any acceptable reservoir which is not a part of the system. All spore lots must be prepared using glass distilled water. * Do not use deionized water.

b. **Phosphate buffer (3 M, pH 7.1)**

Dissolve 306.9 g of K_2HPO_4 and 168.6 g KH_2PO_4 in 1 L distilled water. If necessary, adjust pH by dropwise addition of either 0.1 N HCl or NaOH depending on pH reading. Sterilize at 121°C for 15 minutes or filtering through a 0.2 μm filter.

c. **Ethyl alcohol (USP grade, 200 proof)**

Dehydrated Alcohol, USP, Ethyl Alcohol, 200 Proof Punctilious®, (Ethyl Alcohol [Ethanol] CAS #64-17-5, Warner-Graham Company, 160 Church Lane, Cockeysville, MD 21030). For a 50% solution, mix 1 part of ethyl alcohol with 1 part glass distilled water. Prior to use, filter sterilize through a 0.2 μm filter.

d. **Polyethylene glycol, Mol. Wt. 4000 (Baker Chemicals). Sterilize (121°C for 5 minutes) in a covered beaker prior to use.**

e. **Butterfield's Phosphate Buffer, sterile**

33.323 Supplies

a. **Sterile Roux bottles**
b. **Sterile glass beads, 4 mm diameter**
c. **Sterile 100 ml graduated glass stoppered cylinders or volumetric flasks**
d. **Sterile centrifuge tubes, 40 ml (Nalgene 3118 or equivalent)**
e. **Sterile pipettes, 10 ml and 1 ml graduated to the tip**
f. **Sterile, clear glass vials 51 x 15 mm with deep seated screw caps**
g. **Pressure sensitive labels not to exceed 2" x 1/2"**
h. **Acetate shrink-wrap material for sealing 15 x 51 mm glass vials or equivalent closure material**

*** Resins of some systems produce quaternary ammonium compounds**

which interfere with the analysis.

i. Forceps
j. Permanent marking pen
k. Antibiotic discs: Neomycin - 5 μg
l. Sterile cotton swabs on hollow plastic tubes
m. Sterile, plastic 60 X 15 mm petri plates (Falcon Cat. No. 1007 or equivalent)

33.33 Media

a. Brain Heart Infusion broth (BBL or equivalent); reconstitute according to manufacturer's directions, dispense 10 ml/tube and sterilize at 121°C for 15 minutes.

b. Blood agar plates (Columbia Blood Agar Base, 5% HRBC).

c. A-K Sporulating agar No. 2.

i. Agar slants - reconstitute A-K Sporulating Agar No. 2 according to manufacturer's directions with extra 0.5% Purified Agar (Difco or equivalent), sterilize by autoclaving at 121°C for 15 minutes and prepare slants.

ii. Roux bottles - add 300 ml reconstituted A-K Sporulating Agar No. 2 with extra 0.5% purified Agar. Sterilize (121°C for 15 minutes) and allow medium to harden in Roux bottles placed in a horizontal position.

d. Mueller-Hinton Agar (Acumedia Manufacturers Inc., Baltimore, MD); reconstitute according to manufacturer's directions, dispense 100 ml/flask and sterilize (121°C for 15 minutes).

33.34 Test Organism

Bacillus megaterium ATCC 9885 (American Type Culture Collection, Rockville, MD)

33.341 Purity and Biochemical Properties of *Bacillus megaterium*

a. Reconstitute a lyophilized culture in Brain Heart Infusion broth and incubate at 37°C for 18 h. Streak blood agar plates with the broth culture, incubate at 37°C for 18 h and check for culture purity.
b. Streak the culture for isolation onto two Columbia Agar plates with 5% defibrinated horse blood. Incubate at 37°C

for 18 h.

c. Prepare a Gram stain of three well isolated colonies. All cultures should be Gram positive.

d. Stain a drop of the spore suspension with malachite green and counterstain with carbol-fuchsin solution. The spores will appear green, whereas the vegetative cells will appear red or pink.

e. Use one Columbia Agar plate with 5% defibrinated horse blood from the culture to test for presence of catalase. *Bacillus* are catalase positive.

f. Use colonies from the other plate to check biochemical characteristics of the culture by inoculating O-F glucose, Voges-Proskauer, and mannitol broths. Incubate at 35°C for 18 h.

The biochemical patterns of *B. megaterium* should agree with the following chart:

Catalase	Gram stain	Spore forming	O-F glucose	Voges-Proskauer	Mannitol
+	+	+	O	-	A

(+) = positive; (-) = negative; (F) = fermentative; (O) = oxidative; (A) = acid.

g. If the test organism does not meet all the above criteria, replace with a new ATCC culture of the test organism.

33.342 Preparation of *Bacillus megaterium* Spore Suspension

a. After the culture meets all biochemical criteria, pick several well isolated colonies from the plates and streak A-K Sporulating Agar No. 2 slants (one per Roux bottle) and incubate the slants at 37°C for 18 h.

b. After incubation, put 4-6 sterile glass beads and 2-3 ml sterile distilled water into each tube and gently shake for 2 minutes to dislodge organisms from agar slants.

c. Aseptically transfer the suspension from slants to a Roux bottle containing A-K Sporulating Agar No. 2 and spread with the help of glass beads. (Multiple cultures may be prepared and pooled for transfer to Roux

bottles).

d. Incubate the Roux bottles horizontally for 18-24 h at 37°C and then at room temperature for the remainder of 1 week (6 days).

e. Harvest the growth from the Roux bottles by the use of 20-30 sterile glass beads and approximately 25 ml of sterile distilled water per bottle. Gently agitate bottles to dislodge bacterial growth. (While harvesting care must be taken not to break the agar).

f. Aseptically transfer the bacterial suspension into sterile centrifuge tubes (40 ml volume) and heat the tubes in boiling water (100°C) for 10 minutes.

g. Wash the heated suspension three times with sterile distilled water by centrifuging and decanting in the following manner:

i. Centrifuge at 5°C for 20 minutes at 20,000 x G.
ii. Pour off supernatant.
iii. Resuspend the pellet in 20 ml sterile distilled water.
iv. Repeat Steps i, ii and iii two more times.

h. Wash and coat a Virtis jar with a mixture of sterile phosphate buffer and sterile polyethylene glycol in the following manner:

Mix 34.1 ml of sterile phosphate buffer and sterile 11.8 g of polyethylene glycol in a 100 ml glass stoppered volumetric flask and shake vigorously. Bring to volume with sterile distilled water. Pour the mixture into a Virtis jar and place the jar on the homogenizer. Blend for 5 minutes at 5,000 RPM. Discard the mixture. Repeat the process.

i. Prepare a fresh solution of sterile buffered polyethylene glycol (34.1 ml of phosphate buffer and 11.8 g of polyethylene glycol) in a 100 ml glass stoppered sterile volumetric flask. Add 25 ml of the washed spore mixture and bring to volume with sterile distilled water. Shake vigorously. Pour the mixture into a coated Virtis jar and homogenize for 5 minutes at 5,000 RPM.

j. Dispense the mixture equally into four sterile centrifuge tubes and centrifuge in a swinging bucket rotor at 1,500 x G (3,000 RPM in H-4 Rotor in Sorvall

RC5C) for 2 minutes at room temperature.

k. A two-phase system with an interface will be formed in the centrifuge tube. Being careful not to disturb or disperse the interface layer, transfer the spore containing, upper phase using a 10 ml pipette to a second set of sterile centrifuge tubes.

l. Centrifuge the tubes at 20,000 x G for 20 minutes at 5°C. Pour off the supernatant. Resuspend the pellet in each tube with 20 ml sterile distilled water and pool the contents of all tubes into a sterile container.

m. Pipette 25 ml aliquots of spore suspension into each sterile centrifuge tube. Centrifuge tubes at 20,000 x G for 20 minutes at 5°C. Repeat the process five times after decanting the supernatant and re-suspending the pellet in 20 ml of sterile distilled water.

n. After the last wash step, resuspend each spore pellet in 20 ml 50% ethyl alcohol. Pool all spore suspensions into a sterile bottle containing 15-20 sterile glass beads. Store the stock suspension at 35-40°F (2-4.4°C). (Properly preserved stock spore suspension may be used indefinitely).

33.343 Preparation of Working Spore Suspension of *B. megaterium*

a. To determine the number of spores/ml in each new spore stock suspension, prepare tenfold serial dilutions (10^{-2}-10^{-10}) of the suspension using Butterfield's Phosphate Buffer. (Pipet 1.0 ml of well mixed spore stock suspension (use vortex mixer) into 9 ml buffer and then make serial dilutions up to 10^{-10}.).

b. Using separate pipettes, pipette 1.0 ml of each dilution into triplicate 100 x 15 mm plates.

c. Pipette 15 ml molten Plate Count Agar (cooled to 48 $\pm$ 1°C) into each plate. Mix by swirling or tilting plates to disperse the inoculum evenly throughout the agar. Incubate for 48 h at 37 $\pm$ 1°C.

d. Count colonies (30-300) in triplicate plates on a Quebec Colony Counter. Record and average the number of colonies/ml for each dilution. Determine the number of colony forming units (cfu)/ml of the stock solution.

e. To prepare the final spore suspension at a concentration of 1 x 10^6 cfu/ml in 50% ethyl alcohol from the stock

spore suspension, use the following formula:

Concentration of stock spore suspension (cfu/ml)	=	Dilution factor	X	Desired concentration of working spore suspension (cfu/ml)

Example:

Stock spore suspension = 1×10^9 spores/ml
Desired concentration of working spore suspension = 1×10^6 spores/ml:

$(1 \times 10^9 \text{ cfu/ml}) = (x)\ (1 \times 10^6 \text{ cfu/ml})$

$$\frac{(1 \times 10^9 \text{ cfu/ml})}{(1 \times 10^6 \text{ cfu/ml})} = x$$

$x = 1000$

In this example, the stock spore suspension must be diluted 1:1000 (1 part stock spore suspension plus 999 parts diluent) in 50% ethyl alcohol to prepare the 1 x 10^6 spore/ml concentration.

33.344 Packaging of *B. megaterium* Spore Suspension

a. Dispense 4.0 ml of the working spore suspension (1 x 10^6 cfu/ml in 50% ethyl alcohol) into each (51 x 15 mm) clear glass vial with leak-proof screw caps.

b. After capping the vials, seal with shrink-seal, or equivalent material to prevent leakage or dehydration.

c. Label the vials with the following information on a transparent mylar pressure sensitive label, or equivalent:

i. "CAST Spores"
ii. *B. megaterium* ATCC 9885
iii. Lot Number
iv. Packaging Date

NOTE: Under FSIS contract, CAST spores are produced commercially. After these spores meet all quality control specifications they are used in slaughter plants.

33.35 Preparation of CAST Plates

a. Weigh and add 38 g of Mueller-Hinton Agar powder to 1 L distilled water. Heat while stirring and bring to a boil. Sterilize the medium at 121°C for 15 minutes and then mix it thoroughly. Allow agar to cool to 48°C in a water bath. Continue mixing during cooling and dispensing.

b. Using a sterile agar delivery system, deliver 6.0 ml of the agar to each 60 x 15 mm plate. Distribute the agar evenly to cover entire surface of the plate. Allow the agar to harden on a flat, level surface.

c. Label the lid of each plate using a label containing the following information:

   i. "CAST PLATE"
   ii. Lot Number
   iii. Expiration Date

d. Refrigerate plates in sealed double plastic bags to prevent moisture evaporation. These plates can be used for a period of 90 days.

NOTE: Under FSIS contract, CAST plates are produced commercially. After these plates meet all quality control specifications they are used in slaughter plants.

33.36 Performing the CAST Test

33.361 Sample Condition

a. Assure that the samples are received at a temperature of 4°C or below.

b. Identify samples according to standard operating procedures.

NOTE: CAST test should only be used on kidney tissue of bob veal calves.

33.362 Procedure

a. Allow frozen samples to thaw completely at room temperature for a sufficient period of time such that ice crystals are no longer present within the sample.

b. Open a sterile cotton swab pack, remove one swab, and

insert the sharp end of the swab about 1/2" to 3/4" into the kidney tissue.

c. Move the swab shaft back and forth several times to macerate the tissue, disrupting tissue cells and releasing tissue fluid. Remove the swab shaft.

d. Reverse the swab and insert the cotton tip into the tissue opening, twisting to make sure that the cotton tip is in good contact with the macerated tissue.

e. Allow swabs to remain in the tissues for a minimum of 30 minutes.

f. Allow refrigerated plates to warm to room temperature for about 10 minutes before streaking. Check each plate for absence of contamination, cracking of agar or dryness.

g. Lift the plate cover slightly and mark an "X" reference mark on the outer side wall of the plate. Place the covered plate bottom side down on the work place surface with the reference mark at 12 o' clock position. With a fine-tip permanent marking pen, start at the "x" and draw a line across the bottom of the plate dividing it into two equal sections.

h. Check for seal integrity of vials containing spores.

i. Shake the *B. megaterium* spore vial and dip a sterile swab in the solution. Gently touch the swab to the side of the vial to remove excess fluid. Replace the screw cap on the vial.

j. Streak the surface of the agar plates with the swab from a point marked on the side of the plate moving up and down and from left to right. Turn the plate 1/4 turn and streak again.

k. Repeat this streaking process 2 more times. Finally turn the plate 1/2 turn and streak. (Use a separate swab for each plate)

l. Place a neomycin 5 μg disc on the agar surface near the vertical line on a plate.

m. Remove the swab from the tissue, break the shaft approximately two inches from the swab end.

NOTE: If the swabs appear dry, reinsert them in the tissue

and squeeze the tissue around the swab to absorb tissue fluids. For small portions of dry muscle tissue, moisten swab with distilled water prior to insertion.

n. Gently place the swab on the surface of the plate with the broken end of the shaft near the neomycin 5 μg disc making sure not to break the agar surface. Make sure the swab has uniform contact with the agar.

NOTE: Swabs from two different tissues or carcasses may be placed on each plate provided they are properly identified as to location on the plate.

If two tissue swabs are used per plate, place cotton tips in a "rabbit ears" configuration (Fig. 2).

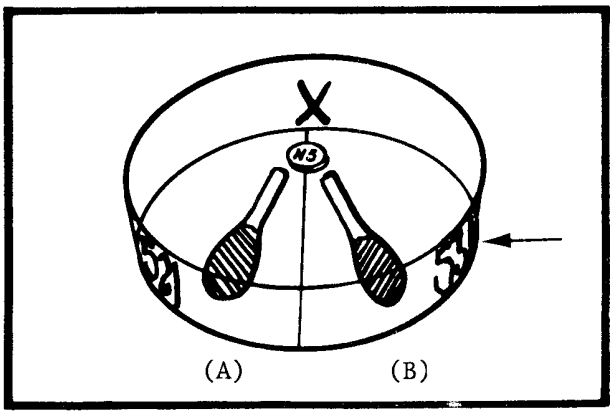

Figure 2. Swab placement on plate

o. Incubate the plates upright at 44 ± 1°C for 16-18 h.

p. Store samples under refrigeration until the test is completed.

33.363 Results and Interpretation

a. Remove plates from the incubator and remove the swabs.

b. Measure the zone of inhibition around the N5 disc with a mm ruler. The ZI should be 24-29 mm wide. If the ZI is not 24-29 mm in width, the test is inconclusive and

should be repeated.

c. Observe the plates for inhibition of *B. megaterium* growth surrounding the swabs.

i. If a zone of inhibition is observed, the test is positive. Measure the width of the zone and record results.

ii. If no zone of inhibition is observed, the test is negative. Record the result.

33.37 Selected References

Johnston, R. W., R. H. Reamer, E. W. Harris, H. G. Fugate, and B. Schwab. 1981. A new screening method for the detection of antibiotic residues in meat and poultry tissues. J. Food Prot. 44:828-831.

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United States Department of Agriculture. 1984. Performing the Calf Antibiotic and Sulfa Test. Food Safety and Inspection Service, Administrative Management, Training and Development Division, College Station, TX.

PART C

33.4 TENTATIVE CONFIRMATION OF CAST RESULTS FOR SULFONAMIDE RESIDUES IN MEAT AND POULTRY TISSUE

B. P. Dey, Sandra L. Kamosa and Clarence A. White

33.41 Background

The Calf Antibiotic and Sulfa Test (CAST) is presently being used for detecting sulfonamide residues in bob veal calves. The test as performed by inspectors is as follows: a sterile cotton tipped applicator (swab) is inserted into the kidney sample of an animal and left for 30 minutes to absorb tissue fluids. A *Bacillus megaterium* spore suspension is applied to CAST agar plates by a sterile swab. The swab from the kidney is then placed on the agar plate and incubated at 44°C for 16-24 h. The plate is then examined for a zone of inhibition (ZI) around the swab. In the case of an 18 mm or greater zone of inhibition, the carcass is subjected to further laboratory analysis. The muscle, liver and kidney tissues from the suspect carcass are sent to the laboratories for analysis. This procedure describes a modified CAST method with sensitivity equal or better than commercial CAST for verifying field results in 5-6 h with inclusion of another plate for confirming the presence of sulfonamide residues in suspected samples at the same time.

33.42 Equipment, Reagents and supplies

33.421 Equipment

a. Laminar Flow Hood or equivalent clean room
b. Sorvall RC5C Refrigerated Centrifuge, Sorvall Rotor SS-34 and Sorvall Swinging Bucket Rotor HB-4 or equivalent. Must operate at 20,000 x G at a constant 5°C and also with a swinging bucket rotor at 1,500 x G at room temperature or equivalent.
c. Virtis homogenizer, Model 60K or equivalent
d. Sterile Virtis jars
e. Vortex mixer or equivalent
f. Incubators: one capable of maintaining a constant 37°C and the other 44 ± 1°C
g. Precision water bath (48 ± 1°C) with cover (Model 183) or equivalent
h. Quebec Colony Counter or equivalent
i. Fisher-Lilly Antibiotic Zone Reader (Fisher Scientific, Cat. No. 07-906)

33.422 Reagents

a. **Distilled water:**

The distilled water must be prepared using an all glass still (Corning Megapure 6L or equivalent) and stored in a glass or any acceptable reservoir which is not a part of the system. All spore lots must be prepared using glass distilled water. * Do not use deionized water.

b. **Phosphate buffer (3 M, pH 7.1)**

Dissolve 306.9 g of K_2HPO_4 and 168.6 g KH_2PO_4 in 1 L distilled water. If necessary, adjust pH by dropwise addition of 0.1 N HCl or NaOH. Sterilize at 121°C for 15 minutes or filter through a 0.2 μm filter.

c. **Ethyl alcohol (USP grade, 200 proof)**

Dehydrated Alcohol, USP, Ethyl Alcohol, 200 Proof Punctilious®, (Ethyl Alcohol [Ethanol] CAS #64-17-5, Warner-Graham Company, 160 Church Lane, Cockeysville, MD 21030). For a 50% solution, mix 1 part of ethyl alcohol with 1 part glass distilled water. Prior to use, filter sterilize through a 0.2 μm filter.

d. **Polyethylene glycol, Mol. Wt. 4000 (Baker Chemicals). Sterilize (121°C for 5 minutes) in a covered beaker prior to use.**

e. **Bromcresol Purple (0.04%) solution. Dissolve 0.1 g Bromcresol Purple dye with 18.5 ml of 0.01 N sodium hydroxide, add 231.5 ml of distilled water.**

f. **Bacto-Dextrose (Difco, Detroit, MI; Cat. No. 0156-17-4)**

g. **p-aminobenzoic acid (Fisher Scientific Co. NJ; Cat. No A-41-70522)**

h. **Butterfield's Phosphate Buffer, sterile**

33.423 Supplies

a. **Sterile Roux bottles**
b. **Sterile glass beads, 4 mm diameter**
c. **Sterile 100 ml graduated glass stoppered cylinders or volumetric flasks**

*

Resins of some systems produce quaternary ammonium compounds which interfere with the analysis.

d. Sterile centrifuge tubes, 40 ml (Nalgene 3118 or equivalent)
e. Sterile pipettes graduated to the tip, 10 and 1 ml
f. Sterile, clear glass vials 51 x 15 mm with deep seated screw caps
g. Pressure sensitive labels not to exceed 2" x 1/2"
h. Acetate shrink-wrap material for sealing 51 x 15 mm glass vials or equivalent closure material
i. Forceps
j. Permanent marking pen
k. Antibiotic discs: Neomycin - 5 μg
l. Sterile cotton swabs on hollow plastic tubes
m. Sterile, plastic 60 X 15 mm plates (Falcon Cat. # 1007 or equivalent)

33.43 Media

Proceed exactly as that described in Section 33.33.

33.44 Test Organism

Bacillus megaterium ATCC 9885 (American Type Culture Collection, Rockville, MD)

33.441 Purity and Biochemical Properties of *Bacillus megaterium*

Proceed exactly as that described in Section 33.341.

33.442 Preparation of *B. megaterium* Spore Suspension

Proceed exactly as that described in Section 33.342

33.443 Enumeration of *B. megaterium* Spores in Working Suspension

Proceed exactly as that described in Section 33.343 except prepare the final spore suspension such that it contains 1 x 10^7 cfu/ml.

33.444 Packaging of *B. megaterium* Spore Suspension

a. Dispense 4.0 ml of the working spore suspension (1 x 10^7 cfu/ml in 50% ethyl alcohol) into sterile 51 x 15 mm clear, glass vials with deep seated, leak-proof screw caps.

b. After securely capping spore vials, seal with shrink-seal, or equivalent closure material, to prevent leakage or dehydration.

c. Label the vials with the following information on a transparent mylar pressure sensitive label:

i. "CAST Spores"
ii. *B. megaterium* ATCC 9885
iii. Date

NOTE: *B. megaterium* spores (1×10^7 cfu/ml) can be obtained from EDITEK, Burlington, NC, by special order.

33.45 Preparation of Plates

33.451 Preparation of Modified CAST (M-CAST) Plates

a. Weigh and add 38 g of Mueller-Hinton Agar (Acumedia) powder to each liter of glass distilled water. Weigh and add 8 g dextrose to the mixture. Add 70 ml Bromcresol Purple solution (0.04%) to the mixture. Heat while stirring and bring to boil.

b. Cool to 48°C and adjust the pH to 7.2 ± 0.1. Sterilize at 121°C for 15 minutes and mix thoroughly. Allow the agar medium to cool to 48°C in a water bath.

c. Continue mixing during cooling.

d. Add 1 ml of *B. megaterium* spore suspension (1×10^7 cfu/ml) to every 100 ml of the medium and mix thoroughly.

e. Aseptically dispense 8 ml of the seeded agar to each 100 x 15 mm plate.

f. Distribute the agar evenly to cover entire surface of the plate. Place plates on a flat, level surface and allow the agar to harden.

g. Label the side of each plate with a marker with the following information:

i. "M-CAST PLATE"
ii. Date

h. Refrigerate plates in sealed double plastic bags to prevent moisture evaporation. These plates can be used for a period of 15 working days.

33.452 Preparation of Modified CAST Plus (M-CAST+) Plates

a. Weigh and add 38 g of Mueller-Hinton Agar (Acumedia) powder to 1 L glass distilled water. Weigh and add 8 g dextrose to the mixture. Add 70 milliliters of Bromcresol Purple solution (0.04%) to the mixture. Heat while stirring and bring to a boil.

b. Add 200 mg of p-aminobenzoic acid to the medium.

c. Cool to 48°C and adjust pH to 7.2 ± 0.1. Sterilize at 121°C for 15 minutes and mix thoroughly. Cool the medium in a 48°C water bath.

d. Continue mixing during cooling.

e. Add 1 ml of *B. megaterium* spore suspension (1 x 10^7/ml) to every 100 ml of the medium and mix thoroughly.

f. Aseptically dispense 8 ml of the seeded agar to each 100 x 15 mm plate.

g. Distribute the agar evenly to cover entire surface of the plate. Place plates on a flat surface and allow the agar to harden.

h. Label the side of each plate with a marker with the following information:

   i. "M-CAST+ PLATE"
   ii. Date

i. Refrigerate plates in sealed plastic (Ziplock®) bags to prevent moisture evaporation. These plates can be used for a period of 15 working days.

33.46 Performing the Test

33.461 Sample Condition

a. Assure that the samples are received at a temperature of 4°C or below.

b. Identify samples according to standard operating procedures.

33.462 Procedure

a. Allow frozen samples to thaw completely at room temperature for a sufficient period of time such that

ice crystals are no longer present within the sample.

b. Open a sterile cotton swab pack, remove both swabs, and insert the sharp end of the swabs shaft 1/2" to 3/4" into the kidney tissue.

c. Move the swab shafts back and forth several times to macerate the tissue, disrupting tissue cells and releasing tissue fluid. Remove the swab shafts.

d. Reverse the swabs and insert the cotton tips into the tissue opening, twisting to make sure that the cotton tip is in good contact with the macerated tissue.

e. Allow swabs to remain in the tissue for a minimum of 30 minutes.

f. Leave refrigerated plates (M-CAST and M-CAST+) at room temperature for about 20-30 minutes to warm up. Discard plates which are contaminated, dried or cracked.

g. Place a neomycin 5 μg (N5) disc and a sulfamethazine 2 μg (S2) disc on separate M-CAST and M-CAST+ plates (control plates) in use each day the test is performed. Make sure that the distance between the two discs is 35-40 mm.

h. Remove the swabs from the tissue, break the shafts approximately two inches from the swab end.

NOTE: If the swabs appear dry, reinsert them in the tissue and squeeze the tissue around the swab to absorb tissue fluids. For small portions of dry muscle tissue, moisten swab with distilled water prior to insertion.

i. Gently place one of the swabs on an M-CAST plate and the other swab on an M-CAST+ plate making sure not to break the agar surface. Make sure the swab has uniform contact with the agar.

NOTE: Properly identified, four (4) swabs from 4 samples can be placed on each plate provided the cotton tip end of one lies next to the shaft of another as shown in Fig. 1.

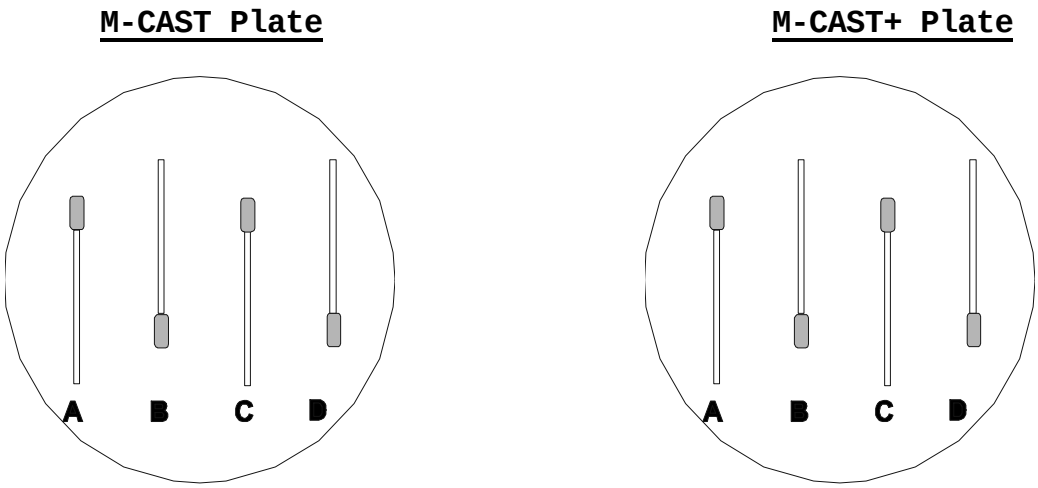


Fig. 1 . Placement of swabs on M-CAST and M-CAST+ plate

j. Incubate plates with sample swabs and the control discs (N5 and S2) upright at 44 ± 1°C for 5-6 h.

k. Refrigerate sample until the test is complete.

33.47 Results and Interpretation

a. Remove the plates from incubator and remove swabs.

b. Measure the ZI around the N5 and S2 discs on the control plates with a mm ruler or by a zone reader. The N5 zone should measure between 20-26 mm on both M-CAST and M-CAST+ plates. There should be a 16-19 mm zone by the S2 disc on the M-CAST plate only, where as there will be no zone by the S2 disc on the M-CAST+ plate. If the observed ZI are not in agreement with the above, repeat the test.

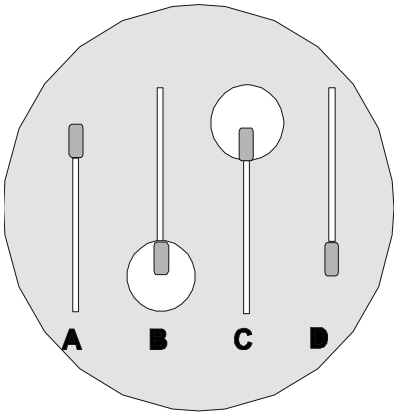
c. Measure the zone of inhibition surrounding each swab corresponding to a sample on each plate (from right to left).

NOTE: It is essential to read test results within 6 h. As the inhibitory effect by bacteriostatic drugs such as sulfonamides diminishes, organisms temporarily inhibited recover over time causing reduction in the zone of inhibition as incubation time increases.

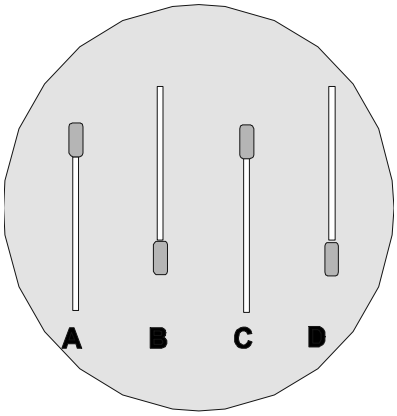
d. i. Samples with sulfonamide residue appear as illustrated below:

M-CAST plate: Zone of inhibition (Samples B and C)
M-CAST+ plate: No zone of Inhibition (Samples B and C)

M-CAST Plate



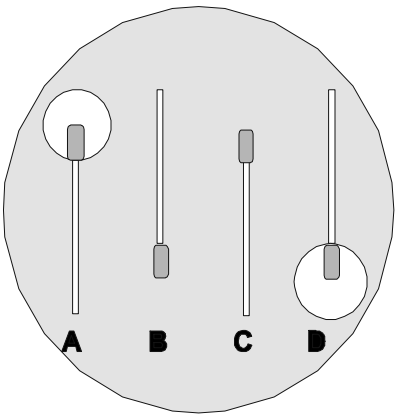
M-CAST+ Plate



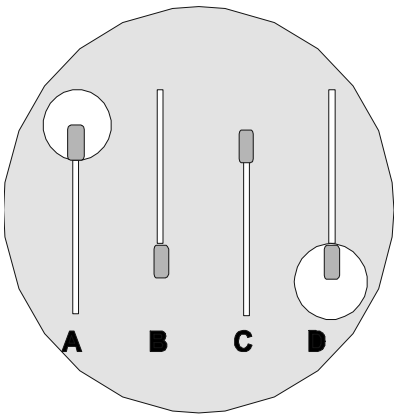
ii. Samples free of sulfonamide residue, but containing antibiotics, appear as illustrated below:

M-CAST plate: Zone of inhibition (Samples A and D)
M-CAST+ plate: Zone of Inhibition (Samples A and D)

M-CAST Plate



M-CAST+ Plate



33.48 Quality Control

a. Test organism must be evaluated for purity and proper biochemical patterns.

b. Freshly prepared plates must be tested with the N5 and S2 discs to assure proper performance.

c. Plates must not be used for more than 15 working days past preparation.

d. Extreme caution should be taken in adding para-amino benzoic acid because the chemical at a higher concentration than the recommended level is toxic to the test organism.

e. New chemicals/reagents and agar should be checked to assure quality.

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PART D

33.5 DETECTION OF ANTIMICROBIAL RESIDUE BY FAST ANTIMICROBIAL SCREEN TEST (FAST)

B. P. Dey, Clarence A. White and Nitin H. Thaker

33.51 Introduction

The Fast Antimicrobial Screen Test (FAST), an in-plant screen test, was developed in 1989 to improve the capability of the Antibiotic Residue Detection Program. FAST has higher sensitivity and can detect a wider range of antibiotics and sulfonamides than STOP and CAST. The test has been introduced in 50 bovine slaughter establishments. It is also being evaluated in swine species. If found suitable in both bovine and swine, it may be used in all species of food animals for detecting antimicrobial residues. Besides improving efficiency, this test would be used uniformly for detecting antibiotic and sulfonamide residues in food animal carcasses.

The test as performed by inspectors is as follows: a sterile cotton tipped applicator (swab) is inserted into the kidney sample of an animal and left for 30 minutes to absorb tissue fluids. The agar plates are surface streaked with *Bacillus megaterium* spore suspension on a sterile cotton swab. The swab from the kidney is removed, broken as close to the cotton tip as possible, and placed onto the agar plate and incubated at 44°C. The plate is examined for a zone of inhibition (ZI) around the swab at 6 and 18 h. In the case of inhibition at 6 h, the plate is further examined at 18 h for confirmation. If there is clear inhibition, muscle, liver and kidney tissues from the suspect carcass are collected and further analyzed for confirmation at an FSIS laboratory. When no inhibition is seen at 6 h, the carcass is free of antimicrobial residues at detectable levels. The test allows screening and releasing a large number of residue free carcasses within a work shift.

33.52 Equipment, Reagents and Supplies

33.521 Equipment

a. Laminar Flow Hood or equivalent clean room
b. Sorvall RC5C Refrigerated Centrifuge, Sorvall Rotor SS-34 and Sorvall Swinging Bucket Rotor HB-4 or equivalent. Must operate at 20,000 x G at a constant 5°C and also with a swinging bucket rotor at 1,500 x G at room temperature or equivalent.
c. Virtis homogenizer, Model 60K or equivalent

d. Sterile Virtis jars
e. Vortex mixer or equivalent
f. Incubators: one capable of maintaining a constant 35°C and the other at 44 ± 0.5°C
g. Precision water bath (with cover (Model 183) or equivalent
h. Quebec Colony Counter or equivalent
i. Fisher-Lilly Antibiotic Zone Reader (Fisher Cat.# 07-906)

33.522 Reagents

a. Distilled water:

The distilled water must be prepared using an all glass still (Corning Megapure 6L or equivalent) and stored in a glass or any acceptable reservoir which is not a part of the system. All spore lots must be prepared using glass distilled water. * Do not use deionized water.

b. Phosphate buffer (3 M, pH 7.1)

Dissolve 306.9 g of K_2HPO_4 and 168.6 g KH_2PO_4 in 1 L distilled water. If necessary, adjust pH by dropwise addition of 0.1 N HCl or NaOH. Sterilize at 121°C for 15 minutes or filtering through a 0.2 μm filter.

c. Ethyl alcohol (USP grade, 200 proof)

Dehydrated Alcohol, USP, Ethyl Alcohol, 200 Proof Punctilious®, (Ethyl Alcohol [Ethanol] CAS #64-17-5, Warner-Graham Company, 160 Church Lane, Cockeysville, MD 21030). For a 50% solution, mix 1 part of ethyl alcohol with 1 part glass distilled water. Prior to use, filter sterilize through a 0.2 μm filter.

d. Polyethylene glycol, Mol. Wt. 4000 (Baker Chemicals). Sterilize (121°C for 5 minutes) in a covered beaker prior to use.

e. Bromcresol Purple (0.04%) solution. Dissolve 0.1 g Bromcresol Purple dye with 18.5 ml of 0.01 N sodium hydroxide, add 231.5 ml of distilled water.

f. Bacto-Dextrose (Difco, Detroit, MI; Cat. # 0156-17-4) or equivalent

g. Butterfield's Phosphate Buffer, sterile

* Resins of some systems produce quaternary ammonium compounds which interfere with the analysis.

33.523 Supplies

a. Sterile Roux bottles
b. Sterile glass beads, 4 mm diameter
c. Sterile 100 ml graduated glass stoppered cylinders or volumetric flasks
d. Sterile centrifuge tubes, 40 ml (Nalgene 3118 or equivalent)
e. Sterile pipettes, 10 ml and 1 ml graduated to the tip
f. Sterile, clear glass vials 51 x 15 mm with deep seated screw caps
g. Pressure sensitive labels not to exceed 2" x 1/2"
h. Acetate shrink-wrap material for sealing 15 x 51 mm glass vials or equivalent closure material
i. Forceps
j. Permanent marking pen
k. Antibiotic discs: Neomycin - 5 μg
l. Sterile cotton swabs on hollow plastic tubes
m. Sterile, plastic 60 X 15 mm plates (Falcon Cat. No. 1007 or equivalent)

33.524 Media

a. Brain Heart Infusion broth (BBL or equivalent); reconstitute according to manufacturer's directions, dispense 10 ml/tube and sterilize (121°C for 15 min).

b. Blood agar plates (Columbia Blood Agar Base, 5% HRBC).

c. A-K Sporulating Agar No. 2.

i. Agar slants - reconstitute A-K Sporulating Agar No. 2 according to manufacturer's directions with extra 0.5% purified Agar (Difco or equivalent), sterilize by autoclaving at 121°C for 15 minutes and prepare slants.

ii. Roux bottles - add 300 ml reconstituted A-K Sporulating Agar No. 2 with extra 0.5% purified agar. Sterilize (121°C for 15 minutes) and allow medium to harden in Roux bottles placed in a horizontal position.

d. Mueller-Hinton Agar (Acumedia Manufacturers Inc., Baltimore, MD); reconstitute according to manufacturer's

directions, dispense as desired and sterilize (121°C for 15 minutes).

33.53 Test Organism

Bacillus megaterium ATCC 9885 (American Type Culture Collection, Rockville, MD)

33.531 Purity and Biochemical Properties of *Bacillus megaterium*

a. Reconstitute a lyophilized culture in Brain Heart Infusion broth and incubate at 37°C for 18 h. Streak blood agar plates with the broth culture and incubate plates at 37°C for 18 h. After incubation check for culture purity.

b. Streak the culture for isolation onto two Columbia Agar plates with 5% defibrinated horse blood. Incubate at 37°C for 18 h.

c. Prepare a Gram stain of three well isolated colonies. All cultures should be Gram positive.

d. Stain a drop of the spore suspension with malachite green and counterstain with carbol-fuchsin solution. The spores will appear green, whereas the vegetative cells will appear red or pink.

e. Use one Columbia Agar plate with 5% defibrinated horse blood from the culture to test for presence of catalase. *Bacillus* are catalase positive.

f. Use the other plate to check biochemical characteristics of the culture by inoculating O-F glucose, Voges-Proskauer, and mannitol broths. Incubate at 35°C for 18 h.

The biochemical patterns of *B. megaterium* should agree with the following chart:

Catalase	Gram stain	Spore forming	O-F glucose	Voges-Proskauer	Mannitol
+	+	+	O	-	A

(+) = positive; (-) = negative; (F) = fermentative; (O) = oxidative; (A) = acid.

g. If the organism does not meet all the above criteria, replace with a new ATCC culture of the test organism.

33.532 Preparation of *Bacillus megaterium* Spore Suspension

a. After the culture meets all biochemical criteria, pick several well isolated colonies from the plates and streak A-K Sporulating Agar No. 2 slants (one per Roux bottle) and incubate the slants at 37°C for 18 h.

b. Add 4-6 sterile glass beads and 2-3 ml sterile distilled water to each slant and gently shake for 2 minutes to dislodge organisms.

c. Aseptically transfer the slant suspensions to a Roux bottle containing A-K Sporulating Agar No. 2 and spread with the help of sterile glass beads. Multiple cultures may be prepared and pooled.

d. Incubate the Roux bottles horizontally for 18 h at 37°C and then at room temperature for the remainder of 1 week (6 days).

e. Harvest the growth from the Roux bottles by adding 20-30 sterile glass beads and approximately 25 ml of sterile distilled water per bottle. Gently agitate each bottle to dislodge bacterial growth. (Care must be taken not to break the agar during harvesting).

f. Aseptically transfer the bacterial suspension into sterile centrifuge tubes (40 ml volume) and heat the tubes in boiling water (100°C) for 10 min.

g. Wash the heated suspension three times with sterile distilled water by centrifuging and decanting in the following manner:

   i. Centrifuge at 5°C for 20 minutes at 20,000 x G.
   ii. Pour off supernatant.
   iii. Resuspend the pellet in 20 ml sterile distilled water.
   iv. Repeat Steps i, ii and iii two more times.

h. Wash and coat a Virtis jar with a mixture of sterile phosphate buffer and sterile polyethylene glycol in the following manner:

   Mix 34.1 ml of sterile phosphate buffer and 11.8 g of polyethylene glycol in a 100 ml glass stoppered sterile volumetric flask and shake vigorously. Bring to volume with sterile distilled water. Pour the mixture into a

Virtis jar and place the jar on the homogenizer. Blend for 5 minutes at 5,000 RPM. Discard the mixture. Repeat the process.

i. Prepare a fresh solution of sterile buffered polyethylene glycol (34.1 ml of phosphate buffer and 11.8 g of polyethylene glycol) in a 100 ml glass stoppered sterile volumetric flask. Add 25 ml of the washed spore mixture and bring to volume with distilled water. Shake vigorously. Pour the mixture into a coated Virtis jar and homogenize for 5 minutes at 5,000 RPM.

j. Dispense the mixture equally into four sterile centrifuge tubes and centrifuge in a swinging bucket rotor at 1,500 x G (3,000 RPM in H-4 Rotor in Sorvall RC5C) for 2 minutes at room temperature.

k. A two-phase system with an interface will be formed in the centrifuge tube. Being careful not to disturb or disperse the interface layer, transfer the spore containing, upper phase using a 10 ml pipette to a second set of sterile centrifuge tubes.

l. Centrifuge the tubes at 20,000 x G for 20 minutes at 5°C. Pour off the supernatant. Resuspend the pellet in each tube with 20 ml sterile distilled water and pool the contents of all tubes into a sterile container.

m. Pipette 25 ml aliquots of spore suspension into each sterile centrifuge tube. Centrifuge tubes at 20,000 x G for 20 minutes at 5°C. Repeat the process five times after decanting the supernatant and re-suspending the pellet in 20 ml of distilled water.

n. After the last wash step, resuspend each spore pellet in 20 ml 50% ethyl alcohol. Pool all spore suspensions into a sterile bottle containing 15-20 sterile glass beads. Store the stock suspension at 35-40°F (2-4.4°C). (Properly preserved stock spore suspension may be used indefinitely).

33.533 Enumeration of *B. megaterium* Spores in Stock Suspension

a. To determine the number of spores/ml in each new spore stock suspension, prepare tenfold serial dilutions (10^{-2}-10^{-10}) of the suspension using Butterfield's Phosphate Buffer. (Pipet 1.0 ml of well mixed spore stock suspension (use vortex mixer) into 9 ml buffer and then make serial dilutions up to 10^{-10}.).

b. Using separate pipettes, pipette 1.0 ml of each dilution into triplicate 100 x 15 mm plates.

c. Pipette 15 ml molten Plate Count Agar (cooled to 48 ± 1°C) to each plate. Mix by swirling and tilting plates for even dispersal of the inoculum. Incubate the plates at 37 ± 1°C for 48 h.

d. Count colonies (30-300) of triplicate plates on a Quebec Colony Counter. Record and average the number of colonies/ml in each dilution. Determine the number of colony forming units (cfu)/ml of the stock solution.

e. To prepare the final spore suspension at a concentration of 1 x 10^6 cfu/ml in 50% ethyl alcohol from the stock spore suspension, use the following formula:

Concentration of stock suspension (cfu/ml) = Dilution factor X Desired concentration of working spore suspension (cfu/ml)

Example:

Stock spore suspension = 1 x 10^9 spores/ml

Desired concentration of spore suspension = 1 x 10^6 spores/ml:

(1 x 10^9 cfu/ml) = (x) (1 x 10^6 cfu/ml)

$$\frac{(1 \times 10^9 \text{ cfu/ml})}{(1 \times 10^6 \text{ cfu/ml})} = x$$

x = 1000

In this example, the stock spore suspension must be diluted 1:1000 (1 part stock spore suspension plus 999 parts diluent) in 50% ethyl alcohol to prepare the 1 x 10^6 spore/ml concentration.

33.534 Packaging of *B. megaterium* Spore Suspension (Field Use)

a. Dispense 4.0 ml of the working spore suspension (1 x 10^6 cfu/ml in 50% ethyl alcohol) into sterile (15 mm diameter x 51 mm height) clear, glass vials with deep seated, leak-proof screw caps.

NOTE: Under FSIS contract, FAST spores are produced commercially. After they meet all quality control specifications they are used in slaughter plants.

b. After securely capping spore vials, seal with shrink-seal, or equivalent closure material, to prevent leakage or dehydration.

c. Label the vials with the following information on a transparent mylar pressure sensitive label:

i. "FAST Spores"
ii. *B. megaterium* ATCC 9885
iii. Date

33.54 Preparation FAST Plates (Used in the Plant)

a. Weigh and add 38 g Mueller-Hinton Agar (Acumedia) powder to 1 L glass distilled water. Add 7 g dextrose to the mixture. Add 70 ml Bromcresol Purple solution (0.04%) to the mixture. Heat while stirring and bring to boil. After sterilizing at 121°C for 15 minutes, mix the medium thoroughly, and cool it in a 48°C water bath. Continue mixing during cooling and dispensing.

b. Using a sterile agar delivery system, deliver 6.0 ml agar to each 60 x 15 mm plate. Distribute the agar evenly to cover entire surface of the plate. Place plates on flat level surface and allow the agar to harden.

c. Label the lid of each plate using a label, containing the following information:

i. "FAST PLATE"
ii. Lot Number
iii. Expiration Date

d. Refrigerate plates in sealed double plastic bags to prevent moisture evaporation. These plates can be used for a period of 90 days.

NOTE: Under FSIS contract, FAST plates are produced commercially. After these plates meet all quality control specifications they are used in slaughter plants.

33.55 Performing the FAST Test

33.551 Tissue Sample and Conditions

a. The kidney is the target tissue for FAST

b. The kidney and other tissue samples should be received at 4°C or below and identified properly.

33.552 Procedure

a. Allow frozen samples to thaw completely at room temperature for a sufficient period of time such that ice crystals are no longer present within the sample.

b. Open a sterile cotton swab pack, remove one swab, and insert the sharp end of the swab shaft about 1/2" to 3/4" into the kidney tissue.

c. Move the swab shaft back and forth several times to macerate the tissue, disrupting tissue cells and releasing tissue fluid. Remove the swab shaft.

d. Reverse the swab, insert the cotton tip into the tissue opening and twist to make sure that the cotton tip is in contact with the macerated tissue.

e. *Allow swabs to remain in the tissues for a minimum of 30 minutes.

f. Allow refrigerated plates to warm to room temperature for about 10 minutes. Check plates for contamination, cracking or dryness of agar.

g. Lift the plate cover slightly and mark an "X" reference mark on the outer side wall of the plate. Place the covered plate bottom side down on the work place surface with the reference mark at 12 o' clock position. With a fine-tip permanent marking pen, start at the "x" and draw a line across the bottom of the plate dividing it into two equal sections.

h. Shake the *B. megaterium* spore vial and dip a sterile swab in the solution. Gently touch the swab to the side of the vial to remove excess fluid. Replace the screw cap on the vial.

i. Streak the surface of the agar plates with the swab from a point marked on the side of the plate moving up and down and from left to right. Turn the plate 1/4 turn and streak again.

j. Repeat this streaking process 2 more times. Finally turn

the plate 1/2 turn and streak. (Use a separate swab for each plate)

*NOTE: If the swabs appear dry, reinsert them in the tissue and squeeze the tissue around the swab to absorb tissue fluids. For small portions of dry muscle tissue, moisten swab with distilled water prior to insertion.

k. As a control, place a neomycin (N5) 5 μg disc one half inch from the edge of the plate on the agar surface.

l. Remove swab from the tissue and break the shaft approximately two inches from the swab end.

m. Gently place the swab on the agar without breaking the surface. Make sure that swab has uniform contact with the surface.

NOTE: Two (2) swabs from two samples can be placed on one plate as illustrated below in (Figure 1).

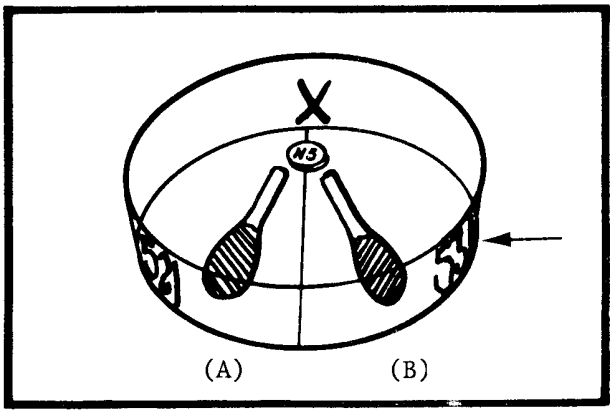

Figure 1. Swab placement on plate

n. Incubate the plates upright at 44 ± 0.5°C for 6 h, up to a maximum of 16-18 h.

o. Store samples in refrigerator until the test is completed.

33.56 Results and Interpretation

a. Remove plates from the incubator and remove the swabs.

b. Measure the ZI around the N5 disc with a mm ruler or a zone reader. The zone should be 20-26 mm wide. If not, the test must be repeated.

c. Observe the plates for inhibition of *B. megaterium* growth surrounding the swabs (Figure 2).

i. Samples with Antimicrobial Chemical Residue

Zone of inhibition around swab "A": Sample A may contain antimicrobial residue, and must be subjected to confirmatory testing procedures.

ii. Samples without Antimicrobial Chemical Residue

No Zone of Inhibition around swab "B" : Sample B is free of antimicrobial residue.

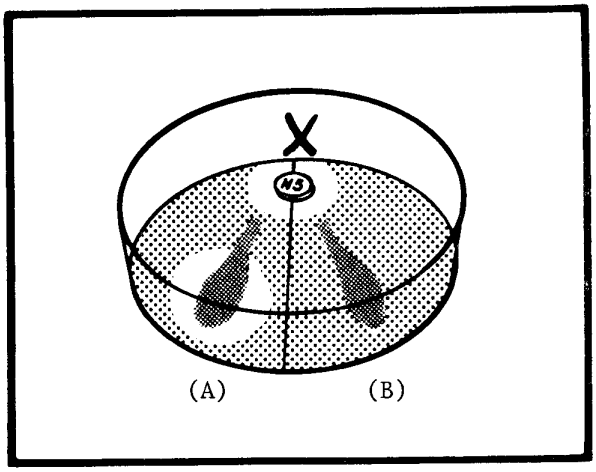

Figure 2. Inhibition of microorganism by swab

33.57 Quality Assurance

a. The FAST plates can be stored at room temperature protected from extremes of heat, cold and moisture.

b. Store spore suspensions under refrigeration condition with cap tightly closed.

c. Store neomycin disc vial in a plastic bag in refrigerator.

d. Do not use outdated plates, spores or N5 discs.
e. Shake the spore vial for even dispersal of spores.

f. Check plates before use for contamination, cracking or drying of agar.

g. Do not to break the agar surface while placing the neomycin disc and the swab.

h. Allow swabs to remain in the tissues for 30 minutes.

i. Read plates any time after 6 h of incubation, up to a maximum of 18 h.

j. Make sure that the incubator temperature is 44 ± 0.5°C.

33.58 Selected References

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PART E

33.6 EVALUATION OF ANTIMICROBIAL RESIDUES IN MEAT AND POULTRY TISSUE BY A MODIFIED FAST ANTIMICROBIAL SCREEN TEST (M-FAST)

B. P. Dey, Richard H. Reamer and Sandra L. Kamosa

33.61 Introduction

The Fast Antimicrobial Screen Test (FAST) developed in 1989, is presently being used in selected bovine slaughter plants. It is expected that the test will be used universally in plants for the detection antimicrobial residue in all species of food animal carcasses. The test as performed by inspectors is as follows: a sterile cotton tipped applicator (swab) is inserted into the kidney sample of an animal and left for 30 minutes to absorb tissue fluids. The agar plates are surface streaked with *Bacillus megaterium* spore suspension using a sterile cotton swab. The swab from the kidney is removed, broken as close to the cotton tip as possible, and placed onto the agar plate and incubated at 44°C. The plate is examined for a zone of inhibition (ZI) around the swab at 6 and 18 h. In the case of inhibition at 6 h, the plate is further examined at 18 h for confirmation. If there is no inhibition at 6 h, the carcasses is released. The test allows screening and releasing a large number of residue free carcasses within a work shift. If there is clear zone of inhibition, muscle, liver and kidney tissues from the suspect carcass are collected and further analyzed for confirmation at an FSIS laboratory. The method described here is a modified FAST procedure for verifying field test results in 6 h with sensitivity equal to the commercial FAST at comparable incubation times.

33.62 Equipment, Reagents and Supplies

33.622 Equipment

a. Laminar Flow Hood or equivalent clean room
b. Sorvall RC5C Refrigerated Centrifuge, Sorvall Rotor SS-34 and Sorvall Swinging Bucket Rotor HB-4 or equivalent. Must operate at 20,000 x G at a constant 5°C and also with a swinging bucket rotor at 1,500 x G at room temperature or equivalent.
c. Virtis homogenizer, Model 60K or equivalent
d. Sterile Virtis jars
e. Vortex mixer or equivalent
f. Incubators: one capable of maintaining a constant 37°C and the other 44 ± 0.5°C
g. Precision water bath (48 ± 1°C) with cover (Model 183) or

equivalent
h. Quebec Colony Counter or equivalent
i. Fisher-Lilly Antibiotic Zone Reader (Fisher Scientific, Cat. No. 07-906)

33.623 Reagents

a. Distilled water:

The distilled water must be prepared using an all glass still (Corning Megapure 6L or equivalent) and stored in a glass or any acceptable reservoir which is not a part of the system. All spore lots must be prepared using glass distilled water. * Do not use deionized water.

b. Phosphate buffer (3 M, pH 7.1)

Dissolve 306.9 g of K_2HPO_4 and 168.6 g KH_2PO_4 in 1 L distilled water. If necessary, adjust pH by dropwise addition of 0.1 N HCl or NaOH. Sterilize at 121°C for 15 minutes or filtering through a 0.2 μm filter.

c. Ethyl alcohol (USP grade, 200 proof)

Dehydrated Alcohol, USP, Ethyl Alcohol, 200 Proof Punctilious®, (Ethyl Alcohol [Ethanol] CAS #64-17-5, Warner-Graham Company, 160 Church Lane, Cockeysville, MD 21030). For a 50% solution, mix 1 part of ethyl alcohol with 1 part glass distilled water. Prior to use, filter sterilize through a 0.2 μm filter.

d. Polyethylene glycol, Mol. Wt. 4000 (Baker Chemicals). Sterilize (121°C for 5 minutes) in a covered beaker prior to use.

e. Bromcresol Purple (0.04%) solution. Dissolve 0.1 g Bromcresol Purple dye with 18.5 ml of 0.01 N sodium hydroxide, add 231.5 ml of distilled water.

f. Dextrose (Bacto Dextrose-Difco, Detroit, MI; Cat. No. 0156-17-4) or equivalent.

g. Butterfield's Phosphate Buffer, sterile

33.624 Supplies

a. Sterile Roux bottles.
b. Sterile glass beads, 4 mm diameter

*

Resins of some systems produce quaternary ammonium compounds which interfere with the analysis.

c. Sterile 100 ml graduated glass stoppered cylinders or volumetric flasks
d. Sterile centrifuge tubes, 40 ml (Nalgene 3118 or equivalent)
e. Sterile pipettes graduated to the tip, 10 and 1 ml.
f. Sterile, clear glass vials 51 x 15 mm with deep seated screw caps
g. Pressure sensitive labels not to exceed 2" x 1/2"
h. Acetate shrink-wrap material for sealing 15 x 51 mm glass vials or equivalent closure material
i. Forceps
j. Permanent marking pen
k. Antibiotic discs: Neomycin (N5)- 5 μg
l. Sterile cotton swabs on hollow plastic tubes
m. Sterile, plastic 60 X 15 mm plates (Falcon Cat. No. 1007 or equivalent)

33.63 Media

Proceed exactly as that described in Section 33.524.

33.64 Test Organism

Bacillus megaterium ATCC 9885 (American Type Culture Collection, Rockville, MD)

33.641 Purity and Biochemical Properties of *Bacillus megaterium*

Proceed exactly as that described in Section 33.531.

33.642 Preparation of *B. megaterium* Spore Suspension

Proceed exactly as that described in Section 33.532.

33.643 Enumeration of *B. megaterium* Spores in Stock Suspension

Proceed exactly as that described in Section 33.533 except prepare the final spore suspension such that it contains 1 x 10^7 cfu/ml.

33.644 Packaging of *B. megaterium* Spore Suspension

a. Dispense 4.0 ml of the working spore suspension (1 x 10^7 cfu/ml in 50% ethyl alcohol) into sterile (51 x 15 mm) clear, glass vials with deep seated, leak-proof screw caps.

b. After securely capping spore vials, seal with shrink-seal, or equivalent closure material, to prevent leakage or dehydration.

NOTE: *B. megaterium* spore (1×10^7 cfu/ml) can be obtained by special order from EDITEK, Burlington, N.C.

c. Label the vials with the following information on a transparent mylar pressure sensitive label:

i. "FAST Spores"
ii. *B. megaterium* ATCC 9885
iii. Date

33.65 Preparation of Plates

a. Weigh and add 38 g Mueller-Hinton Agar (Acumedia) powder to 1 L glass distilled water. Add 8 g dextrose to the mixture. Add 70 ml Bromcresol Purple solution (0.4%) to the mixture. Heat while stirring and bring to boil. Cool (48°C waterbath). Adjust the pH to 7.2 ± 1. After the medium has been sterilized at 121°C for 15 minutes, mix the medium thoroughly.

b. Keep mixing the medium while cooling in a 48°C water bath.

c. Add 1 ml of *B. megaterium* spore suspension (1×10^7/ml) to every 100 ml of the medium and mix thoroughly.

d. Aseptically dispense 8 ml of the seeded agar to each 100 x 15 mm plate.

e. Distribute the agar evenly over the entire plate. Place plate on a flat, level surface and allow agar to harden.

f. Label the lid of each plate using a label containing the following information:

i. "M-FAST PLATE"
ii. Expiration Date

g. Refrigerate plates in sealed double plastic bags to prevent moisture evaporation. These plates can be used for a period of up to 15 working days.

33.66 Performing the Test

33.661 Sample Condition

a. Assure that the samples are received at a temperature of

4°C or below.

b. Identify samples according to standard operating procedures.

NOTE: FAST test is used on the kidney tissue of all bovine species where implemented.

33.662 Procedure

a. Allow frozen samples to thaw completely at room temperature for a sufficient period of time such that ice crystals are no longer present within the sample.

b. Open a sterile cotton swab pack, remove one swab, and insert the sharp end of the swab shaft about 1/2" to 3/4" into kidney tissue.

c. Move the swab shaft back and forth several times to macerate the tissue, disrupting tissue cells and releasing tissue fluid. Remove the swab shaft.

d. Reverse the swab, insert the cotton tip into the tissue opening and twist to make sure that the cotton tip is in contact with the macerated tissue.

e. Leave the swab in the tissues for a minimum of 30 minutes.

f. Allow the plates to warm at room temperature for about 20 minutes. Check plates for contamination, cracking and dryness of agar.

g. As a positive control place a neomycin 5 μg (N5) disc in the center of a plate from the same batch used in the analysis.

h. Remove the swab from the tissues, break the shaft approximately two inches from the swab end.

NOTE: If a swab appears dry, reinsert and squeeze the tissue around the swab to absorb fluid. For a dry muscle tissue, moisten the swab with distilled water prior to insertion.

i. Place the swab on the agar surface gently with uniform contact with the surface.

NOTE: Properly identified, four (4) swabs from 4 samples can

be placed on each plate provided the cotton tip end of one lies next to the shaft of another as shown in Fig. 1.

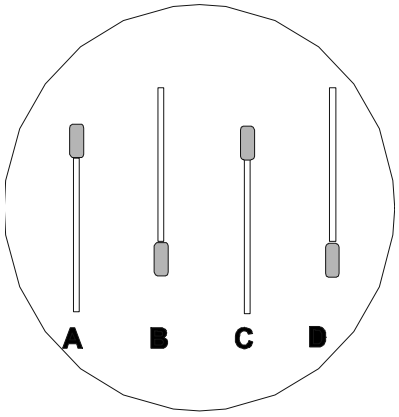

Figure 1. Swab placement on plate.

j. Incubate the plates upright at 44 ± 0.5°C for 6 hours, up to a maximum of 16-18 h.

k. Store samples in refrigerator until the test is completed.

33.663 Results and Interpretation

a. Remove the control and test plates with swabs from incubator and remove swabs.

b. Measure the ZI around the N5 disc on the control plate with a mm ruler or a zone reader. The zone should be 20-26 mm wide. If not, the test should be repeated.

c. Observe the plates for inhibition of *B. megaterium* growth surrounding the swabs (Figure 2).

i. Samples Free of Antimicrobial Chemical Residue

If no zone of inhibition is observed around a swab, the test is negative, .i.e. the samples (A, B, C and D) do not contain an antimicrobial residue (Fig. 2).

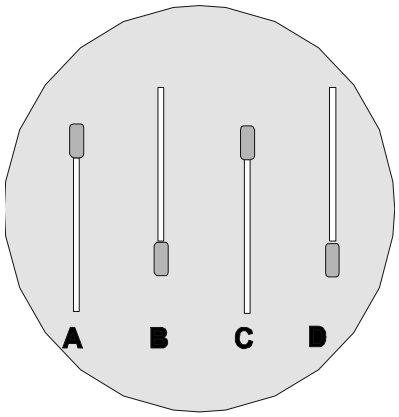

Figure 2. Swabs with no zone of inhibition.

ii. Samples with Antimicrobial Residue

If a zone of inhibition is observed around a swab, the test is positive, i.e. the samples (A and D) may have an antimicrobial residue. Measure the width of the zone.

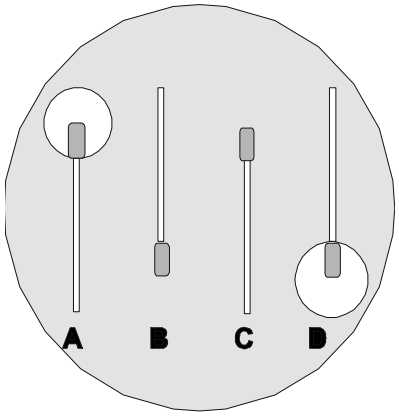

Figure. 3. Positive samples illustrating zone of inhibition around the swabs (samples) A and D.

d. Record and compare result with the field result. Positive samples must be subjected to confirmatory testing.

33.67 Quality Assurance

a. The M-FAST plates wrapped in plastic bags should be stored at refrigerator temperature (4-8°C).

b. Spore suspensions in tightly closed container should be stored at refrigerator temperature (4-8°C).

c. Neomycin disc vial wrapped in a plastic bag should be stored at refrigerator temperature (4-8°C).

d. Observe expiration date of plates. More than 2 week old plates should be discarded.

e. The spore vial should be shaken thoroughly before use.

f. Incubate 1 plate each day at 44°C as control.

g. Check plates before use for contamination, drying or cracking of agar.

h. Allow enough room for each swab placed on a plate.

i. Be careful not to break the agar surface while placing the neomycin disc and the swabs.

j. Leave swabs in the tissues for a minimum of 30 minutes.

k. Read plates any time after 6 h of incubation, up to a maximum of 18 h.

l. Stabilize the incubator temperature at 44 ± 0.5°C.

33.68 Selected References

Dey, B. P., Richard Reamer, and S. Kamosa. 1995. Evaluation of antimicrobial residues in meat and poultry tissues by a modified Fast Antimicrobial Screen Test (M-FAST). Laboratory Communication No. 80. USDA, Food Safety and Inspection Service, S&T, Microbiology Division, Washington, D.C.

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United States Department of Agriculture. 1981. Performing the Swab Test on Premises (STOP) for Detection of Antibiotic Residues in Livestock Kidney Tissue. Handbook. Food Safety and Inspection Service, Administrative Management, Training and Development Division, College Station, TX.

United States Department of Agriculture. 1982. The shelf stable swab test system for detecting antibiotic residues in tissues. Laboratory Communication No. 31. Food Safety and Inspection Service, S&T, Microbiology Division, Washington, D.C.

United States Department of Agriculture. 1994. Fast Antimicrobial Screen Test (FAST): For Detection of Antibiotic and Sulfonamide Residues in Livestock Kidney Tissue. A Self-Instructional Guide. Food Safety and Inspection Service, Administrative Management, Human Resource and Development Division, College Station, TX.

CHAPTER 35. DETECTION OF ANTIBIOTIC AND SULFONAMIDE RESIDUES IN MEAT TISSUE BY COMMERCIALLY AVAILABLE IMMUNOASSAY KITS

Anne M. Dulin, Clarence A. White and Nitin H. Thaker

35.1 Introduction

In recent years, the application of immunochemical methods for detecting veterinary drug residues in animal tissue has increased. These methods are based on highly specific antigen-antibody reactions on a solid phase matrix involving conjugation of an enzyme to the drug analyte and specific antibody directed against the analyte. Advantages of enzyme immunoassays include sensitivity (usually in the ng/ml range), simplicity of test performance, stability of reagents, lack of radioisotope use and associated hazards, potential for automation, and relatively inexpensive equipment.

In direct competitive enzyme immunoassays, enzyme labeled drug antigen and unlabeled drug antigen (sample analyte) compete for limited antibody binding sites. Specific antibody is generally bound to a solid phase support. The amount of enzyme labeled drug that binds to antibody is inversely proportional to the amount of unlabeled drug antigen (analyte) present in the tissue sample, which also competitively binds to the same antibody. Upon addition of substrate to the reaction mixture, to provide a visible indication of the test reaction, the amount of colored end product produced is inversely proportional to the amount of unlabeled drug analyte bound to the antibody. Thus, positive reactions indicating the drug's presence in the sample are generally indicated by no color change, while negative reactions indicating absence of the drug analyte are usually colored products. The exact color of the end product depends upon the specific substrate - chromogen system used in the particular assay. The applications of direct competitive enzyme-linked immunosorbent assays (ELISA) have provided additional support to the FSIS regulatory programs by enabling the detection of drug residues in food animal tissues at appropriate levels.

Presently, there are a number of screen test kits commercially available for detecting the presence of antibiotic and sulfonamide residues. However, regulatory action cannot be based on screen test results alone, since they are not quantitative, do not relate to biological activity of the detected drug and they are not considered to be absolutely definitive and confirmatory in nature. The presence of antibiotic residues, therefore, must be confirmed by bioassay and/or chemical methods, when a chemical method exists.

35.2 Commercially Available Test Kits

Currently there are commercially available, immunoassay, screen test kits, in several different formats, for such antimicrobial agents as:

penicillin (β-lactams)
ceftiofur (cephalosporin)
chloramphenicol
gentamicin
sulfonamides
tetracyclines
neomycin

Many of these test kits, however, were originally developed for specific application in bulk milk tank testing for antimicrobial residues. Before any of these kits can be used in an FSIS Laboratory for testing meat tissue extracts for antimicrobial residues, they must first be thoroughly evaluated to determine their suitability and applicability with regard to appropriate performance characteristics. They must perform in a manner to meet minimum sensitivity detection levels for the drug in question relative to that drug's established tolerance level, be specific, show excellent lot-to-lot reproducibility, have stability over the reported shelf life of the kit, and also have very low (0-5%) false positive and false negative reaction rates. Non-government users of this Guidebook must assume individual responsibility for evaluating commercial test kits for their applicable suitability with regards to the above performance parameters.

35.3 Equipment

This generic list applies to all test kits. Depending on the exact kit used, other supplies might be required.

a. Tekmar stomacher®, Model 400 (Tekmar Company, Cincinnati, OH)
b. Eppendorf centrifuge, Model 5412 (Thomas Scientific Co., Swedesboro, NJ)
c. Timer
d. Tekmar strainer bags, 18 oz capacity. (Tekmar Company, Cincinnati, OH)
e. Micro centrifuge tubes, 1.5 ml volume. (Thomas Scientific Co., Swedesboro, NJ)

35.4 Reagents

a. 0.1 M phosphate buffer, pH 8.0 (± 0.1). Dissolve 16.73 g dibasic potassium phosphate and 0.523 g monobasic potassium phosphate in distilled water and

dilute to 1 liter with distilled water. Check pH of the nonsterile buffer before autoclaving. If necessary, adjust by the dropwise addition of 0.1 N HCl or NaOH. Autoclave for 15 minutes at 121°C and 15 lbs pressure.

b. 0.1 M phosphate buffer, pH 4.5 (± 0.1). Dissolve 13.6 g monobasic potassium phosphate in distilled water and dilute to l liter with distilled water. For pH adjustment, proceed as in (a) above.

c. 0.1 M phosphate buffer, pH 6.0 (± 0.1). Dissolve 11.2 g monobasic potassium phosphate and 2.8 g dibasic potassium phosphate in distilled water and dilute to l liter with distilled water. For pH adjustment, proceed as in (a) above.

d. U. S. Pharmacopeia (USP) antibiotic and sulfonamide standard reference materials

35.5 Tissue Extraction Procedure

This procedure generally applies to all test kits:

a. Weigh out 10 g of sample (muscle, kidney, or liver tissue) into a sterile container.

b. Place the sample into a labeled Tekmar strainer bag.

c. Add 40 ml of appropriate phosphate buffer for the antibiotic or sulfonamide residue under evaluation.

d. Place strainer bag in a Tekmar stomacher® and stomach for 30 seconds for kidney or liver and 60 seconds for muscle tissue.

e. Allow the extract to settle for 45 minutes.

f. Place 1.5 ml of the settled extract into a labeled micro centrifuge tube.

g. Centrifuge for 10 minutes in an Eppendorf micro centrifuge at maximum speed.

h. Pipette supernatant fluid into another labeled test tube avoiding any fat and debris.

i. The 1:5 extracts prepared for bioassay analysis (Chapter 34) can be used instead of performing steps a through e.

35.6 Performing the Assay

Perform the assay procedure according to the specific test kit manufacturer's directions if no modifications were found to be necessary, or according to any specific instructions provided by the Microbiology Division, OPHS, for a particular test kit, when extensive protocol modifications were necessary.

35.7 Reporting and Confirmation of Screen Test Results

All positive screen test results should initially be reported as a presumptive positive finding. All samples presenting a positive screen test result must be subjected to confirmatory testing by the bioassay procedure or an appropriate chemical analysis procedure, if available for that particular drug, to confirm the drug's identity and determine it's quantitation. All sulfonamides must be confirmed by appropriate chemical methods. Final violative result reports must be based on confirmed drug quantitative levels present above that of established tolerance levels for that drug in a specific animal slaughter class.

35.8 Quality Assurance Procedures

a. Maintain a written log of all kits purchased, used and appropriate dates.

b. Test kits must be stored under refrigeration (4-8°C). Do not freeze.

c. Upon receipt of new test kits, perform positive and negative control testing at appropriate drug concentration levels.

d. Do not mix reagents and test components from kits with different serial numbers or from different manufacturers kits that detect the same analyte.

e. Do not use kits past their expiration date.

f. Use a separate pipet and test device for each sample.

g. Before performing the test, allow all reagents to reach room temperature. If the room temperature is not within the range of 18-29°C (65-85°F), perform test in another area within the proper temperature range.

h. Observe all test time intervals accurately by using a timer.

i. Two weeks before test kit expiration, perform positive

and negative control tests at appropriate drug levels to assure proper kit performance as expiration approaches.

j. U.S. Pharmacopeia (USP) standards of antibiotics and sulfonamides at appropriate quantitative levels should be used.

k. Record the results of all positive and negative control tests in a log book.

35.9 Selected References

Agarwal, V. K. 1992. Analysis of Antibiotic/Drug Residues in Food Products of Animal Origin. Plenum Press, New York, NY 10013.

Boison, J. O., and J. D. MacNeil. 1995. New test kit technology, p. 77-119. In H. Oka, H. Nakazawa, K. Harada and J. D. MacNeil (ed.), Chemical Analysis for Antibiotics Used in Agriculture. AOAC International, Gaithersburg, MD 20877.

CHAPTER 36. Equipment Calibration, Maintenance, and Performance Verification. Revision 1, 7/11/00

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36.1 Introduction

The guidelines for equipment (i.e. maintenance, calibration, and performance verification) covered in this chapter include the criteria for testing equipment and analytical instruments. Also included is guidance for monitoring and controlling environmental conditions including sanitation, safety, and discard procedures for hazardous material. The quality control parameters that are used in the analysis of a food product for specific microorganisms, species, and residues are included with the method.

The Microbiologist-in-Charge or Branch Chief ensures that all quality assurance and quality control procedures are consistently followed by everyone in the laboratory operation. Compliance with these procedures is verified by the internal audits conducted yearly by the Quality Assurance Manager.

Any deviation from an expected quality control result (nonconformance) is documented and verified by the individual responsible for the analysis. The unit supervisor must be informed. The nonconformance is recorded in the appropriate log along with any corrective action taken. It is the unit supervisor's responsibility to review all logs weekly. Daily verification means normal work days. If a piece of equipment is not in service it is so labeled and records so indicate. Non-working days (i.e., weekend, holidays) are noted.

The following are taken from the ISO/IEC 17025, Food Microbiology ALACC standards, or manufacturer requirements that have been tailored and/or expanded to meet the specific needs of the laboratory.

36.11 Equipment Manuals

a. Master copies of all available equipment manuals are stored and filed in a manner that allows easy retrieval.

b. For all testing equipment not in this chapter a working copy of the appropriate manual(s) containing the operating procedures, care, and maintenance is located near each piece of equipment.

36.12 Equipment Logs/Records

a. A log is maintained for and near each piece of equipment. The log includes:
- the name of the equipment
- the manufacturer's name, the serial number, or other unique identification
- the date received and placed in service (if available)
- the current location, where appropriate
- the condition when received (e.g. new, used, reconditioned)
- a copy of or the location of the manufacturer's instructions
- a copy of or the location of the dates and results of calibrations and/or verifications and the date of the next calibration and/or verification
- details of maintenance performed to date and planned for the future
- the history of any damage, malfunction, modification, or repair.

b. Each event relative to a piece of equipment is recorded in the log, showing the date, the event, any corrective action taken, the name or initials of the person making the entry.

c. All equipment records and maintenance logs are maintained for 3 years past last entry.

36.2 Temperature Control Equipment

36.21 Autoclaves

36.211 Temperature Calibration and Verification

a. All autoclaves are calibrated at installation and annually using a certified/traceable thermometer to assure stability of temperature.

b. To verify autoclave performance, a biological indicator spore vial or strip is added to each fully loaded autoclave once per week. Manufacturer's instructions for followed. (An unautoclaved vial or strip, incubated as a positive control, should show growth, and the autoclaved item should not.)

c. To verify autoclave performance daily each autoclave is equipped with an automatic temperature recorder. This chart/record is used to demonstrate proper time and temperature of each load.

d. Each chart is identified with the autoclave number, date, product, run number, time into autoclave, time at desired temperature, and time out of autoclave.

e. Each chart is reviewed at the end of a run and initialed by the operator to make sure that the temperature and time used conform to the directions for sterilizing that product or material. Any problems are noted in the autoclave log along with the corrective action taken.

36.212 Operation

a. A copy of the operating manual, a protocol for each type of material being processed, and an equipment log is located near each autoclave.

b. Temperature sensitive autoclave tape, or equivalent, is placed on all autoclaved containers to validate that the load was processed.

c. Insulated autoclave gloves, or equivalent, are kept near the autoclaves at all times.

36.213 Maintenance

a. Each autoclave will be serviced at 6-month intervals by a qualified contractor. In addition, each autoclave must have an annual temperature validation against a certified thermometer and a temperature uniformity check.

b. The "strainer" in the steam exhaust line of the autoclave is checked and cleaned weekly.

c. The autoclave is kept clean and free of debris to provide maximum heat transfer.

d. A log is maintained for each autoclave documenting all services performed and temperature validations.

e. The supervisor examines and initials each log weekly to ensure that it is correct and complete.

36.22 Incubators

36.221 Temperature Calibration and Verification

a. Each incubator will be calibrated for stability and uniformity of temperature at installation.

b. To verify performance the incubator temperature is recorded AM and PM using a certified/traceable device (i.e. thermometer, temptale®, thermocouple, etc).

c. Any nonconforming temperature is noted in the incubator log along with the cause, if identified, and any corrective action taken.

36.222 Operation

a. Incubators should be located where ambient temperature variation is minimal.

b. The temperature of a cabinet type incubator should not vary more than ± 1°C. A walk-in incubator may be hard to control closer than ± 2°C.

36.223 Maintenance

a. Incubators are cleaned and sanitized biannually to prevent the accumulation of mold or other microorganisms.

b. The over all condition of the incubator (i.e. door gaskets, blower fan, etc.) is checked annually. A record of all maintenance, repairs, etc. is kept in the incubator log.

c. If a container of water has to be added to an incubator to maintain humidity, a non-volatile microbial inhibitor can be added to prevent build-up of microorganisms. The container is cleaned and sanitized monthly.

36.23 Water Baths and Heating Blocks

36.231 Temperature Calibration and Verification

a. All water baths and heating blocks will be calibrated for stability and uniformity of temperature at installation.

b. To verify temperature performance a certified/traceable thermometer is placed in the bath or block and the temperature is recorded at the time of use.

c. Water baths used as close tolerance incubators should have a built-in water circulation system and a cover. The temperature is maintained within a temperature range of ± 0.5ºC. The temperature is checked at each use.

d. Most block heaters used in microbiology have a built-in thermostat that can be adjusted from ambient to approximately 115 ± 0.5ºC. The temperature of block heaters will be checked and recorded daily.

36.232 Operation

a. Operate the water bath or heating block according to the manufacturer's instructions.

b. For the most accurate temperature reading make sure the recording thermometer is not contacting the sides of the equipment.

36.233 Maintenance

a. All water baths are emptied, cleaned, and sanitized at least monthly.

b. Records of all maintenance, performance deviations, and corrective actions are maintained.

36.24 Refrigerators and Freezers

36.241 Temperature Calibration and Verification

a. All refrigerators and freezers are calibrated for stability and uniformity of temperature at installation.

b. To verify temperature performance the analyst checks and records the temperature daily using a certified/traceable device.

36.242 Operation

a. Freezer temperatures are maintained at or below –10ºC.

b. Ultra low freezer temperatures are maintained at or below –70 or –90ºC.

b. Refrigerators are maintained at a temperature within a range of 2-8ºC.

36.243 Maintenance

a. The overall condition of each freezer and refrigerator (i.e. door gaskets, blower fan, etc.) is checked annually. A log is kept for each refrigerator and freezer. The log contains the dates of all scheduled maintenance, any problems encountered, and any corrective action taken.

b. Where applicable, freezers and refrigerators are defrosted, cleaned, and sanitized at least once a year. (e.g. Neither self defrosting units nor cascade units generally need defrosting.)

c. The following procedures are applicable to all the ultra-low freezers in the laboratories. These do not preclude the addition of other cleaning/maintenance steps that may be specified for individual freezers

1. Air filters/coils shall be checked and cleaned quarterly.

2. Ice build-up inside door gaskets and seals shall be removed promptly. Any seals that allow significant ice build-up over a thirty-day period shall be replaced.

3. Defrosting and cleaning of the interior of the box need only be performed when the freezer is down for repairs.

36.25 Hot Air Ovens

36.251Temperature Calibration and Verification

a. All hot air ovens will be calibrated for stability and uniformity of temperature at installation.

b. To verify temperature performance the analyst records the temperature daily or at the time of use with a certified/traceable device.

36.252 Operation

a. The materials placed in the oven to dry are well separated to allow heat penetration.

b. Follow manufacturer's instructions for operation.

c. Keep at least 1 pair of insulated autoclave gloves, or equivalent, near the oven at all times.

36.253 Maintenance

a. Ovens are cleaned and sanitized at least annually.

b. The over all condition of the oven (i.e. door gaskets, door latches, burners, etc.) are checked annually. All maintenance observations, performance deviations, and corrective actions taken are recorded in the oven log.

36.3 Measuring Equipment

36.31 Laboratory Balances Calibration and Verification

a. All balances will be calibrated using certified/traceable weights annually.

b. To verify performance, a mass measurement is recorded daily using a single weight in the desired range.

36.311 Operation

a. Balances are placed on solid surfaces to guard against drafts and vibrations.

b. Balances and any associated weighing equipment and supplies are located in clean, dry areas. These criteria are especially important for analytical balances.

c. Boats or special papers can be used for weighing. Avoid spills and creation of aerosols.

d. All laboratory balances, top loading and analytical, are appropriately sensitive for their intended purpose.

e. If a balance is equipped with a leveling device care is taken to ensure that the balance is level before use.

36.312 Maintenance

a. All balances are professionally cleaned and calibrated annually using certified/traceable weights.

b. Balances are cleaned after each use.

c. A log is maintained for each balance showing the daily checks, all cleaning, maintenance, performance deviations, and any corrective actions taken.

36.32 pH Meter Calibration and Verification

a. To verify the performance of a pH meter a calibration is recorded daily using standard buffers. If pH readings are going to be taken intermittently throughout the day the pH meter is re-calibrated with fresh portion of buffers before each use.

b. Calibrate the instrument using two standard buffers that bracket the desired pH value of the test material (e.g. pH 4.0 and 7.0 or 7.0 and 10.0).

c. Ensure that the acceptance criteria for calibration, usually found in the manufacturer's instruction manual, have been met prior to use, and record all the calibration information.

36.321 Operation

a. The buffer aliquot used for the calibration is discarded after each use.

b. The calibration temperature should approximate that of the test solution. The most desirable temperature range for determining pH is 20ºC to 30ºC. It is preferable to use a temperature compensating probe, otherwise temperature corrections shall be made according to the manufacturer's instructions.

c. Reference buffers are labeled with identification/number, date received, and expiration date.

36.322 Maintenance

a. A professional will service all pH meters annually. A certificate of calibration/service is required.

b. Electrodes are cleaned after each use. Electrodes are stored as recommended by the manufacturer. Electrodes should never be allowed to dry out.

c. A log is maintained for each pH meter. Dated entries are made each time the pH meter is used, the buffers or electrodes are changed, and the instrument is serviced. Observed performance deviations are noted along with corrective actions taken.

36.33 Water Activity (a_w) Calibration and Verification

Follow the instructions in the manufacturer's operating manual for calibration, maintenance, and test procedure.

36.331 Operation

a. The test method and operation of this instrument is discussed in Chapter 2 of the MLG.

b. Temperature is very important when determining a_w. A small change in temperature can produce a large change in vapor pressure. Therefore the instrument, the reference salts, and the sample should be at the same temperature.

c. A sample should not be left in the instrument after a reading has been taken. When a sample is loaded, avoid tipping or moving the instrument.

d. To ensure a correct reading, fill the disposable cup no more than half full

e. Wipe any excess sample from the top rim of the cup before placing it in the unit to prevent contamination of the unit. If a spill occurs, the unit must be cleaned and re-calibrated.

36.332 Maintenance

a. The Hydrodynamics Instrument shall have the sensors checked at least once a year following the instruction manual. At any time, if the data of a salt standard deviates significantly from the expected results, check the suspect sensor and if found to be defective discard or return it to the manufacturer for re-calibration.

b. Maintain a log for the instrument documenting the date used, all repairs, readings of standard salt solutions, all performance deviations, and any corrective actions taken.

36.34 Micropipettor Calibration and Verification

a. Delivery volumes are verified monthly using a mass/volume measurement near mass/volume used. The following is an example of how to meet the mass/volume criteria.

Single volume pipette
5 reps/mg at set volume (ul)

Multivolume pipette
5 reps at low, mid, and high volumes = 20, 50 and 100% maximum volume. Record the average at each setting.

Multichannel pipette
Conduct a visual inspection of the draw, and take cumulative readings. Again 5 reps (at 20, 50, and 100% of max, if adjustable).

b. If performance verification fails re-calibrate following manufacturer instructions or return to the manufacturer for re-calibration.

36.341 Operation

a. This is a precision instrument that must be maintained and used with care.

b. Follow the manufacturer's instructions for use.

c. Select an appropriate pipette and tip combination.

36.342 Maintenance

a. Keep the pipettes clean and store them according to manufacturer's instructions.

b. Keep a record of all maintenance, service, calibration, and verification measurements.

36.35 Automated Pumps/Washing Equipment/Vial Fillers Calibration and Verification

a. If the equipment is used to dispense a designated volume, it is calibrated using a mass/volume measurement (see section 36.34 a) at installation.

b. If the delivery volume is constant then the performance verification is met by the daily calibration. If the volume is changed then the performance must be verified for each volume used.

36.351 Operation

a. When using sterile media, use aseptic technique at all times prior to and during a filling operation.

c. Aluminum foil or equivalent autoclave material may be used to wrap equipment for sterilization.

36.352 Maintenance

a. All equipment is cleaned and sanitized after each use.

b. The over all condition of the equipment (i.e. switches, spindles, hoses, etc.) is checked annually. All maintenance observations, performance deviations, and corrective actions taken are recorded in a log.

36.4 Microscope Calibration and Verification

a. All microscopes will have the stage micrometer calibrated at installation.

36.41 Operation

The manufacturer's instructions will be followed when using and adjusting any microscope.

36.42 Maintenance

a. Each microscope is professionally serviced annually.

b. The eyepiece and objective lens is cleaned after each use.

36.5 Automated Equipment

a. Unattended operation increases the importance of strict adherence to instrument operation, maintenance, and calibration instructions.

b. A standard operating procedure is followed for each instrument to ensure the maintenance and calibration is adequate for its intended use.

c. Quality control requirements for certain instrument components (e.g. ovens, incubators, and refrigerators) are included in Section 36.2 of this chapter.

36.51 Spiral Platers Calibration and Verification

a. Spiral platers will be calibrated for use by comparing to conventional plating method at the time of installation.

b. To verify the performance of a spiral plater check the siphon condition daily (see manufacture's instructions), volume dispersal monthly, and compare with conventional plating method annually.

36.511 Operation

Follow manufacturer's instruction for proper operation.

36.512 Maintenance

Spiral platers are cleaned and sanitized after each use by following the manufacturer's instructions.

36.52 Spectrophotometer Calibration and Verification

a. All spectrophotometers will have the wavelength calibrated by the manufacturer at installation.

b. To verify the performance of a spectrophotometer a blank reading will be recorded daily.

36.521 Maintenance

Spectrophotometers are cleaned according to manufacturer's recommendation.

36.53 Hydrometer Calibration

Calibrate to chemical compound annually.

36.6 Laminar Flow Hood/Biohazard Cabinet/Safety Cabinet Calibration and Verification

a. Safety cabinet and laminar flow hoods are services at installation and annually.

b. To verify performance, with each use check the sterility of the hood/cabinet using an open media control. In addition, check the airflow monthly using an appropriate monitor.

36.61 Maintenance

a. Hoods/cabinets are serviced annually.

b. Hoods/cabinets are cleaned and sanitized after each use.

36.7 Centrifuge Maintenance

a. A professional will service all centrifuge equipment on an annual basis. The laboratory will clean and sanitize each centrifuge monthly.

b. Rotors on ultra high centrifuge are maintained annually. The usage of rotor is maintained.

36.8 Measurement Traceability and Calibration of Reference Standards.

Equipment	Requirement	Frequency
Calibrated thermometer*	Calibration Standard reverification	every 5 years
Reference thermocouples	boiling water & ice point	annually
Working thermometers (Including Infra Red)	Calibration Standard traceable calibration	annually
Working thermocouples	Calibration Standard traceable calibration or ref. Thermocouple	annually
Weights*	Recertification to Calibration Standard weights	every 5 years
Balances	Calibration Standard traceable calibration	annually
Timers	national time standard	annually
Volumetric glassware (non class A)	mass, traceable to Calibration Standard weights	annually
Autoclaves	Calibration Standard traceable thermometers or thermocouples	annually

*All thermometers and weights must be calibrated and traceable to national and/or international calibration standards, such as NIST or SI units, etc.

36.9 Microbiology Supplies

36.91 Consumables

Laboratory consumables consist of those items used during the test method and then disposed of after use. These items would include but are not limited to disposable pipettes, petri dishes, scalpels, weigh boats, stomacher/whirlpak bags, or any other item consumed during the course of the test.

These items must be shown to be clean, sterilized, and accurate. The laboratory can satisfy this requirement by having a manufacturer's certificate for each lot to demonstrate performance. The mass/volume delivery of each lot of pipettes shall be verified. The laboratory will maintain the certificates.

36.92 Re-Usables

Laboratory re-usables consist of those items that are used during the analysis and are then cleaned, sterilized, and used again. These items include, but are not limited to, glass pipettes, hockey sticks, test tubes, glassware (non class A), plastic ware, stainless instruments, blenders, knives, or other reusable materials.

Items that have been cleaned and sterilized shall be clearly labeled (e.g. autoclave tape). Cutting utensils can be washed, flamed, and cooled just prior to use.

36.93 Reference Culture/Material

Certified reference cultures (CRC) must be traceable to a nationally or internationally recognized type culture collection (e.g. ATCC). Reference cultures (RC) from laboratory sources must be identified relative to standard reference sources.

These reference cultures must be handled to maintain their biochemical reaction and physiological characteristic integrity. All RC and CRC must not be transferred more than 5 times from the original source. After the fifth transfer the laboratory may purchase another culture from a type culture collection or re-identify the culture for key biochemical and physiological characteristics using nationally or internationally recognized reference sources. Alternatively, the type culture may be grown, then freeze dried or stored frozen and then used periodically, thus, extending the length of time required before repurchase or re-identification.

Stock cultures must be maintained as indicated in the specific chapters of this guidebook. Working stocks are used for quality control and cannot be sub-cultured more than five times. Commercially prepared lyophilized cultures traceable to ATCC can also be used. Records shall clearly show the cross-reference between the identification of each lot of media and the samples analyzed with that media.

36.94 Water Still/DI/RO Units (Laboratory Grade Water)

a. Only water that has been treated to be free from traces of dissolved metal, bactericidal, and inhibitory compounds should be used to prepare culture media, reagents, and dilution blanks. Inhibitor free water is referred to as microbiologically suitable (MS) water. The following tests are performed on the water source to ensure that the water is inhibitor free. Records of the following parameters will be kept.

Weekly testing (or prior to use):
- >1.0 megohms-cm resistance at 25^{o} C.

Monthly testing:
- Total Residual Chlorine must be < 0.01 mg/l
- Aerobic Plate Count must be < 1,000 colony forming unit (cfu)/ml

Annual testing:
- Heavy Metals (Cd, Cr, Cu, Ni, Pb, and Zn-single) must be < 0.05 mg/L
- Heavy Metals (total) must be < 10 mg/L

The suitability of water for microbiological analyses must pass the test for toxicity annually.

b. The DI/RO system-cartridge is replaced as recommended by the manufacturer.

c. Stills are cleaned as recommended by manufacturer.

36.10 Laboratory Maintenance Requirements

36.101 Work Surfaces

a. Prior to processing a sample or initiating culture work, the area must be thoroughly cleaned and sanitized with a suitable EPA registered disinfectant. The area must be thoroughly cleaned and sanitized again at the end of a work segment (e.g. sample preparation, plating, transfers, etc.) and/or the end of the day.

b. When working with pathogenic materials use a solution of 70% ethyl alcohol, 70-90% isopropyl alcohol, or an EPA registered commercial disinfectant (i.e. Lysol, hypochlorite, etc.) prepared at the manufacturer's recommended concentration. If there is a potential for contamination by *Clostridium botulinum* toxin, the 70% ethanol or the hypochlorite solution is adjusted to pH 11.0.

c. Only ethyl alcohol is used in areas where antibiotic residue testing is being done to avoid chance contamination with the phenolic or the hypochlorite solutions.

36.102 Biohazard Material

a. Immediately after use all items are placed in a suitable container with a disinfectant solution prepared at the manufacturer's recommended concentration or directly into a biohazard bag. All items are terminally sterilized at 121ºC for at least 45 minutes.

c. Remove and discard all implements after sterilization. Follow local regulations for final disposal.

36.11 Nonconforming Equipment (Defective)

All equipment shall be properly maintained. Any item of the equipment that has been subjected to overloading or mishandling, or which gives suspect results, or has been shown to be defective, shall be taken out of service. The equipment will be clearly identified and wherever possible, stored at a specific location until it has been repaired and shown by calibration, verification or test to perform satisfactorily. The laboratory shall examine the effect of this defect on previous test results.

36.12 Selected References

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O'Leary, W. M. (ed.). 1977. Practical Handbook of Microbiology. 2nd Edition. CRC Press, 2000 Corporate Blvd., Boca Raton, Fl 33431.

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AOAC International, AOAC INTERNATIONAL Accreditation Criteria for Laboratories Performing Food Microbiological Testing (ALACC)., 1999.

ISO/IEC 17025:1999 GENERAL REQUIREMENTS FOR THE COMPETENCE OF TESTING AND CALIBRATION LABORATORIES

ISO 7218, Microbiology of food and animal feeding stuffs-General rules for microbiological examinations, second edition, 1996-02-15.

Nordic Committee on Food Analysis, Quality Assurance Guidelines for microbiological laboratories, Report no. 5, 2nd edition, 1994.

United States Department of Agriculture
Food Safety and Inspection Service
Office of Public Health and Science
Laboratory QA/QC Division
950 College Station Road
Athens, GA 30605

Microbiology Laboratory Guidebook
Notice of Change

Chapter new, **revised**, or archived: MLG 4.02

Title: Isolation and Identification of *Salmonella* from Meat, Poultry, and Egg Products

Effective Date: 10/25/02

Description and purpose of change(s):

The Microbiology Laboratory Guidebook (MLG) chapters are currently under revision. The formatting is being changed to meet the requirements of the laboratory's document control system. Additional content is being added to meet the requirements of ISO 17025. The chapter has been revised to include a statement of the method detection limits, a section on safety precautions and a revised section on quality control practices. Previous pen-and-ink changes are also incorporated into the revision.

United States Department of Agriculture
Food Safety And Inspection Service, Office of Public Health and Science

MLG 4.02		Page 1 of 16
Title: Isolation And Identification of *Salmonella* From Meat, Poultry And Egg Products		
Revision:02	Replaces: MLG Chapter 4 Revision 1	Effective: 10/25/02

Procedure Outline

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- 4.1.1 General
- 4.1.2 Limits of Detection

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- 4.8.1 Somatic (O) Antigen Agglutination Tests
- 4.8.2 Flagellar (H) Antigen Agglutination Tests

4.9 Storage of Cultures

4.10 References

4.1 Introduction

4.1.1 General

This method describes the analysis of various meats, meat products, sponge and rinse samples, eggs, and egg products for *Salmonella*. It is not intended for the isolation and identification of *Salmonella* Typhi.

Success in isolating *Salmonella* from any food can be related to a number of factors including food preparation procedures, the number of organisms present, sample handling after collection, etc. With raw meat samples the competitive flora may be the most important factor. It varies from sample to sample and from one kind of meat to another.

Another consideration is whether the examination is for routine monitoring or epidemiological purposes. The analyst may choose to augment the method for epidemiological purposes with additional enrichment procedures and culture media, two temperatures of incubation, intensified picking of colonies from plates, and/or rapid screening methods.

All isolates must be identified as *Salmonella* biochemically and serologically.

Unless otherwise stated all measurements cited in this method have a tolerance range of ± 2%.

4.1.2 Limits of Detection

The *Salmonella* detection limit for this method has been determined to be less than 1 colony forming unit (cfu)/g in a 25 g sample.

4.2 Safety Precautions

Salmonella are generally categorized as BioSafety Level 2 pathogens. CDC guidelines for manipulating Biosafety Level 2 pathogens should be followed whenever live cultures of *Salmonella* are used. A Class II laminar flow biosafety cabinet is recommended for procedures in which infectious aerosols or splashes may be created. All available Material Safety Data Sheets (MSDS) must be obtained from the manufacturer for the media, chemicals, reagents and microorganisms used in the analysis. The personnel who will handle the material should read all MSDS sheets.

4.3 Quality Control Procedures

4.3.1 Method Controls

Include at least three method controls in all analyses. These controls must include a *Salmonella* spp. H_2S-negative culture, a *Salmonella* spp. H_2S-positive culture and an uninoculated media control. To facilitate identification of control isolates, the laboratory may use strains of uncommonly found serogroups. S. Abaetetuba, serogroup F, is suggested as a readily available, H_2S-positive culture that is not commonly found in meats or meat products. *Salmonella* serotype Choleraesuis is typically negative for H_2S production. These cultures may be obtained from ATCC. Other serotypes may be found that have aberrant H_2S-negative strains. The inoculum level for the positive controls should approximate 30 to 1000 cfu per sample. A 1 microliter loopful of a suspension of a fresh culture equivalent to a 0.5 McFarland Standard may be used for this purpose. Alternatively, commercially prepared bacterial pellets containing concentrations of 100 to 1000 cfu/pellet may be used according to the manufacturer's instructions. The control cultures should be inoculated into either a meat matrix or the matrix that is being analyzed. Incubate the controls along with the samples, and analyze them in the same manner as the samples. Confirm at least one isolate from each positive control sample. In the absence of a positive test sample, control cultures may be terminated at the same point as the sample analyses.

4.3.2 Specific Procedure Controls

The biochemical and serological tests used for confirmation of the sample isolates require the use of appropriate controls to verify that the results are valid. *Salmonella* 'O' antisera should be tested with QC control sera before initial use, and with a saline control for each test. Biochemical kit and rapid test manufacturers may specify control cultures for use with their products. If not specified, quality control procedures for biochemical tests and test media should include cultures that will demonstrate pertinent characteristics of the product.

4.4 Equipment, Reagents, Media and Test Kits

All of the materials listed below may not be needed. Media and reagents specific to the biochemical test method that is used will be needed in addition to the materials listed below. See Section 4.7.

4.4.1 Equipment

a. Sterile tablespoons, scissors, forceps, knives, glass stirring rods, pipettes, petri dishes, test tubes, bent glass rods ("hockey sticks") as needed
b. Blending/mixing equipment: Sterile Osterizer-type blender with sterilized cutting assemblies, and blender jars or Mason jars and adapters for use with Mason jars; *or* a Stomacher™ (Tekmar or equivalent) with sterile Stomacher bags
c. Sterile Stomacher™ 3500 bags, plain, clear polypropylene autoclave bags (ca. 24" x 30 - 36"), or Whirl-Pak™ bags (or equivalent)
d. Incubator, 35 ± 1°C
e. Incubator or water bath, 42 ± 0.5°C
f. Water bath, 48-50°C
g. Glass slides, glass plate marked off in one-inch squares or agglutination ring slides
h. Balance, 2000 g capacity, sensitivity of 0.1 g
i. Inoculating needles and loops
j. Vortex mixer

4.4.2 Reagents

a) Crystal violet dye, 1% aqueous solution, steamed
b) Butterfield's phosphate diluent
c) Saline, 0.85%
d) Saline, 0.85% with 0.6% formalin for flagellar antigen tests
e) Calcium carbonate, sterile
f) *Salmonella* polyvalent O antiserum
g) *Salmonella* polyvalent H antiserum
h) *Salmonella* individual O grouping sera for groups A-I (antisera for further O groups are optional)
i) (Optional) Oxoid *Salmonella* Latex Test (Unipath Company, Oxoid Division, Ogdensburg, NY) or equivalent
j) Additional reagents as needed for biochemical tests

4.4.3 Media

a) Buffered peptone water (BPW)
b) TT broth (Hajna)

c) Modified Rappaport Vassiliadis (mRV) broth, Rappaport-Vassiliadis R10 broth, or Rappaport-Vassiliadis Soya Peptone Broth (RVS)
d) Brilliant green sulfa agar (BGS; contains 0.1% sodium sulfapyridine)
e) Xylose lysine Tergitol™ 4 agar (XLT4) or Double modified lysine iron agar (DMLIA)
f) Triple sugar iron agar (TSI)
g) Lysine iron agar (LIA)
h) Trypticase soy broth (TSB) or Tryptose broth
i) Trypticase soy agar (TSA)
j) Nutrient agar slants
k) Nutrient broth, semi-solid
l) Tryptic soy agar with 5% sheep blood agar
m) Additional media as needed for biochemical tests

4.4.4 Cultures

At least one H_2S-positive strain of *Salmonella* and one H_2S-negative strain of *Salmonella* are required for method controls.

4.4.5 Commercially Available Test Kits (optional)

Any screening method under consideration for *Salmonella* testing must meet or exceed the following performance characteristics: sensitivity ≥ 97%, specificity ≥ 90%, false-negative rate ≤ 3%, and false-positive rate ≤ 10%.

4.5 Isolation Procedures

4.5.1 Sample Pooling

NOTE: Follow sample pooling instructions in specific program protocols. Otherwise, do not use sample pooling.

When examining products that are expected to be *Salmonella*-free, sample pooling can save valuable time, labor and materials. There are several ways this can be done. Pooling at the non-selective enrichment step is appropriate when the likelihood of finding *Salmonella* is unlikely or when, if a positive is found, it is not important to know which particular sample contained the organism.

a. Those samples that are to be examined only for *Salmonella* may be pooled in the blenders. Up to 30 samples may be combined, depending on the capacity of the blenders and culture flasks. The proportions of sample volume to BPW must be maintained at the 1 to 10 ratio (1 part sample to 9 parts BPW). The BPW should be pre-warmed to the temperature of incubation.

b. When food homogenates (Section 4.5.9) are pooled, the culture flask should be warmed in a water bath to bring the contents up to incubation temperature before placing it in the incubator.

c. Incubation of large pools should be prolonged to two days if growth is not apparent in one day. Subculture 5 ± 0.5 ml of the incubated non-selective broth pool into 100 ±1 ml of TT (Hajna), 1 ± 0.1 ml into 100 ± 1 ml of mRV, and proceed as usual.

In cases in which it is important to identify particular samples that may contain *Salmonellae*, it is still possible to take advantage of labor-saving by pooling. In such cases, the samples may be started in the usual way in non-selective broth. After incubation, up to ten of these cultures may be pooled in selective enrichment broth. Maintain the 0.5 to 10 ratio for inoculation of TT broth and the 0.1 to 10 ratio for inoculation of mRV broth. The remaining non-selective broths (or portions of them) are refrigerated. The total volume of selective enrichment broth used will be the same, but the number of plates to be streaked is reduced. If a positive pool is found, all the pooled samples are started individually in selective enrichment broth by going back to the refrigerated non-selective broths.

4.5.2 Breading Mixes, Dehydrated Sauces and Dried Milk

For dehydrated sauces, dried milk, and breading mixes add BPW as described for powdered egg in Section 4.5.8.

4.5.3 Ready-to-Eat Foods

Follow program requirements for preparing sample and sub-sample composites. Outbreak investigation requirements may differ, in which case, follow the client specifications for those samples.

a. Weigh 325 g of the composite sample into a Stomacher bag (or sterile blender jar if required by the client or sample type).

b. Add approximately one third to half of 2925 ml of ambient temperature sterile buffered peptone water. Blend or stomach approximately 2 minutes, and then add the remainder of the 2925 ml of BPW.

c. Incubate at 35 ± 1°C for 20-24 h.

d. Transfer 0.5 ± 0.05 ml of incubated broth into 10 ml TT and 0.1 ± 0.02 ml into 10 ml of mRV broth.

e. Incubate the enrichment broths at 42 ± 0.5°C for 22-24 h, or in a water bath at 42 ± 0.5°C for 18-24 h.

f. Streak above enrichments on BGS and either DMLIA or XLT4 agar plates. Use one 10-microliter loopful for each plate. Do not subdivide plates for streaking multiple samples; streak the entire agar plate with a single sample enrichment.

g. Incubate at 35 ± 1°C.

h. Examine in 18-24 h. Select colonies. Refer to Section 4.6 et seq.

i. Re-incubate all plates for an additional 18-24 h. Reexamine initially negative plates and pick colonies as above. Reserve, under refrigeration, all plates from which colonies were picked. If suspect *Salmonella* colonies do not confirm, reexamine the plates from which they were picked, and if appropriate, re-pick colonies for confirmation. See Section 4.6.1.b.

4.5.4 Fermented Products

Follow the procedure for ready-to-eat foods (Section 4.5.3) except:

a. Blend/stomach the sample with 10 g of sterilized calcium carbonate.

b. Use buffered peptone water that contains 1 ml of a 1% aqueous solution of crystal violet per liter.

4.5.5 Raw Meat

If the sample is not already ground, in some cases it may be best to mince it with scissors or leave it whole (e.g. chicken wings) to avoid jamming blender blades with skin or connective tissue. Whirl-Pak™ bags can be used in culturing these samples.

a. Weigh 25 ± 0.5g of meat into a sterile blender jar, other sterile jar or a Whirl-Pak™ or Stomacher™ bag. HACCP program samples collected using a sampling ring are allowed a weight range of 25 ± 2.5 g.

b. Add 225 ml of BPW. Stomach or blend, as required, for approximately two minutes or shake thoroughly.

c. Incubate at 35 ± 1°C for 20-24 h.

d. Transfer 0.5 ± 0.05 ml into 10 ml TT broth and 0.1 ± 0.02 ml into 10 ml mRV broth.

e. Incubate at 42 ± 0.5°C for 22-24 h.

f. Streak on DMLIA or XLT4 and BGS agar plates. Use one loopful of inoculum for each plate. Do not subdivide plates for streaking multiple samples; streak the entire agar plate with a single sample enrichment.

g. Incubate at 35 ± 1°C.

h. Examine in 18-24 h. Select colonies. See Section 4.6 et seq.

i. Re-incubate all plates for an additional 18-24 h. Reexamine initially negative plates and pick colonies as above. Reserve, under refrigeration, all plates from which colonies were picked. If suspect *Salmonella* colonies do not confirm, reexamine the plates from which they were picked, and if appropriate, re-pick colonies for confirmation. See Section 4.6.1.b.

4.5.6 Carcass Sponge Samples

a. Add 50 ml of BPW to the sample bag containing the moistened sponge to bring the total volume to 60 ml. Mix well.

b. Incubate at 35 ± 1°C for 20-24 h.

c. Follow the procedures in Section 4.5.5, d-i.

4.5.7 Whole Bird Rinses

Due to differences between sample types/sizes (e.g. chicken vs. turkey carcasses), follow instructions given in the specific program protocol. For chicken carcasses, aseptically drain excess fluid from the carcass and transfer the carcass to a sterile Stomacher™ 3500 bag, a plain, clear polypropylene bag (ca. 24" x 30-36"), or equivalent. Pour 400 ml (or other volume specified in program protocol) of Buffered Peptone Water (BPW) into the cavity of the carcass contained in the bag. Rinse the bird inside and out with a rocking motion for one minute (ca. 35 RPM). This is done by grasping the broiler carcass in the bag with one hand and the closed top of the bag with the other. Rock with a reciprocal motion in about an 18-24 inch arc, assuring that all surfaces (interior and exterior of the carcass) are rinsed. Transfer the sample rinse fluid to a sterile container.

Use 30 ml of the sample rinse fluid obtained above for *Salmonella* analysis. Add 30 ml of sterile BPW, and mix well. Incubate at 35 ± 1°C for 20-24 h, and then proceed according to 4.5.5 (d-i).

NOTE: If analyses other than *Salmonella* are to be performed, the carcass may be rinsed in Butterfield's Phosphate Diluent instead of BPW. In this case, add 30 ml of 2X BPW to 30 ml of carcass-rinse fluid, mix well, and continue as above.

4.5.8 Liquid, Frozen, Cooked or Dried Egg Samples

a. Mix the sample with a sterile spoon, spatula, or by shaking.

b. Aseptically weigh a minimum of 100 g of egg sample into a sterile blender jar, other sterile jar, or a Whirl-Pak™ or Stomacher™ bag containing 900 ml of sterile BPW. If a special sample or specification requires a sample size other than 100 g, the ratio of egg sample to BPW is to be maintained at 1:10.

c. Mix the inoculated BPW well by shaking, stomaching, or blending.

d. With dried egg samples, gradually add BPW to the sample. Add a small portion of sterile BPW and mix to obtain a homogeneous suspension. Add the remainder of the BPW. Mix until a lump-free suspension is obtained.

e. Incubate at 35 ± 1°C for 20-24 h, and then proceed according to 4.5.5 (d-i).

4.5.9 Sanitation Series Food Homogenates (optional)

To isolate salmonellae from food samples homogenized as outlined in MLG 3 Section 3.3.1, as part of a sanitation test series, use the 10^{-1} food homogenate dilution (See also this chapter, Section 4.5.1 Sample Pooling).

a. Weigh 250 g of food homogenate into a sterile jar (this contains 25 g of product).

b. Add 25 ml of 10x BPW (broth made to ten time's normal strength).

c. Incubate 24-26 h at 35 ± 1°C.

d. Transfer 0.5 ± 0.05 ml into 10 ml of TT broth and 0.1 ± 0.02 ml into 10 ml of mRV broth.

e. Incubate at 42 ± 0.5°C for 22-24 h.

f. Streak on DMLIA or XLT4 and BGS agar plates. Use one loopful for each plate. Do not subdivide plates for streaking multiple samples; streak the entire agar plate with a single sample enrichment.

g. Incubate 18-24 h at 35 ± 1°C.

h. Select colonies. See Section 4.6 et seq.

i. Re-incubate all plates for an additional 18-24 h. Reexamine initially negative plates and pick colonies per Section 4.6. Reserve all plates from which colonies were picked. If suspect *Salmonella* colonies do not confirm, reexamine the plates from which they were picked, and if appropriate, re-pick colonies for confirmation. See Section 4.6.1.b.

4.5.10 Most Probable Numbers (MPN) Determination

Due to differences between sample types (e.g. whole chicken rinse vs. ground beef) and sample sizes (e.g. 25 g vs. 325 g) follow MPN instructions given in the specific program protocol. See also MLG Appendix 2, Most Probable Number Tables.

4.6 Examination of and Picking Colonies from Plating Media

4.6.1 Picking Colonies

a. After the recommended incubation interval, examine the selective-differential agar plates for the presence of colonies meeting the description for suspect *Salmonella* colonies. Pick well-isolated colonies.

- BGS. Select colonies that are pink and opaque with a smooth appearance and entire edge surrounded by a red color in the medium. On very crowded plates, look for colonies that give a tan appearance against a green background.
- XLT4. Select black colonies or red colonies with or without black centers. The rim of the colony may still be yellow in 24 h; later it should turn red.
- DMLIA. Select purple colonies with or without black centers. Since salmonellae typically decarboxylate lysine and ferment neither lactose nor sucrose, the color of the medium reverts to purple.

b. Pick up to three colonies from each plate, if available. (NOTE: Before any sample is reported as *Salmonella*-negative, a total of three typical colonies, if available, from each selective agar plate must be examined). Pick only from the surface and center of the colony. Avoid touching the agar because these highly selective media suppress growth of many organisms that may be viable.

If there are typical colonies on a plate, that are not well isolated, pick from the typical colonies and re-streak directly to selective agar plates. Alternatively, place a loopful of growth into a tube of TT or mRV broth and incubate overnight, then re-streak to selective agars.

4.6.2 Screening Media

a. Inoculate TSI and LIA slants in tandem with a single pick from a colony by stabbing the butts and streaking the slants in one operation. If screw cap tubes are used, the caps must be loosened. Incubate at 35 ± 1°C for 24 ± 2 h.

b. Examine TSI and LIA slants as sets. Note the colors of butts and slants, blackening of the media and presence of gas as indicated by gas pockets or cracking of the agar. Note also the appearance of the growth on the slants along the line of streak. Discard, or re-streak for isolation, any sets that show "swarming" from the original site of inoculation. Discard sets that show a reddish slant in lysine iron agar. Isolates giving typical *Salmonella* spp. reactions and isolates which are suggestive, but not typical of *Salmonella* spp. should be confirmed by a combination of biochemical and serological procedures. Refer to Table 1 for a summary of TSI-LIA reactions. The motility testing in the last column of the table is optional.

c. (Optional: for some biochemical test kits) Streak a TSA + 5% sheep blood agar plate from either the TSI or LIA slant. Incubate 18-24 h at 35 ± 1°C.

4.7 Biochemical Procedures

Commercially available biochemical test kits, including automated systems may be used for biochemical identification. If the VITEK® test kit is used, the cytochrome oxidase and gram stain tests are optional. Alternatively, use traditional methods of biochemical identification. Refer to AOAC Official Method 967.27 or "Edwards and Ewing's Identification of Enterobacteriaceae", 4th Edition, for biochemical reactions of *Enterobacteriaceae* and for fermentation media and test procedures.

Table 1.

Triple Sugar Iron Agar			Lysine Iron Agar		Polyvalent Sera		Disposal
Butt	Slant	H_2S	Butt	H_2S	O	H	
Y	R	+	P	+	+	+	B. & M. T.
Y	R	+	P	+	+	-	B. & M. T.
Y	R	-	P	-			B. & M. T.
Y	R	-	Y	-	+	+	* B. & M. T.
Y	R	-	Y	-	-	-	Discard
Y	R	+	Y	+/-			B. & M. T.
Y	Y	-	Y or P	-			Discard
Y	Y	+	P	+			** B. & M. T.
NC	NC						Discard

Y = Yellow; R = Red; P = Purple; B. & M. T. = Biochemical and motility tests; NC = No change in color from uninoculated medium.

* *Salmonella* Typhisuis (found seldom in swine in U.S.)
** *Salmonella enterica* subsp. *arizonae* or *S. enterica* subsp. *diarizonae*

4.8 Serological Tests

4.8.1 Somatic (O) Antigen Agglutination Tests

At a minimum, isolates should be tested with polyvalent O antiserum reactive with serogroups A through I. Following a positive reaction with polyvalent O antiserum, it is necessary to type the isolate using individual *Salmonella* antisera for O groups A through I.

Testing for O groups A through I should encompass the majority of the *Salmonella* serotypes commonly recovered from meat and poultry products. Occasionally, however, an isolate will be recovered which is typical of *Salmonella* biochemically and is serologically poly H-positive, but is non-reactive with antisera to groups A through I. Such an isolate should be reported as "*Salmonella* non A-I" or "*Salmonella* O group beyond I".

Use growth from either the TSI or LIA slant. Test first with polyvalent O antiserum. Include a saline control with each isolate. If there is agglutination with the saline control alone (autoagglutination), identify such a culture by biochemical reactions only. If the saline control does not agglutinate and the polyvalent serum does, test the culture with *Salmonella* O grouping antisera. Record positive results and proceed to H agglutination tests.

4.8.2 Flagellar (H) Antigen Agglutination Tests

Inoculate trypticase soy broth or tryptose broth. Incubate at 35 ± 1°C overnight or until growth has an approximate density of three on the McFarland scale. Add an equal amount of saline containing 0.6% formalin and let sit one hour. Remove one ml to each of two 13 x 100 mm test tubes. To one of the tubes, add *Salmonella* polyvalent H serum in an amount indicated by the serum titer or according to the manufacturer's instructions. The other tube serves as an autoagglutination control. Incubate both tubes at 48-50°C in a water bath for up to 1 h. Record presence or absence of agglutination.

If desired, use Spicer-Edwards pooled serum or H typing serum. Find details in "Edwards and Ewing's Identification of Enterobacteriaceae" (Ewing, 1986).

The Oxoid *Salmonella* Latex Test, or equivalent, may be used as an optional method for H antigen agglutination testing. Follow the manufacturer's instructions. If a suspect *Salmonella* isolate is negative by the latex test, perform the poly H tube agglutination test described above.

4.9 Storage of Cultures

Do not store cultures on TSI agar because this tends to cause roughness of O antigens. For short-term (2-3 months) storage, inoculate a nutrient agar slant, incubate at 35 ± 1°C overnight and then store at 4-8°C.

Store "working" *Salmonella* stock cultures on nutrient agar slants. Transfer stocks monthly onto duplicate nutrient agar slants, incubate overnight at 35 ± 1°C, and then store them at 4-8°C. Use one of the slants as the working culture. Use the other slant for sub-culturing to reduce the opportunity for contamination. Cultures may be subcultured up to 5 times. After this period the culture must be re-confirmed biochemically or a new culture initiated.

For long term storage freeze cultures using cryo-beads, i.e. Cryostor™ or equivalent, lyophilize or use the procedure that follows. Subculture *Salmonella* isolates by picking a colony with an inoculating needle and stabbing it into semi-solid nutrient broth (0.75% agar). Incubate at 35 ± 1°C overnight, and then seal with hot, paraffin-soaked corks. Household wax is better than embedding paraffin because it stays relatively soft at room temperature making the corks easy to remove. Store the cultures in the dark at room temperature. Such cultures will remain viable for several years.

4.10 Selected References

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United States Department of Agriculture
Food Safety and Inspection Service
Office of Public Health and Science
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Microbiology Laboratory Guidebook
Notice of Change

Chapter new, **revised**, or archived: MLG 5.03

Title: Detection, Isolation, and Identification of *Escherichia coli* O157:H7 and O157:NM (Nonmotile) from Meat Products

Effective Date: 10/25/02

Description and purpose of change(s):

The Microbiology Laboratory Guidebook method chapters are currently under revision. The formatting is being changed to meet the requirements of the laboratory's document control system. Additional content is being added, i.e. Section 5.1.2. Limits of Detection, to meet the requirements of ISO 17025. Safety Precautions are also included in the revised chapters.

United States Department of Agriculture
Food Safety And Inspection Service, Office of Public Health and Science

MLG 5.03		Page 1 of 13
Title: Detection, Isolation and Identification of *Escherichia coli* O157:H7 and O157:NM (Nonmotile) from Meat Products		
Revision: 03	Replaces: MLG Chapter 5 Revision 2	Effective 10/25/02

Procedure Outline

5.1 Introduction
- 5.1.1 General
- 5.1.2 Limits of Detection

5.2 Safety
5.3 Quality Control Practices
5.4 Equipment, Materials, Media, Reagents and Test Kits
- 5.4.1 Equipment
- 5.4.2 Media, Reagents and Cultures
- 5.4.3 Test Kits

5.5 Detection Procedure
5.6 Isolation Procedure
5.7 Identification and Confirmation
5.8 Storage of Cultures
5.9 Selected References

5.1 Introduction

5.1.1 General

The following method is used for the analysis of raw and ready-to-eat meat products for *Escherichia coli* O157:H7 and O157:NM (O157:H7/NM). The method is based on enrichment in a selective broth medium, application of a rapid screening test, immunomagnetic separation (IMS) in paramagnetic columns, and plating on a highly selective medium.

The following definitions are used for reporting purposes. A potential positive sample causes a positive reaction on the screen test kit. A presumptive positive sample has typical colonies, observed on Rainbow Agar, and reacts specifically with O157 antiserum. A sample is a confirmed positive sample for E.coli O157:H7 or E. coli O157:NM when the isolate is confirmed biochemically and serologically, and the presence of Shiga toxin(s) or Shiga toxin gene(s) is demonstrated.

Unless otherwise stated all measurements cited in this method have a tolerance of ± 2%.

5.1.2 Limits of Detection

This test has been shown to consistently detect less than 1 colony forming unit (cfu)/g in a 65 g sample.

5.2 Safety

E. coli O157:H7/NM is a human pathogen with a low infectious dose. (Ingestion of 100 cells can cause disease.) The use of gloves and eye protection is mandatory and all work surfaces must be disinfected prior to and immediately after use. Laboratory personnel must abide by CDC guidelines for manipulating Biosafety Class II pathogens. A Class II laminar flow biosafety cabinet is recommended for activities with potential for producing aerosols of pathogens. All available Material Safety Data Sheets (MSDS) should be obtained from the manufacturer for the media, chemicals, reagents and microorganisms used in the analysis. The personnel who will handle the materials should read all MSDS sheets.

5.3 Quality Control Practices

a. Rainbow® Agar plates have a shelf life of 2 weeks.

b. All media and E-Buffer must be pre-warmed to 18-35ºC prior to use.

c. The recommended fluorescent strain of *E. coli* O157:H7 must be used in this procedure to monitor for cross contamination. The protocol for the use of fluorescent strains of *E.coli* O157:H7 as positive controls follows:

   Wild-type strains of *E. coli* O157:H7 transformed with pGFP produce a green fluorescent protein. As a result of this transformation, fluorescent strains of *E. coli* O157:H7 possess the unique property of expressing bright green fluorescence visible in the dark when illuminated by long-wave UV light. This property, which sets them apart from typical *E. coli* O157:H7, makes them useful positive controls for analyses of meat samples for *E. coli* O157:H7/NM. At different steps in the procedure, both test samples and (fluorescent) positive controls can be tested for the bright green fluorescence as a Quality Control measure to make sure that positive sample isolates actually came from the test sample and not from accidental contamination by the positive control cultures.

Results of studies done at the FSIS Beltsville Microbial Pathogens Laboratory showed that these fluorescent cultures can be subjected to *E. coli* O157:H7/NM isolation and identification procedures without losing their fluorescent properties. These strains retain their fluorescent properties when grown in SOB media with added ampicillin (SOB + A). These cultures must be transferred every 7 days to fresh SOB + A media, according to the protocol outlined below. The fluorescent colonies are ready to be used as positive controls on day 3 of the following protocol, and for the next 6 consecutive days without losing their fluorescent properties. If these cultures are not needed on a continuous basis, they can be stored at refrigeration temperatures on SOB + A agar plates in zip-lock bags or sealed with parafilm® for 1 month and then transferred, or started up again 2 days before needed. *Strict adherence* to the protocol described below is essential, in order to ensure that the fluorescent strains do not lose their ability to express green fluorescence.

i. Test the fluorescent *E. coli* O157:H7 strain (FSIS culture # EC 465-97 or the currently designated control strain) on SOB + A agar plate for fluorescence by illuminating colonies under long-wave UV light in the dark.

ii. Select only fluorescing colonies and inoculate into 10 ml of SOB + A broth in a tube. Incubate at 35 ± 2°C overnight.

iii. Streak the culture from the SOB + A broth onto a SOB + A agar plate. Incubate at 35 ± 2°C overnight.

iv. Examine colonies on the plate for fluorescence. The fluorescent colonies are ready to be inoculated into modified EC broth + novobiocin (mEC+n) at this stage. These cultures on SOB + A agar plates can be stored refrigerated and be used as positive controls for 6 more days. Incubate the inoculated mEC+n positive control culture at 35 ± 2°C overnight, along with the test samples.

v. Continue analysis per Sections 5.5-5.7 and test the Blood Agar Plates of the fluorescent positive controls and any positive sample cultures for fluorescence.

5.4 Equipment, Materials, Media, Reagents and Test Kits

5.4.1 Equipment

a. Balance, sensitivity of 0.1 g
b. Stomacher™ 400 or 3500 with appropriate sizes of sterile Stomacher™ bags, with or without mesh. (Tekmar Co., Cincinnati, Ohio), or equivalent
c. Incubator, static 35 ± 2°C
d. Micropipettors to deliver 15-1000 µl with sterile disposable filtered micropipet tips
e. Mechanical Pipettor with 1.0 ml, 5.0 ml, 10.0 ml sterile pipettes
f. Inoculating loops, "hockey sticks" or spreaders, and needles
g. UV light (long-wave, e.g. VWR # 36553-124, or equivalent)
h. Filter unit, 0.2 µm, nylon, sterile
i. Infrared thermometer
j. LabQuake® Agitator (or equivalent) with clips to hold microcentrifuge tubes
k. Sterile disposable 12 x 75 mm polypropylene tubes (e.g. Fisher # 14-956-1B, or equivalent)
l. Microcentrifuge and sterile 1.5 ml microcentrifuge tubes
m. Sterile 50 ml conical tubes (e.g. Falcon® # 2070, or equivalent) or sterile bottles
n. Sterile 40 µm Cell Strainer (Falcon® # 2340, or equivalent)
o. MACS® Large Cell Separation Columns (Miltenyi Biotec # 422-02, or equivalent)
p. OctoMACS® Separation Magnet (Miltenyi Biotec # 421-09, or equivalent)
q. Multistand to support OctoMACS® Separation Magnet (Miltenyi Biotec # 423-03, or equivalent)
r. Tray, autoclavable, approximately 130 mm x 83 mm (e.g. VWR # 62663-222, or equivalent) for use with the OctoMACS®

5.4.2 Media, Reagents and Cultures

a. Modified EC broth with novobiocin (mEC+n) (or equivalent)
b. Rainbow® Agar O157 (Biolog Inc., Hayward California, 94545) containing 10 mg/L novobiocin plus 0.8 mg/L potassium tellurite, or equivalent selective medium
c. Tryptic soy agar with 5% sheep blood

d. SOB + A Medium
e. E Buffer, approximately 7 ml per sample [Buffered Peptone Water, Bovine Albumin Sigma # 7906 (or equivalent) , and Tween-20®, or equivalent]
f. Disinfectant (Lysol® I. C., 2.0%, or equivalent)
g. Dynal® # 710.04 anti-*E. coli* O157 antibody-coated paramagnetic beads (Dynal Inc., Lake Success, NY 11042), or equivalent
h. *E. coli* O157:H7 strain 465-97 (positive control used throughout method)
i. *E. coli* ATCC strain 25922 (negative control for bead capture and screen tests)

5.4.3 Test Kits

a. The screening test for the detection of *E. coli* O157:H7/NM should meet or exceed the following performance characteristics:

Sensitivity	≥98%
Specificity	≥90%
False Negative Rate	≤ 2%
False Positive Rate	≤10%

b. *E. coli* O157:H7 latex agglutination test kit (RIM® *E. coli* O157:H7 Latex Test Kit, REMEL, 12076 Santa Fe Drive, Lenexa, KS 66215, or equivalent)

c. Biochemical test kits and systems [Vitek® GNI and GNI Plus cards (bioMerieux Vitek, Inc., 595 Anglum Drive, Hazelwood, MO 63042-2395), or equivalent]

d. Shiga Toxin test kit [Premier® EHEC, cat. # 608096 (Meridian Diagnostics, Inc., 3471 River Hills Dr., Cincinnati, OH, 45244), or equivalent

5.5 Detection Procedure

a. Sample Preparation

i. Raw ground beef microbiological testing programs.
Randomly collect five 65 ± 2 g sub-samples (total of 325 ± 10 g) that are representative of the entire sample. Place each 65 ± 2 g sub-sample in a

sterile Strainer Stomacher™ bag. Add 585 ml mEC+n broth and pummel for two minutes in a Stomacher™.

ii. Cooked meat patties and semi-dry and dry fermented sausages. Randomly prepare five 65 ± 2 g sub-samples (total of 325 ± 10 g) that are representative of the entire sample. When appropriate, sample representative portions from both the outer surface (shell) and inner section (core) of RTE products, especially semi-dry and dry fermented sausages. Place each 65 ± 2 g sub-sample in a sterile Strainer Stomacher™ bag. Add 585 ml mEC+n broth and pummel for two minutes in a Stomacher™.

iii. Outbreak-related samples. Randomly collect thirteen 25 ± 1 g sub-samples (total of 325 ± 13 g) that are representative of the entire sample. Place each 25 ± 1 g sub-sample in a sterile Strainer Stomacher™ bag and add 225 ml of mEC+n broth. Pummel for 2 minutes in a Stomacher™.

b. Incubate all bags (static) with their contents for 20 to 24 h at 35 ± 2°C. Include a positive, negative, and uninoculated medium control for each group of samples tested. Use the fluorescent *E. coli* O157:H7 strain (FSIS culture # EC 465-97) as a positive control and *E. coli* ATCC strain 25922 as the negative control.

c. From the enrichment cultures in the Stomacher™ bags, perform the screening test for *E. coli* O157:H7/NM following the manufacturer's instructions. The enrichment culture may be analyzed immediately upon removal from the incubator without waiting for tempering to room temperature. To prevent clogging the pipette tip, be sure to collect the appropriate size sample from the enrichment culture outside the inner strainer bag.

d. Samples negative by the screening test can be reported as negative for *E. coli* O157:H7/NM and discarded.

e. Samples positive by the screening test should be reported as potential positives. Begin isolation procedures from the enrichment culture in the Stomacher™ bag.

5.6 Isolation Procedure

Note: Steps a.-l. may be performed in a sequence that is convenient to the laboratory personnel.

a. Prepare E Buffer by mixing 0.5 g Bovine Albumin and 50 µl Tween-20® into 100 ml Buffered Peptone Water (BPW). Filter sterilize (0.2 µm) and store at 2-8°C.

b. Remove Rainbow® Agar plates from 2-8°C storage, allowing 3 plates for each screen-positive culture and each control. Be sure that plates have no visible surface moisture at the time of use. If necessary, dry plates (e.g. for up to 30 minutes in a laminar flow hood with the lids removed) prior to use. Dried plates that are not used should be labeled "dried", placed in bags and returned to 2-8°C.

c. Remove a bottle of E Buffer from 2-8°C storage. Decant 7 ml of E Buffer for each culture and each control into a sterile tube or bottle and allow it to warm to at least 18°C. (Return the stock E Buffer to 2-8°C.)

d. For each positive control, negative control and screen-positive culture to be analyzed, order and label 50 ml conical centrifuge tubes so that the positive control is first, followed by the negative control, then all cultures. Maintain this order for subsequent steps.

e. For each positive control, negative control, and screen-positive culture, label two sterile 1.5 ml microcentrifuge tubes (for step g and step s), one 50 ml conical centrifuge tube (for step h.) and two 12 x 75 mm capped tubes (one for step p.). For each pair of 12 x 75 mm tubes, label one tube and add 0.9 ml E Buffer (for step q.).

f. Prepare the Dynal #710.04 *E. coli* O157:H7 immunomagnetic bead suspension by following Table 1 below. Be sure to include the positive and negative controls in the total number of cultures. Use the bead suspension immediately (step g), or hold at 2-8°C. Return the stock vial of Dynal #710.04 *E. coli* O157:H7 immunomagnetic beads to 2-8°C.

g. Vortex the bead solution briefly (2-3 seconds), then add 50 µl to a labeled

microcentrifuge tube (from step e), one for each control and screen-positive culture. Use immediately or hold these tubes at 2-8°C.

h. Place a 40 µm Cell Strainer on a labeled 50-ml conical centrifuge tube (from step e.). Pipet 5 ± 1 ml of each control and enrichment culture into the respective Cell Strainer and collect at least 1.0 ml of filtrate.

i. Do not proceed with more than the number of tubes that the OctoMacs® magnet(s) will hold. Transfer 1.0 ml of a filtrate (step h.) to the corresponding microcentrifuge tube containing the immunomagnetic bead suspension (step g.) and place in the clips of the LabQuake® tube agitator. Rotate the tubes for 10-15 min at 18-30°C.

j. Attach the OctoMACS® Magnet to the Multistand.

k. Position a tray on the base of the Multistand so that it will collect the filtrate passing through the columns. Add approximately 300 ml of 2% Lysol® I. C. (or equivalent) disinfectant to cover the bottom of the tray.

l. Label and place the appropriate number of Large Cell Separation columns on the OctoMACS® Magnet. Insert columns from the front making sure the column tips do not touch any surfaces. Leave the plungers in the bags at this time to maintain sterility.

m. Transfer at least 0.5ml E Buffer to the top of each column and let the buffer run through.

n. Resuspend, then transfer each culture and control from step i. to its corresponding column.

o. After a culture or control has drained through, wash the column by applying 1.0 ml of E Buffer to each column and allow to drain. Repeat 3 more times for a total of 4 washes.

p. After the last wash has drained, remove the column from the OctoMACS® Magnet and insert the tip into an empty labeled 12 x 75 mm tube (from step e.).

Apply 1.0 ml of E Buffer to the column, and using the plunger supplied with the column, *immediately* flush out the beads into the tube. Use a smooth, steady motion to avoid splattering. Cap the tubes. Repeat this for each column. If the OctoMACS® magnet is to be used for a second set of cultures, it must be decontaminated as described in step u, below. Repeat steps j.-s. for the additional cultures.

q. Vortex the tubes from step p. briefly to resuspend the beads. Make a 1:10 dilution of each treated bead suspension by adding 0.1 ml of the bead suspension to a 12 x 75 mm labeled tube containing 0.9 ml E Buffer (from step e.).

r. Vortex briefly to maintain beads in suspension and plate 0.1 ml from each tube (from step p. and step q.) onto a labeled Rainbow® Agar plate. Use a hockey stick or spreader to spread plate the beads, being careful not to spread the beads against the edge of the plate.

s. Vortex the tubes containing undiluted beads (from step p.) and transfer to a labeled microfuge tube (from step e.) and centrifuge at least one minute using a bench-top microcentrifuge to concentrate the beads. Withdraw and discard the supernatant without disturbing the beads. Add 0.1 ml of E Buffer to the beads, resuspend the beads and transfer the beads to a labeled Rainbow® Agar plate. Spread plate the beads as described in step r.

t. As soon as there is no visible moisture on the agar surface, invert plates and incubate for 24-26 h at 35 ± 2°C.

u. Decontaminate the OctoMACS® Magnet by applying 2% Lysol® I. C. (or equivalent) disinfectant directly to the surface. After approximately ten minutes, rinse with deionized or tap water. Allow the unit to air-dry or use absorbent paper towels to dry the unit.

Table 1.

# of Cultures	*ul of Beads**	*ul of E-Buffer*	*# of Cultures*	*ul of Beads**	*ul of E-Buffer*
1	15	135	26	145	1305
2	20	180	27	150	1350
3	25	225	28	155	1395
4	30	270	29	160	1440
5	35	315	30	165	1485
6	40	360	31	175	1575
7	45	405	32	180	1620
8	50	450	33	185	1665
9	55	495	34	190	1710
10	60	540	35	195	1755
11	65	585	36	200	1800
12	70	630	37	205	1845
13	75	675	38	210	1890
14	80	720	39	215	1935
15	85	765	40	220	1980
16	90	810	41	230	2070
17	95	855	42	235	2115
18	100	900	43	240	2160
19	105	945	44	245	2205
20	110	990	45	250	2250
21	120	1080	46	255	2295
22	125	1125	47	260	2340
23	130	1170	48	265	2385
24	135	1215	49	270	2430
25	140	1260	50	275	2475

* Dynal® anti-*E. coli* O157:H7 antibody-coated paramagnetic beads (vortex briefly before use)

5.7 Identification and Confirmation

a. After incubation, *E. coli* O157:H7 colonies have black or gray coloration on Rainbow® Agar. When *E. coli* O157:H7 colonies are surrounded by pink or magenta colonies, they may have a bluish hue. Mark colonies typical of *E. coli* O157:H7 and perform latex agglutination assays for O157, following manufacturer's instructions. Streak all latex positive colonies, up to a total of five from each sample (one per sub-sample, if possible) onto Blood Agar plates. Incubate Blood Agar plates for 16-24 h at 35 ± 2°C.

Note: If no typical colonies are present, hold the original Rainbow® plates at 20-24°C for an additional 6-24 h then re-examine for typical colonies.

b. After incubation, examine the Blood Agar plates for purity under visible light, and evidence of cross contamination with the positive control by using long wave UV light. Only the positive control culture, *E. coli* O157:H7 strain 465-97, should fluoresce. If the Blood Agar plates appear pure and uncontaminated, perform the following confirmatory tests:

i. Biochemical confirmation.
Inoculate Vitek-GNI or GNI Plus cards or use an equivalent biochemical identification testing system. The cytochrome oxidase and gram stain tests are optional.

ii. Serological confirmation.
To confirm the absence or presence of O157 and H7 antigens, use an *E. coli* O157:H7 latex test agglutination kit (RIM® *E. coli* O157:H7 Latex Test Kit, or equivalent). Use growth from the Blood Agar plate (from step b).

iii. Shiga toxin/toxin genes confirmation.
The presence of Shiga toxin(s) in a culture isolate should be confirmed by the use of a toxin assay, e.g., Meridian Premier® EHEC Kit, or equivalent. When Shiga toxin(s) is (are) not demonstrated, detection of one or more toxin genes by PCR should be used for confirmation. The positive control culture, *E. coli* O157:H7, is toxin-negative.

c. If the isolate confirms as an *E. coli* O157:H7, or *E. coli* O157:NM (or H-indeterminate) and the Shiga toxin(s) and/or one or more toxin genes are present, the sample will be treated as positive for *E. coli* O157:H7, and regulatory action will be taken. The cultures will also be tested by pulsed-field gel electrophoresis (PFGE) for potential epidemiological association.

5.8 Storage of Cultures

For storage requirements of the fluorescent *E. coli* O157:H7 strain (FSIS culture # EC 465-97 or the currently designated control strain), refer to Section 5.3.c. of this chapter.

Store other "working" *E. coli* stock cultures on nutrient agar slants. Transfer stocks monthly onto duplicate nutrient agar slants, incubate overnight at 35 ± 1°C, and then store them at 4-8°C. Use one of the slants as the working culture. Use the other slant for sub-culturing to reduce the opportunity for contamination. Cultures may be subcultured up to 5 times. After this period the culture must be re-confirmed biochemically or a new culture initiated.

For long term storage freeze cultures using cryo-beads i.e. Cryostor™ or lyophilize.

5.9 Selected References

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United States Department of Agriculture | Food Safety and Inspection Service | Office of Public Health and Science | Laboratory QA/QC Division 950 College Station Road Athens, GA 30605

Microbiology Laboratory Guidebook
Notice of Change

Chapter **new**, revised, or archived: MLG 8A.00

Title: FSIS Procedure for the Use of *Listeria monocytogenes* BAX® Screening Test

Effective Date: 4/29/02

Description and purpose of change(s):

The use of a rapid screening procedure potentially reduces report-out time for true negative samples by 24 hours. FSIS has validated use of this commercial PCR based screening procedure for processed meat and poultry products. All samples identified as presumptively positive for *Listeria monocytogenes* by these tests are subject to cultural confirmation as described in this chapter and MLG 8 Isolation and Identification of Listeria monocytogenes from Red Meat, Poultry, Egg, and Environmental Samples.

United States Department of Agriculture
Food Safety And Inspection Service, Office of Public Health and Science

MLG 8A.00		Page 1 of 4
Title: FSIS Procedure For the Use of *Listeria monocytogenes* BAX® Screening Test		
Revision: Original	Replaces: NA	Effective: 4/29/02

Procedure Outline

8A.1 Introduction
- 8A.1.1 General
- 8A.1.2 Limits of Detection

8A.2 Safety Precautions

8A.3 Quality Control Procedures
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8A.4 Equipment, Reagents, and Media

8A.5 Sample Preparation and Primary Enrichment

8A.6 Secondary Enrichment and Direct Plating

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8A.8 Cultural Confirmation

8A.9 Interpretation of Results

8A.10 Completion of Testing if BAX® Unavailable

8A.11 Selected References

8A.1 Introduction

8A.1.1 General

This method describes the use of a commercial PCR based screening procedure as described in MLG 8 Section 8.4.5. to screen-test processed meat and poultry products for the presence of *Listeria monocytogenes.* All samples identified as presumptively positive for *Listeria monocytogenes* by these tests are subject to cultural confirmation.

8A.1.2 Limits of Detection

For this method, *L. monocytogenes* detection limits are determined to be better than 1 cfu/g in a 25g sample.

8A.2 Safety Precautions

CDC guidelines for the handling of BioSafety Level 2 organisms should be followed whenever live cultures of *Listeria monocytogenes* are used. All available Material Safety Data Sheets (MSDS) must be obtained from the manufacturer for the media, chemicals, reagents, and microorganisms

used in the analysis. The personnel who will handle the material should read all MSDS sheets, and all MSDS requirements should be followed.

Pregnant women and potentially immunocompromised individuals must be prohibited from laboratory rooms or areas where *L. monocytogenes* isolation or identification procedures are in progress. Although a properly sanitized laboratory area should not harbor *L. monocytogenes* or other pathogens, supervisors should use their own discretion in allowing high-risk individuals into these areas when not in use for these activities.

8A.3 Quality Control Procedures

8A.3.1 Culture Controls

a. At least one *L. monocytogenes* positive control strain is required. Appropriate cultures include ATCC 19111, NCTC 7973 or other *L. monocytogenes* cultures validated to perform in an equivalent manner.

b. At least one *L. innocua* negative control culture is required. Appropriate cultures include L. *innocua* strain ATCC 33090 or other *L. innocua* strains validated to perform in an equivalent manner.

8A.3.2 Sterility Control

Additionally, always prepare one "blank" (incubated but un-inoculated pre-enrichment/ enrichment broth) to provide a sterility control for the process.

8A.4 Equipment, Reagents, and Media

In addition to equipment, reagents, and media used in analysis of samples as described in MLG 8, the following materials will be needed.

a. PCR tube holder (Qualicon)
b. Cell Lysis Tube Cooling Block (Qualicon) held at 4 ± 2°C
c. Techne DB-2A Heating block set at 55 ± 2°C
d. Techne DB-2A Heating block set at 95 ± 2°C
e. Eppendorf Repeater Plus Pipettor (or equivalent) set at 200:l µl, and tips
f. Corning Lambda 20 Pipettor (or equivalent) set at 5 ± l µl, and tips
g. Corning Lambda 200 Pipettor (or equivalent) set at 150 ± l µl, and tips

h. 12 X 75 mm (Falcon 352063, or equivalent) tubes
i. Cell Lysis Tubes and Caps, Cell Lysis Tube Rack and box (Genemate 8 strip tubes, ISC Bioexpress, T-3120-5)
j. Pipettor and 5 ml pipettes
k. BAX® Assay for Screening *L. monocytogenes* (Qualicon # 17710609) held at 4 ± 2°C
l. MOPS-BLEB medium
BBL Listeria enrichment broth (BBL #12333, or equivalent)
MOPS free acid (Sigma #1254, or equivalent)
MOPS sodium salt (Sigma #M9381, or equivalent)

8A.5 Sample Preparation and Primary Enrichment

Perform sample preparation and pre-enrichment in as described in MLG 8, Section 8.5.1 and 8.5.2.

8A.6 Secondary Enrichment and Direct Plating

a. Transfer 0.1 ± 0.02 ml of the UVM enrichment to 10 ± 0.5 ml of MOPS-BLEB. Incubate inoculated MOPS-BLEB tubes at 35 ± 2°C for 18-24 h.

b. Streak a MOX plate. Streak a loopful or a drop approximating 0.1 ml of the UVM over the surface of the plate. Alternatively, dip a sterile cotton-tipped applicator or equivalent into the UVM and swab 25-50% of the surface of a MOX plate. Use a loop to streak for isolation from the swabbed area onto the remainder of the plate. Incubate the MOX at 35 ± 2°C for 26 ± 2 h.

8A.7 The BAX® System for Screening L. monocytogenes Test Procedure

Follow the current BAX® User's Guide for preparing reagents, performing the test, and reading the results. The equipment must be set up, and operated, and all records must be documented, according to laboratory work instructions.

8A.8 Cultural Confirmation

a. Streak a MOX plate using a loopful of the MOPS-BLEB, or by streaking a drop approximating 0.1 ml or aseptically dip a sterile cotton-tipped applicator or equivalent into the MOPS-BLEB and swab 25-50% of the surface of a MOX plate. Use a loop

to streak for isolation from the swabbed area onto the remainder of the plate. Incubate the MOX at 35 ± 2°C for a minimum of 24 h.

b. Proceed with all isolation and purification procedures as per MLG 8, Sections 8.5.4.a, 8.5.6, and 8.6.

8A.9 Interpretation of Results

a. Samples that test BAX®-negative will be reported as negative if the concurrent 24 h Direct Plating is also negative. Cultural analysis will continue on samples that are BAX®-negative but have typical colonies on the 24 h Direct Plating MOX plates, or have a BAX®-positive, BAX®-indeterminate or BAX® signal-error result.

b. In analytical runs where the positive control tests negative, either the reserve samples will be retested or the laboratory shall complete the cultural method by streaking all samples and controls from MOPS-BLEB medium onto MOX plates. Proceed with all isolation and purification procedures as per MLG 8, Sections 8.5.6 and 8.6.

8A.10 Completion of Testing if BAX® Unavailable

If circumstances (e.g. a power outage or equipment failure) do not allow testing using the BAX® system, the laboratory shall complete the cultural method by streaking all samples and controls from MOPS-BLEB medium onto MOX plates. Proceed with all isolation and purification procedures as per MLG 8, Sections 8.5.6 and 8.6.

8A.11 Selected References

Centers for Disease Control and Prevention and National Institutes of Health (CDC/NIH). 1999. BioSafety in Microbiological and Biomedical Laboratories, 4th ed. U.S. Government Printing Office, Washington, D.C. (also found on the internet at:

http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm)

BAX® System PCR Automated Detection for Bacterial Screening User Guide, Dupont Qualicon.

CHAPTER 1. SAMPLE PREPARATION FOR MEAT, POULTRY AND PASTEURIZED EGG PRODUCTS

Charles P. Lattuada and B. P. Dey

1.1 Introduction

The purpose for the microbiological examinations of meat and poultry products is to obtain information. This information gathering may follow a qualitative or quantitative analytical format. The format followed is called the sampling plan. Many microorganisms are present in very low numbers and require one or more enrichment steps. If cell injury is anticipated, a non-selective enrichment frequently is used to resuscitate cells, followed by a more selective enrichment.

The analyst must study all records and correspondence before examining the sample. Care must be exercised in maintaining and handling the sample to insure that it is the same one that was collected, that it has not been tampered with, and that its condition is the same as it was at collection. The reserve sample must be stored properly to maintain its integrity in case additional analyses are required.

An analyst must be keenly aware that during all steps of the analysis, it is important to minimize the growth of non-critical microorganisms and to prevent entrance of environmental contaminants. The organism(s) isolated must come from the test sample and not from an outside source. These facts cannot be over-emphasized and can be accomplished only if strict attention is paid to the following rules:

The sampling operation must be well organized, with all supplies and equipment properly positioned before starting.

Ideally, sampling should be done in an area free of air currents following good aseptic procedures.

All work surfaces must be clean and sanitized.

Implements used for sampling must be sterile before use and protected from outside contamination during use.

The outside of the immediate container must be thoroughly sanitized.

Any laboratory person processing samples must be very familiar with aseptic techniques and the principles of sterilization, sanitization and disinfection. The person assigned to the sampling task should know the sampling protocol to be used and have a

reference copy at hand in case questions arise.

1.2 Sanitizing the Work Area

The work area must be clean and free from dust; detergent sanitizers are satisfactory for cleaning. Before work begins, the work area should be cleaned and a sanitizer/disinfectant applied liberally and given time to act. Quaternary ammonium compounds, sodium hypochlorite and phenolic compounds all are suitable for this purpose. The manufacturer's instructions regarding the concentration needed and the time required for the compound to act should be followed.

1.3 Sterilization of Instruments

a. All instruments and containers to be used in the sample analysis must be sterile. Any sterilization procedure may be used that is compatible with the material to be sterilized. Sterilization implies the total destruction of all viable organisms as measured by an appropriate culturing method.

b. An exception can be made, if necessary, when the number of instruments is limited (ie. chisels) and the testing protocol does not include sporeforming microorganisms. In which case, the instruments first are washed with soap and water, rinsed and inspected to be sure there is no organic matter in crevices or hinges, then they may be steamed for 30 minutes in an instrument sterilizer or placed in boiling water for two minutes.

c. Do not dip instruments into alcohol and flame them as a substitute for heat sterilization. It is not a substitution for the methods given above.

1.4 Disinfection of Outer Surface of the Immediate Container

a. The outside covering of the intact immediate container must be decontaminated to the greatest extent possible and particularly in the area where an opening will be made to expose the contents.

b. Hydrogen peroxide, tincture of iodine or 2500 ppm sodium hypochlorite solution may be used for this purpose. Allow time for the disinfectant to act before opening the container. Aseptically remove any residual disinfectant to prevent its entering the container when an opening is made.

1.5 Cutting and Weighing Samples

a. The sample should never be touched with bare hands. During the process of sanitizing the immediate container, the analyst should put on a pair of sterile gloves for handling samples.

b. Sterile instruments should be used for cutting, removing and manipulating all samples.

c. The sample must be taken aseptically according to the sampling protocol and placed in the proper sterile container for the next processing step. The remainder of the sample must be secured with an appropriate sterile closure that will preserve the sterility and integrity of the sample reserve. The sample reserve must be held according to the sampling protocol.

d. If the sample is to be weighed, the balance on which samples are weighed must be placed in an area that is clean and free of strong air currents.

e. If at all possible, the product should be weighed directly into the sterile container that will be used for dilution, mixing, blending and/or stomaching.

f. When weighing is complete, clean and disinfect the area with the same product used initially for disinfecting the work area. All instruments, containers, gloves and other materials that may have been in contact with the product must be incinerated or terminally sterilized before cleaning or disposal.

1.6 Selected References

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CHAPTER 2. PHYSICAL EXAMINATION OF MEAT AND POULTRY PRODUCTS

Charles P. Lattuada and B. P. Dey

2.1 Introduction

Microorganisms associated with meat and poultry products can be placed in three categories, beneficial, spoilage and pathogenic. Each product has a characteristic microbial profile called its "normal flora". Frequently information on changes in the "normal flora" can be obtained rapidly by simple observations. These observations can be grouped into a category called organoleptic observations. The term "organoleptic" refers to the use of the senses in determining the acceptability of a product. This would also include a direct microscopic examination.

Organoleptic analyses are of particular importance during investigations of certain food production problems such as detecting deleterious pre- or post-processing changes of canned products. Changes brought about by abusive handling and storage also may be detected by organoleptic observation.

In order to make a valid judgment, based upon one or more organoleptic observations, the analyst must know the physical characteristics of a "normal" product. This knowledge can be gained by experience and specialized training. Each laboratory should have Standard Operating Procedures (SOPs) describing the organoleptic standards for the acceptance or rejection of samples.

When judging a product to be abnormal, if possible, the decision should be based on a comparison of the suspect product with one that is normal, if readily available. This minimizes the subjectivity of the decision that a product has an "off odor", "off color", or other sensory abnormality. Tasting products as part of a microbiological examination is a dangerous practice and should be avoided. When the question to be answered is related to spoilage, odor is of primary importance; chemical and/or bacteriological results are corroborative and substantiating.

2.2 Examination

The following guideline establishes a standardized inter-laboratory procedure for characterizing samples.

a. Appearance: Changes in color; degradation of fat; presence of foreign materials such as metal, hair, feathers, sand, charcoal, etc.

b. Texture: Change in consistency; development of slime; breakdown of structure (proteolysis), etc.

c. Odor: Examples of words used to describe off-odors are: sour (acidic), moldy, musty, fishy, rancid, fruity, yeasty (beer-like) and putrid. However, if the analyst cannot decide how to classify an odor it is acceptable and appropriate to say simply: "off-odor" or "taint". Notations as to whether the off-odor is strong or slight are also in order.

2.21 Odor Examination By a Panel

In some cases results of odor examinations are equivocal and an odor detection panel, consisting of at least three members must be formed. The purpose of this panel is to evaluate aroma only, and its judgement must not be swayed by appearances. Only people with a good sense of smell can be assigned to it. The coordinator, who is not a panel member, will prepare the samples and ensure that the following procedures are followed:

a. The test must be conducted in a well-ventilated area free of strong odors.

b. At least 15 - 20% of the samples in the test group should be normal, wholesome, product-counterparts of the samples being examined. The normal controls should be as similar to the test product as possible with respect to ingredients, processing, packaging, size, age and handling procedures.

c. All samples should be presented to the smell panel in sequentially coded glass jars or polyethylene bags of the same size and shape, similar in weight and at the same temperature (usually 35°C). Both the normal and questionable products should be presented in a random order with a rest between samples. Do not decontaminate cans by flaming since heating and/or burning the contents could alter or mask any other odors that might be present.

d. Before beginning the examination, the panel members should smell and discuss the characteristic aroma of a normal product. They should be made aware that it is for general reference only, since normal products may vary slightly in odor and intensity. They then should rest until the samples are presented to allow recovery of the sense of smell which tires easily.

e. During the actual sample analysis, each panel member should remove the jar lid or open the bag, sniff the contents without glancing at them, replace the lid/close the bag and return the container to the panel coordinator. The panelist's sensory perceptions should

be entered on a score pad containing a list of appropriate terms with notations about whether the odor was strong or weak.

f. During the examination the panel members must not comment, exclaim or use body language that conveys their impression of the odors to other members of the panel.

Caution: It is not to be assumed that a smell panel composed of laboratory personnel will have the degree of skill attained by professional odor analysts. The purpose of a panel of laboratory personnel is to detect the odors of decomposition or product contamination with an odorous compound.

2.3 Determination of pH in Meat and Poultry Products

Potentiometric measurements should be used to determine the pH of a food product. The accuracy of most pH meters is approximately 0.1 pH units and reproducibility should be approximately ± 0.005 pH units. Both the glass and reference electrode are usually housed in a single tube, called the combination electrode. To obtain accurate results the same temperature should be used for standardization with the buffers and the sample. Measurements should be taken within the temperature range of 20 to 30°C.

2.31 Equipment and Reagents

a. Blender
b. Beaker, 100 ml
c. Separatory funnel
d. pH meter, suitable for reading pH from 0 to 14 in 0.1 unit increments. A rugged, designated combination electrode should be used for pH measurement of meats and poultry. A flat combination electrode works well for determining the surface pH of canned foods.
e. Distilled water
f. Certified buffer solutions of pH 7.00, and either pH 4.00 or 10.00. The buffers chosen should bracket the desired pH.

2.32 Procedure

a. Calibrate the pH meter, according to manufacturer's instructions, using certified buffers pH 7.00 and either pH 4.00 or 10.00.

b. Most products will be solid and require blending. A 1:5 or 1:10 dilution should be made with distilled water in a clean blender jar. Blend to a thin uniform consistency and perform the pH measurement. If fat or oil causes fouling of the electrode, transfer a portion

of the homogenate to a separatory funnel and draw off a portion of the aqueous phase. On certain products centrifugation may be required in order to recover a measurable aqueous phase.

c. Adjust the temperature control on the pH meter to that of the sample (ideally 25°C) and immerse the pH electrode into the liquid phase.

d. A surface electrode may be used with certain low fat products that present a flat, solid core surface. If a surface measurement is taken, ensure that the electrode has good contact with the product surface.

e. Record pH to the nearest 0.1 unit.

2.4 Determination of Water Activity (A_w) of Meat and Poultry Products

The free moisture level in food is called water activity (a_w). This is the water available to support microbiological growth in the food. It can be lowered by dehydration or by the addition of binding agents such as salt or sugar. The growth of different types and genera of microorganisms is controlled by the water activity level in a specific product. Much information exists on the water activity limits of growth for microorganisms. For example, the limit of growth for *Clostridium botulinum* occurs between an a_w of 0.935 and 0.945. Canned foods with an a_w of $\leq$0.85 are exempt by the FDA from the canned food regulations and cured meats without nitrates must have an a_w of $\leq$0.92. It is important, therefore, that the a_w in foods be measured very accurately. A detailed list of growth limiting a_w values can be found in Chapter 8 of the Compendium of Methods for the Microbiological Examination of Foods.

Measurement of the a_w in a food sample is affected by both time and temperature. It is dependent upon allowing enough time for the water vapor of the sample to reach equilibrium with the air space in a closed container, such as a closed jar, at a constant temperature. When incubation is required for equilibration, it is absolutely necessary to maintain accurate temperature control of the food samples inside the incubator used for a_w. It is equally important to allow ample time for the humidity of the air space above the sample to reach equilibrium with the food sample.

2.41 Decagon

The Decagon CX-2 will measure a_w in less than 5 minutes. The instrument has rapid vapor equilibration, does not require temperature equilibration and requires only a small sample (approximately 5 grams of food). The instrument does not have to be calibrated, but quality control samples, consisting of deionized water and various salt slushes, must be included in an analysis. When a very wet sample and a very dry one follow one another, two interim readings should be taken of the second sample before collecting data with the third reading. When a reading is completed,the instrument will "beep" continuously. The only reported material to interfere with a Decagon reading is propylene glycol. Foods containing propylene glycol should not be analyzed by this method.

2.42 Equipment and Materials

a. Decagon, Model CX-2 manufactured by Decagon Devices, Inc., Pullman, WA 99163-0835.
b. Blender and blending jars
c. Transfer pipettes

2.43 Procedure

a. In order to obtain a representative sample, approximately 100-200 grams of food should be blended.

b. Remove at least two samples, approximately 5 grams each, for a_w determination; the cup should never be filled above the fill level line molded into the side of the plastic cup.

c. Follow the manufacturer's directions contained in the Decagon Manual very carefully when performing this analysis.

d. Saturated salt solutions should be used for reference controls. The following saturated salt mixes and their expected a_w at 25°C normally are used:

NaCl ---------0.755
KBr ----------0.811
KCl ----------0.845
$(NH_4)H_2PO_4$-----0.934

Note: Never leave a sample in the instrument after a reading has been taken.

2.44 American Instrument Electronic Hydrometer

Another method for determining a_w is the American Instrument Electronic Hydrometer. Reportedly, it is an accurate instrument for measurement of the a_w in food products, provided the manufacturer's directions are followed carefully. The instrument measures the changes in electrical resistance of specially coated lithium chloride sensors. The electronic part of the instrument is very rugged and needs no special care. The sensors, like pH electrodes, are very sensitive and can be affected permanently by water condensation, desiccation, corrosive chemicals such as mercury vapor, unstable hydrocarbons such as ketones; halogen gases; and sulfur compounds such as hydrogen sulfide and sulfur dioxide. Sensors can be affected reversibly by polar vapors such as ammonia, amines, alcohols, glycols and glycerols. The response of sensors will return to normal, from slightly higher readings, if the polar vapors are removed by aeration.

2.45 Equipment and Materials

a. American Instrument Electronic Hydrometer (Model No. 30-87 or equivalent) manufactured by Newport Scientific, Inc., 8246E Sandy Court, Jessup, MD 20794.

b. Sensors, Color Code-Gray, (Cat-No. 4822W) for the above instrument, available from the same manufacturer. The Company makes different types of sensors for different ranges of humidities. This sensor is the one most commonly used in meat and poultry product analyses. They have an a_w range of about 0.81 to 0.99. Each sensor is unique and comes with its own factory calibration curve. When purchasing gray sensors specify that the a_w readings between 0.90 - 0.94 be inside the linear portion of the calibration curve. Also request that the correction factor of each sensor at 30°C (86°F) be incorporated into each calibration curve.

c. Sensor lids and 8-gang switch box. These socket type lids normally fit into the rims of standard pint size canning jars. The 8-gang switch box allows measurement of eight samples at a time. The sensor connectors should be labeled 1 to 8 to correspond to the switch position.

d. A forced-air incubator should be used to hold the samples at 30 ± 0.5°C. If necessary, cut a 1.5" diameter hole in the incubator to introduce the electrical leads for the eight sensors into the incubator. Be sure to fill the hole with sealant.

e. Clean and dry standard pint-size glass canning jars, without chips or cracks on the rims, for the samples.

f. Pipettes

g. Preparation of a saturated ammonium phosphate, monobasic, $[(NH_4)H_2PO_4]$ slush

$(NH_4)H_2PO_4$, reagent grade	200 g
Merthiolate	25 mg
Glass distilled water	

Place the ammonium phosphate and merthiolate in a new or clean pint-size jar, slowly add glass-distilled water (approximately 2-3 ml at a time), and stir vigorously with a spoon until approximately one half of the crystals are dissolved. Care must be taken to avoid splashing the salts onto the sides and rims of the jar.

Incubate the salt slushes at 30°C for 2-3 days to establish equilibrium.

h. Preparation of saturated potassium dichromate (K_2CrO_4) slush

Use the same procedure as above. Omit the merthiolate.

i. Store the salt slushes indefinitely in a 30°C incubator at all times except to install or remove sensors.

j. The a_w of the salt slushes should be (measured with a calibrated gray sensor):

$(NH_4)H_2PO_4$ slush	0.929 at 30°C
K_2CrO_4 slush	0.865 at 30°C

2.46 Procedure

a. Follow the manufacturer's directions very carefully when using this method.

b. Test each sensor first in $(NH_4)H_2PO_4$ and then in K_2CrO_4 salt slush and record the results on the analysis sheet. The sample test results will be recorded on the same sheet. Do not use sensors that differ from the expected value of the salt slush by more than a_w 0.01 unit.

c. If the a_w is going to be measured in other than the range specified for the grey sensor, be sure to use the appropriate sensor and prepare salt slushes appropriate for the expected range. A table of other salt slushes can be found in Chapter 8, "Measurement of water activity (a_w) and acidity", in the Compendium of Methods for the Microbiological Examination of Foods.

2.5 Selected References

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Prior, B. A. 1979. Measurement of water activity in foods: A review. J. Food Prot. 42:668-674.

Troller, J. A., and V. N Scott. 1992. Measurement of water activity (a_w) and acidity, p. 135-151. *In* C. Vanderzant and D. F. Splittstoesser (ed.), Compendium of Methods for the Microbiological Examination of Foods. 3rd Edition. Amer. Publ. Hlth. Assoc. Washington, D.C.

CHAPTER 3. EXAMINATION OF FRESH, REFRIGERATED AND FROZEN PREPARED MEAT, POULTRY AND PASTEURIZED EGG PRODUCTS

Charles P. Lattuada, Larry H. Dillard and
Bonnie E. Rose

3.1 Introduction

The laboratory methods contained in this section of the Guidebook are used to detect and, when desired, quantitate selected microorganisms in samples collected in federally inspected meat, poultry and egg processing establishments. They generally follow the Compendium of Methods for the Microbiological Examination of Foods and AOAC International's Official Methods of Analysis. The methods presented in this section may be used to analyze samples of:

a. fresh, frozen, smoked, cured or dehydrated meat and poultry products;

b. prepared/ready-to-eat products such as pot pies, luncheon meats, dinners, battered or breaded meat and poultry products;

c. refrigerated meat or poultry salads;

d. dehydrated soups and sauces containing the requisite amount of meat or poultry;

e. meat snacks, hors d'oeuvres, pizza and specialty items;

f. various ingredients incorporated with meat and poultry products such as spices, vegetables, breading material, milk powder, dried egg, vegetable proteins;

g. pasteurized egg products;

h. environmental samples from areas in which any of the above are processed or manufactured.

The quantity and types of mesophilic microorganisms present in or on any of these products offer a means of evaluating the degree of sanitation used during the process. If the results obtained for coliforms, *Escherichia coli*, and *Staphylococcus aureus* are unusually high, they might result in some type of official follow-up action. Any such follow-up analysis will use the appropriate Final Action Method found in the latest edition of Official Methods of Analysis of AOAC International or any of its supplements. Pertinent sections in the 16th Edition are:

- **Aerobic Plate Count (APC): 966.23**
- **Coliform Group and *E. coli*: 966.24**
- ***S. aureus*: 987.09**

3.11 Comparison With the AOAC Method

The procedures in the following sections of this Chapter are either the same as those published by the AOAC or generally follow an AOAC method. The following is a listing of deviations:

a. The procedure for determining numbers of coliform and *E. coli* differ from the AOAC procedure as follows:

i. Use a single tube of laurel sulfate tryptose broth (LST) per dilution, rather than three tubes per dilution.
ii. Incubate inoculated LST and EC broths for 24 ± 2 h.
iii. Consider the presence of gas in LST and EC broths as positive for coliform and *E. coli* respectively, with no further testing required.

b. The procedure for the enumeration of *S. aureus* differs from the AOAC procedure in that only one tube, instead of three, per dilution is used to determine the estimated count.

3.12 General Guidelines for Testing Fresh or Prepared Foods

a. Do not combine the components of composite items such as frozen dinners into a single sample. To the greatest extent possible, examine as separate samples the vegetable or non-meat portion(s) and the meat portion.

b. The quantity, condition and suitability of the sample are very important.

i. The quantity should be sufficient to perform the analysis and have a reasonable amount in reserve for repeat testing.
ii. The condition of receipt should be in keeping with good microbiological practices for the analysis(es) requested.
iii. The sample should be, to the greatest extent possible, representative of the whole of the original product at the time the sample was taken.
iv. When appropriate and if possible, samples should be received at the laboratory in their original unopened package(s) (intact sample).

3.13 Tests Covered in This Section

a. Aerobic plate count
b. Coliform and *E. coli* quantitative estimates
c. *S. aureus*

3.2 Equipment and Materials

a. Balance, capacity ≥2 kg, sensitivity ± 0.1 g
b. Blender and sterile blender jars
c. Stomacher™ and sterile stomacher bags
d. Incubators at 35 ± 1.0°C, and 20 ± 1.0°C
e. Water bath at 45.5 ± 0.05°C
f. Water bath at 37 ± 1.0°C
g. Manual or Automatic colony counter and tally register
h. Sterile, disposable/reusable dishes, pans or trays for sample cutting
i. Sterile forceps, spoon, knife, scissors and other sterile sampling equipment
j. Sterile 1, 5 and 10 ml pipettes
k. Sterile 100 x 15 mm petri dishes
l. Transfer loop, 3 mm
m. Microscope and clean slides
n. Refrigerated centrifuge
o. Refrigerator
p. pH meter

3.21 Media

a. Plate count agar (PCA) in containers suitable for making pour plates
b. Laurel sulfate tryptose (LST) broth with fermentation tubes
c. EC broth with fermentation tubes
d. Surface dried Baird-Parker plates (egg tellurite glycine pyruvate agar, ETGPA)
e. Brain heart infusion (BHI) broth
f. Trypticase soy broth with 10% sodium chloride and 1% sodium pyruvate (PTSBS)
g. Toluidine blue DNA agar

3.22 Reagents

a. Butterfield's phosphate diluent
b. Gram stain reagents
c. Desiccated rabbit plasma (coagulase) EDTA
d. Tris Buffer
e. Ammonium sulfate [$(NH_4)_2SO_4$], reagent grade
f. Triton X-100
g. 3M trichloroacetic acid solution
h. 1N HCl solution

3.3 Preparation and Dilution of Samples

See Section 1.3 - 1.5 (Sterilization of Instruments, Disinfection of Containers, and Cutting and Weighing Samples)

3.31 Food Homogenates

a. Using sterile spoons, forceps, scissors, etc., aseptically weigh 50 ± 0.1 g of the sample into a sterile blender jar or stomacher bag.

b. If the sample is frozen, remove portions, whenever possible, without thawing the larger sample and weigh 50 ± 0.1 g of the sample into a sterile blender jar or stomacher bag. It is well known that freeze/thaw cycles are damaging to bacteria. This is particularly important when a re-examination of the product may be necessary. Otherwise, partially thaw the sample at 2-5°C for about 18 h, or by placing the sample in a watertight container and immersing it in cold water for 1-2 h.

c. Add 450 ml sterile Butterfield's phosphate diluent and stomach for 2 minutes, or blend at high speed for two minutes. The total volume in the blender jar must completely cover the blades. This becomes the 1:10 dilution.

d. Permit the foam to settle; then pipet 10 ml of the blended 1:10 dilution into a 90 ml dilution blank to make the 1:100 dilution. Repeat this procedure to prepare serial dilutions of 10^{-3}, 10^{-4}, etc. Shake all dilutions 25 times in a one foot arc. Use a separate 10 ml pipette to prepare each dilution. Pipettes must deliver accurately the required volumes. Do not deliver less than 10% of a pipette's volume. For example, to deliver one ml, do not use a pipette of more than 10 ml volume.

e. The analyst should strive to minimize the time from when the sample is stomached or blended until all the dilutions have been placed in or on the appropriate medium; ideally this time should not exceed 15 minutes whenever possible.

f. If the sample consists of less than 50 g, weigh about half the sample, and add the amount of diluent required to make a 1:10 dilution (nine times the weight of the portion of sample used) and proceed as above.

g. Hold reserves of each sample at or below -15°C (5°F), unless the product is stored normally at ambient

temperature or unless a specific protocol specifies otherwise. Samples should be held until a determination is made that a repeat test is not necessary or for the length of time designated by the testing protocol.

3.32 Whole Bird Rinse

a. Since there are differences between sample types and sizes (eg. chicken vs. turkey carcasses), be sure to check the specific program protocol before using this procedure.

b. Aseptically transfer the carcass to a sterile Stomacher 3500 bag (or equivalent), draining as much excess fluid as possible during the transfer.

Note: Larger (24 x 30-36 in.) bags will have to be used with turkeys.

c. Add 400 ml (chickens) or 600 ml (turkeys) of Butterfield's Phosphate Diluent (BPD) to the carcass in the bag. Pour approximately one half the volume into the interior cavity of the bird and the other half over the skin. Note: If *Salmonella* is the ONLY target analyte, Buffered Peptone Water (BPW) may be substituted for the BPD.

d. Rinse the bird, inside and out, with a rocking motion for 1 min at a rate of approximately 35 forward and back swings per minute. This is done by grasping the carcass in the bag with one hand and the closed top of the bag with the other. Rock with a reciprocal motion in an 18-24 inch arc, assuring that all surfaces (interior and exterior) are rinsed.

e. Aseptically remove the carcass from the bag, draining excess rinsed liquid into the bag, dispose of the carcass, and culture the bird rinse liquid according to protocol directions.

3.33 Egg Products

a. Liquid eggs must be held at 4.4°C (40°F) or below for valid analysis.

b. Frozen samples must be thawed as rapidly as possible in a water bath at 45°C.

c. Exposed or leaking samples should not be analyzed.

d. Mix the sample with a sterile spoon, spatula, or by

shaking.

e. Aseptically weigh a minimum of 100 g of egg sample into a sterile blender jar or sealable bag containing 900 ml of the appropriate enrichment or buffer. If a specific protocol requires a sample size greater than 100 g, the 1:10 ratio must be maintained in the same enrichment or buffer.

f. Mix the 1:10 sample enrichment/buffer well by shaking, stomaching, or blending.

g. Dried egg samples should be rehydrated slowly by gradually adding the enrichment/diluent to the sample. This is done by adding a small portion of liquid to the sample and mixing aseptically to obtain a homogeneous suspension. Repeat this procedure three times and then add the remainder of the liquid. Mix until a lump-free suspension is obtained.

h. Incubate or transfer to the appropriate enrichment medium and incubate according to the protocol(s) being used.

3.4 Aerobic Plate Count (APC)

a. Pour Plates (Reference AOAC 966.23 C)

i. Using the dilutions prepared in section 3.3, pipet 1 ml from the 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} etc. dilutions into each of four petri dishes, two for each incubation temperature. Plate additional dilutions when expecting higher bacterial levels.

ii. Use separate sterile pipettes for each dilution.

iii. Add molten Plate Count Agar cooled in a water bath to 45 ± 1°C. Uniformly mix the agar and the inoculum by gently swirling or tilting each plate, taking care not to generate bubbles.

iv. Allow the agar to harden and then place one series of duplicate plates in a 35 ± 1°C incubator for 48 h. Incubate the other series at 20 ± 1°C for four or five days.

v. Use a colony counter and count colonies on the duplicate plates in a suitable range (30-300 colonies per plate). If plates do not contain

30-300 colonies, record the dilution counted and the number of colonies found. Average the counts obtained from duplicate plates, multiply by the dilution factor and report this number as the aerobic plate count per gram or milliliter at the incubation temperature used.

b. Alternate Methods - AOAC

i. Aerobic Plate Count in Foods: Hydrophobic Grid Membrane Filter Method* (AOAC 986.32)

ii. Dry Rehydratable Film (Petrifilm Aerobic Plate™) Method* (AOAC 990.12)

iii. Spiral Plate Method* (AOAC 977.27)

*Since these methods are available commercially, the manufacturer's directions should be followed.

3.5 Coliform Group and *Escherichia coli*

a. Estimated Count Procedure (Reference AOAC 966.24)

i. Using the dilutions prepared in section 3.3, pipet 1 ml from the 10^{-1}, 10^{-2}, 10^{-3} etc. dilutions into LST broth, one tube per dilution. Inoculate additional dilutions when expecting higher bacterial levels. The highest dilution of sample must be sufficiently high to yield a negative end point.

ii. Use separate sterile pipettes for each dilution.

iii. Incubate the tubes of LST broth at 35°C for 24 ± 2 h.

iv. Examine each tube for gas formation as evidenced by displacement of fluid in the inverted tubes or by effervescence when tubes are shaken gently.

v. Consider any tube of LST broth displaying gas as coliform positive, and report the number of coliform per gram in accordance with the highest dilution with gas. When a "skip" occurs, report by using the missing estimate (for example: If the 10^{-1}, 10^{-2}, and 10^{-4} dilutions produce gas but the 10^{-3} dilution tube is non-gassing, report "1,000 coliforms per gram.")

b. Fecal Coliform (*E. coli*) Estimated Count Procedure (Reference AOAC 966.24)

i. Use a 3 mm calibrated loop to transfer one loopful

from every gas-positive LST broth tube to a correspondingly marked tube of EC broth.

ii. Incubate the EC tubes in a 45.5 ± 0.05°C covered water bath for 24 ± 2 h. Submerge the EC tubes in the bath so that the water level is above the level of medium in the tubes.

iii. Record every tube producing gas, as evidenced by displacement of liquid in the inverted tube or by effervescence when tubes are shaken gently.

iv. Report the number of *E. coli* per gram in accordance with the highest dilution displaying gas. When a "skip" occurs, report by using the missing estimate (for example: If the 10^{-1}, 10^{-2}, and 10^{-4} dilutions produce gas but the 10^{-3} dilution tube is non-gassing, report "1,000 *E. coli* per gram.")

c. Alternate Methods - AOAC

i. Coliform and *Escherichia coli* Counts in Foods: Hydrophobic Grid Membrane Filter/MUG Method*
ii. Coliform and *Escherichia coli* Counts in Foods: Dry Rehydratable Film*

*Since these methods are available commercially, the manufacturers's directions should be followed.

3.6 *Staphylococcus aureus*

a. Estimated Count Procedure (Reference AOAC 987.09)

i. Using the dilutions prepared in section 3.3, pipet 1 ml from the 10^{-1}, 10^{-2}, 10^{-3} etc. dilutions into tubes containing 10 ml of Trypticase (tryptic) Soy Broth with 10% sodium chloride and 1% sodium pyruvate (PTSBS), one tube per dilution. Inoculate additional dilutions when expecting higher bacterial levels. The highest dilution of sample must be sufficiently high to yield a negative end point.

ii. Use separate sterile pipettes for each dilution.

iii. Incubate the PTSBS tubes at 35°C for 48 h.
iv. Using a 3 mm calibrated loop, transfer a loopful from each growth-positive tube as well as from the tube of the next highest dilution to previously prepared plates of Baird-Parker agar. Streak in a manner to produce well-isolated colonies.

v. Incubate the Baird-Parker plates at 35°C for 48 h.

vi. Typical *S. aureus* colonies appear as circular, convex, smooth, grey-black to jet-black colonies on uncrowded plates and frequently have an off-white margin surrounded by a zone of precipitation (turbidity) followed by a clear zone. The colonies usually have a buttery to gummy consistency.

vii. Test two or more isolates, from each useable plate meeting the above description (3.6,vi), for coagulase as in Section 3.6 (c).

b. Direct Plating

i. If *S. aureus* counts of 100 cfu per gram or more are expected, direct plating can be done using Baird-Parker agar.

ii. Pipet 0.1 ml from each dilution on previously prepared and dried Baird-Parker agar plates. Use separate accurate pipettes for each dilution.

iii Distribute the inoculum evenly over the surface of the plates using separate, sterile, fire polished, bent-glass rods ("hockey sticks") for each plate. Mark plates according to the dilution used.

iv. Invert plates and incubate at 35°C for 48 h.

v. Select plates containing approximately 20 or more well-isolated typical *S. aureus* colonies. Count plates containing 20-200 colonies. Typical colonies are circular, convex, smooth, grey-black to jet-black and frequently have an off-white margin surrounded by a zone of precipitation (turbidity) followed by a clear zone. The colonies usually have a buttery to gummy consistency.

vi. Select 10 colonies from those counted and inoculate each into separate 13 x 100 millimeter tubes containing 0.2 ml of BHI broth for coagulase testing. Test for coagulase as in 3.6 (c).

vii. Calculate the total number of colonies represented by coagulase positive cultures and multiply by the appropriate sample dilution factor to record the number of coagulase positive staphylococci per gram.

c. Coagulase Test for *Staphylococcus aureus*

i. Use an inoculating needle to obtain a small amount of growth from each suspect colony and place it into 13 X 100 mm tubes containing 0.2 ml of BHI Broth.

ii. A known coagulase positive and a known negative culture should be inoculated into BHI broth at the same time as the samples.

iii. Incubate each tube at 35°C for 18-24 h.

iv. Add 0.5 ml of rabbit plasma with EDTA, reconstituted according to the manufacturer's directions, to the BHI cultures.

v. Mix thoroughly and place the tubes in a 35-37°C. water bath.

vi. Examine these tubes each hour, from one through six hours, for clot formation. Any degree of clotting should be interpreted as a positive reaction.

3.61 Special Sampling Procedure for Fermented Sausage Products

a. Introduction

During the early stages of sausage fermentation, staphylococci can grow extensively if the starter culture is not added or fermentation fails with no concomitant production of lactic acid and drop in pH. Failure can be caused by poor quality starter cultures or the improper use of starter cultures or "back inoculation". *S. aureus* growth is aerobic and usually confined to the outer 1/8 inch of the sausage. Enterotoxin may be formed as a result of this growth.

Coagulase-positive staphylococcal counts on large sticks of salami have been noted to vary widely. On large sticks, some areas may have very few staphylococci while other areas may have levels in excess of 10^6/g. Whenever possible, obtain 1-2 pounds of the suspect sausage. In order to obtain a representative sample, portions should be taken from several different areas and composited for testing.

b. Procedure

i. If the sausage is moldy, wipe the mold off the sausage casing with a piece of sterile tissue paper and proceed.

ii. To collect a sample, use a sterile, sharp knife and cut several thick slices from the sausage near the ends as well as in the middle. Aseptically trim and save the outer 1/8 to 1/4 inch portion of the sausage and label it "shell portion". Even if the amount of sample is limited, do not cut deeper than 1/4 inch.

iii. Working aseptically, blend 25-50 g of the shell portion for enterotoxin testing; the same blended sample can be used to test for viable coagulase-positive *S. aureus* as described in section 3.6.

iv. Analyze the sample by either of the following procedures.

3.62 The (Presumptive) Staphylococcal Enterotoxin Reverse Passive Latex Agglutination Test

The procedure for this test is given in (15.20) and usually is the method of choice.

3.63 Thermonuclease Assay

a. Introduction

This procedure is based on the detection of a heat stable DNase which is produced by most strains of *S. aureus,* including 98.3% of the enterotoxigenic strains. This heat stable DNase is produced in detectable amounts under all conditions which permit the growth of *S. aureus* and the production of enterotoxin. The DNase is able to survive processing conditions which would destroy viable *S. aureus.*

This method can be used to screen large sausages or a large number of samples to identify "hot spots".
It has been shown (Tatini, 1981) that the detection of DNase with this procedure is indicative of *S. aureus* populations of $\geq 10^5$ per gram.

b. Procedure:

i. Blend 20 g of shell, 10 g $(NH_4)_2SO_4$, and 2 ml Triton X-100 in 40 ml of distilled water.

ii. Adjust the pH of this slurry to 4.5-4.8 with 1N HCl.

iii. Centrifuge under refrigeration at 7-10,000 RPM for 15 min.

iv. Decant and discard the supernatant and add 0.05 ml cold 3M trichloroacetic acid for each ml of the original slurry, mix and centrifuge a second time as above.

v. Decant and discard the supernatant. Re-suspend the precipitate in 1 ml of Tris buffer, adjusted to pH 8.5, and then adjust the volume to 2 ml with Tris buffer.

vi. Boil the solution for $\geq$15 but $\leq$90 min, cool and store under refrigeration until needed.

vii. Cut 2 mm diameter wells into air dried Toluidine Blue DNA Agar.

viii. Dispense the food extract into one or more wells using a Pasteur pipette. Do not overfill the well.

ix. Incubate these plates, agar side down, at 37°C for 4 to 24 h.

x. Any pink halo, extending 1 mm beyond the well is considered positive for thermonuclease.

3.7 Selected References

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CHAPTER 6. ISOLATION, IDENTIFICATION, AND ENUMERATION OF *CAMPYLOBACTER JEJUNI/COLI* FROM MEAT AND POULTRY PRODUCTS

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6.1 Introduction

Procedures for the recovery of *Campylobacter* spp. from foods are evolving and no single method can be recommended for testing a wide variety of foods. Isolation of *Campylobacter jejuni* and *Campylobacter coli* is achieved both with and without selective broth enrichment. The procedures outlined below are among the most promising for the isolation and enumeration of these bacteria from raw/cooked meat and poultry products.

Campylobacters are sensitive to freezing and die off at room temperature. Samples intended for *Campylobacter* examination should be transported and held at 4°C. Sample analysis should begin as soon as possible since campylobacters can be overgrown by contaminating psychrotrophic bacteria. If freezing of samples cannot be avoided, cryoprotective agents should be used. Stern and Kotula, 1982, reported improved recovery of *C. jejuni* from ground beef stored frozen in 10% dimethyl sulfoxide or glycerol. Blankenship et al., 1983, found that brucella broth supplemented with 10% polyvinyl pyrrolidine was suitable for transporting frozen swab samples (from freshly processed poultry carcasses) to a central laboratory for analysis.

Campylobacters are microaerophilic and certain environmental stresses such as exposure to air, drying, low pH, and prolonged storage can be detrimental to their survival. Use of oxygen-quenching agents, a microaerobic atmosphere, and antibiotics that suppress competitors, significantly improve *Campylobacter* recovery.

6.2 Equipment, Reagents, and Media

6.21 Equipment

a. Phase-contrast microscope with 100X oil immersion objective
b. Agitating incubator(s)/water bath(s) at 37 ± 1.0°C and 42 ± 1.0°C
c. 42 ± 1.0°C incubator (static)
d. Balance, sensitivity of 0.1 g
e. Quart-size Qwik Seal® bags (Reynolds Metals Co., Richmond, VA; # RS78)
f. Anaerobic jars (vented or non-vented)

g. CampyPak Plus™ (BBL 71045)
or
Gas Generating Kits for *Campylobacter* (Oxoid BR56 for 3.0-3.5 liter jars, or BR60 for 2.5-3.0 liter jars)
h. Vacuum pump and gauge with appropriate tubing and connectors for evacuation of vented anaerobic jars
i. Gas cylinder containing a mixture of 5% O_2, 10% CO_2, and 85% N_2 with appropriate tubing and connectors for gassing vented anaerobic jars and Qwik Seal® bags
j. Regulator for gas cylinder compatible with Compressed Gas Association (CGA) connection on cylinder
k. Filter paper (for glycerol humectant and oxidase test)
l. Petri dishes (100 x 15 mm disposable)
m. Platinum or sterile plastic inoculating loops and needles
n. Microscope slides, cover slips, and immersion oil
o. 0.2 μm sterile membrane filters
p. 16 x 150 mm and 16 x 125 mm screw-cap test tubes
q. 250-ml screw-cap bottles
r. Sterile swabs or bent glass rods ("hockey sticks")
s. Sterile forceps and scissors
t. Sterile pipettes
u. Large sterile plastic bags
v. Stomacher™ 400, and Stamacher™ 400 bags
w. Centrifuge, rotor, and 250-ml sterile centrifuge bottles
x. Sterile cheesecloth-lined funnels

6.22 Reagents

a. Glycerol
b. 3% Hydrogen peroxide solution
c. Cephalothin antibiotic susceptibility discs (30 μg)
d. Nalidixic acid antibiotic susceptibility discs (30 μg)
e. Oxidase reagent (1% Tetramethyl-p-phenylenediamine dihydrochloride solution)
f. *Campylobacter* latex test kit (optional presumptive identification)

6.23 Media

a. Hunt Enrichment Broth (HEB)
b. 0.1% peptone water
c. Modified *Campylobacter* Charcoal Differential Agar (MCCDA)
d. Brucella-FBP (BFBP) Broth
e. Semisolid Brucella Glucose Medium
f. Brucella-FBP (BFBP) Agar
g. Enriched Semisolid Brucella Medium (optional)

6.3 Isolation and Enumeration

a. Place 25 g meat or swab samples into 100 ml of HEB in a Reynolds quart-size Qwik Seal® bag. Place the Qwik Seal® bag inside a Stomacher™ 400 bag for reinforcement and stomach for 2 minutes. Flatten the Qwik Seal® bag against the lab bench edge to remove as much air as possible without spilling the contents, then seal the bag, leaving a 1/2 inch opening at one end. Aseptically insert the tip-end of a sterile 10 ml pipette (or equivalent) into the bag through this opening. Be sure that the mouth-end of the pipette contains a sterile cotton filter. Connect the mouth-end of the pipette to the microaerobic Campy gas mixture (5% O_2, 10% CO_2, and 85% N_2) with sterile rubber tubing equipped with a sterile filter (a sterile filter can be made out of an autoclaved, shortened 25 ml volumetric pipette stuffed with glass wool). Slowly inflate the bag to capacity with the Campy gas mixture and continue to fill until excess gas flows from the bag. Then allow a small amount of gas to escape to provide for expansion, before securing the remainder of the seal. Proceed to step d.

b. Place a raw whole chicken carcass or meat pieces (up to 3 lb) in a large sterile plastic bag such as a Stomacher™ 3500 bag, and add 200 ml 0.1% peptone water. Twist bag to seal and shake contents for 2 minutes. Tilt the bag and hold back the meat pieces, allowing the rinse liquid to flow to one corner. Sanitize bag corner with 1000 ppm hypochlorite solution or 70% ethanol, then rinse in sterile distilled water. Aseptically cut the corner of the bag and pour the rinse through a sterile cheesecloth-lined funnel into a sterile 250 ml centrifuge bottle. Centrifuge at 16,000 x g for 15 minutes. Discard the supernatant and suspend the pellet in 10 ml 0.1% peptone water. For detection, inoculate 1 ml of rinse concentrate into 100 ml HEB in a Qwik Seal® bag. Then follow gassing steps as outlined, beginning with the third sentence of step a. above.

c. If enumeration is desired, prepare a three tube MPN series using HEB. Choose test dilutions and HEB volumes based on the expected numbers of campylobacters in the meat species being tested. For example, for poultry rinse samples (prior to centrifuging) begin by adding three 10 ml portions of the rinse to three 90 ml bottles of HEB. (Alternatively, Qwik Seal® bags may be used here [see step a. above]). Then add 1 ml portions of the rinse to each of three 9 ml tubes of HEB. Prepare serial dilutions of the rinse in 0.1% peptone water. Prepare subsequent MPN tubes by transferring 1 ml portions of the

decimal dilutions into 9 ml tubes of HEB in triplicate. Place all bottles and tubes in anaerobic jars. See step g. for jar gassing methods. Follow incubation steps beginning with step d. below. Use tubes or bottles found to contain confirmed *Campylobacter* to calculate MPN (refer to appropriate tables).

d. Incubate gassed Qwik Seal® bags or anaerobic jars containing bottles or tubes at 37 ± 1.0°C, shaking at 100 rpm for 4 h.

e. After the 4 h incubation at 37 ± 1.0°C, aseptically add additional sterile cefoperazone solution to bring the final concentration in each enrichment vessel to 30 mg/L. Reestablish the microaerobic atmosphere and increase the temperature to 42 ± 1.0°C. Continue the incubation for 20 h shaking at 100 rpm.

f. Swab/streak enrichments directly and at a 1:100 dilution onto MCCDA plates (for cooked products, a 1:50 dilution may be plated). Prepare the dilution by swirling a swab in the broth and twisting it against the side of the vessel to remove excess liquid. Break off the swab tip into a tube containing 9.9 ml of 0.1% peptone water and vortex. Inoculate the plates by placing a swab into the enrichment or dilution and removing excess liquid as above. Swab approximately 40% of the MCCDA plate, then streak from the swabbed area to yield isolated colonies. Alternatively, 0.1 ml portions of the enrichments or dilutions may be plated by spreading with a sterile bent glass rod. This plating technique may be used provided isolated colonies result.

g. Incubate the MCCDA plates at 42 ± 1.0°C for 24 h in an anaerobic jar under microaerobic conditions. Add about 4 drops of a humectant such as glycerol to a filter paper and place it in the jar to diminish typical confluent and swarming growth of *Campylobacter*. If no growth is achieved after 24 h, reincubate the plates for an additional 24 to 48 h to attempt recovery. The microaerobic conditions can be achieved in the jar by either of the following methods:

   i. Evacuate the air from a vented anaerobic jar to a partial vacuum of 20 inches of Hg and fill the jar with a gas mixture of 5% O_2, 10% CO_2, and 85% N_2. Repeat the evacuation-replacement procedure a total of three times to assure proper atmospheric conditions.

ii. CampyPak Plus™ (BBL) or Gas Generating Kits for *Campylobacter* (Oxoid). Follow the manufacturer's instructions on use and disposal of the kit materials. Keep jars away from flames when opening.

NOTE: Gas generator envelopes should be used if non-vented anaerobic jars are the only type available. Evacuation-replacement gassing of vented anaerobic jars is very economical.

To facilitate lid removal from a vented anaerobic jar, first release pressure by opening clamped tubing on port or by depressing the valve stem.

6.4 Identification of *Campylobacter*

Campylobacter colonies on MCCDA are smooth, shiny, and convex with a defined edge, or flat, transparent or translucent, and spreading with an irregular edge; colorless to grayish or light cream; and usually 1 to 2 mm in diameter but may be pinpoint to several mm in diameter. Plates of *Campylobacter* colonies may be stored up to 48 h refrigerated under microaerobic conditions if isolates cannot be picked immediately.

Use a platinum or plastic needle to pick three suspect *Campylobacter* colonies for each sample from the MCCDA plates and transfer each to 10 ml of brucella-FBP (BFBP) broth. Since campylobacters can vary greatly in colonial morphology, it is advisable to similarly culture at least one or all colony types present on the plates to assure the target is not overlooked. Alternatively, direct screening of colonies by phase-contrast microscopy can be done prior to picking isolates. To culture isolates, incubate the BFBP tubes with caps loosened for 24 to 48 h at 42 ± 1.0°C in an atmosphere of 5% O_2, 10% CO_2, and 85% N_2. Do not vortex culture tubes of *Campylobacter*, this will introduce oxygen into the media.

Perform the following identification tests on each BFBP broth culture:

a. Examine a wet-mount preparation of the BFBP broth culture with a phase-contrast microscope using a 100X oil immersion objective. Young cells of *Campylobacter* appear as narrow curved rods (0.2 to 0.8 μm wide by 1.5 to 5 μm long). The organisms show rapid movement with darting or corkscrew-like motility. Pairs of cells can resemble the silhouette of a gull's wing span or the letter S. Longer chains can appear helically curved, and multispiralled filamentous elongated forms may exist. Cells grown for more than 72 h may become non-culturable and coccoid. Campylobacters are Gram negative, but Gram staining may

be omitted since cell morphology and motility are more significant in the identification of these organisms. (Carbol fuchsin [0.5%] is used instead of safranin as a counter stain to improve Gram stain results.) Continue confirmation of those BFBP cultures that exhibit typical *Campylobacter* morphology.

b. Inoculate the top 10 mm layer of a tube of semisolid brucella glucose medium with several drops of the above BFBP broth culture. Incubate tubes with caps loosened in an anaerobic jar under microaerobic conditions at 42 ± 1.0°C for 1 to 3 days.

   i. Glucose fermentation test: Campylobacters are nonfermentative, so the color of the medium will remain red-orange. A positive reaction shows a yellow color (acid with phenol red indicator) in the semisolid brucella glucose medium.

   ii. Catalase test: After reading the results of the glucose fermentation test, add 1 ml of 3% hydrogen peroxide to the semisolid brucella glucose medium culture, let sit for two to three minutes, then gently invert the tube to distribute the reagent. Examine after 1 to 10 minutes for formation of bubbles, indicating a positive reaction. *C. jejuni* and *C. coli* are catalase positive.

c. Add about six drops of the BFBP broth culture to a BFBP agar plate, and spread the inoculum over the surface with a sterile swab or a bent glass rod. Aseptically place a disc of nalidixic acid (30 μg) and a disc of cephalothin (30 μg) on each plate. Press each disc with sterile forceps to adhere it to the agar surface. Incubate the plates in an anaerobic jar at 42 ± 1.0°C for 1 to 3 days in a microaerobic atmosphere.

   i. Susceptibility to nalidixic acid and cephalothin: Observe the growth patterns surrounding the antibiotic impregnated discs. *C. jejuni* and *C. coli* are sensitive to nalidixic acid, and a clear zone of inhibition will exist around the disc. A zone of any size indicates sensitivity. The organisms are both resistant to cephalothin, so growth will be present right up to the disc. Lawns of *Campylobacter* growth may be very light and can be difficult to see, so it is helpful to tilt the plate at an angle under a light for viewing.

   ii. Oxidase test: Place a 2 cm square piece of filter

paper in an empty petri dish and add 1 to 2 drops of oxidase reagent to the paper. Heavily smear cells from the above BFBP agar plate onto the reagent-impregnated paper in a spot 3 to 5 mm in diameter using a platinum or plastic loop. The test is positive if the cell mass turns dark purple within 30 seconds. Alternatively, the Difco DrySlide™ oxidase test may be used. Campylobacters are oxidase positive.

d. Optional tests

Other biochemical tests useful for differentiation of catalase-positive campylobacters include nitrate and nitrite reduction, H_2S production, growth in 1% glycine, growth in 3.5% NaCl, and growth at 25, 30.5, 37, and 42°C. *C. jejuni/coli* grow well at 42°C and are curved or S-shaped with darting, corkscrew-like motility. Biochemically, they are catalase positive, oxidase positive, nonfermentative, nalidixic acid sensitive, and cephalothin resistant. Distinguishing between *C. jejuni* and *C. coli* is usually not necessary in a food microbiology laboratory since both are causes of human campylobacteriosis. The few existing tests to separate these species are not dependable. Hippurate hydrolysis appears to be the most reliable and useful test for this purpose. A convenient rapid disk method is available (Cacho et al., 1989). *C. jejuni* is positive for this test, while *C. coli* yields a negative reaction.

6.5 Multiple Start Days

Analysis should begin on a Monday, Tuesday, Wednesday, or Thursday to avoid weekend work. Samples received on a Friday should be analyzed immediately or begun on Saturday; starting either day will require weekend work. Follow the table below according to the day analysis is to begin.

	Analysis To Be Done On Days				
Starting Date	Enrichment	Plating	Pick Colonies	Inoculate Biochemicals	Read/ Perform Tests
MON	MON	TUE	WED	THU	FRI
TUE	TUE	WED	THU	FRI	MON
WED	WED	THU	FRI	MON	WED
THU	THU	FRI	MON	TUE	THU
FRI	FRI	SAT	MON	TUE	THU
SAT	SAT	SUN	MON	TUE	THU

6.6 Storage and Transport of Stock Cultures

Inoculate overnight BFBP broth cultures into tubes of Brucella broth with 0.15% agar. Loosen the screw-caps and incubate for 24 to 48 h at 42 ± 1.0°C in an atmosphere of 5% O_2, 10% CO_2, and 85% N_2. Store refrigerated under this atmosphere for up to a month without serial passage. Cultures in this medium can be transported by mail. Seal tightened caps with adhesive tape to prevent leakage during shipment.

Cultures grown in enriched semisolid brucella medium may be stored under atmospheric conditions at room temperature with caps tightened, for at least three weeks. This medium is also suitable for transporting cultures by mail.

Cultures may also be preserved frozen. To prepare these stocks, swab 6 drops of a 24 h BFBP broth culture onto a BFBP agar plate and incubate microaerobically at 42 ± 1.0°C for 24 to 48 h. Then remove the plate growth with a swab and suspend the cells in 4 ml of Brucella broth with 15% sterile glycerol. The suspension can be stored frozen at -70°C in 1 ml portions for 6 months or longer. Thawing and refreezing these stocks will usually result in loss of viability.

6.7 Media Quality Control

Pay strict attention when preparing all media to assure proper supplement additions. Ingredients, reagents, and media that are past expiration date should be discarded. It is important to discard all unused liquid media more than one month old and all plating media more than two weeks old, since absorbed oxygen will generate peroxides which can be detrimental to campylobacters. Store all media refrigerated, tightly sealed, and shielded from light.

Inoculated media controls should be incubated with each batch of tests to assure proper media formulation and atmospheric conditions. When enriching, include a Qwik Seal® bag of HEB inoculated with an actively growing BFBP broth culture of *C. jejuni* as a control. Similarly, in each anaerobic jar, include an appropriate agar plate or broth inoculated with a known *C. jejuni* strain. Use of positive and negative controls for all biochemical tests is also recommended. An uninoculated control of all test media should also be included to allow assessment of sterility and any changes that may occur in the medium.

Listed below are some recommended controls for the *Campylobacter* biochemical tests:

a. Glucose fermentation test:
   Inoculate a semisolid brucella glucose tube with an *Escherichia coli* strain and incubate aerobically to generate a positive reaction. Inoculate a *C. jejuni* strain and incubate microaerobically to yield a negative reaction.

b. Catalase test:
   Use a *C. jejuni* strain as a positive control and a *Streptococcus* spp. as a negative control.

c. Susceptibility to nalidixic acid and cephalothin:
   Use a *C. jejuni* strain to demonstrate the desired sensitive/resistant pattern.

d. Oxidase Test:
   Use a *C. jejuni* strain as a positive control and an *E. coli* strain as a negative control.

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CHAPTER 7. ISOLATION AND IDENTIFICATION OF *AEROMONAS* SPECIES FROM MEAT AND POULTRY PRODUCTS

Bonnie E. Rose and Anita J. G. Okrend

7.1 Introduction

Members of the genus *Aeromonas* typically are aquatic bacteria and sometime pathogens of fish and cold-blooded vertebrates that inhabit wet environments. Nevertheless, aeromonads are isolated (often in considerable numbers) from various foods of animal origin. These include seafood, raw milk, beef, pork, lamb, and poultry. They grow readily at refrigeration temperatures. Production of enterotoxins can be demonstrated using various laboratory assays, and indirect epidemiological evidence suggests that members of the genus *Aeromonas* have been involved in sporadic human gastroenteritis outbreaks involving seafood. However, no fully confirmed foodborne outbreak has been described in the scientific literature.

The method presented describes procedures for isolation and identification of species of the *Aeromonas hydrophila* group which consists of *A. hydrophila*, *A. sobria* and *A. caviae*. A procedure for detection of hemolysin(s) is also provided. Burke et al., 1983, reported a 97% correlation between hemolysin production and enterotoxin production among *Aeromonas* species.

7.2 Equipment, Reagents and Media

7.21 Equipment

(isolation/identification)

a. Incubator, static 28 ± 1°C
b. Osterizer-type blender with sterilized cutting assemblies and adapters for use with Mason jars, or Stomacher™ (Tekmar) with sterile Stomacher™ bags
c. Sterile bent glass rods ("hockey sticks")

(hemolysin test)

d. Incubator, static 37°C
e. Microtiter plate reader equipped to read at 540 nm
f. Centrifuge capable of 12,000 RPM

g. Shaker incubator (30°C; 210 RPM)
h. Screw-cap Erlenmeyer flasks, 125 ml
i. Sterile screw-cap centrifuge tubes: 15 ml conical and 50 ml round bottom
j. 96-well microtiter plates
k. Membrane filters, 0.2 µm
l. Bench top clinical centrifuge

7.22 Reagents

(isolation/identification)

a. Butterfield's phosphate diluent (BPD)
b. Mineral oil, sterile
c. N,N-dimethyl-p-phenylenediamine monohydrochloride (1% aqueous solution)

(hemolysin test)

d. Rabbit blood, defibrinated
e. Phosphate buffered saline (PBS)
f. Distilled water, sterile

7.23 Media

(isolation/identification)

a. Tryptic soy broth plus 10 µg/ml ampicillin (TSBA)
b. Starch-ampicillin (SA) agar
c. Triple sugar iron (TSI) agar
d. Nutrient agar
e. Mannitol fermentation broth with Andrade's indicator
f. Arginine decarboxylase broth (Moeller)
g. Ornithine decarboxylase broth (Moeller)
h. Decarboxylase broth base (Moeller)
i. Glucose fermentation broth with Andrade's indicator
j. Bile esculin agar

(hemolysin test)

k. Brain heart infusion (BHI) broth

7.3 Isolation Procedure

Serial dilutions of meat samples may be surface-spread directly on SA agar. However better recovery of *Aeromonas* will be achieved by

using enrichment procedures, particularly when the aeromonads have been freeze-injured or are low in number.

a. Blend 25 g of meat in 225 ml TSBA with a blender or Stomacher™ for 2 minutes. Incubate at 28°C for 18 to 24 h.

b. After incubation prepare serial dilutions of the enrichment cultures in BPD. Transfer 0.1 ml of the 10^{-4} to 10^{-6} dilutions onto the surface of SA plates. Evenly spread the inoculum with sterile bent glass rods. The plates must be free of surface moisture if single colonies are to be obtained. Incubate the plates at 28°C for 18 to 24 h.

c. Pick three typical colonies per sample from the SA agar plates to TSI agar and nutrient agar slants. Incubate overnight at 28°C. *Aeromonas* colonies are typically 3 to 5 mm in diameter and appear yellow to honey-colored on SA agar.

7.4 Identification

a. Read the TSI reactions. *Aeromonas* reactions on TSI are as follows: acid butt, acid or alkaline slant, H_2S negative, positive or negative gas production.

b. Perform the oxidase test on the nutrient agar slants. Add a few drops of a N,N-dimethyl-p-phenylenediamine monohydrochloride solution (prepared fresh daily) to the growth on the nutrient agar slant. Oxidase positive cultures develop a pink color which successively becomes maroon, dark red, and black in 10 to 30 min. All aeromonads are oxidase-positive and fermentative.

c. Transfer all oxidase-positive fermenters from the TSI agar slants to the following media for biochemical confirmation: mannitol fermentation broth, arginine decarboxylase broth, ornithine decarboxylase broth, glucose fermentation broth, and bile esculin agar. After inoculation, layer the decarboxylase media with sterile mineral oil and incubate at 28°C for 48 h. Incubate the remainder of the confirmation media at 28°C for 24 h.

d. Record the biochemical characteristics of each isolate. All aeromonads produce acid from mannitol and are arginine positive, ornithine negative. Species of the *A. hydrophila* group can be differentiated according to the biochemical characteristics shown below:

Test (Substrate)	*A. hydrophila*	*A. sobria*	*A. caviae*
Gas from Glucose	+	+	-
Esculin hydrolysis	+	-	+

NOTE: Esculin hydrolysis imparts a dark brown color to the medium.

e. Transfer isolates of suspected *Aeromonas* that are to be tested for hemolysin production from TSI agar to nutrient agar slants and incubate overnight at 28°C.

7.5 Hemolysin Test

The hemolysin test described below is based on that of Burke et al., 1983 and 1984.

7.51 Preparation of Culture Filtrate

a. Transfer growth from the nutrient agar slant to BHI broth (25 ml broth in a 125 ml Erlenmeyer flask). Incubate overnight at 30°C on a shaker incubator at 210 RPM.

b. Centrifuge the broth culture at 11,950 RPM (SS-34 Dupont-Sorvall rotor) for 30 minutes. Decant and save the supernatant liquid; discard the cell pellet.

c. Filter sterilize the supernatant through a sterile disposable membrane filter (0.2 µm).

d. Hold the sterile culture filtrate at 4°C until needed, and test it for hemolysin activity within 24 h of preparation.

7.52 Preparation of Rabbit Erythrocyte Suspensions

a. Centrifuge 10 ml of defibrinated rabbit blood in a 15-ml conical centrifuge tube at 2400 RPM in a bench top clinical centrifuge for 5 minutes.

b. Remove the supernatant and white blood cell layer by suction and discard.

c. Add 10 ml of cold PBS to the packed erythrocytes, mix gently, and centrifuge as described above. Discard supernatant.

d. Wash the erythrocytes in PBS two more times, as described above.

e. After the final wash, note the volume of packed erythrocytes in the centrifuge tube. Prepare a 10% and a 1% erythrocyte suspension in PBS. Hold the two suspensions at 4°C until needed (use within 24 h).

7.53 Preparation of Hemoglobin Standard Curve

a. Transfer 1 ml of the 10% erythrocyte suspension into 8 ml of sterile distilled water. Shake the mixture until all cells are lysed. Add 1 ml of 10X PBS to obtain a 1% hemoglobin solution.

b. Add 1% hemoglobin solution and 1% erythrocyte suspension to conical centrifuge tubes in the following volumes:

Volume (ml)	% hemoglobin										
	0	10	20	30	40	50	60	70	80	90	100
Hemoglo -bin	0	.1	.2	.3	.4	.5	.6	.7	.8	.9	1.0
Erythro -cytes	1.0	.9	.8	.7	.6	.5	.4	.3	.2	.1	0

c. Centrifuge tubes at 2400 RPM for 5 minutes in a clinical centrifuge. Transfer 0.5 ml of supernatant from each tube into wells of a 96-well microtiter plate. Hold the plate for the hemolysin test (Section 7.54).

7.54 Hemolysin Test

a. Add 1 ml of sterile culture filtrate (Section 7.51) to 1 ml of the 1% erythrocyte suspension (Section 7.52) in a conical centrifuge tube and mix gently.

b. Incubate at 37°C for 1 h, then incubate for an additional 1 h at 4-5°C.

c. Centrifuge at 2400 RPM for five minutes.

d. Transfer 0.5 ml of supernatant to the 96-well plate containing the standards (Section 7.53).

e. Read the plate on a microtiter plate reader at 540 nm.

f. A positive hemolysin test is defined as the production of an O.D. reading $\geq$ the O.D. of the 20% hemoglobin standard in the standard curve prepared above in Section 7.53.

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CHAPTER 9. ISOLATION & IDENTIFICATION OF PATHOGENIC *YERSINIA ENTEROCOLITICA* FROM MEAT AND POULTRY PRODUCTS

Jennifer L. Johnson

9.1 Introduction

Yersinia enterocolitica and other *Yersinia* species such as *Y. frederiksenii* and *Y. kristensenii* are ubiquitous in the natural environment, and may be recovered from water, soil, animals, and food. There is considerable variation within the species *Y. enterocolitica*, and member organisms range from the so-called "*Y. enterocolitica*-like" organisms and "environmental" strains of *Y. enterocolitica* to strains capable of causing serious disease in humans. Hogs have been shown to be a reservoir for certain types of pathogenic *Y. enterocolitica* and pork products have been implicated in human disease. The presence of pathogenic *Y. enterocolitica* on food products is a special concern since those organisms are capable of growth at refrigerator temperatures.

Pathogenic *Y. enterocolitica* organisms are significant causes of human disease in many parts of the developed world. Epidemiological evidence from Belgium, Norway, Denmark, The Netherlands, Japan, Canada, and elsewhere strongly implicates consumption of pork products in human disease. In fact, disease due to *Y. enterocolitica* in the United States may be on the rise, and more information on contamination of meat (especially pork) and poultry is needed.

The term "pathogenic serotype", when used in reference to *Y. enterocolitica*, typically refers to one of 11 O-antigen groups in the *Y. enterocolitica* serotyping scheme. Some strains belonging to these serotypes have been implicated in human disease and have demonstrated pathogenicity in animal models or tissue culture cell invasiveness tests. Until recently, serotypes O:4,32; O:8; O:13a,13b; O:18; O:20; and O:21 have accounted for the majority of pathogenic serotypes recovered in the U.S. Only recently have serotype O:3 organisms been identified as a common cause of yersiniosis in the United States of America. In a recent American survey of hospitalized gastroenteritis patients, 92% of the *Y. enterocolitica* isolates were serotype O:3 while 5% were serotype O:5,27. Serotypes O:3, O:9, and O:5,27 are well-established human pathogens in other areas of the world. The so-called "North American serotypes" of *Y. enterocolitica* (serotypes

O:8, O:13, and O:21) represent a genetically distinct lineage from that of the other pathogenic serotypes.

While the term "pathogenic serotype" is in common usage, several authors have stated that terms such as "pathogenic phenotype", "pathogenic bio-serotype", and "pathogenic bio-serogroup" are more descriptive since they differentiate between pathogenic and nonpathogenic members of a generally pathogenic serotype. Biogrouping, the phenotypic characterization of *Y. enterocolitica*, can serve as a useful indication of the likely pathogenicity of a given strain. Testing for markers of pathogenicity like calcium dependence, crystal violet dye binding, auto-agglutination, and pyrazinamidase activity provide additional information. Markers are not perfectly correlated with pathogenicity but provide useful information under conditions where animal testing is undesirable or impractical.

Virulence in *Y. enterocolitica* is mediated by both chromosomal and plasmid-borne genes. While chromosomal determinants are stable, plasmids containing virulence genes may be lost during culture and confirmational procedures. Temperatures above 30°C are known to cause the loss of virulence plasmids in pathogenic *Y. enterocolitica*, but plasmid loss may also occur under other, less well-defined, circumstances.

Numerous enrichment schemes have been described for the recovery of *Yersinia enterocolitica* from meat samples. These enrichment procedures include cold enrichment for up to a month, direct selective enrichment, or two-step pre-enrichment/selective enrichment procedures. It appears that some enrichment procedures are better suited for the recovery of pathogenic *Y. enterocolitica* than others, though recovery may be influenced by the type of meat product. Even when using an enrichment and plating scheme reported to give good recovery from a particular meat product, considerable variation in recovery may be observed. Methods reported to provide good recovery of pathogenic *Y. enterocolitica* in one part of the world may not work so well in another geographical area, possibly due to differences in levels of *Y. enterocolitica* and competing flora.

Recovery of pathogenic *Y. enterocolitica* is contingent upon a number of factors including: the level of background flora on the product; the amount of background flora coming through enrichment and plating; the level of pathogenic *Y. enterocolitica* present on the sample; the numbers of non-pathogenic *Y. enterocolitica* and non-pathogenic *Yersinia* spp. present on the product; and loss of

virulence factors during enrichment and plating. Furthermore, a recovery method which gives good recovery of one serotype of pathogenic *Y. enterocolitica* may not be suited to other serotypes. In order to recover any of the important pathogenic serotypes of *Y. enterocolitica* which might be present, multiple enrichment broths and plating media are usually recommended for the recovery of the organism from naturally-contaminated foods.

As there is no "universal" enrichment scheme capable of reliably isolating all important pathogenic serotypes of *Y. enterocolitica*, recovering serotypes O:3, O:8, and O:5,27 necessitates the use of parallel procedures. This protocol specifies the use of three separate enrichment procedures in combination with two selective/differential agars. Even with the use of multiple cultural enrichment schemes, however, shortcomings of conventional cultural procedures for the recovery of pathogenic *Y. enterocolitica* undoubtedly result in an under-estimation of the prevalence of this organism in foods and in clinical specimens. A study reported that while 18% of raw pork products were found to contain *Y. enterocolitica* serotype O:3 by two cultural procedures, use of a genetic probe on plated enrichments gave a detection rate of 60%. One of the main difficulties encountered during conventional cultural isolation of pathogenic *Y. enterocolitica* appeared to be overgrowth of small numbers of pathogenic *Y. enterocolitica* by nonpathogenic yersiniae and other microorganisms. The use of conventional cultural procedures for the detection and recovery of pathogenic *Y. enterocolitica* by FSIS sets the stage for a move towards use of genetically-based detection methods.

A great deal of effort must be expended in the recovery and characterization of presumptively-pathogenic *Y. enterocolitica*. Sequential levels of characterization tests include: identification of presumptive *Yersinia*, speciation to *Y. enterocolitica*, biogrouping the *Y. enterocolitica*, followed by testing for pathogenicity markers. *Y. enterocolitica* is more active biochemically at 25°C than at 35-37°C, meaning that disparate results for a given test may be obtained depending on incubation temperature. This characteristic, coupled with the known temperature-sensitivity of the *Y. enterocolitica* virulence plasmid, makes strict adherence to temperature and time requirements a necessity. A word to the reader: although the extensive characterization protocol appears intimidating, the vast majority of non-*Y. enterocolitica* are effectively eliminated with minimal work by the first tier of testing.

The enrichment and characterization procedures described in this protocol are well-documented in the literature. The inclusion of these procedures in the latest edition of the "Compendium of Methods for the Microbiological examination of Foods" is further evidence of their acceptance by the scientific community.

9.2 Equipment, Reagents and Media

9.21 Equipment

a. Sterile scissors, forceps, knives, pipettes, hockey sticks, and other supplies
b. Balance (sensitivity of ± 0.1 g)
c. Inoculating needles and loops
d. Vortex mixer
e. Stomacher™ and sterile stomacher bags
f. Freezer (-70°C)
g. Stereomicroscope and oblique lighting (optional)
h. Incubators capable of holding temperatures at 4 ± 1°C, 25 ± 1°C, 28 ± 1°C, 30 ± 1°C, 32 ± 1°C, 35 ± 1°C and 37 ± 1°C.

9.22 Reagents

a. 0.25% KOH in 0.5% NaCl aqueous solution
b. Crystal violet (85 µg/ml aqueous solution)
c. Sterile mineral oil
d. 1% Ferrous ammonium sulfate (prepare fresh on day of use)
e. Kovacs' reagent
f. Voges-Proskauer (VP) test reagents
g. Oxidase reagent or reagent-impregnated disc/strip
h. Glycerol (sterile)
i. 1 N HCl solution

9.23 Media

a. Irgasan-Ticarcillin-Cholate (ITC) broth
b. Trypticase Soy Broth (TSB)
c. Bile-Oxalate-Sorbose (BOS) broth
d. 0.01 M Phosphate Buffered Saline (PBS, pH 7.6)
e. Cefsulodin-irgasan-novobiocin (CIN) agar (MUST BE MADE ACCORDING TO FORMULATION IN APPENDIX)
f. Salmonella Shigella Deoxycholate Calcium (SSDC) agar
g. Kligler's Iron agar (KIA) slants

h. Simmon's Citrate agar slants
i. Christensen's urea agar slants
j. Lysine decarboxylase medium (0.5% lysine)
k. Ornithine decarboxylase medium (0.5% ornithine)
l. CR-MOX (Congo Red Magnesium Oxalate) agar
m. Methyl Red-Voges Proskauer (MR-VP) broth
n. β-D-Glucosidase test medium
o. Purple broth with 1% filter-sterilized salicin
p. Purple broth with 1% filter-sterilized xylose
q. Purple broth with 1% filter-sterilized sucrose
r. Purple broth with 1% filter-sterilized trehalose
s. Purple broth with 1% filter-sterilized rhamnose
t. Esculin agar slants
u. Sterile Saline (0.85% NaCl)
v. Tween 80 agar (lipase test agar)
w. DNase test agar
x. Tryptophan broth (indole test medium)
y. Pyrazinamide agar slants
z. Veal infusion broth
aa. Trypticase Soy agar or Brain Heart Infusion agar plates

NOTE: Formulations for all the very specialized media and reagents used for the isolation and identification of *Yersinia* are presented at the end of this chapter.

9.3 Isolation Procedures

9.31 Preparation of Sample Homogenate

a. For meat samples other than surface samples: Add 25 g of sample to 100 ml of 0.01 M Phosphate Buffered Saline (PBS: pH 7.6). Homogenize for 2 minutes in a Stomacher™. Allow homogenate to stand undisturbed at room temperature for 10 minutes to allow settling of large meat particles.

b. For carcass surface samples: Add PBS to surface sample so as to prepare a 2:1 ratio of volume to surface area (e.g. add 100 ml PBS to a 50 cm^2 sample). Homogenize for 2 minutes in a Stomacher™. Allow homogenate to stand undisturbed at room temperature for 10 minutes.

9.32 Enrichment & Plating Procedures

In order to improve the chances of recovering pathogenic *Y. enterocolitica*, three enrichment procedures (ITC, TSB/BOS, and PBS) should be used. Although this will increase a laboratory's work-load, it is the best way to insure that any serotype of pathogenic *Y. enterocolitica* present in the product will be recovered. ITC broth provides good recovery of serotype O:3 and probably serotype O:9 *Y. enterocolitica*. TSB/BOS permits recovery of serotype O:8. PBS-cold enrichment has been shown to recover serotype O:5,27. KOH treatment of *Y. enterocolitica* enrichment cultures decreases background flora. Two selective plating media, SSDC and CIN agars, are recommended for the isolation of pathogenic *Y. enterocolitica*. Figure 1 illustrates the enrichment procedures which are included in this protocol.

a. ITC broth: Transfer 2 ml of sample homogenate supernatant into 100 ml ITC broth contained in an Erlenmeyer flask. Incubate at 25°C for 2 days. Spread-plate 0.1 ml onto SSDC agar and incubate the plates at 30°C for 24 h. Spread-plate 0.1 ml onto CIN agar, and incubate the plates at 32°C for 18 h. Also, remove 0.5 ml of the ITC enrichment, treat it with KOH, then streak onto CIN. Reincubate the ITC enrichment at 25°C for another 24 h. After the plate incubation is complete, examine the plates as described below. If colonies having typical *Y. enterocolitica* morphology are not visible on the plates, the ITC culture should be plated out as before.

b. TSB/BOS: Transfer 20 ml of sample homogenate supernatant into 80 ml TSB. Incubate at 25°C for 24 h. Transfer 0.1 ml of the TSB culture into 10 ml BOS. Incubate at 25°C for 3 days. Spread-plate 0.1 ml onto SSDC agar and incubate the plates at 30°C for 24 h. Spread-plate 0.1 ml onto CIN agar, and incubate the plates at 32°C for 18 h. Also, remove 0.5 ml of the BOS enrichment, treat it with KOH, then streak onto CIN. Reincubate the BOS enrichment culture at 25°C for 2 additional days, then plate as before.

c. PBS: Refrigerate the remainder of the PBS homogenate at 4°C for 14 days. Spread-plate 0.1 ml onto CIN agar, and incubate the plates at 32°C for 18 h. Also, remove 0.5

ml of the PBS enrichment, treat it with KOH, then streak onto CIN. Also, use KOH treatment with plating onto CIN.

d. KOH treatment: Add 0.5 ml of enrichment culture to 4.5 ml KOH/NaCl. Vortex briefly (3-4 sec) and IMMEDIATELY streak a loop-full of the KOH-treated broth onto CIN agar (Do NOT use KOH treatment in combination with SSDC agar).

9.33 Selection of Colonies from Plating Media

Due to the fact that SSDC and CIN agars are not completely inhibitory to non-yersiniae, a variety of non-*Yersinia* organisms may be recovered from these agars. Some of these organisms (e.g. strains of *Citrobacter* and *Enterobacter*) have a colonial morphology similar to that of *Y. enterocolitica*. Care must be exercised in the selection of suspect colonies from SSDC and CIN agars in order to minimize picking non-yersiniae. It may be helpful for the analyst to compare colonies growing on sample plates to colonies on the positive control plates. Colony appearance can change over time so strict adherence to time/temperature recommendations is necessary.

a. SSDC: On SSDC, *Y. enterocolitica* colonies are typically round, about 1 mm in diameter and opaque or colorless. When observing plates through a stereomicroscope with oblique transillumination, look for irregular colony edges with a finely granular colony center (never iridescent). Non-yersiniae present either an entire edge or a coarser pattern or both.

b. CIN: On CIN, typical *Y. enterocolitica* colonies have a red bulls-eye which is usually very dark and sharply delineated. The bulls-eye is surrounded by a transparent zone with varying radii, with the edge of the colony either entire or irregular; colony diameter is ca. 1-2 mm (larger colonies are usually not *Yersinia*). Again, the use of a stereomicroscope and oblique transillumination may facilitate examination of plates.

9.4 Identification and Confirmation Procedures

9.41 Identification of *Yersinia*

Select a colony on CIN or SSDC having morphology typical of *Y. enterocolitica* and emulsify colony in about 1 ml of sterile saline (0.85%). Use this to first inoculate a slant of Simmon's Citrate Agar, then inoculate Kligler's Iron Agar, and a tube of urea agar. Repeat until 5 colonies having morphology typical of *Y. enterocolitica* have been selected from each plate of selective agar. Table 1 presents the testing scheme to which isolates recovered from SSDC and CIN will be submitted.

a. Simmon's Citrate: Only Streak-inoculate the slant of a tube of Simmon's Citrate agar; do NOT stab the butt. Incubate at 28°C for 24 h. Presumptive *Y. enterocolitica* are citrate negative (-) and the citrate slant will remain the original green color (a positive (+) reaction is characterized by the agar turning a vivid blue color).

b. Kligler's Iron Agar: Stab-inoculate the butt and streak the slant. Incubate at 28°C for 18-24 h. Presumptive *Y. enterocolitica* should present an alkaline (red) slant and acid (yellow) butt, without gas or H_2S on KIA.

c. Christensen's urea agar: Streak the slant with a heavy inoculum load; do NOT stab the butt. Incubate at 28°C for 24-72 h. Presumptive *Y. enterocolitica* are (+) for urease and will turn the agar to an intense red-pink color.

9.42 Confirmation and Biogrouping of *Yersinia enterocolitica*

Any organism which is citrate negative (-), urease positive (+), and gives an alkaline slant/acid butt without gas or H_2S on KIA should be submitted to further testing. Inoculum for further testing may be obtained from the KIA slant; the KIA slant should then be refrigerated pending the test results. THE TESTS LISTED BELOW ARE ALL NECESSARY TO CONFIRM AND BIOGROUP POTENTIALLY-PATHOGENIC *Y. enterocolitica*. Do NOT attempt to biogroup any isolate until the results are available from ALL tests! Similarly, do NOT discard any culture until ALL tests have been completed. See Holt et al., 1994, for additional information on speciating *Yersinia*.

a. Oxidase test: Test colony growth from the KIA slant of any presumptive *Y. enterocolitica* isolates using oxidase reagent or commercially-available, reagent-impregnated test strips/discs. *Yersinia* are oxidase negative (-).

b. Lysine and ornithine decarboxylase: Inoculate one tube each of lysine decarboxylase medium and ornithine decarboxylase medium; overlay each inoculated tube with sterile mineral oil (4-5 mm deep layer). Incubate at 28°C for 4 days. *Y. enterocolitica* are LYS negative (-) and ORN positive (+).

c. Rhamnose, sucrose, xylose, and trehalose utilization: Inoculate one tube of each of these carbohydrate broths, and incubate at 25°C for 10 days, reading after 1,2,3,7, and 10 days. *Y. enterocolitica* are rhamnose negative (-) and sucrose positive (+). Xylose and trehalose reactions vary between biogroups.

d. Salicin utilization: Inoculate a tube of salicin broth, and incubate at 35°C, reading after 1,2,3, and 4 days. Salicin reactions vary between biogroups.

e. Esculin hydrolysis: Inoculate a tube of esculin agar. Incubate at 25°C for 10 days, reading after 1,2,3,7 and 10 days. Blackening indicates esculin hydrolysis. Esculin reactions vary between biogroups of *Y. enterocolitica*.

f. Indole test: Inoculate a tube of Tryptophan broth (indole test medium). Incubate (with loosened caps) at 28°C for 48 h. Add 0.5 ml of Kovacs' reagent, mix gently, then allow tubes to stand about 10 minutes. A dark red color developing below the solvent layer is evidence of a positive (+) test while the color will remain unchanged in a negative (-) test. Indole test results vary with biogroup of *Y. enterocolitica*.

g. VP test: Inoculate a tube of MR-VP broth, and incubate at 25°C for 24 h. After incubation, add 0.6 ml α-naphthol to the tube, and shake well. Add 0.2 ml 40% KOH solution with 0.3% creatine and shake. Read results after 15 minutes and 1 hour. Development of a pink to ruby red color is a positive test. Results vary with biogroup.

h. β-D-Glucosidase test: Emulsify culture in saline to McFarland 3 turbidity. Add 0.75 ml of culture suspension to 0.25 ml of β-D-glucosidase test medium. Incubate at 30°C overnight (16-20 h). A distinct yellow color indicates a positive reaction. Results vary with biogroup.

i. Lipase test: Inoculate *Y. enterocolitica* isolate onto a plate of Tween 80 agar (more than one isolate may be tested per plate). Incubate at 28°C, and examine after 2 and 5 days. Lipase activity is evidenced by an opaque halo surrounding the streak, and varies with biogroup.

j. Deoxyribonuclease (DNase) test: Inoculate *Y. enterocolitica* strain onto a plate of DNase test agar by streaking the medium in a band (about 3/4 inch length streak). Four or more strains may be tested per plate. Incubate plates at 28°C for 18-24 h. Following incubation, examine plates as follows. For DNase test agar, flood plate with 1 N HCl. A zone of clearing around a colony indicates a positive test. Observe for clear zones surrounding the streak (no clearing or a uniformly opaque agar indicates a negative reaction). DNase test agars containing toluidine blue or methyl green may also be used; follow manufacturer's instructions for interpreting results.

k. Pyrazinamidase test: Inoculate strains over entire slant of pyrazinamide agar and incubate at 25°C for 48 h. Flood slant surface with 1 ml of freshly prepared 1% (w/v) aqueous solution of Fe^{+2} ammonium sulfate. Read after 15 minutes; a pink to brown color indicates PYR positive (+); (presence of pyrazinoic acid) while no color development is observed with PYR negative (-) strains. Pathogenic strains are PYR negative (-).

9.43 Testing for Pathogenicity Markers

Presumptive pathogenic *Y. enterocolitica* are LYS negative (-), ORN positive (+), sucrose positive (+), salicin negative (-) and esculin negative (-). Once the results from all the biogrouping tests are available, Table 2 should be consulted for information on biogroup designation. *Y. enterocolitica* isolates belonging to Biogroups 1B, 2, 3, 4, or 5 should be subjected to further testing for pathogenicity markers.

a. Auto-agglutination in MR-VP broth: Inoculate 2 tubes of MR-VP broth; incubate one at 25°C for 24 h, and the other at 35°C for 24 h. After incubation, the tube incubated at the lower temperature should exhibit turbidity from cell growth. The tube which had been incubated at 35°C should show agglutination (clumping) of bacteria along the walls and/or bottom of tube and clear supernatant fluid. Test is plasmid-dependent.

b. Congo red binding/crystal violet binding: Grow isolates in TSB at 25°C for 16-18 h, then dilute in saline to obtain about 10^4 cfu/ml and dilute to 10^{-5}. Spread-plate 10 µl of diluted suspension on CR-MOX plates. Incubate plates at 37°C for 24 h. A predominance of tiny red colonies is indicative of a positive response for both congo red binding and calcium dependency (some large colorless colonies [CR-MOX negative] may be present due to loss of the virulence plasmid). Perform crystal violet binding on the same agar by flooding each plate with about 8 ml of crystal violet (85 µg/ml), allowing this to stand for 2 minutes, then decanting off the dye. If desired, plates may be observed with a stereo dissecting microscope at 40X magnification. Examine colonies as soon as possible as color tends to fade with time; positive isolates display small, intensely purple colonies. CR-MOX permits demonstration of calcium dependency, Congo red binding, and crystal violet dye binding. Test is plasmid-dependent.

9.5 Method Quality Control Procedures

Due to the variety of bio-serogroups of *Y. enterocolitica* which can be found on meat and poultry, a cocktail of control cultures (including serotypes O:3 and O:8) should be used as a positive control. In addition, an uninoculated media control should be utilized for each of the different enrichment media.

Inoculate control strains into separate tubes of TSB. Incubate at 25°C for 18-24 h. In order to provide ca. 30-300 cfu/ml, make a 10^{-7} dilution of each culture in sterile saline. Add 1 ml of the 10^{-7} dilution of each culture to a single bottle containing 50 ml PBS. Mix well. From this point forward, treat the PBS/*Y. enterocolitica* positive-control cocktail as a sample, following the instructions given above in Section 9.32. Confirm

at least one isolate (of each morphological type present on each of the agars) recovered from the positive-control sample.

9.6 Storage of Isolates

9.61 Maintenance of *Y. enterocolitica* Control Strains

Because of the possibility of plasmid loss in virulent *Y. enterocolitica*, it is recommended that control strains of *Y. enterocolitica* be immediately subcultured upon receipt (incubating at temperatures below 30°C), then preserved in a frozen state.

Inoculate a tube of veal infusion broth with each control strain. Incubate for 48 h at 25°C. Add sterile glycerol to a final concentration of 10% (e.g. 0.3 ml in 3 ml veal infusion broth), dispense into several sterile vials, and freeze immediately at -70°C. Preparation of a batch of vials for each strain is recommended so that one vial can be held in reserve to serve as a source of inoculum for preparation of a new batch of frozen stocks.

When a fresh culture of a control strain is needed, a small portion of frozen suspension may be removed aseptically and transferred to a tube of TSB. Incubation should be at 25°C for 24 h, followed by streaking onto a non-selective agar such as TSA or BHI agar with incubation at 25°C for 24 h.

Strains may be kept on TSA or BHI slants at 4°C for short periods of time, but it is not recommended that such strains be transferred due to the possibility of plasmid loss.

Periodically, control cultures should be tested for pathogenicity markers as described above. Cultures which have lost the virulence plasmid should be destroyed, and replaced by a fresh subculture from the frozen stock preparation.

9.62 Maintenance of Isolates During Confirmation

Due to the possibility of plasmid loss during extensive subculturing (even at temperatures below 30°C), it is recommended that presumptive *Y. enterocolitica* isolates be frozen following *Y. enterocolitica* confirmation testing.

From the KIA slant of a presumptive *Y. enterocolitica* isolate, inoculate a tube of veal infusion broth.

Incubate for 48 h at 25°C. Add sterile glycerol to a final concentration of 10%, and freeze immediately at -70°C.

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Figure 1. Enrichment schemes used for the recovery of pathogenic *Y. enterocolitica* from meat or poultry samples.

Homogenize Sample in PBS

2 ml into 100 ml ITC broth	20 ml into 80 ml TSB	remainder of homogenate
2 days 25°C	1 day 25°C	14 days 4°C
--Onto SSDC 24 h 30°C	--0.1 ml TSB culture + 10 ml BOS 25°C 3 days	--Onto CIN --KOH Onto CIN
--Onto CIN 18 h 32°C	--Onto SSDC --Onto CIN	
--KOH treatment Onto CIN	-KOH treatment Onto CIN	
After 1 additional day[a] of broth incubation	After 2 additional days of broth incubation	
--Onto SSDC	--Onto SSDC	
--Onto CIN	--Onto CIN	
--KOH treatment	--KOH treatment	

Onto CIN Onto CIN

[a] Plating should only be done if colonies having typical *Y. enterocolitica* morphology are not present on plates inoculated on previous day.

Table 1. Sequence of Confirmation, Biogrouping, and Pathogenicity-marker Tests used for *Y. enterocolitica*

Yersinia Confirmation Tests	Simmons' Citrate slant	Kligler's Iron Agar slant & butt	Christensen's urea agar
	28°C, 24-72 h	28°C, 18-24 h	28°C, 18-72 h
	Citrate (-) (green) little/no gas	Alk/Acid no H_2S	Urea (+) (pink)
Y. enterocolitica Confirmation Tests	Oxidase Lysine decarboxylase Ornithine decarboxylase Rhamnose utilization Sucrose utilization		
Y. enterocolitica Biogrouping Tests	Lipase DNase Indole Xylose VP β-D-Glucosidase		

	Pyrazinamidase	
	Salicin;	Esculin
	Trehalose;	Nitrate Reduction
Pathogenicity-Marker Tests	Autoagglutination in MR-VP broth	
	Congo Red Binding	
	Crystal Violet Binding	

Table 2. Biogrouping Scheme for *Yersinia enterocolitica* [a]

	Biogroups[b]					
	1A	1B[c]	2[c]	3[c]	4[c]	5[c]
Lipase (Tween-esterase)	+	+	-	-	-	-
Esculin/salicin 24 h[d]	+,-	-	-	-	-	-
Indole	+	+	(+)[e]	-	-	-
Xylose	+	+	+	+	-	V[f]
Trehalose/NO_3[g]	+	+	+	+	+	-
Pyrazinamidase	+	-	-	-	-	-
β-D-Glucosidase	+	-	-	-	-	-
Voges-Proskauer	+	+	+	+[h]	+	(+)
DNase	-	-	-	-	+	+

[a] Modified from Wauters et al., 1987.

[b] Reactions from tests incubated at 25-28°C, with the exception of β-D-Glucosidase which was incubated at 30°C and salicin which was incubated at 35°C. Incubation at other temperatures may result in different results and biogroupings.

[c] Biogroup contains pathogenic strains.

[d] Esculin and salicin reactions for a given strain of *Y. enterocolitica* are nearly always identical so they are listed together in this table.

[e] Indicates a delayed positive reaction.

[f] Indicates variable reactions.

[g] **Trehalose and nitrate reduction reactions for a given strain of *Y. enterocolitica* are nearly always identical so they are listed together in this table.**

[h] **Rarely, a serotype O:3 strain may be negative for VP.**

ADDENDUM

Formulations for Media and Reagents for *Yersinia enterocolitica* Isolation and Identification

β-D-Glucosidase test

Add 0.1 g 4-nitrophenyl-β-D-glucopyranoside to 100 ml 0.666 M NaH_2PO_4 (pH 6.0), dissolve, then filter-sterilize.

BOS broth

$Na_2HPO_4*7H_2O$	17.25 g
Na oxalate	5.0 g
Bile salts No. 3 (Difco)	2.0 g
NaCl	1.0 g
0.1% solution of $MgSO_4*7H_2O$	10.0 ml
Distilled deionized H_2O	639.0 ml

Combine ingredients and mix until dissolved, adjust pH to 7.6 with 5 N HCl, then autoclave at 121°C for 15 minutes.

Add the following filter-sterilized solutions:

100 ml of 10% sorbose
100 ml of 1.0% asparagine
100 ml of 1.0% methionine
10 ml of 2.5 mg/ml metanil yellow
10 ml of 2.5 mg/ml yeast extract
10 ml of 0.5% Na pyruvate
1 ml of 0.4% solution of Irgasan DP300 (2,4,4'-trichloro-2'-hydroxydi

Adjust pH to 7.6 with either 5 N NaOH or HCl as required. Store at 4°C for up to 7 days.

On day of use, add 10 ml of 1.0 mg/ml Na furadantin (from stock solution stored at -70°C) to the above complete base. Aseptically dispense 10 ml portions into sterile tubes.

CIN agar

MUST CONTAIN Cefsulodin at 4 mg/L:

This formulation is commercially available from Difco; premixes available from other manufacturers contain different levels of cefsulodin.

Oxoid special peptone	20.0 g
Yeast extract	2.0 g
Mannitol	20.0 g
Na pyruvate	2.0 g
NaCl	1.0 g
0.1% aqueous stock solution of $MgSO_4*7H_2O$	10.0 ml
Na deoxycholate	0.5 g
Oxoid No. 4 (L11) agar	12.0 g
Distilled deionized H_2O	748.0 ml

Bring to a boil in order to dissolve agar completely (do NOT autoclave). Cool to around 80-85°C.

Add 10 ml of Irgasan DP300 (2,4,4'-trichloro-2'-hydroxydiphenyl ether, Ciba Geigy) solution (0.04% in 95% ETOH). Shake vigorously to disperse ethanol. Cool in a water bath to ca. 50-55°C.

Add 1 ml of 5 N NaOH, then 10 ml of each of the following aqueous, filter sterilized (0.22 µm pore size) stock solutions:

neutral red (3 mg/ml)
crystal violet (0.1 mg/ml)
cefsulodin (0.4 mg/ml)
novobiocin (0.25 mg/ml).

[Stock antibiotic solutions are stored at -70°C and thawed at room temperature just before use]

Adjust final pH to 7.4 with 5 N NaOH. Store prepared plates at around 20-25°C for up to 9 days.

CR-MOX agar

Tryptic soy agar	40.0 g
Distilled deionized H_2O	825.0 ml

Mix and autoclave at 121°C for 15 minutes. Cool basal medium to 55°C.

Add the following solutions:

a) 80 ml of 0.25 M sodium oxalate (Sigma) solution (sterilized by autoclaving at 121°C for 15 minutes)
b) 80 ml of 0.25 M magnesium chloride solution (sterilized by autoclaving at 121°C for 15 minutes)
c) 10 ml of 20% D-galactose solution (sterilized by autoclaving at 115°C for 10 minutes)
d) 5 ml of 1% Congo red solution (sterilized by autoclaving at 121°C for 15 minutes)

Mix well and dispense into 15 X 100 mm petri dishes. Store prepared media in plastic bags at 4°C for up to 3 months.

DNase test Agar

Tryptose	20.0 g
Deoxyribonucleic acid	2.0 g
Sodium chloride	5.0 g
Agar	15.0 g
Distilled water	1.0 L

Suspend all ingredients and heat to boiling to dissolve completely. Sterilize in the autoclave at 121°C for 15 minutes, final pH = 7.3. Dispense into sterile Petri dishes.

Esculin agar

Polypeptone (Oxoid)	10.0 g
Esculin	1.0 g
Ferric ammonium citrate	1.0 g
Agar	5.0 g
Distilled deionized H_2O	1.0 L

Mix well. Dispense into tubes, and autoclave at 121°C for 15 minutes.

Indole test medium

Prepare a 1% solution of Bacto Peptone (Difco) OR 1% Trypticase peptone (BBL) OR use Tryptone Water (Oxoid). Dispense 5 ml quantities into tubes. Sterilize by autoclaving at 121°C for 15 minutes.

ITC broth

Tryptone	10.0 g
Yeast extract	1.0 g
$MgCl_2*6H_2O$	60.0 g
NaCl	5.0 g
0.2% (w/v) malachite green solution (aqueous)	5.0 ml
$KClO_3$	1.0 g
Distilled deionized H_2O	1.0 L

Mix above ingredients, autoclave at 121°C for 15 minutes, cool. Then add,

a) 1 ml of Ticarcillin solution (1 mg/ml in H_2O; filter-sterilized) (Ticarcillin available from Sigma)
b) 1 ml of Irgasan DP300 (1 mg/ml in 95% ethanol); AKA 2,4,4'-trichloro-2'-hydroxydiphenyl ether (CIBA-Geigy, Basel)
c) Mix well. Dispense 100 ml into sterile 100 ml Erlenmeyer flasks (it is important to minimize the surface area:volume ratio). Store at 4°C for up to 1 month.

Kligler's iron agar (KIA) slants

Polypeptone peptone	20.0 g
Lactose	20.0 g
Dextrose	1.0 g
NaCl	5.0 g
Ferric ammonium citrate	0.5 g
Sodium thiosulfate	0.5 g
Agar	15.0 g
Phenol red	0.025 g
Distilled water	1.0 L

Heat with agitation to dissolve completely. Dispense into 13 X 100 mm screw-cap tubes and autoclave for 15 minutes at 121°C. Cool and slant to form deep butts. Final pH = 7.4.

KOH solution

NaCl	5.0 g
KOH	2.5 g
Distilled deionized H_2O	1.0 L

Dispense 4.5 ml amounts in small screw-cap tubes, and sterilize at 121°C for 15 minutes. Tighten caps when cool. Make only a small number of tubes at a time since pH decreases with storage time; store at 4°C for no more than 7 days.

Pyrazinamide agar

Tryptic soy agar (Difco)	30.0 g
Pyrazine-carboxamide (Merck)	1.0 g
0.2 M Tris-maleate buffer (pH 6)	1.0 L

Mix well, dispense 5 ml amounts in tubes (160 X 16 mm). Autoclave at 121°C for 15 minutes. Slant for cooling.

SSDC agar

SS agar (quantity per liter as stated by a particular Manufacturer)

Yeast extract	5.0 g
Na deoxycholate	10.0 g
$CaCl_2$	1.0 g
Distilled deionized H_20	1.0 L

Adjust pH to 7.2 to 7.3 Bring agar almost to a boil on a hot plate (Do NOT autoclave). Temper agar to 55-60°C, mix and pour while still warm, making thick plates. Store prepared plates for 7 days at 20-25°C in the dark. Do NOT store at 4°C.

Tween 80 agar (Lipase test agar)

Peptone	10.0 g
NaCl	5.0 g
$CaCl_2*H_2O$	0.1 g
Agar	15.0 g
Distilled deionized H_2O	1.0 L

Sterilize agar base by autoclaving at 121°C for 15 minutes. Temper to 45-50°C.

Sterilize Tween 80 by autoclaving at 121°C for 20 minutes.

Add sterile Tween 80 to tempered agar base to give a final concentration of 1% (v/v). Mix well. Dispense into Petri dishes, and allow to solidify.

Veal infusion broth

Veal, infusion from	500.0 g
Proteose peptone # 3	10.0 g
NaCl	5.0 g
Distilled water	1.0 L

Heat with agitation to dissolve all ingredients. Dispense 7 ml portions into 16 X 150 mm tubes and autoclave at 121°C for 15 minutes. Final pH = 7.4.

CHAPTER 10. EXAMINATION OF HEAT PROCESSED, HERMETICALLY SEALED (CANNED) MEAT AND POULTRY PRODUCTS

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10.1 Introduction

Thermally processed meat and poultry products in hermetically sealed containers include both shelf stable products as well as those that must be kept refrigerated (i.e. perishable product). There are a wide variety of packages designed to totally exclude air. These include traditional rigid containers, such as metal cans and glass jars; semi-rigid containers such as plastic cans, bowls and trays; and flexible containers such as retortable pouches and bags. The microbiological examination of these food products requires knowledge and a thorough understanding of food microbiology, food science, and packaging technology and engineering. Many books and scientific articles are available on the processing and the laboratory testing of these products. Individuals who perform these analyses should be familiar with the current procedures and methods. Some of these references are listed in section 10.6.

10.2 Important Terms and Concepts

a. Shelf Stability (commercial sterility):

The term "shelf stability" traditionally has been used by the Agency and is synonymous with the terms "commercial sterility" or commercially sterile". Shelf stability is defined in CFR title 9, part 318, Subpart G, 318.300 (u) of the Food Safety and Inspection Service (meat and poultry) USDA regulations. Shelf stability (commercial sterility) means "the condition achieved by application of heat, sufficient, alone or in combination with other ingredients and/or treatments, to render the product free of microorganisms capable of growing in the product at non-refrigerated conditions (over 50°F, 10°C) at which the product is intended to be held during distribution and storage". Such a product may contain viable thermophilic spores, but no mesophilic spores or vegetative cells. These products usually are stable for years unless stored at temperatures of 115-130°F (46-55°C) which may allow swelling or flat sour spoilage to

occur because of germination and growth of the thermophilic spores. Many low acid canned meat/poultry products contain low numbers of thermophilic spores. For this reason, samples of canned foods are not routinely incubated at 55°C because the results usually will be confusing and provide no sound information. Canned food lots that are to be held in hot vending machines or are destined for tropical countries are exceptions to this rule.

b. Hermetically Sealed Container:

A container that is totally sealed to prevent the entry or escape of air and therefore secure the product against the entry of microorganisms.

c. Adventitious contamination:

Adventitious contamination may be defined as the accidental addition of environmental microorganisms to the contents of a container during analysis. This can occur if the microbiologist has not sterilized the puncture site on the container surface or the opening device adequately, or is careless in manipulating equipment or cultures. Strict attention to proper procedures is required to avoid this type of contamination.

d. Cured Meat/Poultry Products:

Many canned meat/poultry products contain curing salts such as mixtures of sodium chloride and sodium nitrite. When included in a canned meat/poultry product formulation, sodium chloride and sodium nitrite inhibit the outgrowth of bacterial spores, particularly clostridial spores. Lowering the pH and increasing the sodium chloride concentration enhance the inhibitory action of sodium nitrite. Thus, most canned, cured meat/poultry products are minimally heat processed and are rendered shelf stable by the interrelationship of heat, pH, sodium chloride, sodium nitrite and a low level of indigenous spores. Spoilage in canned cured meat/poultry products attributed to underprocessing is rare. When it occurs, it is usually the result of improper curing rather than inadequate heating. The heat processes used for canned, cured, shelf stable meat/poultry products are unique in that they usually

are not designed to destroy mesophilic bacterial spores but merely to inhibit their outgrowth.

e. Uncured Meat/Poultry Products:

Canned uncured meat/poultry products are given a much more severe heat treatment than canned cured products. The treatment given to canned uncured meat/poultry products is commonly referred to as a "full retort cook".

10.21 Classification of Containers

a. Metal and plastic cans with metal double sealed end(s):

Cans must be at room temperature for classification. Cans are classified as NORMAL if both ends are flat or slightly concave; FLIPPER when one end of a normal-appearing can is struck sharply on a flat surface, the opposite end "flips out" (bulges) but returns to its original appearance with mild thumb pressure; SPRINGER if one end is slightly convex and when pressed in will cause the opposite end to become slightly convex; SOFT SWELL if both ends are slightly convex but can be pressed inward with moderate thumb pressure only to return to the convex state when thumb pressure is released; HARD SWELL if both ends are convex, rigid and do not respond to medium hard thumb pressure. A can with a hard swell will usually "buckle" before it bursts. Hard swollen cans must be handled carefully because they can explode. They should be chilled before opening except when aerobic thermophiles are suspected. Never flame a can with a hard swell, use only chemical sanitization.

b. Glass jars:

Classify glass jars by the condition of the lid (closure) only. Do not strike a glass jar against a surface as you would a can. Instead shake the jar abruptly to cause the contents to exert force against the lid; doing so occasionally reveals a flipper. Scrutinize the contents through the glass prior to opening. Compare the contents of the abnormal/questionable jar with the contents of a normal jar (e.g., color, turbidity, and presence of gas bubbles), and record observations.

c. Flexible containers (pouches):

Pouches usually are fabricated from laminates consisting of two or more layers (plies) of material. Retortable pouches are the most common type of flexible container used for canned, shelf-stable products. Most pouches are 3-ply: an outer ply of polyester film, a middle ply of aluminum foil, and an inner ply of polypropylene. The polyester functions as the heat resistant, tough protective layer; the aluminum foil as a moisture, gas and light barrier; and the polypropylene functions as the food contact surface and the film for heat sealing. The polypropylene also provides added strength, and protects the aluminum film against corrosion by the food product. Not all retortable pouches contain an aluminum foil ply. Pouches and paperboard containers used for non-retorted, shelf stable products (e.g. pH-controlled and hot-filled product) or aseptically filled containers may be quite different from retortable pouches in construction. Pouches and other flexible containers are either factory-formed and supplied ready for filling, or are formed by the processor from roll stock.

10.22 Container Abnormalities

To determine the cause of product abnormalities, both normal and abnormal containers from the same production lot should be examined. All observed microbiological results should be correlated with any existing product abnormalities (Section 10.46 a) such as atypical pH, odor, color, gross appearance, direct microscopic examination, etc. as well as the container evaluation findings (Section 10.46, b,c). Non-microbial swells (such as hydrogen swells) are usually diagnosed by considering all product attributes because culture results are negative or insignificant.

a. Metal cans, plastic containers and glass jars:

Conditions such as "swells" are defined in Section 10.21 (a). The defects and abnormalities associated with these containers have been extensively detailed by others. Rather than include extensive descriptions for each of them in this section, the analyst is referred to several excellent references presented in Section 10.6. These references provide detailed information on the numerous defects and abnormalities that can occur with these containers. The analyst should be familiar with these conditions before beginning any analysis of a

defective or abnormal container. The effect of processing failures, such as overfilling, closure at low temperature or high altitude; container damage; and storage temperature changes, must be taken into consideration as the analyst evaluates possible causes for the defect or abnormality. For quick reference, a Glossary of Terms is provided in Appendices I and II.

b. Pouches:

A Glossary of Terms for these containers can be found in Appendix III. It is imperative to follow uniform procedures (Section 10.46,c) when examining defective or abnormal pouches. The APHA, 1966 reference (Section 10.6) provides detailed information on the analysis of pouch defects.

10.3 Analysis of Containers

The number of containers available for analysis will vary. However, it is important that the number be large enough to provide valid results. Unless the cause of spoilage is clear cut, at least 12 containers should be examined. With a clear cut cause, one half this number may be adequate. If abnormal containers have been reported, but are not available for analysis, incubation of like-coded containers may reproduce the abnormality. The "normal" cans should be incubated at 35°C for 10 days prior to examination. Incubation temperatures in excess of 35°C should not be used unless thermophilic spoilage is suspected. This incubation may reproduce the abnormality, and thereby document progressive microbiological changes in the product. Examine the incubated cans daily. Remove any swells from the incubator as they develop and culture them along with a normal control. After the 10 day incubation period, cool the cans to room temperature and reclassify. Swollen, buckled and blown containers should NOT be incubated but analyzed immediately along with a normal control. All steps in the analysis should be conducted in sequence according to protocol.

10.31 Physical Examination of Metal and Plastic Containers

a. Before opening, visually examine the double end seam(s) and side seam (if present) for structural defects, flaws and physical damage; record pertinent observations.

b. Run thumb and forefinger around the inside and outside of the double seams for evidence of roughness, unevenness, or sharpness.

c. Using a felt marker, make three slash marks at irregular intervals across the label and the code-end seam. Remove the label and copy any label code-numbers to the side of the container along with a mark indicating the code end of the can. Correlate any stains on the label with suspicious areas on the side panel (can body) by returning the label to its exact position relative to the slash marks.

d. Examine all non-seam areas of the can and ends for any evidence of physical damage. If the code is embossed, carefully examine it for any evidence of puncturing. Circle any suspect and/or defective areas with an indelible pen and record this information on the work sheet. For an illustration of these defects see the APHA, 1966 reference (Section 10.6).

10.32 Physical Examination of Glass Jars

a. Before opening, remove the label and, using a good light source such as a microscope light, examine the container for apparent or suspected defects. Microorganisms may enter jars through small cracks in the glass. Make note of any residue observed on the outer surface and the location.

b. Test the closure gently to determine its tightness. After sampling has been completed, examine the lid (closure) and the glass rim (sealing surface) of the jar. Look for flaws in the sealing ring or compound inside the closure; for food particles lodged between the glass and the lid; and for chips or uneven areas in the glass rim.

10.33 Physical Examination of Pouches

a. Pouches should be examined using an illuminated 5X magnifier.

b. Hold the pouch in one hand, examine it for abnormalities, such as swelling, leakage, overfilling, and defects such as delamination and severe distortion. Record any pertinent observations.

c. Hold the pouch at both ends and examine both sides for noticeable cuts, cracks, scratches, food residues, punctures, missing labels, foreign materials or other abnormalities.

d. Carefully examine all seal areas for incomplete fusion. Pay attention to such defects as entrapped product, wrinkles, moisture and foreign material in the seal. Particular attention should be given to the final or closing seal.

e. All actual and suspected defects should be circled with an indelible marking pen for more detailed examination after all sampling is complete.

10.4 Analysis of the Contents

Processing errors occur infrequently with canned products, but may result in the improper processing of large quantities of product. Swollen cans, for instance, may signal a microbial spoilage problem. Each abnormality in a "canned" product must be investigated thoroughly and correctly. The following procedures should be followed carefully.

10.41 Equipment and Material

a. Incubators 20°, 35° & 55 ± 1°C
b. Vertical laminar flow hood
c. Microscope, microscope slides & cover slips
d. pH meter equipped with a flat electrode
e. Felt-tip indelible marker
f. Illuminated 5X magnifier
g. Sterile Bacti-disc cutter or other suitable opening device
h. Large, sterile plastic or metal funnel
i. Large autoclavable holding pans
j. Sterile towels
k. Clean laboratory coat and hair covering(s)
l. Sterile wide bore pipettes or 8 mm glass tubing with cotton plugs
m. Sterile serological pipettes with cotton plugs
n. Safety aspiration device for pipetting (e.g. pro-pipette)
o. Sterile petri dishes, beakers, and large test tubes
p. Sterile triers, cork borers, scissors, knives and 8" forceps. Triers can be made from the tail piece of

chrome finish sink drain pipe, 1 1/2" in diameter, flanged on one end and sharpened on the other end.

q. Sterile cotton swabs with wooden handles in glass test tubes, one per tube, or commercially sterilized swabs in paper sleeves
r. Sterile gloves
s. Small wire basket to hold pouches in an upright position
t. Seam analysis tools (micrometer, calipers, saw, countersink meter, metal plate scissors, nippers).
u. Vacuum gauge
v. Light source such as a microscope light
w. Sonic cleaning apparatus
x. Transparent acrylic plate with a hole and tubing to a vacuum source
y. Bituminous compound in strips (tar type strips usually available in hardware stores) stored in the 35°C incubator
z. Seamtest Type U (Concentrate), Winston Products Co., Inc Box 3332, Charlotte, N.C., Dilute 1:300 with distilled water for use.
aa. Wooden dowels, 1/2" diameter
bb. Gas cylinder clamp
cc. Abrasive chlorinated cleaner or a scouring pad

10.42 Media and Reagents

a. Modified Cooked Meat Medium (MCMM) STEAM JUST BEFORE USE
b. Brom Cresol Purple Broth (BCPB) or Dextrose Tryptone Broth
c. Plate Count Agar
d. APT Agar
e. KF Broth
f. Strong's Sporulation Medium
g. Gram stain reagents
h. Spore stain
i. Dishwashing detergent
j. Chlorine solution, (Commercial Bleach with approximately 5% available chlorine diluted 1:100 with 0.5 M phosphate buffer, pH 6.2)

10.43 Preparation

a. The Analyst

i. The analyst must wear a clean full length laboratory coat.

ii. Hair must be completely covered with a clean, disposable operating room type hair cover. A surgical face mask should be worn; if the analyst has facial hair such as beards and sideburns, the mask must completely cover it.

iii. Hands, forearms and face should be washed with germicidal soap and water.

iv. The analyst should wear safety glasses or goggles, preferably in combination with some type of face shield when opening swollen cans or cans suspected of being contaminated with ***Clostridium spp***.

b. Preparing the Environment

i. If possible, the analysis should be done in a vertical laminar flow hood. If a hood is not available, the area used must be clean and draft-free.

ii. Flat cans should be opened in the laminar flow hood.

iii. Swells may explode or spew, therefore they should be opened outside the hood and the container transferred to the hood only after it is opened and all gas released.

iv. Disinfect the work surface before beginning any work.

c. Preparing Metal Cans Prior to Opening

i. Scrub the non-coded end of the metal can with abrasive cleaner or a scouring pad. This removes bacteria-laden oil and protein residues. Rinse well with tap water. Cans with an "easy open" end usually are coded on the bottom. Record the code exactly and prepare the code end as described above.

ii. Sanitize the cleaned end with chlorine solution (Section 10.42 j) either by placing clean tissues over the end and saturating it with chlorine solution or by immersing the end in a shallow pan containing the solution. Allow a 15-minute contact

time; wipe dry with sterile towels or tissue. (An alternative sanitization procedure which can be used on Normal-appearing cans ONLY is to heat the entire can surface using a laboratory burner or a propane torch until the metal becomes slightly discolored from the heat.) Proceed as outlined in Section 10.44.

d. Preparing Jars Prior to Opening

i. Scrub the surface of the jar closure with abrasive cleaner or scouring pads. Rinse well with tap water.

ii. Sanitize the jar closure with chlorine (Section 10.42 j) either by placing clean tissues over the closure and saturating it with chlorine solution or immersing the closure in a shallow pan containing the solution. Allow a 15-minute contact time; wipe dry with sterile towels or tissue.

e. Preparing Plastic Containers Prior to Opening

i. Scrub the bottom surface of the container with abrasive cleaner or scouring pads. Rinse well with tap water.

ii. Sanitize the bottom with chlorine solution (Section 10.42 j) by placing clean tissues over the bottom and saturating it with chlorine or immersing the bottom of the container in a shallow pan containing the solution. Allow a 15-minute contact time; then wipe dry with sterile towels or tissue.

f. Preparing Normal and Abnormal-Appearing Flexible Retortable Pouches Prior to Opening

i. Clean the outside of the pouch with a sanitizer and rinse well.

ii. Sanitize the entire pouch in a suitably sized pan with chlorine solution (Section 10.42 j). Allow a 15-minute contact time; then wipe dry with sterile towels or tissue.

g. Preparing Swollen Cans Prior to Opening

i. Scrub the non-coded end of the chilled metal can with an abrasive cleaner or a scouring pad. This removes bacteria-laden oil and protein residues. Rinse well with tap water.

ii. Sanitize the cleaned end with chlorine solution (Section 10.42 j) either by placing clean tissues over the end and saturating it with chlorine solution or immersing the end in a shallow pan containing the solution. Allow a 15-minute contact time; then wipe dry with sterile towels or tissue.

h. Opening Devices

i. The preferred type of opening device is the adjustable Bacti-disc cutter (available from the Wilkens-Anderson Company, 4525 W. Division Street, Chicago, IL.; a similar device is available from the American National Can Co., 1301 Dugdale Rd., Waukegan, IL. Order Number WT2437). The opener should be pre-sterilized or heated in a flame to redness. If this type of device is not available, individually packaged and heat sterilized regular, all metal, kitchen-type can openers may be used. The advantage of the Bacti-disc type opener is that it causes no damage to the double seam (simplifying later examination) and the size of the opening can be adjusted.

ii. Sometimes a large can (e.g. a #10 size can) may be difficult to open. The analyst could be exposed to pathogens or their toxins if the can is not properly secured. The container can be held tightly with a gas cylinder clamp secured in an inverted position in a shallow metal drawer or tray lined with a large disposable poly bag or an autoclavable tray to contain any overflow. Place the #10 container against the clamp and secure the strap. Rotate the can and continue cutting until the opening is completed. The metal tray and liner may be removed for cleaning and the clamp is autoclavable.

10.44 Sampling

a. Normal-Appearing Metal Cans and Jars with Metal Closures

i. Prepare the area and can or jar closure as described in section 10.43.

ii. Shake the container to distribute the contents.

iii. Use a sterilized opening device to cut the desired size entry hole. Transfer samples immediately to the selected media with a sterile pipette or swab and proceed as outlined in Section 10.45.

iv. Aseptically transfer a representative amount of the product to a sterile test tube or other sterile container as a working reserve. Use a pipet or sterile spoon to accomplish this.

v. Caution: The contents from overfilled cans may flow out of the hole onto the surrounding lid surface at the time of opening. This material can then drain back into the can when the opening device is removed. Should this occur, terminate the analysis.

b. Normal and Abnormal-Appearing Plastic Containers

i. Immediately after removing the container from the chlorine solution and wiping the excess liquid, use a very hot, sterilized opening device to cut the desired size entry hole. Transfer samples immediately to the selected media with a sterile pipette or swab and proceed as outlined in Section 10.45.

ii. Aseptically transfer a representative amount of the product to a sterile test tube or other sterile container as a working reserve. Use a pipet or sterile spoon to accomplish this.

c. Normal and Abnormal Appearing Flexible Retortable Pouches

i. Place the disinfected pouch upright in a sterile beaker and cut a two inch strip about one quarter

of an inch under the seam edge using a sterile scissors. If possible, use a pipette to remove some of the pouch contents, otherwise use a swab. Transfer the samples immediately to the selected media with a sterile pipet or swab, proceed as in section 10.45.

ii. Aseptically transfer a representative amount of the product to a sterile test tube or other sterile container as a working reserve. Fold the edge of the opened pouch over against itself several times and secure with tape until the microbiological analysis is complete.

d. Swollen Cans

i. Cans displaying a hard swell should be chilled before opening. Most foods spoiled by ***Bacillus stearothermophilus*** will not produce gas (flat sour spoilage). However, if nitrate or nitrite is present in the meat/poultry product, gas may be produced by this microorganism. Cold usually will kill ***B. stearothermophilus*** resulting in no growth in Bromcresol Purple Broth. If possible, save one or two cans and store without refrigeration.

ii. NEVER FLAME A SWOLLEN CONTAINER - IT MAY BURST. Place the container to be opened in a large, shallow, autoclavable pan. The side seam, if present, should be facing away from the analyst. A container with a hard swell may forcefully spray out some its contents, posing a possible hazard to the analyst if the contents are toxic. Therefore, these cans should be considered a biohazard and precautions must be taken to protect the analyst. Protective gloves should be worn and the lab coat should be tucked inside the cuffs of the gloves or at least secured around the wrist. Some type of facial shield is also recommended.

iii. Place the sanitized container into a biohazard bag and cover with a sterile towel or invert a sterile funnel with a cotton filter in the stem over the can. Place the point of the sterile opening device in the middle of the container closure. Make a small hole in the center of the sterilized end/closure. Try to maintain pressure over the

hole. Release the instrument slowly to allow gas to escape into the towel or funnel.

iv. After the gas pressure has been released, enlarge the opening to the desired size to permit sampling and aseptically remove some of the container contents. Sample as outlined in (a) above.

10.45 Culturing

a. Inoculation of Culture Media

i. The sampling and transfer processes must be conducted aseptically; care must be taken to prevent contamination during the various manipulations.

ii. Transfer the sample at once to the selected media, inoculating each tube at the bottom. Whenever possible, use a pipet and pro-pipette to remove 1-2 ml of product for inoculating each tube of medium. When the nature of the meat/poultry product makes it impossible to use a pipet, use a sampling swab (holding it by the very end of the shaft) to transfer 1-2 g of the product to each tube. This is accomplished by plunging the swab into the product, then inserting the swab as far as possible into the appropriate tube of medium and breaking off the portion of the shaft that was handled. Use one swab for each tube of medium. When inoculating MCMM, force the broken swab to the bottom of the tube by using the tip of another sterile swab.

iii. For each sample, inoculate 2 tubes of MCMM which were steamed (or boiled) for 10 minutes and cooled just before use and 2 tubes of Bromcresol Purple Broth. If a tube of KF medium is inoculated at the same time, the presence of enterococci can be determined rapidly.

iv. As a process control, place uninoculated swabs into each of two tubes of MCMM and BCP and one swab into KF broth (if used). Additionally, label two uninoculated tubes of each medium to serve as controls. If multiple samples are cultured at the same time, only one set of control tubes are needed for each medium and each temperature.

v. After all tubes have been inoculated with a sample, aseptically transfer approximately 30 ml or a 30 g portion of the container contents to a sterile tube, Whirl-Pak® or jar for retention as a working reserve sample. Appropriately label the container and store it in a refrigerator at approximately 4°C.

vi. Finally, transfer a portion of the container contents to a sterile Petri plate, clean jar or beaker for pH, microscopic, organoleptic and other relevant analyses (10.46).

vii. Cover the hole made in the container with several layers of sterile aluminum foil, secure the foil with tape and then store the container in a refrigerator at approximately 4°C. This serves as the primary reserve. Re-enter it only as a last resort. If the sample is a regulatory sample, chain of custody records must be maintained on it.

b. Incubation of Culture Media

i. Incubate one tube each of MCMM and BCP at 35°C and one tube each at 55°C. If used, incubate the tube of KF medium at 35°C. For the MCMM and BCP controls, incubate one tube at 35° and one at 55°C.

ii. Observe all tubes at 24 and 48 h. Tubes incubated at 35°C that show no growth should be incubated for 5 days before discarding. Tubes incubated at 55°C should be incubated for 3 days before discarding. Subculture any questionable tubes, especially if the product under examination contributes turbidity.

c. Identification of Organisms

i. Use conventional bacteriological procedures to characterize the type(s) of microbial flora found in the contents of the container.

ii. Use descriptive terms such as: mixed culture or pure culture, anaerobic or aerobic growth, spore former or non-sporeformer, mesophile or thermophile, cocci or rods.

iii. Cultures should be examined using a Gram stain. Gram stains should be done only on 18-24 h cultures. Record the morphological types observed and their Gram reaction. If the container contents are examined microscopically using a methylene blue stain, record those observations as well. If endospores are present, the spore stain can be used for better definition of spore type and placement.

iv. Record all biochemical test results in addition to any characteristic growth patterns on differential and/or selective media.

v. MCMM tubes showing a bright yellow color with visible gas bubbles, and containing gram positive or gram variable rods should be suspected of containing gas-forming anaerobes. If ***Clostridium botulinum*** is suspected, sub-cultures should be made and incubated for 4-5 days. The original tube should be reincubated to check for spores. After 4 - 5 days incubation, test the cultures for toxin by the mouse bioassay (see Chapter 14).

10.46 Supportive Determinations

a. Examination of Container Contents

i. Determine the pH of the sample (10.45, a, vii) using a flat electrode. Disinfect the electrode after taking this measurement.

ii. If applicable, determine the water activity of the sample (Section 2.4).

iii. Examine the sample microscopically by making a simple methylene blue or crystal violet stain. A Gram stain is of no value since the age of the cells is not known and Gram-stain reactions may not be dependable in the case of old cells. Prepare a spore stain if the contents of a swollen container show signs of digestion and few bacterial cells.

iv. Note abnormalities observed in the container contents such as off-odors, off-color, changes in consistency and texture when compared with normal product. DO NOT TASTE!

b. Examination of Metal and Plastic Cans

NOTE: Whenever possible a "normal" companion can should be examined along with the abnormal one.

i. After a reserve sample has been taken and all examinations are complete, discard any remaining product into an autoclavable bag and terminally sterilize.

ii. Disinfect the inside of the container with a phenolic disinfectant and carefully clean it with a stiff brush or use an ultra sonic bath. Do not autoclave the container since this may destroy any defects.

iii. Examine the interior lining of metal containers for blackening, detinning and pitting.

iv. The container code should have been recorded prior to analysis; if it was not, do so now. Sometimes embossed codes are poorly impressed and can be revealed by rubbing a pencil on a paper held over the code. If this does not work, place a thin smooth piece of paper over the code, hold securely and rub the paper with a clean finger in order to impress the paper. Rerub the paper with a finger coated with graphite. This is superior to using a pencil to rub the code. If that fails, rub the code with carbon paper. Place transparent adhesive tape over the code and rub the tape with the back of a fingernail. Lift the tape and transfer it to any document requiring the can code. The latter two techniques allow a record to be kept of any partial numbers or symbols. It is also possible to wait until the can is emptied, then view the reverse of the code from the inside. If needed, the code can be viewed in a mirror.

v. When leakage from double seams or side seams is suspected, remove excess metal from the opened end, leaving a 0.5 - 1 cm flange. Dry thoroughly,

preferably overnight, in the 55°C incubator. Add leak detection liquid (10.41z) to the can to a depth of 2-4 cm. Place a microleak detector on the open end of the container. The leak detector consists of a transparent acrylic plate with a vacuum gauge and connector for a vacuum source. Place a gasket (cut pieces of an automobile tire inner tube will do) between the apparatus and the can. If the fit is not tight (e.g., end seam is bent), use modeling clay to fill in the gaps. Large cans without beading or thin metal cans having a wider diameter than height may collapse when vacuum is applied. To prevent this from happening, use 1/2" wooden dowels cut to the appropriate length to support the can sides. Bituminous compound on the dowel ends will hold them in place. Generally, 4 dowels are sufficient for a #10 can. Apply the gasket and any bituminous compound, to the open can end and fit the leak detector plate in place. Connect the vacuum and apply 10 inches vacuum to the can. Swirl the liquid to dissipate bubbles formed by gases dissolved in the liquid. Examine seams by covering them with the diluted Seamtest. Leaks are identified by a steady stream of bubbles or a steadily increasing bubble size. After carefully examining all seams for leaks, increase the vacuum to 20 inches vacuum and re-examine the seams. Leave the can under vacuum until a leak appears or for a maximum of 2 h, and examine at half-hour intervals. Mark the location of leaks on the can's exterior using a marking pen. When reporting, note which seam, and the distance from the side seam or some other appropriate reference point. If no leaks were found, note test conditions (time and amount of vacuum drawn).

vi. Perform a tear-down examination of the double seams. The following references in Section 10.6 will guide you through this process: APHA, 1966; Food Processors Institute, 1988; Double Seam Manual; Evaluating a Double Seam, FDA Bacteriological Analytical Manual, 1992.

vii. The tightness of double seams formed by plastic cans and metal can ends may be evaluated by comparing the actual seam thickness to the

calculated thickness of the plastic flange, neck, or metal end. This would include three thicknesses of plastic and two of metal. Also, assess tightness by inspecting the pressure ridge, since it reflects the compression of the plastic body wall. The pressure ridge should be visible and continuous. Each packer may have different specifications for the finished seams; if necessary, the analyst must call the in-plant inspector and ask for specifications for the container of interest.

c. Examination of Pouches

i. The best way to determine if a pouch has leaked is by the type of microorganisms recovered.

ii. The pouch should be examined microscopically looking for points of light coming through the film. These are potential leakage sites.

10.47 Interpretation of Results

Use Tables 2, 3 and 4 to arrive at possible causes of spoilage based on all laboratory results. Caution: The tables are based on a single cause of spoilage. If there are multiple causes, the tables may not help.

10.5 Examination of Canned, Perishable Meat/Poultry Products

Perishable meat and poultry products, such as hams, luncheon meats, and loaves are packaged in hermetically-sealed containers and then heat-processed to internal temperatures of not less than 150°F (65.5°C) and usually not greater than 160°F (71°C). "Perishable, Keep Refrigerated" must appear on the label of these products. Although they are not shelf stable, good commercial processing usually will destroy vegetative bacterial cells. The combined effects of sodium nitrite, salt, refrigeration, and low oxygen tension retard the outgrowth of the few vegetative cells and/or spores that may survive the process. Such products can retain their acceptable quality for 1 to 3 years when properly processed and refrigerated.

10.51 Analysis of Containers

See Sections 10.3 - 10.33

10.52 Analysis of the Contents

a. Equipment and Material

See Section 10.41

b. Media and Reagents

See Section 10.42

c. Preparation

See Section 10.43

d. Sampling

i. Using procedures already described (Section 10.44) remove approximately 50 g of sample with a sterilized trier, large cork borers, scissors, knife or forceps.

ii. Place the sample into a sterile blender jar or Stomacher bag, add 450 ml of sterile Butterfield's Phosphate Diluent and homogenize for 2 minutes. This is a 1:10 dilution; make additional dilutions through at least 10^{-4}. Proceed with the culturing steps given in Section 10.52 (e, f & g).

iii. After sampling, cover the container opening with sterile aluminum foil several layers thick and secure with tape. Place the opened sample unit in the freezer until the analysis is complete.

e. Aerobic Plate Counts

i. Pipet 1 ml of each dilution prepared in 10.52 (d) into each of two sets of duplicate pour plates according to the instructions given in Section 3.4.

ii. Prepare one dilution set with Plate Count Agar. Incubate this set at 35°C for 48 h.

iii. Substitute APT agar for the Plate Count Agar in the other set of plates. Incubate this set at 20°C for 96 h.

iv. Count and record the results from both sets as described in Section 3.4.

f. Gas-Forming Anaerobes (GFAs)

i. Steam tubes of MCMM for 10 minutes and cool just prior to use.

ii. Inoculate each tube with l ml of each dilution prepared in 10.52 (d). Begin with the 1:10 dilution and continue with subsequent dilutions. Use a separate pipet for each dilution. Dilutions must be sufficiently high to yield a negative endpoint. Be sure that the inoculum is deposited near the bottom of the tube.

iii. Incubate these tubes for 48 h at 35°C, but read daily.

iv. Consider any MCMM tubes showing a bright yellow color, containing visible gas bubbles, and containing gram positive or gram variable rods as positive for GFAs.

v. Based upon the highest dilution showing these organisms, report the approximate number of gas-forming anaerobes per gram, calculated as the reciprocal of the highest positive dilution. If skips occur, disregard the final actual dilution and calculate the end point at the dilution where the skip occurred. This is only an approximation of the gas forming anaerobe count. A minimum of three tubes per dilution and an MPN table must be used for a more accurate determination.

vi. If *Clostridium botulinum* is suspected, representative tubes that have not been opened should be reincubated for a total of 4 - 5 days and then tested for botulinum toxin using the mouse bioassay (Chapter 14).

g. Enterococci

i. Transfer 1 ml of each dilution prepared in 10.52(d) to individual tubes of KF broth. Use a separate pipette for each dilution. Begin with the 1:10 dilution and continue with each subsequent

dilution. Dilutions must be sufficiently high to yield a negative end point.

ii. Incubate these tubes at 35°C for 48 h. Tubes showing a yellow color, turbidity and buttoning of growth are presumptive positives.

iii. Confirm all presumptive positives microscopically. Either wet mounts examined under low light or gram stained preparations are suitable for these microscopic determinations. Microscopic determinations yielding cells with ovoid streptococcal morphology shall be considered confirmed positive.

iv. Report the approximate number of enterococci per gram, calculated as the reciprocal of the highest positive confirmed dilution. If skips occur, disregard the final actual dilution and calculate the end point at the dilution where the skip occurred. This is only an approximation of the number of enterococci. A minimum of three tubes per dilution and an MPN table must be used for a more accurate determination of organisms as described in 10.43-10.45 and Tables 2, 3 and 4.

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Appendix I

Glossary of Metal/Plastic Can Seam Terminology for Container Components and Defects

The same terms that are used to describe an all-metal seam apply equally well to the metal end/plastic body seam.

Base Plate: Part of a closing machine which supports cans during seaming operation.

Beaded Can: A can which is re-enforced by having ring indentations around the body. The bead tends to keep the can cylindrical and helps to eliminate paneling of the can body.

Body: Principal part of a container - usually the largest part in one piece containing the sides (thus sidewall or body wall).

Body Hook: Can body portion of double seam. Prior to seaming, this portion was the flange of the can.

Bottom Seam: Factory end seam. The double seam of the can end put on by the can manufacturer.

Buckling: A distortion in a can end.

Can Size: Two systems are commonly used to denote can size:

i. An Arbitrary system (1, 2, etc.) with no relation to finished dimension.

ii. A system indicating the nominal finished dimensions of a can; e.g. "307 x 512." In this example, the first group of digits ("307") refers to the can's diameter and the second set ("512"), the can's height. The first digit in each set represents inches, and the next two digits represent sixteenths of an inch. Hence, the example can has a diameter of 3-7/16 and a height of 5-12/16 (or 5-3/4) inches.

Chuck: Part of a closing machine which fits inside the countersink and in the chuck wall of the end during seaming.

Closing Machine: Also known as a double seamer. Machine which double seams the lid onto the can bodies.

Compound: Rubber or other material applied inside the end curl to aid in forming a hermetic seal when the end is double seamed on the can body.

Contamination in Weld Area: Any visible burn at one or more points along the side seam of a welded can. This is a major defect.

Countersink: On a seamed end, the perpendicular distance from the outermost end panel to the top seam.

Cover: Can end placed on can by packer. Also known as top, lid, packer's end, canner's end.

Cover Hook: That part of double seam formed from the curl of the can end.

Cross Over: The portion of a double seam at the lap.

Cross Section: Referring to a double seam, a section through the double seam.

Curl: The semi-circular edge of a finished end prior to double seaming. The curl forms the cover hook of the double seam.

Cut Code: A break in the metal of a can due to improper embossing-marker equipment.

Cut-Over: During certain abnormal double seaming conditions, the seaming panel becomes flattened and metal is forced over the seaming chuck forming a sharp lip at the chuck wall. In extreme cases the metal may split in a cut-over.

Dead-Head: An incompletely rolled finished seam. Also known as a skip, skid or spinner.

Double Seam: The joint between the end and the can body formed by rolling the curl under the flange (1st operation) and then pressing the metal together (2nd operation).

Droop: A smooth projection of double seam below the bottom of a normal seam. While droops may occur at any point of the seam, they usually are evident at the side seam lap. A

slight droop at the lap may be considered normal because of additional plate thickness incorporated into the seam structure.

Excessive Slivers: One or more slivers which are 1/32" or longer. This is a minor defect of welded cans.

Factory End: Bottom or can manufacturer's end.

False Seam: A seam fault where the end and body hook are not over-lapped (engaged), although they give the appearance of a properly formed seam. Also see Knockdown Flange.

Feather: Beginnings of a cut-over. See Sharp Edge.

First Operation: The first operation in double seaming. In this operation, the curl of the end is tucked under the flange of the can body which is bent down to form cover and body hook, respectively.

Flange: The flared portion of the can body which facilitates double seaming.

Flange Crack: Any crack at the flange or immediately adjacent to the weld of welded cans. This is a major defect.

Headspace: The free space above the contents of a can and the can lid.

Heavy Lap: A lap containing excess solder. Also called a thick lap.

Hook: (i). The bent over edges of a body blank, which form the side seam lock (ii). The body and cover hooks in a double seam.

Internal Enamel: A coating applied to the inside of the can to protect the can from chemical action by the contents or to prevent discoloration. A lacquer is usually clear; an enamel is pigmented and opaque.

Jumped Seam: A double seam which is not rolled tight enough adjacent to the crossover caused by jumping of the seaming rolls at the lap.

Knockdown Flange: A seam defect in which the flange is bent against the body of the can. The cover hook is not tucked

inside the body hook, but lies outside of it. False seams, knockdown flanges and soft crabs are degrees of the same effect. In order to distinguish the degree of the defect, the following terminology is suggested:

False Seam: The cover hook and body hook are not tucked for a distance of less than an inch. Thus it may not be possible to detect a false seam until the can is torn down.

Knockdown Flange: As above, but more than an inch in length. Body hook and cover hook in contact, but not tucked.

Soft Crab: A defect in which the body of the can is broken down and does not contact the double seam. Thus, there is a wide open hole in the can below the double seam where the body was not incorporated into the seam.

Lap: The soldered but not locked portions of a side seam at the ends of the can body before seaming and removing the can from the chuck at completion of the operation.

Lid: See Cover.

Lip, Spurs or Vees: Irregularities in the double seam due to insufficient or sometimes absent overlap of the cover hook with the body hook, usually in small areas of the seam. The cover hook metal protrudes below the seam at the bottom of the cover hook in one or more "V" shapes.

Loss of Overlap: Any observable loss of overlap along the side seam of a welded can. This is a critical defect.

Loose Tin: A metal can which does not appear swollen, but slight pressure reveals a looseness.

Mislock: A poor or partial side seam lock, due to improper forming of the side seam hooks.

Neck: The thickness of the top of the sidewall (body wall) of a plastic tub, one tenth of an inch below the junction of the flange and the sidewall.

Notch: A small cut-away portion at the corners of the body blank. This reduces droop when double seaming.

Oozier: An imperfect can which allows the escape of the contents through the seam.

Open Lap: A lap failed due to various strains set up during manufacturing operations. Also caused by improper cooling of the solder (See Weak Lap). A lap which is not properly soldered so the two halves are not properly joined.

Over Lap: The distance the cover hook laps over the body hook.

Paneling: A flattening of the can side. Also used to define concentric (expansion) rings in can ends.

Peaking: Permanent deformation of the expansion rings on the can ends due to rapid reduction of steam pressure at the conclusion of processing. Such cans have no positive internal pressure and the ends can be forced back more or less to their normal position.

Perforation: Holes in the metal of a can resulting from the action of acid in food on metal. Perforation may come from inside due to product in the can or from outside due to material spilled on the cans.

Pleat: A fold in the cover hook which extends from the edge downward toward the bottom of the cover hook and sometimes results in a sharp droop, vee or spur.

Pressure Ridge: A ridge formed on the inside of the can body directly opposite the double seam, as a result of the pressure applied by the seaming rolls during seam formation.

Pucker: A condition which is intermediate between a wrinkle and a pleat in which the cover hook is locally distorted downward without actual folding. Puckers may be graded the same way as wrinkles.

Sanitary Can: Can with one end attached, the other end put on by the packer after the can is filled. Also known as packer's can or open top can.

Sawtooth: Partial separation of the side seam overlap at one or more points along the side seam after performing the pull test on a welded side seam. This is a critical defect.

Seam Arrowing: A readily visible narrowing of the weld at either end of the can body. This is a major defect.

Seam Width: The maximum dimensions of a seam measured parallel to folds of the seam. Also referred to as the seam length or height.

Seam Thickness: The maximum dimension measured across or perpendicular to the layers of the seam.

Second Operation: The finishing operation in double seaming. The hooks formed in the first operation are rolled tight against each other in the second operation.

Sharp Edge: A sharp edge at the top of the inside portion of the double seam due to the end metal being forced over the seaming chuck.

Side Seam: The seam joining the two edges of a blank to form a body.

Skipper / Spinner: See Deadhead.

Uneven Hook: A body or cover hook which is not uniform in length.

Vee: See Lip.

Weak Lap: The lap is soldered and both parts are together. However, strain on this lap (e.g. by twisting with the fingers) will cause the solderbond to break.

Weld Crack: Any observable crack in a welded side seam. This is a critical defect.

Worm Holes: Voids in solder usually at the end of the side seam. May extend completely through the width of the side seam.

Wrinkle: The small ripples in the cover hook of a can. A measure of tightness of a seam.

Appendix II

Glossary of Glass Container Parts

From a manufacturing standpoint, there are three basic parts to a glass container based on the three parts of glass container molds in which they are made. These are the finish, the body and the bottom.

Finish: The finish is that part of the jar that holds the cap or closure. It is the glass surrounding the opening in the container. In the manufacturing process, it is made in the neck ring or the finish ring. It is so named since, in early hand glass manufacturing, it was the last part of the glass container to be fabricated, hence "the finish". The finish of glass containers has several specific areas as follows:

Continuous Thread: A continuous spiral projecting glass ridge on the finish of a container intended to mesh with the thread of a screw-type closure.

Glass lug: One of several horizontal tapering protruding ridges of glass around the periphery of the finish that permit specially designed edges or lugs on the closure to slide between these protrusions and fasten the number of lugs on the closure and their precise configuration is established by the closure manufacture.

Neck Ring Parting Line: A horizontal mark on the glass surface at the bottom of the neck ring or finish ring resulting from the matching of the neck ring parts with the body mold parts.

Sealing Surface: That portion of the finish which makes contact with the sealing gasket or liner. The sealing surface may be on the top of the finish, or may be a combination of both top and side seal.

Vertical Neck Ring Seam: A mark on the glass finish resulting from the joint of matching the two parts of the neck ring. NOTE: Some finishes are made in a one-piece ring and do not have this seam.

Body: The body of the container is that portion which is made in the "body-mold" in manufacturing. It is the largest part of the container and lies between the finish and the bottom.

The characteristic parts of the body of a glass container are:

Heel: The heel is the curved portion between the bottom and the beginning of the straight side wall.

Mold Seam: A vertical mark on the glass surface in the body area resulting from matching the two parts of the body mold.

Shoulder: That portion of a glass container in which the maximum cross-section or body area decreases to join the neck or finish area. Most glass containers for processed foods have very little neck. The neck would be a straight area between the shoulder and the bottom of the bead or, with beadless finishes, the neck ring parting line.

Side Wall: The remainder of the body area between the shoulder and the heel.

Bottom: The bottom of the container is made in the "bottom plate" part of the glass container mold. The designated parts of the bottom normally are:

Bearing Surface: That portion of the container on which it rests. The bearing surface may have a special configuration known as the "stacking feature" which is designed to provide some interlocking of the bottom of the jar with the closure of another jar on which it might be stacked for display purposes.

Bottom Plate Parting Line: A horizontal mark on the glass surface resulting from the matching of the body mold parts with the bottom plate.

Appendix III

Glossary of terms - Flexible Retortable Pouches.

Adhesive: A substance applied to ply surfaces to cement the layers together in a laminated film: (a). Polyurethane adhesive for the outer layer (b). Maleic anhydride adduct of polypropylene for the inner layer.

Blisters: Bubbles/gaseous inclusions/particulate material, may be present between layers of laminate, usually are found in the seal area.

Bottom of Closing Seal: Portion of closing (packer) seal adjustment to the pouch contents.

Bottom Seal: A seal applied by heat and pressure to the bottom of a flexible pouch.

Cosmetic Seal: Area above the primary seal designed to close the edges of the pouch thus preventing the accumulation of extraneous material.

Cuts, Punctures, Scratches: Mechanical defects that penetrate one or more layers of the pouch.

Delamination: Any separation of plies through adhesive failure. This may result in questionable integrity of the package and safety of the product.

Dirty: Smeared with product or product trapped in top edges (where there are no cosmetic seals).

Disintegrated Container: Evidence of delamination or degradation after retorting.

Final Seal: A seal formed by heat and pressure by the packer after pouch filling and prior to retorting.

Foil Flex Cracks/Foil Roll Holes: Visible cracks in the aluminum foil layer caused by flexing of the pouch or pin holes (roll holes) in the foil caused through manufacture of the aluminum ply.

Foreign Materials: Any material (solid food, condensate, grease, voids, blemishes) that may be entrapped between the plies but usually found in the seal area.

Fusion Seal: A seal formed by joining two opposing surfaces by the application of heat and pressure.

Hard Swell or Blown: Distention or rupture due to internal gas formation.

Inner Ply: Polypropylene coating bonded to the food surface side of the aluminum foil.

Laminate: Two or more layers of material held together by adhesive(s).

Leaker: Product leaking through any area of the pouch.

Outer Ply: The polyester film bonded to the exterior surface of the aluminum foil.

Over Carton: A separate container (usually cardboard) in which the flexible pouch is packaged for additional protection.

Package Dimensions: The measurements of retortable flexible pouches stated as length, the longest dimension (LGT), width the second longest dimension (W), and thickness, the shortest dimension (HGT). All are given as internal measurements.

Pin Holes, Roll Holes: Holes in the aluminum foil layer only, originating during manufacturing; usually do not leak.

Preformed Seals: Seals formed by heat and pressure, by the manufacturer of the pouches, along the sides and at the bottom of the pouches.

Primary Seal: A fusion seal formed by the food processor by applying heat and pressure immediately after filling.

Seal: A continuous joint of two surfaces made by fusion of the laminated materials.

Seal Width: The maximum dimension of the seal measured from the leading outside edge perpendicular to the inside edge of the same seal.

Severely Damaged: Punctures, cuts or ruptures which penetrate all layers of the pouch and expose the product to contamination.

Side Seals: Seals formed by applying heat and pressure to the sides of the pouch's laminates to form the "preformed pouch".

Tear Nicks or Notch: Notches near the final seal to aid the consumer in opening the pouch.

Wrinkle: A crease or pucker in the seal (Packer or Factory) areas.

Appendix IV

Table 1. Normal pH Values for a Few Representative Canned Meat/Poultry Products.

Kinds of Food	pH
Beans with Wieners	5.7
Beef Chili	5.6
Beef Paté	5.7
Beef Stew	5.4 - 5.9
Beef Taco Filling	5.8
Beef and Gravy	5.9 - 6.1
Chicken Noodle Soup	5.8 - 6.5
Chicken Soup with Rice	6.7 - 7.1
Chicken Broth	6.8 - 7.0
Chicken and Dumplings	6.4
Chicken Vegetable Soup	5.6
Chicken Stew	5.6
Chicken Vienna Sausage	6.1 - 7.0
Chorizos	5.2
Corned Beef	6.2
Corned Beef Hash	5.0 - 5.7
Egg Noodles & Chicken	6.5
Ham	6.0 - 6.5
Lamb, Strained Baby Food	6.4 - 6.5
Pork Cocktail Franks	6.2
Pork with Natural Juices	6.2 - 6.4
Pork Sausage	6.1 - 6.2
Roast Beef	5.9 - 6.0
Spaghetti and Meatballs	5.0
Spaghetti Sauce with Beef	4.2
Stuffed Cabbage	5.9
Sloppy Joe	4.4
Turkey, Boned in Bouillon	6.1 - 6.2
Turkey with Gravy	6.0 - 6.3
Vienna Sausage	6.2 - 6.5
Wieners, Franks	6.2

Appendix V

Table 2. KEY TO PROBABLE CAUSE OF SPOILAGE IN CANNED FOODS

Group 1.- Low-Acid Foods
pH Range 5.0 to 8.0

Condition of cans	Characteristics of Material in Cans						
	Odor	Appearance	Gas (CO_2 & H_2)	pH	Smear	Cultures	Diagnosis
Swells	Normal to "metallic"	Normal to frothy (Cans usually etched or corroded)	More than 20% H_2	Normal	Negative to occasional organisms	Negative	Hydrogen swells
	Sour	Frothy; possibly ropy brine	Mostly CO_2	Below Normal	Pure or mixed cultures of rods, cocci, yeasts or molds	Growth, aerobically and/or anaerobically at 35°C., and possibly at 55°C.	Leakage
	Sour	Frothy; possibly ropy brine, food particles firm with uncooked appearance	Mostly CO_2	Below Normal	Pure or mixed cultures of rods, coccoids, cocci and yeasts	Growth, aerobically and/or anaerobically at 35°C., and possibly at 55°C. (If product received high exhaust, only spore formers may be recovered)	No process given
	Normal to sour-cheesy	Frothy	H_2 and CO_2	Slightly to definitely below normal	Rods, med. Short to med. long, usually granular; spores seldom seen	Gas, anaerobically at 55°C., and possibly slowly at 35°C.	Post-processing temperature abuse Thermophilic anaerobes
	Cheesy to putrid	Usually frothy with disintegration of solid particles	Mostly CO_2; possibly some H_2	Slightly to definitely below normal	Rods; usually spores present	Gas anaerobically at 35°C.	Underprocessing - mesophilic anaerobes (possibility of Cl. botulinum)
	Slightly off – possibly ammoniacal	Normal to frothy		Slightly to definitely below normal	Rods; spores occasionally seen	Growth, aerobically and/or anaerobically with gas at 35°C and possibly at 55°C. Pellicle in aerobic broth tubes. Spores formed on agar and in pellicle.	Underprocessing - B. subtilis type

No vacuum and/or Cans buckled	Normal	Normal	No H_2	Normal to slightly below normal	Negative to moderate number of organisms	Negative	Insufficient vacuum, caused by: 1) Incipient spoilage, 2) Insufficient exhaust, 3) Insufficient blanch, 4) Improper retort cooling procedures, 5) Over fill
Flat cans (0 to normal vacuum)	Normal to sour	Normal to cloudy brine		Slightly to definitely below normal	Rods, generally granular in appearance; spores seldom seen	Growth without gas at 55°C. Spore formation on nutrient agar	Post-Processing temperature abuse Thermophilic flat sours.
	Normal to sour	Normal to cloudy brine; possibly moldy		Slightly to definitely below normal	Pure or mixed cultures of rods, coccoids, cocci or mold	Growth, aerobically and/or anaerobically at 35°C., and possibly at 55°C.	Leakage

Appendix VI

Table 3. KEY TO PROBABLE CAUSE OF SPOILAGE IN CANNED FOODS

Group 3. Semi-Acid Foods
pH Range 4.6 to 5.0

Condition of cans	Characteristics of Material in Cans						
	Odor	Appearance	Gas (CO_2 & H_2)	pH	Smear	Cultures	Diagnosis
Swells	Normal to "metallic"	Normal to frothy (Cans usually etched or corroded)	More than 20% H_2	Normal	Negative to occasional organisms	Negative	Hydrogen swells
	Sour	Frothy; possibly ropy brine	Mostly CO_2	Below Normal	Pure or mixed cultures of rods, coccoids, cocci, yeasts or molds	Growth, aerobically and/or anaerobically at 35°C., and possibly at 55°C.	Leakage
Note: Cans are Sometimes flat	Sour	Frothy; possibly ropy brine, food particles firm with uncooked appearance	Mostly CO_2	Below Normal	Pure or mixed cultures of rods, coccoids, cocci and yeasts	Growth, aerobically and/or anaerobically at 35°C., and possibly at 55°C. (If product received high exhaust, only spore formers may be recovered)	No process given
	Normal to sour-cheesy	Frothy	H_2 and CO_2	Slightly to definitely below normal	Rods - med. Short to med. long, usually granular; spores seldom seen	Gas, anaerobically at 55°C., and possibly slowly at 35°C.	Post-processing temperature abuse Thermophilic anaerobes
	Normal to cheesy to putrid	Normal to frothy with disintegration of solid particles	Mostly CO_2; possibly some H_2	Normal to slightly below normal	Rods; possibly spores present	Gas anaerobically at 35°C. Putrid odor	Underprocessing – mesophilic anaerobes (possibility of *Cl. Botulinum)*

	Slightly off - possibly ammoniacal	Normal to frothy		Slightly to definitely below normal	Rods; occasionally spores observed	Growth, aerobically and/or anaerobically with gas at 35°C and possibly at 55°C. Pellicle in aerobic broth tubes. Spores formed on agar and in pellicle.	Under-processing - *B. subtilis* type
	Butyric acid	Frothy, large volume gas	H_2 and CO_2	Definitely below normal	Rods - bipolar staining; possibly spores	Gas anaerobically at 35°C. Butyric acid odor	Under processing - butyric acid anaerobe
No vacuum and/or Cans buckled	Normal	Normal	No H_2	Normal to slightly below normal	Negative to moderate number of organisms	Negative	Insufficient vacuum, caused by: 1) Incipient spoilage, 2) Insufficient exhaust, 3) Insufficient blanch, 4) Improper retort cooling procedures, 5) Over fill
Flat cans (0 to normal vacuum)	Sour to "medicinal"	Normal to cloudy brine		Slightly to definitely below normal	Rods, possibly granular in appearance	Growth without gas at 55°C. and possibly at 35°C. Growth on thermoacidurans agar	Underprocessing *B. coagulans*
	Normal to sour	Normal to cloudy brine; possibly moldy		Slightly to definitely below normal	Pure or mixed cultures or rods, coccoid, cocci or mold	Growth, aerobically and/or anaerobically at 35°C., and possibly at 55°C.	Leakage

Appendix VII

Table 4. Characteristics of Normal and Abnormal Perishable Canned Meat/Poultry Products

Condition of Cans	Odor	Appearance	pH	Smear	Cultures	Probable Cause
Flat Cans (0 to Normal Vacuum)	Normal	Normal	Normal	Negative to occasional organisms	0 to low # APC, APT agar count	Normal product
0 to degrees of swelling	Sour to off odor	Normal to mushy, possible gel liquification	Slightly to definitely below normal	Mixed culture of rods & enterococci	Low # mesophiles, high # psychrophilic non-spore formers (enterococci, lactobacilli	1. Prolonged storage at low temperatures 2. Abnormal high levels in raw materials 3. Substandard process
Swell	Sour or off odor, possibly putrid	Normal to mushy, possible gel liquification	Slightly to definitely below normal	Mixed culture of rods, cocci	High # mesophilic spore formers and non-sporeformers	Product held without refrigeration
Swell	Normal to sour	Normal	Below normal	Cocci, rods or both	Enterococci, rods or both	Leakage if shell higher than core. Underprocessing if core higher than shell
Swell	Off odor	Normal to off color	Below normal	Rods	Psychrotrophic clostridia (rarely occurs in U.S.).	Low brine levels
Swell	Normal to putrid, depending on length of storage.	Ranges from uncooked appearance to digested	Normal to low, depending on length of storage.	Vary	Vary	Missed processing cycle. Most of these are detected soon after distribution.

CHAPTER 11. TESTS FOR ENZYMES IN MEAT AND MEAT PRODUCTS

Charles P. Lattuada, James G. Eye, John M. Damare and B. P. Dey

11.1 Catalase Test

11.11 Introduction

Tests for catalase in meat are limited to products that have been given a heat treatment since the enzyme normally is present in all raw meat. It is particularly useful for roast beef. This procedure will detect under-processing when the product is scheduled to be heated to 145°F (62.8°C) or higher internal temperature. Tests for catalase in cooked beef are indicative of the presence of somatic catalase. Somatic catalase is destroyed at approximately 145°F and the test indicates whether or not temperatures higher than 145°F were reached.

Detection of catalase in a canned meat product could be indicative of flat sour spoilage. At canning temperatures all somatic catalase should be destroyed, and the presence of the enzyme in a freshly opened can is indicative of bacterial catalase produced by growth.

11.12 Equipment and Supplies

a. Clean plastic teaspoon
b. Clean paper towels
c. Felt-tip marking pen
d. Adhesive tape or paper labels
e. Whirl-Pak® clear plastic bags (3" x 4")
f. Clear plastic Zip-Loc® bags (12" x 12")
g. Clean and sanitized slicing knife
h. Clean and sanitized large spoon or spatula
i. 3% Hydrogen Peroxide - 1 pint
j. Baby Shampoo
k. Active dry baker's yeast

11.13 Procedure

a. Preparation of the Peroxide Reagent

    i. Remove the caps from both the peroxide and the shampoo bottles.

ii. Add one teaspoonful of the shampoo to the pint of hydrogen peroxide (peroxide reagent).

iii. Replace the caps securely on each bottle.

iv. Slowly invert the peroxide reagent bottle 3-4 times to mix the contents.

v. Label the reagent bottle "Prepared Reagent" followed by the date of preparation.

vi. Store the peroxide reagent in a refrigerator, the unused shampoo can be stored on a shelf with the chemicals.

b. Testing the Peroxide Reagent

i. Label a 3" x 4" Whirl-Pak® bag "Reagent Test".

ii. Carefully open the Whirl-Pak® bag and pour approximately 10 granules of the baker's yeast into the bag.

iii. Hold the Whirl-Pak® bag upright and pour approximately ½ inch of the peroxide reagent into the bag.

iv. Securely hold the top of the bag with the fingers of one hand and securely hold the bottom of the bag with the fingers of the other hand. Position the bag so that the fluid/foam level in the bag is aligned along the edge of the work surface. Keep the bag pressed against the edge of the work surface. Carefully pull the bag downward toward the open end to expel all excess air from the bag. Fold the top over several times and secure it with the built-in clips.

v. Securely replace the cap on the peroxide reagent bottle and then use it to support the upright "Reagent Test" bag.

vi. Record the time and then add 5 minutes to it for the "Read Time".

vii. At the read time note whether the bag has abundant foam and is somewhat inflated (Positive Test) or non-foamy and flat (Negative Test). Record this information in the appropriate Quality Control Log. If the peroxide reagent gives a positive test, proceed to the product test, if otherwise, prepare a fresh aliquot of the peroxide reagent first.

c. Roast Beef Cooking Temperature Test

i. Prepare the product for sampling and secure a clean sanitized (145°F + hot water) slicing knife. Dry the knife with a clean, preferably sterilized, paper towel.

ii. Wipe the knife and slicing surface with a 5% hypochlorite solution.

iii. Make a slice through the roast beef at the thickest part of the sample (maximum circumference). Examine the two halves to see if there are areas that appear to be more rare than others.

iv. Label a Whirl-Pak® bag with the sample identification number and then carefully open it.

v. Cut a ¼ inch thick slice from one of the surfaces, lay it down on a sterile surface and carve out a 1" square section from what appears to be the least cooked area of the slice. Using the knife blade, transfer this 1" square to the Whirl-Pak® bag.

vi. Shake the bag to transfer the piece to the bottom of the bag. Cover the piece with Peroxide Reagent and proceed according to steps b. iv through vi, with the exception that the reaction time between the reagent and the sample is extended to 15 minutes.

vii. Record the results on the form that accompanied the sample and proceed as you would with any other positive or negative official sample.

d. Canned Product

i. Label a 12" x 12" zip-lock® bag with the appropriate sample identification number. Do the same for a 3" x 4" Whirl-Pak® bag.

ii. Aseptically open the suspect can and transfer the contents to the large zip-lock® bag. It may be necessary to use a clean and sanitized large spoon or spatula to facilitate this transfer.

iii. Carefully close the zipper, expelling all air in the process.

iv. Carefully manipulate the contents of the zip-loc® bag in a manner to thoroughly mix the contents.

v. Carefully open the zip-loc® bag, and using a clean, sanitized teaspoon, remove a level spoonful of test material from the bag and transfer it to the Whirl-Pak® bag. Reseal the zip-lock bag and set it and the empty container to one side for possible future use.

vi. Add peroxide reagent to the Whirl-Pak® bag with the sub-sample to completely cover the sample and the peroxide reagent fills the bottom third of the bag. Use the teaspoon to evenly disperse the sub-sample throughout the reagent.

vii. Quickly fold the top of the bag four times the width of the tab tape and secure with the side tabs. Proceed according to steps b. iv through vi, with the exception that the reaction time between the reagent and the sample is one minute.

viii. Allow the sample test bag to stand undisturbed for an additional 15 minute period and then make a final reading.

ix. Record the results on the form that accompanied the sample and proceed as you would with any other positive or negative official sample.

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CHAPTER 12. EXAMINATION OF MEAT AND POULTRY PRODUCTS FOR *BACILLUS CEREUS*

Charles P. Lattuada and Dennis McClain

12.1 Introduction

Bacillus cereus is one of the few sporeforming, aerobic bacteria recognized as a bacterial pathogen. It is widespread in soil, milk, the surfaces of meat and poultry, cereals, starches, herbs and spices. Its' role as a food-borne pathogen is relatively recent and somewhat uncommon in the United States. Two distinct types of illness have been attributed to the consumption of food contaminated with ***B. cereus***. The more common manifestation is a diarrheal illness with an incubation time of 8-16 h characterized by abdominal pain and diarrhea. The other is an emetic illness with an incubation time of 1-5 h and characterized by nausea and vomiting. While the emetic type is usually associated with cereal type products such as rice, the diarrheal type is more widely associated with many foods.

B. cereus typically is a very large, aerobic, Gram positive, sporeforming rod with peritrichous flagella. It grows over a wide temperature range (10 to 48°C) with an optimum range of 28 to 35°C. It will grow over a wide pH range (pH 4.9 - 9.3) and in sodium chloride concentrations approximating 7.5%. Microscopically it may be seen in chains. Macroscopically the colonies have a dull or frosted appearance on a nutrient agar plate. Its association with disease is usually related to counts $>10^5$ cfu/g in the suspect food. Since ***B. cereus*** does not ferment mannitol, does produce lecithinase and is resistant to polymyxin, a selective medium consisting of mannitol-yolk-polymyxin (MYP) is commonly used for its isolation. Colonies typically are pink in color and surrounded by a zone of precipitate. An ELISA test is available to detect the diarrheal toxin.

12.2 Equipment, Reagents, Media

12.21 Equipment

a. Balance capable of weighing to 0.1 g
b. Stomacher™ (model 400 by Tekmar, or comparable model), sterile plastic bags (with twist ties or self-sealing)

OR blade-type blender, sterile cutting assemblies and blender jars

c. Sterile supplies, spoons or spatulas, pipettes (1 ml), bent glass rods "hockey sticks", aluminum pie pans (or equivalent)
d. Incubator, 30 ± 1°C
e. Incubator, 35 ± 1°C
f. Light or Darkfield Microscope
g. Platinum inoculating loops, 3 mm diameter
h. Microscope slides and cover slips
i. Meeker/Bunsen burner with tripod, or hot plate
j. Pyrex beaker, 250-300 ml size

12.22 Reagents

a. Butterfield's Phosphate Diluent (BPD) for sample extraction
b. BPD dilution blanks, 9 ml volume
c. Basic fuchsin staining solution, 0.5% aqueous

12.23 Media

a. Plates of Mannitol Yolk Polymyxin (MYP) Agar
b. Nutrient Agar Slants
c. BC Motility Medium
d. Nutrient Agar Plates
e. Blood Agar Plates, 5% Sheep RBC

12.3 Sampling and Dilution Procedure

a. Aseptically composite a 25 g or 25 ml sample in sterile bag or blender jar.

b. Add 225 ml Butterfield's Phosphate Diluent (BPD) to each sample taken.

c. Stomach or blend for 2 minutes and then prepare serial dilutions of 10^{-2} to 10^{-6} in 9 ml BPD dilution blanks.

12.31 Plating and Examination of Colonies

a. Pipette 0.1 ml of the homogenate (10^{-1}) and spread it over the entire surface of duplicate, predried MYP plates with a "hockey stick". Repeat the procedure for each of the other dilutions through 10^{-6}. Use a

separate, sterile "hockey stick" for each dilution. Allow the inoculum to dry before incubating the plates.

b. Incubate all plates in an upright position for 20 to 24 h at 30°C.

c. After incubation, examine all plates for colonies that are surrounded by a zone of precipitate (lecithinase production) against an eosin pink to lavender agar background (non-fermentation of mannitol). If the areas of lecithinase production coalesce between colonies, look for plates with 10-100 colonies. Count all typical colonies and determine the presumptive count per gram. Remember that the count will be tenfold higher than the dilution, because only 0.1 ml was placed on a plate.

12.32 Confirmatory and Differential Procedures/Tests

a. Select 4-6 typical colonies for confirmation. Each of these colonies is subcultured on a predried Nutrient Agar Plate and incubated at 30°C for 24 - 48 h. Note the presence or absence of rhizoid growth on the nutrient agar plate.

b. At the same time inoculate a tryptic soy sheep blood agar plate that has been divided into 4 - 6 segments. A 2 mm loop should be used to deposit the inoculum in the center of the segment. Note the size of the hemolytic zone (and whether it is partial or complete).

c. Motility test - use BC motility medium method by making a center line stab inoculation with a 3 mm loop and incubating the tube at 30°C for 18-24 h. Observe for diffuse growth into the medium away from the stab as an indication of a motile organism.

Alternatively a microscopic motility test may be used. The slide motility test is done by adding 0.2 ml of sterile water to a nutrient agar slant and then inoculating the aqueous phase with a 3 mm loopful of a 24 h slant culture. Incubate for 6-8 h at 30°C. Place a loopful of the liquid culture on a glass slide and overlay with a cover slip. *B. cereus* and *B. thuringiensis* are actively motile while *B. anthracis* and the rhizoid strains of *B. cereus* are non-motile.

d. Rhizoid growth - to test for rhizoid growth, inoculate several well isolated areas of a predried Nutrient Agar Plate. Use a 3 mm inoculating loop to make a point of contact inoculation. Incubate the plate in an upright position at 30°C for 24-48 h. If hair-like projections (rhizoids) develop outward from these colonies, the isolate is *B. cereus* var. *mycoides* and not considered to be a human pathogen.

e. Protein toxin crystal stain - Make a smear on a microscope slide with sterile water from a 2-3 day old nutrient agar plate or slant. Allow the slide to air dry and then gently heat fix it. After cooling, flood the slide with methanol, wait 30 seconds and pour it off. Then flood the slide with 0.5% aqueous solution of basic fuchsin. Gently heat the slide until steam is observed, remove the heat, wait 1-2 minutes and repeat the procedure. Let the slide cool and rinse well with water. Examine under oil immersion for free spores and darkly stained, diamond shaped, toxin crystals. Toxin crystals should be present if the cells have lysed and free spores are observed. The presence of toxin crystals is strongly indicative that the organism is *B. thuringiensis*.

f. Other Tests - If further biochemical testing is warranted, consult either Bergey's Manual of Systematic Bacteriology or the Compendium of Methods for the Microbiological Examination of Foods.

12.33 Interpretation of Test Results

a. *B. cereus* usually is: lecithinase positive, strongly hemolytic on sheep blood agar, actively motile, does not produce rhizoid colonies and does not produce protein toxin crystals (diamond shaped).

b. Other lecithinase positive or weakly positive cultures may be *B. cereus* var. *mycoides*, *B. thuringiensis*, or *B. anthracis*. Caution: non-motile, non-hemolytic colonies could be *B. anthracis* and should be handled with special care.

12.4 Method Quality Control Procedures

A minimum of three method control cultures is recommended for use whenever a new batch of medium is made or acquired as well as each time that an analysis is performed. These controls should consist of at least one strain each of *B. cereus*, *B. cereus* var. *mycoides*, and *B. thuringiensis*. This also will assist the analyst in becoming more familiar with the morphological and cultural differences of these *B. cereus* variants.

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CHAPTER 13. EXAMINATION OF MEAT AND POULTRY PRODUCTS FOR *CLOSTRIDIUM PERFRINGENS*

Ann Marie McNamara and Charles P. Lattuada

13.1 Introduction

Clostridium perfringens is a spore-forming, anaerobic bacterium that is widespread in soil, water, foods, spices, and the intestinal tract of humans and animals. Viable, sporulating strains that produce typical foodborne illness belong to Type A and produce an enterotoxin that causes typical symptoms of acute abdominal pain and diarrhea. Symptoms of nausea, vomiting and fever are rare. Symptoms usually appear 8-12 (range 6-24) hours after ingestion of a contaminated food, usually cooked meat or poultry. The infectious dose for humans is high, generally considered to be 10^6 - 10^7 cells/g. In foodborne disease outbreaks, findings of hundreds of thousands or more organisms per gram of food supports a diagnosis of ***C. perfringens*** foodborne illness when appropriate clinical and epidemiological evidence exists. There are four other types of ***C. perfringens***: types B, C, D and E. Some strains of type C produce an enterotoxin that causes a rare form of necrotic enteritis that is often fatal and rarely seen outside of New Guinea.

This method for isolating and identifying ***C. perfringens*** in foods is a modification of the ***C. perfringens*** method found in the Compendium of Methods for the Microbiological Examination of Foods, 3rd Edition (Labbe & Harmon, 1992).

For use in the FSIS Nationwide Microbiological Baseline Data Collection Programs and product surveys, the following "presumptive" isolation and enumeration method will suffice. This method is considered to be a "presumptive" method because other species of ***Clostridia*** besides ***perfringens*** can reduce sulfite and produce black colonies which are egg-yolk positive in TSC and EY-free TSC agar (Labbe and Harmon, 1992). Additionally, some strains of ***C. perfringens*** may not produce a halo surrounding their black colonies, so all black colonies should be counted whether a halo is present or not (Labbe and Harmon, 1992). For outbreak investigations or investigation of epidemiologically-linked cases, the more lengthy and time-consuming confirmation method should be used.

All samples should be shipped as refrigerated samples (0 - 10°C); this is particularly important with outbreak samples. Samples should be analyzed promptly upon laboratory receipt (Labbe and Harmon, 1992). *C. perfringens* in foods stored for prolonged periods of time or frozen many lose viability. If frozen samples must be shipped, food samples should be treated with buffered glycerol salt solution to give a 10% final concentration of glycerol. Samples should be shipped on dry ice and be stored frozen at -55°C to -60°C until the samples are analyzed.

13.2 Equipment, Reagents and Media

13.21 Equipment

a. Incubator at 35 ± 1°C
b. Anaerobic containers
c. Anaerobic gas mixture consisting of 90% N_2 + 10% CO_2
d. Colony counter with a piece of white tissue paper over the counting background area to facilitate counting black colonies
e. Stomacher™ 400 and sterile stomacher bags or Blender and sterile blender jars
f. Vortex mixer
g. Water bath 46 ± 1°C
h. Sterile, bent, glass rods ("hockey sticks")

13.22 Reagents

a. Nitrate reduction reagents (Method 1)
b. 0.1% peptone water diluent
c. Phosphate-buffered saline (PBS)
d. Physiological saline (0.85% sodium chloride)
e. Butterfield's Phosphate Diluent
f. Buffered Glycerol Salt Solution (for frozen samples)

13.23 Media

a. Tryptose Sulfite Cycloserine (TSC) agar
b. EY-free TSC agar
c. Trypticase Peptone Glucose Yeast Extract Broth (buffered)
d. Fluid Thioglycollate Medium
e. Motility-Nitrate Medium (buffered)
f. Lactose Gelatin Medium

g. Spray's Fermentation Medium (1% salicin, or 1% raffinose)

13.3 Presumptive Test

13.31 Sample Preparation

a. Meat Samples:

i. Label a sterile stomacher bag so that it corresponds to the label on the sample bag.

ii. Aseptically remove portions of the sample at random to obtain 25 grams. Place these portions in the sterile stomacher bag.

iii. Add 225 ml Butterfield's Phosphate Diluent (BPD) to the stomacher bag of each sample taken.

iv. Stomach for 2 minutes. Prepare serial dilutions of 10^{-2} to 10^{-6}.

b. Poultry Samples:

i. Prepare serial dilutions of 10^{-1} to 10^{-3} of the whole bird rinse.

13.32 Enrichment and Plating

a. Make duplicate spread plates on thin (6-7 ml) TSC with egg yolk agar base, using 0.1 ml/plate of undiluted sample rinse/extract as well as each dilution.

b. Equally distribute the inoculum using sterile "hockey sticks". Use a new sterile "hockey stick" for each dilution.

c. After the inoculum has dried slightly, overlay the surface with approximately 10 ml or more of egg yolk free TSC agar. Allow the plates to solidify before placing them, lid side up, in an anaerobic jar. Flush jar 3 or 4 times with 90% N_2 + 10% CO_2 leaving this atmosphere in after the last flush, or alternatively use a system which catalytically removes oxygen.

d. Incubate all plates for 24 h at 35°C.

13.33 Examination of Plates

a. After incubation, count the number of presumptive *C. perfringens* colonies. These colonies will be black and usually surrounded by a 2-4 mm opaque zone (halo).

b. Multiply the number of colonies counted by 10 (since only 0.1 ml used) and then multiply by the appropriate dilution factor to obtain your total count.

13.4 Confirmatory Procedure (for epidemiologically linked cases)

13.41 Colony Selection

a. Select 10 representative black colonies from each TSC agar plate counted and inoculate each into a freshly boiled (deaerated) and cooled tube of fluid thioglycollate broth.

b. Incubate for 4 h in a water bath at 46°C or overnight at 35°C. After incubation prepare a Gram stain from each tube and examine microscopically. *C. perfringens* organisms are short, fat Gram positive rods. Endospores are rarely produced in fluid thioglycollate medium.

c. If contaminants are observed, re-streak the contaminated culture onto the surface of a TSC (with egg yolk) agar plate (do not overlay) and incubate anaerobically before proceeding with any confirmatory tests. Surface colonies will appear as yellowish-grey colonies measuring approximately 2 mm in diameter. If re-streaking was done, it is necessary to repeat a. and b. of Section 13.41 (above).

13.42 Confirmatory Tests

a. Motility - nitrate reduction test

i. Stab inoculate each tube of motility-nitrate medium with two, 2 mm loopfuls of the fluid thioglycollate medium culture.

ii. The medium contains 0.5% each of glycerol and galactose to improve the consistency of the nitrate

reduction reaction with different strains of the organism.

iii. Incubate the inoculated medium at 35°C for 24 h and check motility. Since *C. perfringens* is non-motile, growth should occur only along the line of inoculum and not diffuse from the stab line.

iv. Test for reduction of nitrate to nitrite. A red or orange color indicates reduction of nitrate to nitrite. If no color develops, test fluid thioglycollate for residual nitrate by addition of powdered zinc.

b. Lactose gelatin medium

i. Stab inoculate each tube of lactose gelatin medium with two, 2 mm loopfuls of the fluid thioglycollate medium culture.

ii. Incubate at 35°C for 24 to 48 h. Lactose fermentation is indicated by gas bubbles and a change in color of the medium from red to yellow. Gelatin usually is liquefied by *C. perfringens* within 24 to 48 h.

c. Carbohydrate fermentation

i. Inoculate 0.15 ml of the fluid thioglycollate broth culture into 1 tube of freshly deaerated Spray's fermentation medium containing 1% salicin, 1 tube containing 1% raffinose, and 1 tube of medium without carbohydrate for each isolate.

ii. Incubate these three media at 35°C for 24 h and then check for production of acid. To test for acid, transfer 1 ml of culture to a test tube or spot plate and add 2 drops of 0.04% bromthymol blue. A yellow color indicates that acid has been produced.

iii. Reincubate negative raffinose tubes for an additional 48 h and retest for the production of acid.

iv. Salicin is rapidly fermented with the production of acid by culturally similar species such as *C. paraperfringens*, *C. baratii*, *C. sardiniense*, *C. absonum*, and *C. celatum*, but usually not by *C. perfringens*.

v. Acid is produced from raffinose within 3 days by *C. perfringens* but is not produced by culturally similar species.

13.43 Quantitation of *C. perfringens* Populations Based on Confirmed Anaerobic Plate Counts

a. Cultures obtained from presumptive *C. perfringens* black colonies on selective, differential TSC or EY-free TSC medium are confirmed as *C. perfringens* if they are:

i. nonmotile
ii. reduce nitrate
iii. ferment lactose
iv. liquefy gelatin within 48 h
v. produce acid from raffinose.

b. Calculate the number of confirmed *C. perfringens* per gram of food sample as follows:

i. Average the paired plates counted, then adjust the average presumptive plate count to 1.0 ml by multiplying by 10.

ii. Multiply the adjusted presumptive plate count by the reciprocal of the dilution plated to arrive at the total of presumptive *C. perfringens* colonies.

iii. The confirmed colony count is then determined by using the ratio of the colonies confirmed as *C. perfringens* to the total colonies tested.

13.5 Quality Control

a. The following authentic, reference cultures can be used as control organisms in the above procedures:

C. perfringens ATCC 13124
C. absonum ATCC 27555

b. The expected reactions produced by these control organisms are as shown in the following table:

Organism	Motility	H_2S	Gelatin liq.	Nitrate reduct.	Lactose ferm.	Salicin ferm.	Raffinose ferm.
C. perfringens ATCC 13124	-	+	+	±	+	-	d
C. absonum ATCC 27555	±*	+	d	+	+	+w	-

*** usually + in young cultures; d = delayed; w = weak**

3.6 Selected References

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CHAPTER 15. IMMUNOASSAYS FOR THE DETECTION AND QUANTITATION OF STAPHYLOCOCCAL ENTEROTOXINS FROM MEAT AND POULTRY PRODUCTS AND/OR BROTH CULTURE FLUIDS

Richard P. Mageau

15.1 Introduction

Some strains of coagulase positive *Staphylococcus aureus* are endowed with the genetic capacity to produce certain extracellular proteins which, when ingested, cause a severe gastrointestinal disturbance. These proteins are known as staphylococcal enterotoxins. There are five distinct, major, serological types of enterotoxins currently recognized as significant and they are designated as serotypes A, B, C (C_1, C_2, C_3), D and E. In 1995 a new serotype, SEH, was identified and reported in the literature, however, it's significance to foodborne illness is still undetermined. When an enterotoxigenic strain of *Staphylococcus aureus* becomes established in a food product, environmental growth conditions may become optimum to allow for high proliferation of the organism and resulting production of the enterotoxin. Ingestion of this food usually results in a foodborne illness. For regulatory and epidemiological purposes in investigating foodborne illnesses it is important to be able to recognize the presence and serotype of staphylococcal enterotoxins in a suspect food product.

Recent advances and refinements in the development of immunoassays and immunological reagents, specifically with regard to the staphylococcal enterotoxins, have allowed the completion and implementation of assays for quantitative detection of these toxins. These new assays provide advantages of increased sensitivity, reduced analysis time, and a capability for greater sample number analyses due to the reduction of high labor intensive operations associated with procedures previously employed. The following provides a detailed description of two immunoassay procedures which are to be used by the Field Service Laboratories for the determination of the major staphylococcal enterotoxins in various meat and poultry product samples and/or broth culture fluids. The procedure described in PART A is to be used only as a presumptive, qualitative screen test. The procedure described in PART B is to be used as the confirmative test which will provide quantitative and qualitative information.

PART A

15.2 (Presumptive) Staphylococcal Enterotoxin Reverse Passive Latex Agglutination Test

15.21 Introduction and Principles

A Staphylococcal Enterotoxin - Reverse Passive Latex Agglutination (SET-RPLA) test for the qualitative determination of enterotoxin serotypes A, B, C and D is commercially available. This test system is available as a complete, stable kit form. The test kit was evaluated by the Immunology Section of the Microbiology Division and was found to be suitable for use as a presumptive, qualitative screen test on meat sample extracts or broth culture filtrates. The SET-RPLA test was found to be specific and capable of detecting each homologous enterotoxin down to at least 1 ng/ml of sample extract fluid.

A latex agglutination test employed for presumptive screen testing of meat and poultry food samples for staphylococcal enterotoxins should meet or exceed the following performance characteristics:

Sensitivity	≥99% *
Specificity	≥99%
False Negative Rate	≤ 1%
False Positive Rate	≤ 1%
Efficiency	≥99%

* All at a toxin concentration level of ≥1 ng/ml of sample extract fluid and/or Protein A concentration level of <50 ng/ml of sample extract fluid.

The test functions on the principle of using individual suspensions of red latex particles which are each sensitized with specific antibody against a particular enterotoxin serotype. The presence of homologous enterotoxin will then cause visible agglutination of the specific antibody sensitized latex particles after an appropriate incubation period. The absence of toxins or the presence of heterologous toxin serotypes will not cause agglutination of the latex particles. The presence or absence of visible agglutination is discerned by observing the characteristic settling pattern of the red latex particles on the bottom of the reaction well.

The following details provide all the necessary instructional information to perform the SET-RPLA. These instructions are to be used in place of the instruction sheet supplied with the test kit.

15.22 Equipment and Supplies

a. **Rainin Pipetman, Model P-200 adjustable digital microliter pipette and RC-20 disposable microliter pipette tips. (Rainin Instrument Co., Woburn, MA.)**
b. **Minishaker for microtiter plates, Cat. #002-963-0900 (Dynatech Laboratories, Inc., Alexandria, VA).**
c. **Microtiter Test Reading Mirror, Cat. #001-010-4900 (Dynatech).**
d. **Microtiter plates, 96 well, "V" bottom, polystyrene, Cat.#001-010-2602 and lids for above plate, Cat. #001-010-5550 (Dynatech).**
e. **Eppendorf Repeater Pipette, Cat. #G20551 with accessory of 1.25 ml capacity Combitips, Cat. #G20552B (Daigger Scientific Co.).**
f. **Waring blender and appropriate blending vessel.**
g. **Centrifuge, refrigerated, capable of operation at 32,000 X G and appropriate centrifuge tubes resistant to chloroform.**
h. **Kimwipes®.**
i. **Glass separatory funnels, with stopper, 125 ml size.**

15.23 Chemicals and Reagents

a. **NaCl (Fisher, S-271).**
b. **Chloroform† (Fisher, C-298).**
c. **SET-RPLA test kit consisting of the following items:**

    i. **Vials of antibody sensitized latex suspensions of Anti A, Anti B, Anti C, Anti D, and Control latex (unsensitized).**

    ii. **Vials of enterotoxin† reference standards of A, B, C, and D serotypes.**

    iii. **Vials of buffered diluent.**

NOTE: Store entire kit at 4°C when not in use. DO NOT FREEZE.

15.24 Preparation of Stock Reagent Solutions

0.2 M Sodium chloride solution at pH 7.5.

Add 11.69 grams of NaCl to 1 liter of distilled water. Dissolve the salt completely and adjust pH to 7.5 with use of 0.1 N NaOH solution.

15.25 Sample Preparation for Enterotoxin Analysis

a. Meat Food Products

i. Blend 20 grams of meat sample together with 40 ml of 0.2 M NaCl solution, pH 7.5, at high speed in a Waring blender for 3 minutes.

ii. Centrifuge the resulting slurry at 32,000 X G for 15 minutes in a refrigerated centrifuge.

iii. Pour off the supernatant fluid and adjust the pH to 7.5 with 1 or 0.1 N NaOH solution.

iv. In a separatory funnel, in a chemical fume hood with the exhaust on, extract the supernatant fluid with a 1/3 volume (about 10 ml) of cold chloroform by shaking vigorously and letting stand for 15-30 minutes.

v. Pour the supernatant - chloroform mixture into chloroform resistant centrifuge tubes and centrifuge the mixture at 32,000 X G for 15 minutes in a refrigerated centrifuge.

vi. Pour both the supernatant layers through a double layer of kimwipes® back into a clean separatory funnel (make sure solid particles are retained by the Kimwipes®) and <u>without</u> further shaking allow the two layers to settle and clearly separate.

vii. Discard the chloroform (lower layer), and collect the clear meat extract (upper layer) free of any chloroform into a clean tube and use in the immunoassay. Keep the extract refrigerated until actually used in the performance of the assay.

b. Culture Fluids

i. Occasionally it may be of interest to determine if an isolated, coagulase positive, culture of *S. aureus* is capable of producing one or more enterotoxins (enterotoxigenic). This can be accomplished by first growing the pure culture for 24 h at 37°C in a medium such as Brain Heart Infusion Broth on a shaker at 150 RPM.

ii. Centrifuge the 24 h broth culture at 15,000 X G for 15 minutes and obtain the cell free culture fluid.

iii. Make a 1:100 dilution of the culture fluid in the buffered diluent supplied in the SET-RPLA kit. Use this diluted culture fluid directly in the assay to determine the presence of enterotoxins.

15.26 Performance of the SET-RPLA Test

a. Obtain the SET-RPLA test kit from the refrigerator, allow to equilibrate to room temperature and see that all the necessary kit components are present.

b. For the first time that the kit is used, rehydrate each of the lyophilized enterotoxin standards (A, B, C, and D) with the appropriate volume (given on kit instruction sheet or vial label) of buffered diluent and mix well. They can now be used without any further modifications in all subsequent assay performances.

c. Obtain the meat sample extracts previously prepared and make a 1:2 dilution of each in the buffered diluent in separate tubes. Culture fluids, if any are to be assayed, can be used directly as previously prepared.

d. Obtain a 96 well, "V" bottom, Dynatech microtiter plate and cover from stock supplies.

e. Place 25 µl of buffered diluent in each well of column 1 in rows A, B, C, D, and E using the Pipetman and a disposable tip.

f. Place 25 µl of reference enterotoxin A, B, C, and D into the wells of column 2 in rows A, B, C, and D respectively.

g. Place 25 µl of any one of the reference enterotoxin standards (your choice) in the well of column 2 in row E.

h. Place 25 µl of each test sample extract in each well of a single, respective column in rows A, B, C, D, and E, beginning with column 3.

i. Obtain the individual vials of latex Anti A, Anti B, Anti C, Anti D, and Control latex suspensions and mix each thoroughly but gently to produce uniform latex suspensions.

j. Using an Eppendorf Repeater Pipette and individual 1.25 ml capacity combitips, dispense 25 µl of latex Anti A, Anti B, Anti C, Anti D, and Control latex into each occupied well of rows A, B, C, D, and E respectively.

k. Mount the plate on the carrier of the Minishaker and carefully shake the plate at a "medium" dial setting for 15 seconds to thoroughly mix, but not spill, the contents of each well.

l. Allow the covered plate to remain undisturbed at normal room temperature for 24 h before the final reading is made.

15.27 Test Reading and Sample Interpretation

a. After the appropriate period of time, remove the cover from the plate, mount it on the Microtiter Test Reading Mirror and observe from the bottom of the plate the pattern of settled red latex particles in each well.

b. The pattern of settled red latex particles determines whether or not agglutination has taken place. Nonagglutination is determined by observing that all of the latex particles have settled into a distinct pile at the bottom of the "V" in a particular well; usually referred to as a "button". Agglutination is determined by observing that all the latex particles in a given well are uniformly spread out over the entire surface of the "V" bottom without any distinct pile or "button". The agglutination patterns illustrated on the SET-RPLA kit instruction sheet may be helpful in regard to understanding this.

c. Observe each well and record whether or not agglutination has taken place.

d. To insure that the test is working properly, the following results should be obtained with regard to the controls employed. All wells of column 1 should be negative (no agglutination) as these are negative controls. All wells of column 2 of rows A, B, C, and D should be positive (agglutination) as these serve as positive homologous controls. The single well in column 2 of row E should be negative.

e. If all of the above controls have reacted properly, proceed to the interpretation of sample results. If any controls did not react properly, the test must be considered invalid and the procedure must be repeated and technical assistance should be sought to determine the nature of the problem.

f. Each sample can be interpreted with regards to the presence or absence of enterotoxins by observing the reactions of that sample column with respect to rows A, B, C, D, and E, which, of course, correspond to Anti A, B, C, D, and Control latex respectively. A positive reaction in any well of Anti A, B, C, or D identifies the presence of that particular toxin serotype. The control latex well (row E) should never show agglutination. If the sample column contains no positive wells, then the sample may be considered to be free of enterotoxins A-D and can be reported out as such.

g. If a sample contains enterotoxin it will usually be of only one serotype. The presence of more than one serotype in a food sample or culture fluid is possible but is rather unusual and one should not normally expect to find this.

h. If a sample should produce a positive reaction in Anti A, B, and C wells simultaneously (but not for the Anti D or Control latex wells) this is usually indicative of the presence of Protein A and the sample must be further treated, as described below, before it can be accurately assessed with regards to the presence of enterotoxins.

i. Add normal rabbit serum to a total concentration of 5% (v/v) to a sample extract suspected of containing significant concentrations of Protein A. Allow the sample to incubate for 30 minutes at 37°C. Centrifuge the sample at 10,000 X G for 15 minutes. Obtain the sample supernatant and perform the SET-RPLA test again to determine the presence of enterotoxins. The normal rabbit serum treatment should effectively neutralize the interfering reactivity of the Protein A.

j. All SET-RPLA positive samples or those with questionable results are to be confirmed by the procedure outlined in PART B.

15.28 Quality Control Procedures

a. Store and maintain the SET-RPLA kit components at refrigerator temperature (4 - 8°C) when not in use. DO NOT ALLOW THEM TO FREEZE.

b. Observe the kit manufacturer's expiration date for all test kit components. Kits should not be used beyond their expiration date.

c. Use only "V" bottom microtiter plates to perform the assay.

d. Allow all test components to equilibrate to room temperature prior to performing an analysis.

e. Thoroughly but gently resuspend the settled latex particle reagents in their vials to produce uniform latex suspensions immediately prior to dispensing this reagent in the test.

f. Always run negative and positive enterotoxin controls and control latex (unsensitized) when performing the analysis.

g. All negative and positive controls must give expected correct results before correct interpretation of test sample results can be made.

h. Do not allow the plate to be disturbed once all reagents have been added and properly mixed. Disturbing the plate may cause the settling pattern of agglutinated or

nonagglutinated latex to form abnormally and thus produce erroneous results.

† Safety Caution: Do not dispose of hazardous (chloroform) or biohazardous (enterotoxin) fluids by pouring down the sink drains.

Collect these liquid wastes in separate containers and dispose of according to standard waste management procedures for your laboratory.

Do not allow human exposure to chloroform vapors.

15.29 Selected References

Bergdoll, M. S. 1980. Staphylococcal food poisoning, p. 108-119. *In* H. D. Graham (ed.), The Safety of Foods, 2nd Edition, AVI Publishing Company, Inc., Westport, CT.

Parks, C. E., and R. Szabo. 1986. Evaluation of reversed passive latex agglutination (RPLA) test kits for detection of staphylococcal enterotoxins A, B, C and D in foods. Can. J. Microbiol. 32:723-726.

Sanjeev, S., and P. K. Surendran. 1992. Evaluation of reversed passive agglutination test kits for the detection of staphylococcal enterotoxins A, B, C and D in fishery products. J. Food Sci. Technol. 29:311-312.

PART B

15.3 (Confirmative) Biotin-streptavidin Enzyme Linked Immunosorbent Assay for Staphylococcal Enterotoxins

15.31 Introduction and Principles

Enzyme Immunoassay (EIA) provides an alternative approach to the immunological detection of staphylococcal enterotoxins. EIA offers the major advantages of being more reliable in their reactions than latex agglutination and they can also be used for quantitation of the material under analysis. The Immunology Section of the Microbiology Division developed a Biotin-streptavidin Enzyme Linked Immunosorbent Assay (ELISA) for the quantitative detection of staphylococcal enterotoxin serotypes A, B, C, D, and E. This developed assay makes use of a biotin-streptavidin amplification reaction for the indicator portion of the assay.

The biotin-streptavidin ELISA described in this procedure is one of a solid phase, double antibody, "sandwich" type with a final biotinylated antibody-streptavidin peroxidase reaction to provide visual evidence of the degree of reaction upon substrate addition. The brief functional principles of this assay are as follows. Specific antibody (capture) against a particular enterotoxin serotype is bound to the walls of a microtiter plate (solid phase) and is allowed to react with test material which may contain enterotoxin(s). Only the homologous enterotoxin will react and bind to the wall bound antibody. A second antibody (probe) is introduced into the system with the same specificity as the first wall bound antibody and can now react with previously bound homologous enterotoxin. This second antibody is one which has had biotin chemically introduced into the molecule and is referred to as biotinylated antibody. Five "sets" of specific antibody pairs are simultaneously but individually employed in the assay corresponding to each of the five enterotoxin serotypes in question. A commercial preparation of streptavidin-peroxidase conjugate is next generally introduced into the assay system. This reaction makes use of the natural, very high, chemical binding affinity of biotin and streptavidin. Amplification is achieved by the fact that each molecule of streptavidin can bind four molecules of biotin. The streptavidin-peroxidase introduced into the assay will therefore bind to any biotinylated antibody present. With the final addition of the substrate to the system, the visible evidence of a positive reaction is produced from

conversion of the substrate to a colored end product by the enzyme peroxidase. If homologous toxins are not present, biotinylated antibody does not bind and subsequent reactions cannot take place, which therefore results in no colored change in the added substrate.

The following details provide all the necessary information for the performance of the Biotin-streptavidin ELISA for the quantitative determination of staphylococcal enterotoxin serotypes A, B, C, D, and E from meat and poultry products or broth culture fluids. All samples giving positive or questionable results in the SET-RPLA analysis (PART A) must be subjected to this confirmative Biotin-streptavidin ELISA for a final quantitative determination of enterotoxin presence before the final analytical results are reported.

15.32 Equipment and Supplies

a. Flow (ICN) Laboratories Titertek Multiskan MC Plate Reader, Cat. #78-530-00.
b. Flow (ICN) Laboratories Titertek Microplate Washer, Cat. #78-431-00.
c. Flow (ICN) Vacuum Pump for above washer, Cat. #78-426-00.
d. Flow (ICN) Titertek Multichannel Pipette, 8 channel, adjustable 50-200 µl volume, Cat. #77-859-00.
e. Eppendorf Repeater Pipette (Daigger Scientific Co., Cat. # G-20551) with accessory of 2.5 ml capacity Combitips (Daigger, Cat. #G-20552C) and 5.0 ml capacity Combitips (Daigger, Cat. #G-20552D).
f. Dynatech Laboratories Microelisa Plates, Immulon I, flat bottom, 96 wells, Cat. #11-010-3350 and covers.
g. Incubator, 37°C (any properly operating brand).
h. Centrifuge, refrigerated, capable of operation at 32,000 X G and appropriate centrifuge tubes resistant to chloroform.
i. Microtest Manifold, Wheaton, straight, 8 place with Luer Lock connection (Daigger, Cat. #G-20560A).
j. Kimwipes®.
k. Glass separatory funnels, with stopper, 125 ml size.
l. Waring Blender and appropriate blending vessel.
m. Rainin Pipetman, Model P-200 adjustable digital microliter pipette and RC-20 disposable microliter pipette tips. (Rainin Instrument Co., Woburn, MA.)

15.33 Chemicals and Reagents

a. Na_2HPO_4 (Fisher, Cat. #S-374).
b. NaH_2PO_4 (Fisher, Cat. #S-369).
c. NaCl (Fisher, Cat. #S-271).
d. Citric acid, anhydrous (Fisher, Cat. #A-940).
e. Hydrogen peroxide, 30% reagent grade (Fisher, Cat. #H-323).
f. Tween 80 (Fisher, Cat. #T-164).
g. Sodium azide† (NaN_3), purified (Fisher, Cat. #S-227).
h. Bovine Serum Albumin, powder, fraction V (Sigma, Cat. #A-4503), store in refrigerator.
i. Chloroform† (Fisher, Cat. #C-298).
j. ABTS substrate indicator; 2,2' azino-di-(3-ethyl Benzthiazoline Sulfonic acid), (Sigma, Cat. #A-1888).
k. Streptavidin-peroxidase conjugate, Cat. #43-4323 (Zymed Laboratories, Inc., San Francisco, CA), store in refrigerator.

15.34 Staphylococcal Biochemical Reagents

a. Anti-staphylococcal enterotoxin A, B, C, D, and E antibody stock solutions.
b. Biotinylated anti-staphylococcal enterotoxin A, B, C, D, and E antibody stock solutions.
c. Staphylococcal enterotoxin† A, B, C, D, E standard reference stock solutions.

NOTE: The above 3 sets of items must be stored in the frozen state at all times to maintain stability.

15.35 Preparation of Stock Reagent Solutions

a. 0.15 M Phosphate Buffered Saline at pH 7.2 (PBS).

Add 10.35 grams of NaH_2PO_4 and 4.38 grams of NaCl to 1 liter of distilled water and dissolve completely to prepare the "acid" solution. Add 10.65 grams of Na_2HPO_4 and 4.38 grams of NaCl to 1 liter of distilled water and dissolve completely to prepare the "base" solution. While mixing with a magnetic stirrer and monitoring the pH on a pH meter, add a sufficient quantity of the "acid" solution to the "base" solution to achieve a final, stabilized pH of 7.2. Dispense into glass containers, autoclave at 121°C for 15 minutes and store

at room temperature. It is most convenient to make up this buffer in 5 liter quantities at a time.

b. Phosphate Buffered Saline containing 0.5% Tween 80 (PBS-Tween).

To 1 liter of prepared 0.15 M phosphate buffered saline at pH 7.2 add 0.5 ml of Tween-80 and mix (not on magnetic stirrer) for several hours at room temperature until completely dissolved. Store this prepared solution in the refrigerator (4°C).

c. Phosphate Buffered Saline containing 0.5% Bovine Serum Albumin (PBS-BSA).

To 1 liter of prepared 0.15 M phosphate buffered saline at pH 7.2, add 5 grams of powdered bovine serum albumin and 1 gram of sodium azide (NaN_3) and mix (not on magnetic stirrer) at room temperature until completely dissolved. Store this prepared solution in the refrigerator (4°C).

d. ABTS - H_2O_2 Substrate Buffered Solution.

Prepare a 0.1 M citric acid stock solution by dissolving 1.92 grams of anhydrous citric acid in 100 ml of distilled water. Prepare a 0.1 M dibasic sodium phosphate stock solution by dissolving 1.42 grams of Na_2HPO_4 in 100 ml distilled water. Add sufficient quantities of these two stock solutions together while mixing with a magnetic stirrer and monitoring the pH on a pH meter to prepare 100 ml of a 0.1 M citrate-phosphate buffer at a final stabilized pH of 4.0.

To 100 ml of the above prepared 0.1 M citrate-phosphate buffer add 22 mg of ABTS [2,2' azino-di-(3-ethyl Benzthiazoline Sulfonic acid)] and 15 µl of stock 30% hydrogen peroxide, mix gently by hand (no magnetic stirrer) until completely dissolved. Pass this substrate solution through a 0.45 µm Millex® filter, place in a sterile glass container, and store in the dark at room temperature until needed. This substrate solution should be prepared 24 h in advance of need and may be used as long as it retains its original light green color. A solution which has deteriorated to the

point where it cannot be used is evidenced by a dark azure-green color formation.

e. 0.2 M Sodium Chloride Solution at pH 7.5.

Add 11.69 grams of NaCl to 1 liter of distilled water. Dissolve the salt completely and adjust pH to 7.5 with use of 0.1 N NaOH solution.

15.36 Sample Preparation for Enterotoxin Analysis

Sample extracts for enterotoxin analysis from meat and poultry products or culture fluids are prepared exactly as described under the similar section (15.25 a. or b.) of PART A for SET-RPLA. These should be prepared in advance of the actual ELISA performance and kept refrigerated until needed.

15.37 Performance of the Biotin-streptavidin ELISA

a. Obtain a flat bottom, 96 well Dynatech Immulon I microtiter plate and cover from stock supplies.

b. Dilute the anti-staphylococcal enterotoxin antibody stock solutions in PBS in individual tubes to contain the following amounts of antibody protein as shown below for each respective serotype.

Anti-SEA antibody = 5 µg/ml
Anti-SEB antibody = 5 µg/ml
Anti-SEC antibody = 1 µg/ml
Anti-SED antibody = 5 µg/ml
Anti-SEE antibody = 5 µg/ml

c. Sensitize wells of the Immulon I microtiter plate with antibody for enterotoxin serotypes A, B, C, D, and E by placing 200 µl of the above concentrations of each antibody protein solution (PBS) in the wells of rows A, B, C, D, and E respectively. Leave all wells of column 2 empty.

d. Incubate the covered plate for 3 h at 37°C.

e. Remove the plate from the incubator, remove the cover and mount on the carrier of a Flow Titertek Microplate Washer which has been primed with PBS-Tween and set to deliver 300 µl fluid to each well.

f. Remove the solution from the wells by aspiration with the washer and wash the wells once with 300 µl fluid to each well.

g. Remove the plate from the washer, invert over a sink, hold the plate tightly in one hand and flick several times to remove any remaining excess liquid from the wells.

h. Tap the plate in an inverted position several times on a soft paper towel (Sorgs Laboratory towels) placed on the surface of the lab bench and allow the plate to remain inverted for 1-2 minutes to complete the draining process. Place the plate right-side up and cover until next reagent addition.

i. Block unwanted reactive sites on the plastic wells by filling all wells (including those in column 2) with 250 µl of PBS-BSA per well, dispensed from an 8 place microtest manifold attached to a Cornwall syringe.

j. Replace the cover on the plate and let stand undisturbed overnight at normal room temperature.

k. Wash the wells once by repeating steps (e thru h).

l. With a Pipetman microliter pipette place 200 µl of PBS-BSA to all wells of column 1 and 2 to serve as negative controls.

m. Obtain previously prepared standard reference enterotoxin solutions of serotypes A, B, C, D, and E at concentrations of 1, 5, 10, 25, and 50 ng/ml in PBS-BSA.

n. Place 200 µl of each of the above concentrations of toxins A, B, C, D, and E to the homologous antibody sensitized wells of rows A, B, C, D, and E respectively, beginning with column 3 wells at the lowest concentration.

o. Place 200 µl of each previously prepared sample extract in each well of a single, respective column in rows A, B, C, D, and E, beginning with column 8.

p. Incubate the covered plate for 2 h at 37°C.

q. Wash the wells twice by repeating steps (e thru h).

r. Prepare the following dilutions of biotinylated anti-staphylococcal enterotoxin antibody stock solutions in PBS-Tween in individual tubes as shown below for each respective serotype.

Biotinylated Anti-SEA antibody = 1:5000
Biotinylated Anti-SEB antibody = 1:5000
Biotinylated Anti-SEC antibody = 1:2500
Biotinylated Anti-SED antibody = 1:5000
Biotinylated Anti-SEE antibody = 1:1500

s. Place 200 µl of the above dilutions (PBS-Tween) of each biotinylated antibody serotype to all wells in a respective row of homologous, primary antibody sensitized wells (i.e., Anti-A in row A, Anti-B in row B, etc.).

t. Incubate the covered plate for 2 h at 37°C.

u. Wash the wells three times by repeating steps (e thru h).

v. Prepare a 1:5000 dilution of the commercial Streptavidin-peroxidase conjugate in PBS-Tween in a separate tube.

w. Add 200 µl of the 1:5000 dilution (PBS-Tween) of Streptavidin-peroxidase conjugate to all wells of the plate with the use of an Eppendorf Repeater pipette and a 5 ml capacity combitip.

x. Incubate the covered plate for 30 minutes at 37°C.

y. Wash the wells three times by repeating steps (e thru h).

z. With the use of the Flow 8 channel pipette, add 200 µl of ABTS-H_2O_2 substrate buffered solution to all wells.

aa. Place the cover on the plate and incubate for 30 minutes at 37°C.

bb. Twenty minutes prior to the end of the above incubation period turn on the power to the Flow Titertek Multiskan MC plate reader and allow it to warm up.

cc. After the 30 minutes incubation period of step (aa) is complete, remove the plate from the incubator, remove the cover, and place the plate on the carrier of the Multiskan MC plate reader.

dd. Program the reader for the current date, Mode 1 (single wavelength absorbance), Wavelength Filter #2 (414 nm), push the carrier and plate into the measuring head and blank the instrument (zero O.D. point set) on column 1.

ee. Press the START button and obtain a printed paper strip of the Optical Density (O.D.) values for all of the reaction wells on the plate.

ff. Remove the plate from the reader and visually examine the plate to see that the obvious colored reaction intensities generally correspond to the numerical values on the printed data sheet to assure that the plate has been properly read in the instrument.

gg. Turn off the power to the Multiskan MC plate reader and discard the plate (save the cover for reuse) after completion of the Data Analysis Plotting and Sample Interpretation Section described below.

15.38 Data Analysis, Plotting, and Sample Interpretation

a. All wells in column 1, which serve as the zero-blank negative control, should have no color reaction, indicating a proper lack of non-specific attachment of biotinylated antibody or Streptavidin-peroxidase to the antibody sensitized wells. Under these conditions these wells are excellent controls to blank in (zero point set) the O.D. reading instrument.

b. All wells in column 2 serve as BSA negative controls to assess non-specific attachment of biotinylated antibody and also Streptavidin-peroxidase. Since the wells originally were never sensitized with anti-enterotoxin antibodies but only blocked with BSA, no positive reactions (high O.D. values) should ever be observed.

c. Wells in columns 3, 4, 5, 6 and 7 of rows A, B, C, D and E represent the standard quantitative dose response values of the reaction with regard to enterotoxin serotypes A, B, C, D, and E respectively. The response (O.D.) observed in this ELISA should be one of a direct linear relationship to increased dose concentration of enterotoxin.

d. The remaining wells of individual columns 8-12 for rows A, B, C, D, and E represent reaction values for individual test sample extracts with regards to the presence or absence of enterotoxins A-E respectively.

e. Obtain a piece of 4 cycle semi-logarithmic graph paper. Label the ordinate (10 division to the inch) with O.D. values from 0-2.0 in increments of 0.05. Label the abscissa (4 cycle logs) with enterotoxin concentrations of 0, 1, 5, 10, 25, and 50 ng/ml.

f. Plot the O.D. values against standard enterotoxin concentrations for each individual serotype together on the same piece of graph paper. Draw straight lines from point to point for each homologous set of enterotoxin concentrations. You will now have 5 individual standard curves for enterotoxin serotypes A-E respectively, which will have similar appearances to each other but still be distinctly different. The curves should illustrate the direct linear dose-response relationship in regards to increasing toxin concentration for each serotype.

g. To determine if a test sample contains enterotoxin and its' quantity if present, proceed as follows:

   i. Obtain the O.D. values of individual sample column wells with regards to rows A, B, C, D, and E (which correspond to Anti A, B, C, D, and E antibodies respectively) and determine if any sample O.D. values exceed the 1 ng enterotoxin standard O.D. value for each individual serotype.

   ii. Any sample O.D. value exceeding the 1 ng enterotoxin standard of a given serotype is to be considered as a positive identification reaction for the presence of that enterotoxin serotype in the sample.

iii. Determine the quantitative amount of an enterotoxin which is present by interpolating the O.D. value with regards to concentration from the standard curve for that particular serotype identified and multiply by 3 (food sample) or 100 (culture fluid).

iv. If the sample O.D. value does not fall within the more linear portion (1-25 ng/ml) of the standard curve of a given serotype, then the sample analysis should be repeated using standard dilutions of the original extract in PBS. The dilution factor which produces readable results would then need to be included in the final quantitative calculations.

v. If sample O.D. values are less than those of the 1 ng standards of each serotype, the sample should be considered free of enterotoxins A-E and reported out as such.

h. If a sample is found to contain an enterotoxin, it will usually be of only one serotype. The presence of more than one serotype toxin in a given sample is possible but rather unusual.

i. If a sample is found to produce a strong positive reaction in all the serotype wells, except Anti-D, this usually indicates that the sample contains a significant amount of Protein A and the sample must be treated as described in PART A, 15.27 step i, before a repeat ELISA analysis can be performed to accurately determine the presence of enterotoxins.

j. All enterotoxin positive samples should be reported out by using a statement such as the following. "This food sample was found to contain Staphylococcal enterotoxin serotype _____, at a concentration of _____ ng/g as confirmed by an ELISA procedure." The serotype and quantitative values would, of course, be filled in from your analytical data.

15.39 Quality Control Procedures

a. The assay reagents have been standardized for use only with Dynatech Immulon I microtiter plates. No other plates should be used.

b. All stock reagent solutions must be properly prepared and maintained free of contamination or chemical breakdown.

c. The stock ABTS-H_2O_2 substrate buffered solution should not be used if it has turned to a significantly darker shade of green from that of the original preparation.

d. Be sure the stock, commercial Streptavidin-peroxidase reagent has not deteriorated to the point of producing abnormally low final O.D. readings. Use only an unexpired lot of this reagent.

e. All standard negative and positive enterotoxin control values must be in the correct range before proper interpretation of test sample results can be reliably made.

f. The standard curves generated from the standard enterotoxin concentrations for each serotype should always be of the same general shape and value from run to run. Drastic changes in the shape of these curves usually indicate critical reagent deterioration (or misuse).

g. Standardized reference enterotoxin concentrations must always be carefully and properly prepared from higher concentrated stock solutions to assure reliability of the generated standard curves.

† Safety Caution: Do not dispose of hazardous (chloroform, sodium azide) or biohazardous fluids (enterotoxin) by pouring down sink drains.

Accumulation of sodium azide in lead drains may result in an explosion.

Collect these liquid wastes in separate containers and dispose of according to standard waste management procedures for your laboratory.

Do not allow human exposure to chloroform vapors.

15.4 Selected References

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CHAPTER 16. AGAROSE THIN-LAYER ISOELECTRIC FOCUSING (TLIEF) FOR SPECIES DETERMINATION OF RAW MUSCLE TISSUES

Richard P. Mageau

16.1 Introduction

Improvements in the developed biochemical technique of isoelectric focusing have allowed the application of this technique to be used for species determination of raw muscle tissue. This method provides for the relatively rapid species determination of a large number of samples in a definitive, less subjective manner, in a single analytical run without the use of anti-species sera. The principle of this technique involves the separation and focusing of proteins under an electrical field in a stable pH gradient dependent upon differences in the isoelectric points of the various proteins present. Since various species tissues contain multiple proteins of different isoelectric points, an aqueous extract of a particular species tissue when subjected to TLIEF will produce a stained protein band pattern unique and distinct for that species. By using the method described below, a total of 24 samples (48 if sample filter papers are cut in half along their long axis) may be analyzed in a single determination in one day as to their correct species. The use of this established method is intended to aid in the rapid species analysis of a large influx of raw tissue samples resulting from particular meat species problems which may be encountered in the Agency's inspection system.

16.2 Materials and Equipment

a. Multiphor for high Performance Analytical Electrofocusing in Agarose; to include 2117-301 Multiphor Basic Unit, 2117-107 Analytical Electrofocusing Lid, 2117-701 Capillary Gel Casting Kit, and 1850-100 Agarose-EF Accessory Kit. (LKB Instruments.)
b. 2197-001 D.C. Power Supply for Electrofocusing and Electrophoresis. (LKB Instruments.)
c. 185-101 Multiphor Gelbond film, 124 x 258 mm. (LKB Instruments.)
d. 2030-710 Bayonet female plastic tubing connector and 2030-702 Bayonet male plastic tubing connector. (LKB Instruments.)
e. 2117-109 Multiphor Staining Kit. (LKB Instruments.)
f. 1403 Coomassie Brilliant Blue R-250 dye (Fisher).
g. S-460 D-sorbitol powder, reagent grade (Fisher).

h. A-322 Trichloroacetic acid, reagent grade (Fisher).
i. A-297 5-sulfosalicylic acid, crystal, reagent grade (Fisher).
j. 14-198-5A High pressure hose clamps, 1/4" to 5/8" size (Fisher).
k. K-10 Kerosene (Fisher).
l. 17-0468-01 Agarose IEF (Pharmacia Fine Chemicals).
m. 17-0453-01 Pharmalyte Carrier Ampholyte, pH 5-8 range (Pharmacia).
n. Schleicher and Schuell #470 filter paper, 12.5 x 26 cm size and Schleicher and Schuell #577 filter paper, 12.5 x 26 cm size (PGC Scientific Corp.).
o. W 3237-10 Lauda Brinkman, Model K-4/RD Circulating water bath. (American Scientific Products.)
p. B-1206-2 Whirl-Pak® bags, 3" x 5". (American Scientific Products.)
q. R5316-8 Tygon tubing, formula S-50-HL, 5/16" x 1/16". (American Scientific Products.)
r. Hair dryer (hot and cold).
s. Rubber print roller, 6" wide.
t. Silicone gasket, 0.75 mm thick, overall dimensions of 12.5 x 26 cm, 3 sided of 5 mm width. (Potomac Rubber Co., Inc., Washington, DC.)
u. Water bath and incubator/oven capable of maintaining 65°C.
v. Centrifuge capable of 9,000 x G maximum.
w. Stomacher®

16.3 Procedure

a. Initial Reagent Preparations

i. Fixing solution:

Dissolve 25 g sulfosalicylic acid and 50 g of trichloroacetic acid in distilled water and dilute to a final volume of 500 ml.

ii. Destaining solution:

Mix 700 ml of ethyl alcohol and 200 ml glacial acetic acid together and dilute to a final volume of 2,000 ml with distilled water.

iii. Staining solution:

Completely dissolve 1 g Coomassie Brilliant Blue R250 dye in 500 ml of destaining solution.

iv. Cathode solution: (1 M NaOH, 100 ml)

v. Anode solution: (0.05 M H_2SO_4, 100 ml)

b. Sample Preparation

i. Obtain 1 g of diced, raw, muscle tissue and place in a small whirl-pak® bag together with 9 ml of distilled water.

ii. Thoroughly macerate the tissue by stomaching for 1-2 minutes and then leave overnight at 4°C.

iii. Centrifuge the resulting solution at 9000 x g for 10 minutes at room temperature and apply to sample filter papers when ready to electrofocus.

c. Apparatus Assembly

i. Set up and align the Lauda K-4/RD circulating water bath, LKB 2117 Multiphor Basic unit, and LKB 2197 D.C. Power supply on a laboratory bench such that the water bath is adjacent and convenient to the Multiphor unit and the power supply is on the adjacent side of the Multiphor unit.

ii. When placed on the same table or workbench, the LK-4/RD circulating waterbath causes a vibration problem that may cause the bands on the final agarose gel plate to be irregular. This problem can be corrected by isolating the waterbath, either by moving the waterbath to a separate table or to the floor. In cases where the lab has a raised or suspended floor, the addition of vibration damping elements (Fisher 01-914045) may be necessary to further isolate the vibration.

iii. Install the cooling plate in the Multiphor unit according to LKB instruction manual and attach appropriate, insulated, circulation hoses to the water bath and secure to make leak-proof.

iv. Adjust and calibrate the water bath temperature to assure an adequate supply of water is circulating through the cooling plate at 4°C.

v. Turn on the circulating, calibrated water bath at least 30 minutes prior to the preparation of a gel plate on the day that an analytical run is to be performed.

d. Agarose Gel-plastic Film Preparation

i. Mix 0.3 g Agarose-IEF (Pharmacia) and 3.6 g sorbitol in a conical flask with 27 ml distilled water and heat with stirring in a boiling water bath until all solids are dissolved.

ii. Place the flask containing the dissolved ingredients in a 65°C water bath and allow the solution to cool and equilibrate to 65°C.

iii. Add 1.9 ml of Pharmalyte, pH 5-8 range, ampholyte solution (Pharmacia) with needle and syringe, while gently swirling the 65°C tempered, liquid agarose solution. The final agarose solution is 30 ml total volume with an ampholine concentration of about 2.5% and agarose concentration of 1%. Leave the liquified agarose solution in the 65°C water bath until needed, after completing step (viii).

iv. Obtain a glass plate 125 x 260 mm (LKB 2117-701) Capillary Gel Casting Kit) that has been previously treated with the surface wetting agent Prosil-28 according to product instructions and place a small amount of distilled water on the glass surface.

v. Obtain a sheet of gel-bond film and place it on the wet glass plate such that the hydrophobic side of the sheet is down and in contact with the water and the hydrophilic side is up. Properly align the edges of the film sheet with the edges of the glass plate and remove excess water and air bubbles by rolling the surface of the film sheet with a rubber roller. Carefully remove excess water with absorbent towels.

vi. Place the three-sided, orange, silicone gasket on the film sheet and align the gasket edges with the edges of the film sheet.

vii. Place a 125 x 260 mm Prosil-28 treated glass plate on top of the orange gasket and align the leading edges with the gasket. Place five clamps around the three gasket-glass edges (2 each on long sides and 1 on the short end). When properly set up you will have a glass-film sheet sandwich arrangement which is leak proof on three sides where the gasket is and one open end with a space of about 0.75 mm (equal to gasket thickness) between the bottom of the top glass plate and the top of the gel-bond film sheet.

viii. Place this glass-film sheet sandwich arrangement in a 60-65°C oven for 10 minutes to warm up along with a 50 cc syringe and 21 gauge needle.

ix. Remove the warm glass-film sheet sandwich from the oven and set-up on a rack near the water bath containing the previously prepared liquid agarose solution at 65°C. Quickly fill a 50 cc syringe fitted with a 1 inch 21 gauge needle with the liquid agarose solution. Insert the needle in the space between the gel bond film sheet and bottom of the top glass plate. Rapidly but evenly inject the liquid agarose solution to fill this space without air bubbles before the agarose solution starts to gel.

x. Allow the agarose filled sandwich to set undisturbed until the agarose has solidified and then place in a refrigerator for 30 minutes to completely solidify the agarose.

xi. Carefully remove the five clamps and the top glass plate from the sandwich and obtain the agarose coated gel-bond film sheet from the bottom glass plate. When properly executed you will have a gel-bond film sheet containing a uniform, bubble free solidified agarose-ampholine layer of approximately 0.75 mm thickness.

xii. Several agarose gel-bond film plates may be prepared at the same time in order to reduce preparation time for future runs. The prepared plates must be preserved until needed by storage in the LKB Humidity Chamber (LKB-2117-110). These chambers are stackable and come in a kit holding up to three gel plates. Plates stored refrigerated for as long as 6 weeks in the humidity chamber show no loss in performance.

NOTE: Do not perform step (xi) above until just prior to starting step (iii) of section (e) below.

e. Isoelectric Focusing of Samples and References

i. Smear a small amount of reagent grade kerosene (Fisher) on the top of the cooling plate (which has 4°C water circulating through it) of the Multiphor unit.

ii. Place an LKB sample position template on top of the kerosene covered cooling plate, position in proper alignment with the cooling plate and smooth out so that no air bubbles are present under the template. Blot excess kerosene from edges of the template with absorbent towels.

iii. Smear a small amount of kerosene on top of the template and place the previously prepared agarose film sheet on top of the kerosene covered template, align edges with the cooling plate, remove any trapped air bubbles and blot excess kerosene from the edges.

iv. Soak filter paper strips (10 x 5 mm) in sample or reference tissue extracts and apply to the surface of the agarose gel near the anode using the visible template under the agarose-film sheet as a guide. A maximum of 24 samples total (including desired reference extracts) may be placed on the agarose surface. Be sure that the sample paper strip is in complete contact with the agarose surface and rinse off the tweezers between the handling of each sample strip with distilled water.

An alternative approach to sample application is to first place 24 blank paper strips in the proper position on the agarose surface and then with the use of a micropipetting device place a standard amount (25 µl) of sample extract on each respective strip. If it is desirable to employ small paper strips (10 x 2.5 mm) to accommodate a larger number of samples (48) for analysis, these strips should have only 10-15 µl sample extract applied to them and care must be taken to not cause overloading and mixing of adjacent samples.

v. Soak electrode filter paper strips with appropriate solutions for cathode (1 M NaOH) and anode (0.05 M H_2SO_4), blot excess off on paper toweling and guided by the visible template apply the wet electrode strips to the surface of the agarose in the proper anode and cathode positions and cut to the proper size of the agar.

vi. Place the LKB electrofocusing lid on the Multiphor unit over the cooling plate in the proper alignment such that the platinum electrode wires are centered and make good firm, complete contact with the respective soaked anode and cathode filter paper strips.

vii. Connect the electrical cables of the electrofocusing lid to the small pins on the front of the Multiphor unit.

viii. Mount the cover by first introducing the hooks on the cover into the rectangular holes on the rear side of the Multiphor unit, lower the cover and press the large electrode pins into the holes on the cover.

ix. Connect the electrical leads from the cover to the proper terminals (check for like charge) on the LKB 2197 D.C. power supply.

x. Turn on the power supply and adjust to provide the following conditions: 10 watts constant power, 700 V constant voltage and current unlimited (wide open) for a period of 45 minutes.

xi. After this period of time, change power to the following conditions: 10 watts constant power, 1000 V constant voltage and current unlimited for a period of 60 minutes.

xii. Turn off power after this period of time, remove the cover and electrofocusing lid and proceed to section (f) below.

f. Fixing, Staining, and Destaining

i. After completing the isoelectric phase of separation in Section 16.3 e, remove the agarose-film sheet, discard the electrode filter paper strips and sample filter paper strips. Place the agarose-film sheet in the LKB staining tray and immerse in fixing solution for 30 minutes with occasional gentle agitation. Perform this and all subsequent steps in a chemical fume hood with the exhaust turned on.

ii. Remove the agarose sheet from the first tray and place in a second tray containing destaining solution. Wash for a 30 minute period changing the fluid once.

iii. Remove the agarose sheet from the destaining solution and place on a glass plate. Place one sheet of Schleicher and Schuell #577 filter paper (12.5 x 26 cm) over the agarose surface so that no air pockets are trapped under the paper. Then place 2 sheets of Schleicher and Schuell #470 (12.5 x 26 cm) on top of the #577 filter paper, followed by a second glass plate and 1 kg weight. Allow sheets to remain in this manner for 15 minutes to effect an initial drying of the agarose gel.

iv. Remove the weight, glass plate, and filter papers (discard). Complete the thorough drying of the agarose gel with a draught of hot air from a hand held hair dryer. The agarose must be completely dry and adhering to the gel-bond sheet as a thin film of its' own before proceeding to the next step.

v. Place the dried agarose-film sheet in the staining solution for 10 minutes.

vi. Remove, drain, and place in destaining solution until background is sufficiently clear.

vii. Remove, drain, and dry to a final state with the hair dryer.

viii. Examine and compare the isoelectric focused protein patterns of the unknown samples to those of the reference tissue extracts used to identify the samples in question. The final dry preparation may be kept without further modifications as a permanent record of sample analysis.

16.4 Quality Control of Key Reagents or Procedures

In order to assure the integrity and reproducibility of the previously outlined TLIEF procedure, special attention should be given to the considerations cited below.

a. Agarose Gel-plastic Film Preparation.

Be sure to maintain the sterility of the stock ampholyte solution by using aseptic techniques and a new sterile needle and syringe to withdraw the necessary volume of ampholyte needed to prepare the liquified agarose solution. Ampholytes are susceptible to microbial contamination and this would destroy their intended function.

b. Do not allow air bubbles to form during the injection of the liquid agarose solution into the glass sandwich. Air bubbles at this stage will produce a void in that area on the solidified agarose sheet. The presence of air bubbles during electrofocusing will cause a discontinuous electrical resistance between the electrodes. This may ultimately result in improper band migration for the applied sample at that point.

c. Isoelectric Focusing of Samples and References.

Extracts from reference tissues should be prepared from relatively fresh tissues. Old tissues stored in the freezer for a period of time beyond 6-12 months begin to demonstrate fewer bands. Reference tissue extracts (controls) should be applied to each agarose sheet used for an analytical determination of unknown samples. Do

not rely on the use of previously prepared, dried, stained sheets of reference tissues for comparative purposes.

d. Fixing, Staining, and Destaining.

Proper staining contrast of the dried agarose sheet and protein bands depends upon complete removal of ampholytes and total drying of the agarose gel prior to staining.

Care should be given to wash well after the fixing step (step i; Section 16.3 f) and not to reuse the same quantity of fixing solution too many times as this will cause a build-up of ampholytes in it. Complete drying must be accomplished in step iv (Section 16.3 f) by careful use of the hot air dryer prior to staining (step v; Section 16.3 f). Destaining (step v; Section 16.3 f) must be accomplished carefully and empirically by frequent examination of the sheet to insure that under or over destaining is not allowed to occur such that all protein bands are optimumly stained and appear readily visible.

16.5 Selected References

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Ukishima, Y., M. Kino, H. Kubota, S. Wada, and S. Okada. 1991. Identification of whale species by thin-layer isoelectric focusing of sarcoplasmic proteins. J. Assoc. Off. Anal. Chem. 74:943-950.

Chapter 18. SPECIES IDENTIFICATION FIELD TESTS (SIFT)

Mark E. Cutrufelli and Richard P. Mageau

18.1 Introduction

A series of individual, serological screen tests has been developed for rapid species verification of raw whole/ground meat tissue or emulsified meat products in field environments. They are collectively referred to as the Species Identification Field Tests (SIFT). The individual tests which comprise SIFT are as follows: ORBIT (Overnight Rapid Bovine Identification Test), PROFIT (Poultry Rapid Overnight Field Identification Test), PRIME (Porcine Rapid Identification Method), SOFT (Serological Ovine Field Test), REST (Rapid Equine Serological Test), and DRIFT (Deer Rapid Identification Field Test).

The basis of these tests is that of an agar-gel immunodiffusion technique using stabilized reference antigen and antibody reagent impregnated paper discs and prepared agar-gel plates that have a printed template for correct placement of test components. Identification of a species tissue is demonstrated by a reaction of complete fusion between sample and reference antigen immunoprecipitin bands which become plainly visible after overnight incubation of the immunodiffusion plate at room temperature. Key components are stable for at least one year when stored under refrigerator conditions. Each test has been shown to have adequate sensitivity and specificity for its intended purpose of the particular species in question. These tests are reliable, practical, economical, and very easy to perform and interpret in any work environment. Individual species tests for beef, pork, poultry and sheep are commercially available as a complete test kit. As a result of an Association of Official Analytical Chemists (AOAC) collaborative study, the method of these tests is an official AOAC first action method.

18.2 Materials and Methods

All materials necessary for the performance of SIFT for beef, pork, poultry and sheep species may be commercially purchased as individual test kits. The method of performing SIFT for beef species detection using an ORBIT test kit is described below. Performance of SIFT for other species, using the other SIFT kits available, would be conducted in an identical manner except for the substitution of the appropriate dye colored - template marked agar-

gel plates and species reference antigen and antibody reagent discs relative to the species being tested. Specific formulations for preparation of the agar-gel plates and the reference antigen and antibody reagent discs for each species SIFT kit are detailed in the individual references cited at the end of this protocol.

18.21 ORBIT Kit Composition is as Follows:

a. ORBIT agar-filled plates with pink dye; pattern for disc placement silk screened on plate bottom.
b. Vial of Anti-Beef Antibody Discs-A-.
c. Vial of Beef Reference Antigen Discs-B-.
d. Vial of Blank Discs-S-.
e. One piece flat black construction paper.
f. Three pieces of white paper.
g. One felt-tip marking pen.
h. Polyethylene sample bags.
i. Three forceps.
j. Hyperion viewer (optional accessory).

18.22 Ground Meat Accessory Kit Composition is as Follows:

a. Wooden applicator sticks - six inches long.
b. Sample cups - silk screen printed with two permanent measurement lines on outside.
c. Forceps.

18.3 Procedure

a. Remove prepared ORBIT agar-gel immunodiffusion plates and reagent discs from the refrigerator and allow equilibration to room temperature.

b. Using the forceps carefully place one anti-beef antibody disc, flat on the agar surface, such that the A lettered circle of the template is completely and evenly covered by the disc.

c. In an identical manner place one beef reference antigen disc over the B lettered circle of the same plate.

d. Sample discs may be prepared from either thawed whole muscle tissue or from ground/formulated meat products:

i. If the sample is whole tissue, make a vertical slice about 38 mm deep in an area which is free of fat or connective tissue. With clean forceps place one blank sample disc halfway into the depth of the slit and gently squeeze the slit closed such that both sides of the disc are in contact with the tissue. Let the disc remain in this position 10 - 30 seconds to absorb tissue fluids and appear obviously wet.

ii. If the sample is of a ground/formulated type, place about 1 gram well packed into the sample cup such that it is filled level with the bottom black measuring line. Add sufficient quantity of cold tap water to fill the beaker level to the top black measuring line. Mix sample and water with a clean wooden applicator stick such that a uniform emulsion results. Tilt the cup 45° and with clean forceps immerse a blank sample disc in the emulsion to a depth necessary for complete saturation. Excess fluid and meat particles are removed from the disc by wiping it on a cup rim during removal.

e. The sample disc, from either type of sample is placed over one of the S lettered circles of the ORBIT plate containing the reference discs.

f. Treat a second sample in an identical fashion and place that disc over the remaining unoccupied S lettered circle of the same plate.

g. Tightly seal the lid on the plate and leave undisturbed overnight (15 - 24 h) at room temperature.

h. The plates are then examined with an indirect white light source against a flat black background. This may be done with a Hyperion viewer or by using black paper taped to and suspended vertically from the rear part of a desk lamp's housing.

i. Examine the plate for the formation of characteristic immunoprecipitin lines in the agar among the four discs to determine which sample contain beef.

18.4 Results

Immunodiffusion reactions for the ORBIT test are interpreted as are those for other SIFT plate reactions. A reference band should always be visible between the reference antigen-B- and reference antibody-A- discs. Complete fusion of this line with a band formed between the antibody-A-disc and the sample-S-discs is indicative of a positive reaction for that sample. Absence of a band between the sample and the antibody disc is read as negative. Any lines formed near the sample disc that are not extensions of the reference band are also negative reactions.

18.5 Quality Control Procedures

a. Maintain storage of unused prepared plates and reagent discs at refrigeration conditions (4°C) in order to assure adequate shelf life and proper reactivity. DO NOT FREEZE.

b. Do not use any kit components beyond their expiration date.

c. Use separate, clean forceps for each individual disc placement to prevent reagent or tissue fluid carry over and cross contamination.

d. Proper disc placement and positioning is critical to obtaining expected reactions.

e. An immunoprecipitin band must always be produced between the reference antigen and antibody discs, as this serves as the positive control and assures the proper reactivity of the test system. If a reference band is not produced, the test system is invalid, samples should not be interpreted and the cause of the failure to produce the reference band must be determined and corrected before subsequent testing can proceed.

f. Do not attempt to read any immunodiffusion plates that have reacted for more than 24 h.

g. The normal room temperature for proper incubation of immunodiffusion plates is considered to be in the range of 70 - 78°F (21.1 - 25.6°C).

18.6 Selected References

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CHAPTER 19. COMPETITIVE ENZYME-LINKED IMMUNOASSAY (CELIA) FOR THE DETECTION AND QUANTITATION OF CHLORAMPHENICOL

Richard P. Mageau

19.1 Introduction and Principles

Enzyme Immunoassays (EIA) have become increasingly popular to detect and quantitate a wide range of biological molecules of interest. The excellent specificity and sensitivity afforded by EIA are two major factors contributing to the development and use of this technique for quantitative detection of low molecular weight haptenic molecules such as antibiotics. The Immunology Section of the Microbiology Division developed and published an original EIA procedure to detect and quantitate the antibiotic chloramphenicol (CA).

The specific type of EIA developed was an indirect Competitive Enzyme-linked Immunoassay (CELIA) system. The principles of this assay are as follows. The binding of the limiting number of specific rabbit CA antibody molecules in liquid phase to solid phase bound CA antigen is competitively inhibited by free liquid phase CA in the sample under assay. Bound antibody (not displaced) is indicated by using an enzyme linked anti-rabbit antibody preparation which is subsequently reacted with an appropriate substrate. Enzyme activity, measured spectrophotometrically, is inversely proportional to the concentration of CA in the sample.

The CELIA procedure for CA when performed on bovine muscle tissue extracts or phosphate buffered saline CA standards has the following characteristics: sensitivity of 1 ng/ml ($P<0.05$), linear quantitative displacement over the range of 1-100 ng/ml, a mean 50% displacement end point of 15 ng/ml and excellent specificity with respect to other antibiotics and related chemicals.

The specific procedure subsequently described provides the complete information necessary to perform the CELIA for CA. This procedure represents a modified version of the originally developed and published manual method. This modified version is automated and employs 96 well microtiter plates and Flow (ICN) automatic plate washing and optical density reading equipment. This automated version affords the potential opportunity for high volume sample analysis and effective cost savings by the reduced use of extremely expensive developmental biochemical reagents.

19.2 Equipment and Supplies

a. Flow (ICN) Laboratories Titertek Multiskan MC plate reader; #78-530-00.
b. Flow (ICN) Laboratories Titertek Microplate Washer; #78-431-00.
c. Flow (ICN) Vacuum pump for above washer; #78-426-00.
d. Flow (ICN) Titertek Multichannel pipette; 8 channel, adjustable 50-200 ul volume; #77-859-00.
e. Eppendorf Repeater Pipette (Daigger Scientific Co.#G20551) with accessory of 2.5 ml capacity Combitips (Daigger #G20552C) and 5.0 ml capacity Combitips (Daigger #G20552D).
f. Dynatech Laboratories Microelisa plates, Immulon I, flat bottom, 96 wells, #11-010-3350 and covers.
g. Incubator, 37°C (any properly operating brand).
h. Stomacher®, Model 80 (Tekmar Co., Cincinnati, OH).
i. Whirl-pak® bags; 75 x 180 cm size.
j. Centrifuge, capable of operation at 15,600 x g (Eppendorf, Model 5412; Brinkman Instruments, Inc.), and appropriate centrifuge tubes.
k. Refrigerator (4°C).
l. Microtest Manifold, Wheaton, straight, 8 place with Luer Lock connection (Daigger #G20560A).

19.21 Chemicals and Reagents

a. Na_2HPO_4 (Fisher, S-374).
b. NaH_2PO_4 (Fisher, S-369).
c. NaCl (Fisher, S-271).
d. Citric acid, anhydrous (Fisher, A-940).
e. Hydrogen peroxide, 30% reagent grade (Fisher, H-323).
f. Tween 80 (Fisher, T-164).
g. Sodium azide†; NaN_3, purified (Fisher, S-227).
h. Bovine Serum Albumin, powder, fraction V (Sigma, A-4503), store in refrigerator.
i. Chloramphenicol, crystalline (Sigma, C-0378), store in refrigerator.
j. ABTS substrate indicator; 2,2' azino-di-(3-ethyl Benzthiazoline Sulfonic acid), (Sigma, A-1888).

19.22 Biochemical Reagents and Supplies

a. Anti-chloramphenicol serum (undilute).
b. Chloramphenicol-BSA conjugated antigen (50 µg/ml stock).

c. Goat anti-rabbit immunoglobulin G horseradish peroxidase (GARP) conjugate; Miles-Yeda, Israel, (undilute).
d. Chloramphenicol negative beef tissue (initial supply only; used to set up tissue-CA standards).
e. Normal Rabbit Serum (undilute).

NOTE: The above 5 items must be stored in the frozen state at all times to maintain stability.

19.23 Preparation of Stock Reagent Solutions

a. 0.15 M Phosphate Buffered Saline at pH 7.2 (PBS)

Add 10.35 grams of NaH_2PO_4 and 4.38 grams of NaCl to 1 liter of distilled water and dissolve completely to prepare the "acid" solution. Add 10.65 grams of Na_2HPO_4 and 4.38 grams of NaCl to 1 liter of distilled water and dissolve completely to prepare the "base" solution. While mixing with a magnetic stirrer and monitoring the pH on a pH meter, add a sufficient quantity of the "acid" solution to the "base" solution to achieve a final, stabilized pH of 7.2. Dispense into glass containers, autoclave at 121°C for 15 minutes and store at room temperature. It is most convenient to make up this buffer in 5 liter quantities at a time.

b. Phosphate Buffered Saline Containing 0.05% Tween 80 (PBS-Tween)

To 1 liter of prepared 0.15 M phosphate buffered saline at pH 7.2 add 0.5 ml of Tween-80 and mix (not on magnetic stirrer) for several hours at room temperature until completely dissolved. Store this prepared solution in the refrigerator (4°C).

c. Phosphate Buffered Saline Containing 0.5% Bovine Serum Albumin (PBS-BSA)

To 1 liter of prepared 0.15 M phosphate buffered saline at pH 7.2, add 5 grams of powdered bovine serum albumin and 1 gram of sodium azide (NaN_3) and mix (not on magnetic stirrer) at room temperature until completely dissolved. Store this prepared solution in the refrigerator (4°C).

d. ABTS - H_2O_2 Substrate Buffered Solution

Prepare a 0.1 M citric acid solution by dissolving 1.92 grams of anhydrous citric acid in 100 ml of distilled water. Prepare a 0.1 M dibasic sodium phosphate stock solution by dissolving 1.42 grams of Na_2HPO_4 in 100 ml distilled water. Add sufficient quantities of these two stock solutions together while mixing with a magnetic stirrer and monitoring the pH on a pH meter to prepare 100 ml of a 0.1 M citrate-phosphate buffer at a final stabilized pH of 4.0.

To 100 ml of the above prepared 0.1 M citrate-phosphate buffer add 22 mg of ABTS [2,2' azino-di-(3-ethyl Benzthiazoline Sulfonic acid)] and 15 µl of stock 30% hydrogen peroxide, mix gently by hand (no magnetic stirrer) until completely dissolved. Pass this substrate solution through a 0.45 µm Millex® filter, place in a sterile glass container, and store in the dark at room temperature until needed. This substrate solution should be prepared 24 h in advance of need and may be used as long as it retains its original light green color. A solution which has deteriorated to the point where it cannot be used is evidenced by a dark azure-green color formation.

e. PBS Chloramphenicol (CA) Standards

Prepare a stock 1 mg/ml chloramphenicol (CA) solution by weighing out 10 mg powdered, pure CA on an analytical balance and placing in 10 ml PBS. Allow the CA to dissolve thoroughly into solution by occasional mixing over a period of 24-48 h, or longer if necessary, due to limited solubility of the CA. From this stock 1 mg/ml CA solution make serial ten-fold dilutions in PBS (10 ml quantities) to produce CA standards at concentrations of 10,000, 1,000, 100, 10, and 1 ng/ml respectively. Store these standards in the refrigerator (4°C) until used in the assay.

f. Tissue Extract CA Standards

Prepare tissue extract from known CA free, raw, bovine muscle tissue by the following manner:

i. Place 5 grams of diced tissue in a 75 x 180 cm Whirl-pak® bag.

ii. Add 10 ml PBS.

iii. Place bag in Model 80 Stomacher® and stomach for 30 seconds.

iv. Remove bag from Stomacher® and leave undisturbed for 1 h at room temperature.

v. Pour off the liquid contents from the extraction bag into a centrifuge tube.

vi. Centrifuge at 15,600 x g for 15 minutes.

vii. Collect the clear supernatant tissue extract. If necessary, filter to remove all debris and lipid particulates, and place in a sterile glass container.

Using the PBS-CA standards prepared in (e) above, make ten-fold dilutions of each needed 10X higher concentration standard into the freshly prepared beef tissue extract to produce CA standards at concentrations of 1,000, 100, 10, and 1 ng/ml respectively. These tissue extract CA standards should be made fresh each time a standard curve is to be run in the CELIA. The tissue extract originally prepared, without CA, should be stored in the refrigerator and may be used for subsequent CA standards preparation as long as the extract shows no evidence of microbial contamination or protein precipitation. Tissue extracts should always be prepared from tissues similar to those being analyzed for the presence of CA with respect to species and organ or tissue type.

19.3 Performance of CELIA for CA

a. Obtain a flat bottom, 96 well Dynatech Immulon I microelisa plate and cover from stock supplies.

b. Prepare a sufficient quantity of the Chloramphenicol-Bovine Serum Albumin (CA-BSA) conjugated antigen for plate well sensitization. Make a small volume dilution

of the stock 50 µg/ml CA-BSA antigen solution in PBS such that a final concentration of 50 ng/ml CA is obtained.

c. By using the 8 channel pipette, place 200 µl of the 50 ng/ml CA-BSA (in PBS) sensitizing antigen solution into all wells except those of column 2. Leave these wells empty for the present time.

d. Place a cover on the plate and allow the CA-BSA antigen to passively absorb to the wells by incubating the plate for 3 h at 37°C.

e. Test sample extractions should now be concurrently started at this stage in the following manner:

   i. Place 5 grams of diced tissue in a 75 x 180 cm Whirl-pak® bag.

   ii. Add 10 ml of PBS.

   iii. Place bag in Model 80 Stomacher® and stomach for 30 seconds.

   iv. Remove bag from Stomacher® and leave undisturbed for 1 h at room temperature.

   v. Pour off liquid contents from the extraction bag into a centrifuge tube.

   vi. Centrifuge at 15,600 x g for 15 minutes. (Eppendorf Model #5412 centrifuge using 1.5 ml volume centrifuge tubes is very convenient for this).

   vii. Place the clear, test sample supernatant extracts in the refrigerator (4°C) until called for in step (p) of this assay procedure.

f. Remove the plate from the incubator [continued from step (d)], remove the cover and mount on the carrier of a Titertek Microplate Washer which has been primed with PBS-Tween and set to deliver 300 µl fluid to each well.

g. Remove the CA-BSA sensitizing antigen solution from the wells by aspiration with the washer and wash the wells <u>once</u> with 300 µl of PBS-Tween per well.

h. Remove the plate from the washer, invert over a sink, hold the plate tightly in one hand and flick several times to remove any remaining excess liquid from the wells.

i. Tap the plate in an inverted position several times on a soft paper towel (Sorg Laboratory Towels) placed on the surface of the lab bench and allow the plate to remain inverted for 1-2 minutes to complete the draining process. Place the plate right-side up and cover until next reagent addition.

j. Block unwanted reactive sites on the plastic wells by filling all wells (including those in column 2) with 250 µl of PBS-BSA per well, dispensed by using a 8 place microtest manifold attached to a Cornwall syringe.

k. Replace the cover on the plate and incubate for 2 h at 37°C.

l. Remove the plate from the incubator, place on the carrier of the washer, aspirate the PBS-BSA blocking solution out of each well and wash the wells twice with 300 µl of PBS-Tween per well.

m. Repeat steps (h) and (i).

n. With an appropriate pipetting device place 150 µl of PBS in the wells of column 1, 2, 3, and 4 of row A and B. Place 150 µl of CA free tissue extract in the wells of column 1, 2, 3, and 4 of row C and D and the wells of column 1 and 2 of row E, F, G, and H. These wells all serve as negative reagent controls (column 1 and 2) or 0 level controls (column 3 and 4).

o. Place 150 µl of PBS CA standards at concentrations of 1, 10, 100, and 1000 ng/ml in wells of column 5 and 6, 7 and 8, 9 and 10, 11 and 12 respectively of rows A and B. Place 150 µl of tissue extract CA standards at concentrations of 1, 10, 100, and 1000 ng/ml in wells of column 5 and 6, 7 and 8, 9 and 10, 11 and 12 respectively of rows C and D. These wells serve to produce the standard CA inhibition curves in PBS (rows A and B) and tissue extract (rows C and D).

p. Place 150 µl of each test sample extract [from step (e)] in 2 adjacent wells (duplicates) of an individual row. All wells of column 3-12 of row E-H are available for use for duplicate analysis of individual test sample extracts (20 test sample capacity/plate). Record in some appropriate fashion the location of each test sample extract within the available wells for sample analysis for future reference.

q. With the use of an Eppendorf Repeater pipette and a 2.5 ml Eppendorf combitip attached, add 50 µl of normal rabbit serum diluted 1:700 in PBS to all wells of column 1. The wells of this column serve as zero blank normal rabbit serum controls, producing no visible reactions and are used to blank in the reader making spectrophotometric measurements of the reactions in all subsequent wells in each row.

r. With the use of the repeater pipette and a new 2.5 ml combitip attached, add 50 µl of anti-chloramphenicol serum diluted 1:700 in PBS to all remaining wells.

s. Carefully mix and distribute the contents in each well by gently rocking the plate and tapping the ends against your fingers. DO NOT allow the contents of any well to spill out as this will invalidate this result.

t. Place the cover on the plate and incubate overnight (16-18 h) in the refrigerator at 4°C.

u. Remove the plate from the refrigerator, allow equilibration to room temperature, place on the carrier of the washer, aspirate the contents out of each well and wash the wells twice with 300 µl PBS-Tween per well.

v. Repeat steps (h) and (i).

w. By using the 8 channel pipette, add 200 µl of goat anti-rabbit immunoglobulin G horseradish peroxidase (GARP) conjugate diluted 1:5000 in PBS-Tween to all wells.

x. Place the cover on the plate and incubate for 2 h at 37°C.

y. Remove the plate from the incubator, place on the carrier of the washer, aspirate the contents out of each well and wash the wells three times with 300 µl of PBS-Tween per well.

z. Repeat steps (h) and (i).

aa. With the use of the 8 channel pipette, add 200 µl of ABTS-H_20_2 substrate buffered solution to all wells.

bb. Place the cover on the plate and incubate for 90 minutes at 37°C.

cc. Twenty minutes prior to the end of the above incubation period, turn on the power to the Titertek Multiskan MC plate reader and allow it to warm up.

dd. After the 90 minute incubation period of step (bb) is complete, remove the plate from the incubator, remove the cover and place the plate on the carrier of the Multiskan MC plate reader.

ee. Program the reader for the current date, Mode 1 (single wavelength absorbance), Wavelength filter #2 (414 nm), push the carrier and plate into the measuring head and blank the instrument (zero O.D. point set) on column 1.

ff. Press the START button and obtain a printed paper strip of the Optical Density (O.D.) values for all of the reaction wells on the plate.

gg. Remove the plate from the reader and visually examine the plate to see that the obvious colored reaction intensities generally correspond to the numerical values on the printed data sheet to assure that the instrument properly read the plate.

hh. Turn off the power to the Multiskan MC plate reader and discard the plate (save the cover for reuse) after completion of the Data Analysis, Plotting, and Sample Interpretation Section described below.

19.4 Data Analysis, Plotting, and Sample Interpretation

a. All wells in column 1, which serve as the zero-blank normal rabbit serum control, should have no color

reaction. This indicates a proper lack of non-specific attachment of rabbit serum or GARP conjugate to the bound CA antigen in the wells. Under these conditions these wells are excellent controls to blank in (zero point set) the O.D. reading instrument.

b. All wells in column 2 serve as BSA negative controls to assess non-specific attachment of anti-CA antibody (and also GARP). Since these wells were never sensitized with CA antigen and only blocked with BSA, no positive reactions (high O.D. values) should be observed. These controls may also be considered as a check on the other half of the primary antigen-antibody component of the assay system initiated in column 1.

c. Wells in columns 3 and 4 of rows A, B, C, and D should demonstrate maximum binding of anti-CA antibody (zero inhibition) and have the highest O.D. values. These represent the zero controls for the standard inhibition curves produced by subsequently increasing concentrations of CA.

d. The remaining wells of rows A and B represent the standard inhibition curve for PBS CA standards and those of rows C and D represent the standard inhibition curve for tissue extract CA standards. The O.D. values in both of these series of wells should decrease with increasing concentrations of CA due to inhibition of binding of anti-CA antibody.

e. The remaining wells of the plate (columns 3-12 of rows E-H) represent reaction values for test sample extracts relative to the presence or absence of CA in the original samples.

f. For each pair or set of wells containing exactly the same test materials, calculate the average O.D. value.

g. Obtain a piece of 5 cycle semi-logarithmic graph paper containing 100 numerical scale divisions. Label the ordinate (100 numerical scale divisions) with O.D. values from 0 to 2.0 in increments of 0.2 units. Label the abscissa (5 cycle logs) with CA concentrations of 0, 1, 10, 100, and 1000 ng/ml.

h. Plot the average O.D. values generated for the PBS CA standards and tissue extract CA standards from 0 to 1000 ng/ml respectively on the graph paper. Draw straight lines from point to point. You will now have two inhibition (displacement) curves for increasing concentrations of CA in PBS or tissue extracts.

i. Examine the two inhibition curves and compare the slopes and overall O.D. values. The PBS CA standard displacement curve represents the basic reaction level of the primary antigen-antibody system influenced only by pure CA. The tissue extract CA standard displacement curve represents the influence of CA and interaction of various proteinaceous materials extracted from the test sample. If the tissue extract CA inhibition curve is significantly different from the PBS CA inhibition curve (which it usually is) use the former for determining positive CA concentration levels in test samples.

j. Calculate the 50% displacement end point for both standard inhibition curves (50% of the 0 standard O.D.). Values in the range of 5 to 20 ng/ml with a mean value of around 15 ng/ml should be obtained as an indication of properly operating displacement systems.

k. To determine if a test sample contains CA and to quantitate the amount, if it is present, proceed as follows:

   i. Obtain the O.D. value for the test sample and determine the relationship to the tissue extract CA standard curve.

   ii. If this value is between 0 and 1 ng/ml (i.e. O.D. greater than the 1 ng/ml standard), the sample is considered to be free of CA.

   iii. If the value falls within the linear portion of the standard curve, from 1-100 ng/ml, the sample is considered to contain CA. To determine the amount of CA present per gram of tissue, interpolate from the curve the ng/ml CA value on the abscissa relative to the particular O.D. obtained for that sample and multiply it by 2. This assumes that all of the CA from the original 5 gram of tissue is extracted into the 10 ml PBS volume and the

resulting dilution therefore is 1:2 rather than the usual 1:3.

iv. If the O.D. value falls beyond the linear portion of the standard curve (ie. O.D. less than the 100 ng/ml standard), the sample is also considered to contain CA but accurate quantitation is not possible from this particular analytical run. More accurate quantitation in this case would be achieved by taking this sample extract, making serial ten-fold dilutions of it in PBS (10^1 -10^6), repeating the CELIA analysis a second time on these dilutions and determining which dilution produced an O.D. value within the linear portion (1-100 ng/ml) of the PBS CA standard curve.

Calculations for this sample would then be reduced to: interpolated CA value of ng/ml from the PBS CA standard curve abscissa x ten-fold dilution factor x 2 = ng CA/gram of tissue.

19.5 Quality Control Procedures

a. The assay reagents have been evaluated for use only with Dynatech Immulon I microtiter plates. No other plates should be used.

b. All stock reagent solutions must be properly prepared and maintained free of contamination or chemical breakdown.

c. All stock immunochemical reagents must be stored in the frozen state at all times to maintain stability.

d. The stock ABTS-H_2O_2 substrate buffered solution should not be used if it has turned to a significantly darker shade of green from that of the original preparation.

e. Be sure the stock, commercial preparation of Goat anti-rabbit immunoglobulin G horseradish peroxidase (GARP) conjugate reagent has not deteriorated to the point of producing improper final O.D. readings. Use only an unexpired lot of this reagent.

f. To insure validity of the quantitative aspects of this assay, extreme care must be exercised to accurately

prepare the standard CA concentrations in PBS and CA free tissue extracts from stock sources of the pure CA drug.

g. The CA free tissue used to prepare extracts for subsequent preparation of the CA tissue extract standards should be initially validated as being free of CA by a reliable procedure.

h. Standard curves for CA in PBS and CA in tissue extracts must always be run in an analytical determination for the presence of CA in test samples.

i. The tissue source used to prepare the CA tissue extract standard curve must be of the same species and organ type as that of the test sample to be quantified.

j. The standard CA inhibition curves should always be quite similar from run to run and the 50% displacement end point should always be in the same general range. Drastic deviations in the above indicates an improperly operating displacement system due to critical reagent deterioration or technical error in the assay set-up and must therefore be corrected.

k. A valid test run can only be assured by the demonstration of proper CA standard inhibition curves for each particular analytical determination.

† Safety Caution: Do not dispose of spent sodium azide PBS-BSA solution by pouring down sink drains.

Collect in separate liquid waste container and dispose of as hazardous waste according to standard waste management procedures for your laboratory.

Accumulation of sodium azide in lead sink drains may result in an explosion.

19.6 Selected References

Campbell, G. S., R. P. Mageau, B. Schwab, and R. W. Johnston. 1984. Detection and quantitation of chloramphenicol by competitive enzyme-linked immunoassay. Antimicrob. Agents Chemother. 25:205-211.

Shekarchi, I. C., J. L. Sever, Y. J. Lee, G. Castellano, and D. L. Madden. 1984. Evaluation of various plastic microtiter plates with measles, toxoplasma, and gamma globulin antigens in enzyme-linked immunosorbent assays. J. Clin. Microbiol. 19:89-96.

CHAPTER 20. QUALITY ASSURANCE PROGRAM TO ENSURE CORRECT PERFORMANCE OF THE FLOW (ICN) TITERTEK MULTISKAN MC PLATE READER

Richard P. Mageau

20.1 Introduction

Due to the increased use of enzyme immunoassay procedures for the analysis of important residues, it is important to assure that the instrument used to measure the data produced in these assays is operating properly. This is especially important with regard to assays that have defined optical density values for positive, negative, and control parameters. Many of the current enzyme immunoassays implemented in the Field Service Laboratories employ the ABTS/H_2O_2 substrate and the Flow (ICN) Multiskan MC Plate Reader to obtain data. This substrate when acted upon by the enzyme peroxidase produces a product which has a maximum absorbance at the 414 nm wavelength (filter #2). There is no way to be certain that the daily optical density readings obtained by the instrument during the performance of an enzyme immunoassay are correct, except perhaps by complacent trust. The easy procedure described in this chapter is an attempt to ensure that the readings generated by the Flow (ICN) Multiskan MC Reader at the 414 nm wavelength filter (#2) are indeed correct and that the instrument is operating properly.

20.2 Procedure

a. Prepare 200 ml of a stock 15% (w/v) solution of nickel sulfate (nickelous sulfate, 6-hydrate, crystal, Baker 2808-1) in distilled water in a volumetric flask. Store this stock solution in an air-tight glass container to prevent evaporation and ensure that deterioration does not occur due to contamination or chemical decomposition.

b. Obtain a Dynatech Immulon I, 96 well microtiter plate.

c. Leave all wells of column 1 empty. Accurately place 200 µl of the stock 15% nickel sulfate solution into all wells of columns 2 and 3 (16 wells total).

d. Turn on the Flow (ICN) reader, allow it to warm up and program it for Mode 1 (singe absorbance) and Filter #2 (414 nm wavelength).

e. Push the plate containing the nickel sulfate wells into the reader, blank the instrument on column 1 and obtain optical density readings for the wells of columns 2 and 3.

f. Calculate the mean O.D. value for the 16 wells of columns 2 and 3.

g. Perform the exact same procedure each month and keep a log book of the monthly mean O.D. values for the 16 nickel sulfate wells.

h. If the instrument is performing correctly there should be no significant change in the monthly mean O.D. values. A significant change (most likely a decrease) in these values indicates a problem with the instrument, probably with regard to the light source (lamp), the 414 nm wavelength filter (#2), or the internal electronics of the instrument itself. A systematic check out of these areas in that order is recommended.

NOTE: Spare lamps, replacement filters, electronic repair/instrument check out, or technical assistance may be obtained from:

ICN Biomedical Instruments
330 Wynn Drive
Huntsville, Alabama 35805
Tele: 1-800-426-8869

Prior to returning the instrument for repair, you must first obtain a Return Goods Authorization (RGA) number by calling the above and making the necessary arrangements.

Chapter 21. ANIMAL SPECIES DETERMINATION, IMMUNOLOGICAL

Richard P. Mageau

PART A

21.1 (Presumptive) Tube Ring Precipitin Test

21.11 Introduction

The accurate identification of animal meat species at a significant level of sensitivity in raw meat and poultry products is an important aspect of the Agency's ability to meet the legislative mandate providing for the assurance of a safe, wholesome, unadulterated and accurately labeled meat and poultry supply to consumers. Raw meat species identification can generally be accomplished by physicochemical procedures such as electrophoresis, isoelectric focusing (see Chapter 16) or high performance liquid chromatography and by immunological procedures such as immunoprecipitin (immunodiffusion) reactions (see Chapter 18) or enzyme-linked immunosorbent assay (ELISA), (see Chapter 17).

The immunological methods described in Parts A, B, and C of this chapter of the Microbiology Laboratory Guidebook concerning raw meat species identification have been selected, adapted and implemented for use in the FSIS Technical Support Laboratories because of their suitability as scientifically sound methods, defendable in a court of law when litigation arises from violative results and their practical working use in high volume, routine sample analysis in regulatory laboratories. The methods in Parts A and B are to be used only as presumptive screen tests and all positive, violative results are to be further subjected to a final confirmation by the procedure described in Part C.

The analytical screen test formerly used by the Technical Support Laboratories for determination of the species of animal tissue in raw meat and poultry products was the Ring Precipitin test. Although this immunoassay was successfully used for many years, it was subject to certain limitations or requirements. These consisted of such factors as unremarkable and variable sensitivity levels for species adulterants in different base meat tissues; the availability of significant quantities of expensive, specific anti-species sera; the exact titration of these antisera against standardized reference 30,000 total protein solutions in a specific timed reaction interval; preparation of crystal clear sample

extracts for test reactivity against the titered, specific antisera in the timed reaction interval; and the known observation that certain meat product ingredients such as spices or soy proteins may interfere with obtaining correct test results when certain samples containing such are analyzed by this standardized procedure. In short, this assay required several immunochemical reagents and much, exact standardization of reagents and test performance in order to insure reliable test results. Although this assay has been replaced for routine use by a commercial Immunostick ELISA screen test procedure described in Part B, the Ring Precipitin test procedure is presented below in detail to provide information as an alternative acceptable method if the need should arise.

21.12 Equipment and Materials

a. Culture tubes, glass, 6 x 50 mm, disposable.
b. Pipettes, Pasteur type, 9" (22.8 cm) and 5-3/4" (14.6 cm), disposable, sterile.
c. Pipettes, calibrated, assorted sizes, sterile.
d. Serum vials, rubber stoppered, 15 and 30 ml size, sterile.
e. Racks for holding 6 x 50 mm culture tubes.
f. Culture tubes, glass, 20 x 150 mm or larger.
g. Filter paper, Whatman #42, 11 cm diameter.
h. Millipore Millex® disposable membrane filter units, Luer-lock fitting, 0.45 or 0.22 µm porosity.
i. Syringes, disposable, assorted sizes.
j. Hypodermic needles, disposable, 20 and 22 gauge by 1" (2.5 cm) long; 19 gauge by 1-1/2" (3.8 cm) long.
k. Centrifuge, preferably refrigerated.
l. Centrifuge tubes, plastic, autoclavable, 50 ml capacity.
m. Spectrophotometer, Bausch & Lomb Spectronic 20.
n. Calworth Stomacher®, Model 80.
o. Whirl-Pak® polyethylene bags, 22.8 x 11.4 cm size.
p. Mechanical Shaker.
q. New Zealand albino rabbits, 2.3 kg.

Precaution: All non-disposable glassware must be thoroughly cleaned in detergent, followed by final distilled water rinse and heating in a drying oven for at least 2 h at 200°C to prevent foreign protein contamination.

21.121 Reagents

a. **Normal Saline (0.85% sodium chloride solution):**

Dissolve 8.5 g NaCl in 1000 ml distilled water.

b. **2X Saline (1.7% sodium chloride solution):**

Dissolve 17 g NaCl in 1000 ml distilled water. Add merthiolate to a final concentration of 1:10,000.

c. **2X Saline Containing 10% Normal Rabbit Serum:**

Add 10 ml of normal rabbit serum to 90 ml of 2X saline (above) and mix thoroughly.

d. **Merthiolated Saline:**

To normal saline add sufficient powdered merthiolate to produce a final concentration of 1:10,000.

e. **Normal Sera:**

Obtain authentic normal horse, beef, pork, sheep, chicken, and turkey sera from a reputable commercial source or by directly bleeding the appropriate animal.

f. **10% Solution of Aluminum Potassium Sulfate in Distilled Water**

g. **Specific Antisera to Animal Species:**

Obtain anti-horse, beef, pork, sheep, chicken, and turkey sera following rabbit immunizations.

h. **Biuret Solution**

21.13 Preparation of Proom's Alum Precipitated (PAP) Antigens for Rabbit Immunizations

The preparation of alum precipitated antigens from the normal serum of various animal species is as follows by the method of Proom (Proom, 1943).

a. **Obtain 25 ml of authentic normal serum of the particular species required and thaw completely from the preserved frozen state.**

b. **To this 25 ml of normal serum add 80 ml of sterile distilled water and 90 ml of 10% aluminum potassium sulfate solution and mix thoroughly.**

c. **Using a pH meter, adjust the pH of the resulting solution to 6.35 very carefully with 5 N NaOH.**

d. **Pour the adjusted solution into centrifuge tubes, centrifuge in the cold at 3,000 RPM for 20 minutes and discard the supernatant fluid.**

e. **To the packed precipitate add 100 ml of merthiolated saline, thoroughly resuspend the precipitate and pour into a large plastic bottle.**

f. **Place this bottle and solution on a mechanical shaker and shake vigorously at room temperature for 25 minutes.**

g. **Pour the solution back into centrifuge tubes and centrifuge as described in Step (d). (Or centrifuge in large bottles.)**

h. **Repeat steps (e thru g) for a total of 4 times.**

i. **After the final centrifugation and liquid discard, add merthiolated saline to the fluffy white precipitate for a final volume of 100 ml and thoroughly resuspend.**

j. **Place 25 ml aliquots of this alum precipitated antigen into sterile serum vials and label the appropriate species represented.**

k. **Store this antigen in the refrigerator until needed for rabbit immunization. DO NOT FREEZE.**

l. **Prepare alum precipitated antigens, as outlined above, for each species of animal to which specific antiserum is required.**

21.14 Antiserum Production

Prepare specific antisera against each species required by the following method:

a. Obtain a healthy 2.3 kg New Zealand albino rabbit and using a syringe fitted with a 20 gauge, 2.5 cm long needle obtain 5 ml of blood from the medial artery of the ear.

b. Separate the serum and test this preimmune serum against the prepared test antigens by the tube ring precipitin test to assure that the rabbit is free of existing antibodies.

c. Using a syringe fitted with a 22 gauge, 2.5 cm long needle inject 0.5 ml of thoroughly mixed, previously prepared alum precipitated antigen of the desired species, intramuscularly into each hind leg of the rabbit (1.0 ml total) as the primary injection.

d. On Day 21 post primary injection, inject 0.5 ml antigen into each leg, as the initial booster.

e. On Day 28 post primary injection, trial bleed the rabbit from the medial artery of the ear, obtain the serum and perform a titration to determine the relative antibody content as described under Section 21.16, Antiserum Titration and Specificity Tests. If the immune serum has a titre of 1:10 or greater, proceed to obtain a large bleeding from the rabbit.

f. If the serum titer of the above trial bleeding is considerably lower than 1:10, proceed to give a second booster injection of antigen as in step (d) on Day 36 post primary injection.

g. After 14 days from this second booster injection of antigen obtain a large bleeding from the rabbit.

h. Large bleedings may be obtained by using a large syringe fitted with a 20 gauge, 2.5 cm needle and bleeding carefully through the medial artery of the ear or by placing the rabbit ventral side up in an appropriate restraining device and performing intracardiac bleeding with a 100 ml disposable syringe fitted with a 19 gauge,

3.8 cm long needle. If the rabbit is to be kept for subsequent bleeding or reimmunizations, DO NOT bleed for more than 35 ml at any one time. Remove the needle and gently aspirate the blood from the syringe into a sterile container.

i. The serum is obtained by allowing the blood to clot at room temperature for 2-4 h, ring the clot from the walls of the container and place in the refrigerator overnight. Decant the serum, centrifuge at 3,000 RPM to remove all RBC's and filter sterilize through a 0.22 µm Millex® membrane filter unit directly into a sterile, rubber stoppered serum vial. Merthiolate may be added to a final concentration of 1:10,000 as a preservative, but only as a last resort in lieu of strict aseptic handling of the serum at all times. More information relative to Steps (h) and (i) may be found in "Methods in Immunology", 1977.

j. Label the vial as to the specific anti-species serum represented and keep refrigerated until further use in the tube ring precipitin test. DO NOT FREEZE.

21.15 Preparation of Normal Serum Antigens for Controls in The Ring Precipitin Test

Antigens to be used for controls and antisera titering in ring precipitin tests are prepared from authentic normal sera obtained from various animal species. Maintain these normal sera in a frozen sterile condition prior to dilution and use.

Since the protein content of sera varies from animal to animal within a species, as well as among species, it is necessary to determine and adjust the amount of antigen used for controls. This is done on the basis of the total protein (TP) content of each normal sera.

The TP content of each sera is determined by the biuret method (Section 21.19). Prepare a 1:500 working dilution of TP using the following formula:

(5 x % TP) - 1 = D_v 500. In which % TP = % TP in serum, and D_v 500 = Volume of normal saline to be added to one volume of serum to attain a 1:500 dilution.

Examples:

Serum A = 7% TP	Serum B = 6.5% TP
(5 x 7) - 1 = 34	(5 x 6.5) - 1 = 31.5
1 ml Serum A + 34 ml	1 ml Serum B + 31.5 ml
Normal Saline = 1:500 TP	Normal Saline = 1:500 TP

From this 1:500 working dilution of TP prepare the following TP dilutions in normal saline: 1:1,500 TP; 1:3,000 TP; and 1:30,000 TP. The 30,000 TP serum antigen will serve as the homologous test antigen, while the 3,000 TP and 1,500 TP serum antigens will serve as heterologous test antigens in the procedures that follow.

Filter these diluted serum antigens through a Millex® filter (0.45 µm) into sterile vials or screw cap tubes. Store these diluted antigens at 4-6°C. DO NOT FREEZE. Discard after 8 weeks, or if cloudy or precipitated.

21.16 Antiserum Titration and Specificity Tests

Since the specific antibody content varies within different lots of a particular prepared anti-species serum, it is necessary to quantitate and standardize this antibody level for use in routine sample analysis by the ring precipitin test. It is also necessary to verify the specificity of the reactivity of an anti-species serum towards its homologous antigen at this time.

a. Using 2X saline containing 10% normal rabbit serum, prepare the following dilution series of the anti-species serum to be titered: undilute; 1:2, 1:3, 1:4, 1:5, 1:6, 1:7, 1:9, 1:10, 1:15, 1:20, and higher if deemed necessary. (Large volumes are not necessary.)

b. Test each of the above dilutions against 30,000 TP homologous serum antigen and 1,500 TP heterologous sera antigens previously prepared using the described Ring Precipitin Test. NOTE EXCEPTIONS: To test anti-bovine and anti-ovine sera with their respective heterologous sheep and beef antigens, use 3,000 TP instead of 1,500 TP. Make the same exception for anti-turkey and anti-chicken sera.

c. Choose as the working dilution of antiserum for subsequent use in routine ring precipitin testing on unknowns the highest dilution of antiserum that gives a positive test with the 30,000 TP homologous antigen

within 6 minutes, and fails to give a positive test with the 1,500 TP heterologous antigen (Note Above Exceptions) within 10 minutes. This establishes the antiserum titer and confirms specificity.

An additional test on specificity may be performed by the agar gel immunodiffusion test using undiluted antiserum and saline extracts of tissues from authentic heterologous and homologous animal species.

d. Prepare a 5-6 ml volume of working dilution of each anti-species sera required in 2X saline containing 10% normal rabbit serum and filter sterilize through Millex® filters (0.22 µm) into sterile 15 ml screw cap vials. Refrigerate at 4-6°C until needed. DO NOT FREEZE. Reconfirm the titer and specificity of the working dilution of antisera against appropriate TP antigens each week and discard the sera upon loss of titer or specificity, or development of autoprecipitation or microbial contamination.

21.17 Sample Extraction

a. Fresh Tissue

Weigh 25 g of fresh tissue, using the inner portion of the piece if possible. Dice the tissue and place into an appropriate receptacle (polyethylene bag or beaker) and add 100 ml normal saline. Allow to stand for 1-1/2 to 2 h at room temperature. Filter 5-6 ml of the extract through three-fold filter paper (Whatman #42) into 20 x 150 mm tubes. The filtrate must be crystal clear, but may be colored from straw to dark red. If the filtrate is not crystal clear, subject it to centrifugation and/or filtration through a Millex® syringe filter unit (0.45 or 0.22 µm pore size). Run the test as soon as possible, before the filtrate becomes cloudy.

b. Partially Cooked or Cured Tissue

When a tissue has been heated above 165-175°F, the proteins become insoluble and cannot be extracted. Frequently, however, an interior section may not have reached the denaturing temperature and will release enough soluble proteins for a test. The same applies to cured products. For cooked, uncured tissues, extract as

for fresh tissue and let stand in the refrigerator at least 18 h, then test aliquots at intervals for 5 days. If no reaction occurs after 5 days' extraction, report sample as not giving an antigenic response. If possible, perform the ELISA cooked meat species procedure (see Chapter 17) to identify and differentiate these non-reactive samples. Use the same procedure for cured tissue, but extract with distilled water instead of saline.

c. Chopped, Ground or Emulsified Tissue

Proceed as for fresh tissue.

d. Alternative Extraction Method

Place 12.5 g of tissue and 50 ml normal saline in a 22.8 X 11.4 cm Whirl-Pak® polyethylene bag. (Do not deviate from above amounts.) Place the bag and contents in a Calworth Stomacher®, model 80, and stomach for the following times found to be optimum for the various types of sample products listed (Table 1):

Table 1. Stomaching Time for Samples

Sample Types	Stomaching Time/seconds
Raw ground meats, emulsions and sausage formulations	0 (manually knead bag and contents)
Raw muscular tissue, diced	5-10 (maximum)
Cooked and cured samples, hard processed meats (salami, bologna, frankfurters, etc)	15-30 (maximum)

After stomaching, allow the bag and contents to sit at room temperature for 15-20 minutes. Proceed to prepare a crystal clear filtrate of this extract in the usual manner outlined for fresh tissue extraction.

21.18 Ring Precipitin Tube Test

In an appropriately marked rack, place one 6 X 50 mm tube for each species for which the sample is to be tested (e.g., horse, beef, pork, sheep, chicken, turkey). Place in each tube about 0.2 ml of the working dilution of respective anti-species serum using individual, sterile Pasteur pipettes. Fill another Pasteur pipette with the unknown tissue extract to be tested. Tilt the tube at a 45^{o} angle and slide the pipette down the side of the tube just above the antiserum. Then allow the extract of the unknown to flow gently over the surface of the antiserum, while withdrawing the pipette, keeping it ahead of the advancing interface. Do not allow the pipette to touch the antiserum, or to disturb the interface. Clean the surface of the tube with moist toweling, then wipe it dry. After 3 to 5 minutes, and again up to 10 minutes, read the tube by indirect light against a black background.

A cloudy white ring at the interface is a positive test. Also test heterologous TP dilutions, and read up to 10 minutes as a test of acceptability of antisera. If the heterologous TP dilution for one species gives a positive test against the serum of another species within 10 minutes, check for possible contamination of the antiserum. (Note: Quality Control Section, 21.110)

Retest the antiserum for specificity and retest the sample, extracting at least two times. If more than one piece of tissue was used, then retest each piece separately using, if possible, the innermost portions of the pieces. If the sample is ground or chopped, retest another extraction of the sample; repeat two times if the reaction indicates possible violation. Record the reaction times.

21.19 Total Protein by Biuret Method

21.191 Biuret Solution

In a one liter volumetric flask place 1.5 g cupric sulfate, and 6.0 g fine crystals of potassium sodium tartrate. Add sufficient distilled water to dissolve. Add slowly with agitation of the flask, 300 ml 2.5 N sodium hydroxide and mix. Add 1 g potassium iodide and shake until dissolved. Dilute to one liter total volume. Discard when black or reddish precipitate forms.

21.192 Method

a. Place 9.5 ml 0.85% NaCl in a test tube. Add 0.5 ml of sample. Rinse out pipette by drawing in and expelling some of the mixture.

b. Into one of 2 test tubes place 2 ml of the diluted sample, above; in the other, 2 ml 0.85% NaCl solution (blank).

c. Add 8 ml biuret reagent (above) to each tube, and mix.

d. Set 100% transmission with "blank" at wavelength 540 nm.

e. Immediately after adding biuret reagent read transmission of sample and obtain concentration from the following (Table 2).

Table 2. Percent protein, as determined by percent transmission of Biuret reaction in Bausch and Lomb Spectronic 20. (Note: Quality Control Section).

% TR* (540 nm)	0	1	2	3	4	5	6	7	8	9
	Percent Protein									
0										
10										
20										
30								13.8	13.4	13.0
40	12.7	12.4	12.0	11.7	11.4	11.1	10.8	10.5	10.2	9.9
50	9.6	9.3	9.0	8.8	8.6	8.3	8.0	7.8	7.6	7.3
60	7.1	6.9	6.6	6.4	6.2	6.0	5.8	5.6	5.4	5.2
70	5.0	4.8	4.6	4.4	4.2	4.0	3.8	3.7	3.5	3.3
80	3.1	2.9	2.8	2.6	2.4	2.3	2.1	2.0	1.8	1.6
90	1.5	1.4	1.2	1.0						

* TR (Transmission)

Example: % transmission = 47. Concentration of protein = 10.5%

21.110 Quality Control Procedures

In order to assure the integrity and reproducibility of the procedures previously outlined, special attention should be given to the following considerations cited for each section listed below:

a. **Normal serum of authentic species: It is absolutely essential that the species authenticity of the normal sera be initially established since these sera serve as the starting material for anti-species sera production and standardized test antigens. This can be accomplished by directly bleeding the live animal species required and preparing the serum from the blood. If a commercial source of normal serum of a particular species is used, it should be verified in a known, correctly functioning, serological test system.**

b. **Total Protein Determinations and TP Dilutions: Care and attention should be given to the correct test performance, data interpretation and calculations to arrive at the total protein content of each normal sera. Caution must also be exercised in the mechanical preparation of the correct TP dilutions of heterologous and homologous sera antigens. Improperly prepared or calculated values for the above will lead to erroneous anti-species sera titration or specificity data. This in turn might render the antisera dilution finally chosen for use, totally ineffective for reacting with an adulterant tissue in an unknown sample.**

c. **Antiserum Titration and Specificity Checks: The most important component of the ring precipitin test system which ultimately is responsible for the successful detection of an adulterant tissue is the standardized anti-species serum. It cannot be stressed too strongly that periodic checks on the performance characteristics of these diluted antisera must be made with the 1,500 TP and 30,000 TP normal serum antigens to assure that the antisera are reacting in the expected manner. Previously titered antisera can on occasion, with age, produce a change in the titration endpoint. Appropriate adjustments in the working dilutions of these antisera would therefore need to be made in order to compensate for this fact.**

d. Biuret Protein Determination Table: It should be noted that the convenient table provided for the determination of protein by the Biuret reaction is valid only if the exact test procedure is followed and the percent transmission values are obtained using a Bausch and Lomb Spectronic 20 spectrophotometer with the standard, round, tube shaped cuvettes. If a different protein determination test or spectrophotometer is to be employed, then a new standard table must first be prepared with the use of known protein standards.

e. Sample Extracts and Anti-species Sera Working Dilutions: Reagents must be crystal clear following Millex® filtrations just prior to performing the ring precipitin test. Any degree of cloudiness will make it more difficult to visualize any reacting immunoprecipitin line at the interface.

f. Overlaying the Working Dilution of Each Respective Anti-species Serum with the Sample Extracts: Overlayering must be done in a careful, gentle manner so as to not create a mixture of the two reagents at the interface. A mixture at the interface will tend to create a broad, diffuse immunoprecipitin band and cause difficulty in visualizing a positive reaction within the specified time period, rather than the usually expected sharp band.

21.111 Selected References

Garvey, J. S., N. E. Cremer, and D. H. Sussdorf. 1977. Methods in Immunology: A Laboratory Text for Instruction and Research, p. 7-38. 3rd Edition. W. A. Benjamin Inc. Reading, MA.

Kenny, F. 1985. A practical species testing programme, p. 155-159. *In* R. L. S. Patterson (ed.), Biochemical Identification of Meat Species. Elsevier Science Publishing Co., Inc. New York, NY.

Proom, H. 1943. The preparation of precipitating sera for the identification of animal species. J. Path. Bact. 55:419-426.

PART B

21.2 (Presumptive) Commercial ELISA Immunostick Screen Test Kit.

21.21 Introduction

Modern developments in immunoassay technology have made available alternative procedures which have the advantage of eliminating or greatly reducing the limitations previously cited for the Ring Precipitin test. One such procedure is the Enzyme-Linked Immunosorbent Assay (ELISA) method, which is now available in a commercialized kit form capable of rapid, specific species identification of raw meat and poultry tissue products, inclusive of all current species of interest to our National Testing Program. The original ELISA raw species test kit was developed and manufactured as a solid phase microwell plate system. The system was subsequently modified slightly by incorporation of NUNC dip-stick paddles (immunosticks) as the solid phase and the use of pre-dispensed, standardized reagents in color coded tubes. It is currently marketed and distributed in the U.S. in a complete (25 test) kit form and is referred to as a commercial ELISA Immunostick Raw Meat Species Screening Test Kit.

This raw meat species screen test is a double antibody "sandwich" ELISA procedure with antibody specificity directed against the various species albumins which are contained in meat tissues. Specific antibody sensitized immunosticks are allowed to capture homologous species albumin from sample tissue extracts, then reacted with the second peroxidase labeled antibody of the same specificity, followed by a final reaction step in ABTS/H_2O_2 chromogen/substrate solution. A short incubation period and a brief tap water rinse is performed between each of the first two steps. A positive reaction, indicating the presence of the test species tissue in the sample, is evidenced by a distinct green color formation in the last reagent tube. Each species kit contains all necessary reagents, controls and accessories to perform the test in an extremely easy fashion with the production of very accurate results.

The Immunology Section of BCB, MD at Beltsville conducted an evaluation of the ELISA Immunostick Screen test kits for all available species. They were found to be very specific, reliable, easy to use and capable of detecting an adulterant tissue at the 1% sensitivity level. It is with the above considerations in mind and the aim of technical improvement over the previous screen test procedure that these commercial Immunostick Screen Tests were

implemented in all FSIS, Technical Support Laboratories for raw meat species determinations. The Immunostick Screen Test is now used in place of the standard Ring Precipitin test procedure. All positive Screen Test results which represent sample violations are to be confirmed in the usual manner by the standard agar-gel immunodiffusion procedure described in Part C.

A commercial ELISA Immunostick Screen Test employed for presumptive identification of species composition of raw meat and poultry tissues should meet or exceed the following performance characteristics:

Sensitivity - produce positive reactions down to the 1% level (W/W) of adulterant or contaminant tissue in a base meat tissue mixture such that a 0% False Negative Rate is observed.

Specificity - produces no positive cross reactions with any heterologous species tissues such that a 0% False Positive Rate is observed.

21.22 Reagents and Equipment

a. Commercial ELISA Immunostick Raw Meat Species Screen Test Kits. Color codes for individual species kits are as follows (Table 1):

Table 1. Color Codes for Commercial ELISA Immunostick Screen Test Kits.

Color Code	Species	Color Code	Species
Red	Beef	Orange	Horse
Yellow	Pork	Lilac	Rabbit
Blue	Poultry*	Grey	Kangaroo
Pink	Chicken*	Brown	Turkey*
Green	Sheep	Various	Mixed

* The ELISA immunostick Poultry screen test does not differentiate between chicken or turkey. If it should become necessary to do

so, this can be accomplished by performing the traditional agar-gel immunodiffusion procedure (Part C), or by using the ELISA immunostick chicken or turkey screen tests. Since these latter two screen tests have less than the required sensitivity, their use should be limited to whole meat or poultry tissues or a mixed meat/poultry emulsion where the poultry component is known to constitute over 5% of the final meat block.

Each individual species kit contains the following items:

i. Twenty-five - color coded, white plastic immunosticks sensitized with specific anti-species capture antibody in tubes of preservative buffer solution.
ii. Twenty-five - color coded tubes containing species specific antibody-enzyme conjugate reagent.
iii. Twenty-five - tubes (non-color coded) containing color development buffer reagent.
iv. One vial of concentrated ABTS color reagent.
v. One vial of aqueous sodium fluoride stop solution.
vi. One vial of positive control solution (homologous species albumin).
vii. Disposable polypropylene pasteur pipettes - NOT TO BE USED.
viii. Product insert test kit instruction pamphlet.

b. Rainin Gilson Pipetman® (P-200) adjustable pipette and appropriate disposable pipette tips.
c. Calworth Stomacher®, Model 80.
d. Whirl-Pak® polyethylene bag, 6 oz size (7.5 x 17 cm).

21.23 Raw Sample Preparation

All types of raw meat and poultry product samples are prepared as follows:

a. Weigh out 1 gram of thawed, diced, raw sample product which is a homogeneous, representative portion of the whole sample.

b. Place in a 6 oz Whirl-Pak® bag.

c. Add 9 ml of distilled water.

d. Place the bag and it's contents in a Model 80 Calworth Stomacher® and stomach for a period of 60 seconds.

e. Allow the extract to settle for 2-3 minutes until a particle free liquid layer is formed in the top portion of the bag's contents. Use this upper liquid layer as the sample extract in the following test procedure.

21.24 Test Procedure

The following procedure is to be used, which represents minor modifications from the original product insert test kit instruction pamphlet. These procedural modifications are designed to improve the accuracy, precision and reproducibility of test results. The subsequent instructions represent the testing of 1 sample through 1 species test procedure. Obviously multiple samples and/or species tests may be performed simultaneously, as long as one is careful to keep track of reaction times, washing steps, various reagent steps, etc. relative to each given test sample.

a. Remove the appropriate color coded species Immunostick tube, antibody-enzyme conjugate reagent tube, and color development buffer tube (a set of 3) from refrigerated storage and allow to equilibrate to room temperature.

b. Label Immunostick caps and all tubes with appropriate sample identification codes.

c. Prepare the color development buffer reagent tube (non-color coded) for later use by adding 40 µl of ABTS concentrate to this tube, replace cap and mix in a gentle but complete manner.

d. Obtain the first color coded Immunostick tube, unscrew the cap and remove the immunostick-paddle, add 200 µl of prepared sample extract to the liquid in the tube, replace the immunostick-paddle in the tube and mix contents by rotating the cap rapidly 4-6 times and tighten the cap. DO NOT INVERT tubes to accomplish mixing at any stage in this procedure. Handle the paddle at all times only by it's attached cap, DO NOT TOUCH paddle with fingers.

e. Allow this tube to stand for 10 minutes at room temperature.

f. Remove the immunostick-paddle and wash the paddle and entire cap completely by placing it under a gentle stream of cold tap water for 10 seconds, then shake to remove excess water.

Note: Water dispensed from a squeeze bottle can also be used to carefully perform this wash step.

g. Place the washed immunostick-paddle into the second color coded tube of antibody-enzyme conjugate reagent, mix contents by rotating the cap rapidly 4-6 times and tighten the cap.

h. Allow this antibody-enzyme reagent tube to stand for 10 minutes at room temperature.

i. Remove the immunostick-paddle and wash the paddle and entire cap completely by placing it under a gentle stream of cold tap water for 30 seconds, then shake to remove excess water.

Note in step (f) above also applies here.

j. Place the washed immunostick-paddle into the final, non-color coded, tube of ABTS prepared (step c) color development buffer reagent, mix contents by rotating the cap rapidly 4-6 times and tighten the cap.

k. Allow the color development reagent tube to stand for 10 minutes at room temperature.

l. Add 200 µl of sodium fluoride stop solution to this color development tube, leave the paddle in, and mix well to stop the reaction.

m. Observe the above tube with the white paddle in it for the presence of any discernable green color in the solution or on the paddle surface. A green color indicates a positive test and the presence of the test species in the original meat sample. A colorless solution around the white paddle indicates a negative test and the absence of the test species in the sample.

All ELISA Immunostick positive species results which represent sample violations are to be confirmed by the traditional agar-gel immunodiffusion procedure as described in Part C.

21.241 Test Controls

The occasional use of positive and negative controls in performing this species screen test will ensure proper quality control and reliable test performance of this method. This should ALWAYS be done initially upon opening and placing into use a brand new kit package.

Each species test kit is supplied with a positive control vial (homologous species albumin solution) for this purpose. The negative control for any one particular species test kit may be obtained by using the positive control solution from any of the other heterologous species test kits: (eg. horse albumin solution should always give negative results in all other species kits except horse).

Control testing may be performed in the following manner:

a. Remove the cap and Immunostick from a tube of an individual test series to be used for control testing.

b. Add 200 µl of negative or positive control solution to the liquid in the tube.

c. Replace the immunostick-paddle in the tube, mix contents by rotating the cap rapidly 4-6 times and tighten the cap.

d. Proceed with the remainder of the test procedure exactly as described above by continuing and completing steps e-m (Section 21.24; Test Procedure). Be sure to initially prepare an ABTS color development buffer reagent tube in the usual manner when you start your control tests.

21.25 Quality Control Procedures

a. Store all kit components at refrigerator temperature (4-8^{o} C) when not in use to preserve and maintain reactivity of immunoreagents.

b. Perform positive and negative control testing of an initially opened kit package and occasionally thereafter to insure proper test performance.

c. Observe the manufacturer's one year expiration date of all test kit components. Kits should not be used beyond the expiration date.

d. The concentrated ABTS color reagent solution tube should be observed over the kit shelf life. If this ABTS concentrate should start to turn a much darker shade of green than when it was originally received, this indicates decomposition, and a new tube of ABTS concentrate should be requested from the vendor.

e. All volumetric additions of sample extracts or reagents to the test procedure should be made only with the Rainin Gilson Pipetman® pipette instrument.

f. Kit components should be allowed to equilibrate to room temperature before commencing test procedure.

g. The 1 gram test sample used for extraction must be representative of the entire original sample in order to insure that test results accurately reflect the true composition of the original sample.

h. Preparation of the color development buffer reagent tube by the addition of ABTS concentrate (step c of Section 21.24, Test Procedure) should only be accomplished just prior to commencing the test procedure. Preparation of this reagent tube should not be done in advance (hours/days) because of the inherent chemical instability of ABTS in buffered substrate for extended time periods.

i. Accurate timings of washing and reaction steps should be performed.

j. Assure that all surfaces of the white immunostick-paddle and cap are adequately washed during the two timed wash steps.

k. Do not use hot or warm water for immunostick-paddle washing, only cold.

l. Since all reactions of this solid phase immunoassay occur on the surfaces of the white immunostick-paddle, it is very important not to touch the paddle surface with fingers or any other physical objects which might interfere with the immunoreaction.

m. When performing different species tests simultaneously on the same sample, be sure to maintain the proper continuity of color coded reagent tubes for each respective test species as you complete the test procedure. (eg. An anti-beef species immunostick (red color code) that has reacted with a beef sample extract if improperly placed in an anti-pork enzyme conjugate reagent tube (yellow color code), will produce a false negative result).

21.26 Technical Assistance

If any problems should arise during the performance of this species screen test or technical assistance is required on any aspect of the procedure, contact the following:

Dr. Richard P. Mageau
Microbiology Staff Officer
USDA, FSIS, OPHS, MD, EMIB
Washington, DC 20250
Telephone (202) 501-7600

21.27 Selected References

Anonymous. 1991. Commercial Immunostick Raw Meat Species Screening Kits; product insert instruction pamphlet.

Fukal, L. 1991. Review Article. Modern immunoassays in meat-product analysis. Die Nahrung 35(5):431-448.

PART C

21.3 (Confirmation) Agar Gel Immunodiffusion Test

21.31 Introduction

The final determination (confirmation) of an adulterant species of animal tissue in raw meat and poultry products is based upon the results of sample analysis by the agar-gel double immunodiffusion procedure presented in Part C. All presumptive positive violative results from the analytical methods in Part A or B are subjected to confirmation by agar-gel immunodiffusion before definitive compliance or legal actions are undertaken.

The agar-gel immunodiffusion procedure described in this section is based upon fundamental principles established previously by Ouchterlony, 1968, and modified for specific application and Agency use by Fugate and Penn, 1971. Agar-gel immunodiffusion is notable for it's qualitative ability to demonstrate similarities and resolve differences in related proteins based upon the formation of specific immunoprecipitin lines resulting from the diffusion of specific antigens and antibodies from wells or troughs cut into an agar matrix after they have reached their optimum proportions. As such, this procedure is ideally suited for meat species protein identification. In addition to being relatively easy to perform and providing results within a 24 hour period, the procedure also has the advantage of generally not being affected by the same factors which tend to produce false positive reactions in other immunoassays such as the Ring Precipitin test. If any false or "non specific" reactions should occur in a double immunodiffusion assay, it is possible to distinguish them from true positive reactions by carefully observing the immunoprecipitin pattern formed and it's relationship to known antigen extracts. The three basic types of reactions usually observed in double immunodiffusion assays are lines of identity, lines of partial identity and lines of non-identity. With a little practice and experience these types of reactions can be easily distinguished and their interpretation in relation to resolving the identity and/or relationships of similar proteins can be made in a definitive and reliable manner.

Although several different patterns of wells or troughs may be generally used in an agar-gel to perform double immunodiffusion reactions, the pattern ultimately employed is usually dependent upon the intended, specific application of the assay. Hvass, 1985, used a relatively simple, common, 7 well , circular pattern to differentiate raw meat species, while Fugate and Penn, 1971, used a

more complicated pattern consisting of 3 antisera troughs and 24 antigen extract wells. The latter was designed with the intention of demonstrating relationships among more than one species on a single plate and also to provide several identical reaction areas on the same plate showing the identity or non-identity relationship of an unknown meat species sample with known reference species tissue extracts. The concept of demonstrating several areas of identical results using several positive and negative controls within the same single reaction system provides almost irrefutable evidence in a court of law when applying this already well recognized immunodiffusion procedure to establish identity of a meat species in a case of fraudulent adulteration.

21.32 Equipment and Materials

a. Dish, Petri, plastic, 15 X 100 mm disposable
b. Pipettes, disposable, capillary, Pasteur type
c. Box, plastic, humidity chamber, or other air tight container used to maintain high humidity.
d. Cutter, agar-gel, or template pattern
e. Flask, side arm
f. Tubing, rubber or neoprene, high vacuum type
g. Tubing, brass (Cork borer), 5/32 x 1-3/4 inch (3.95 x 44.5 mm)
h. Applicators, wooden, cotton tipped
i. Pipettes, graduated, serological, assorted sizes
j. Dishes, staining (only if agar is to be dried and stained)
k. Slides, microscope, 1 x 3 inch (2.54 x 7.62 cm);(only if agar is to be dried and stained)
l. Filter paper, Whatman No. 1 and No. 42
m. Pans, plastic, 6 x 12 x 6 inch (15.2 x 30.5 x 15.2 cm), or other suitable containers (used only if agar is to be air dried and stained).
n. Assorted laboratory flasks, beakers, tubes, etc.

Clean all glassware, rinse in distilled water and heat a minimum of two hours at 200°C in a dry heat oven to eliminate contamination from prior use.

21.33 Reagents

a. Normal saline, (0.85 percent sodium chloride solution):

Dissolve 8.5 g NaCl in 1000 ml distilled water.

b. Buffered saline (0.85 percent sodium chloride solution, pH 7.2 phosphate buffered):

To 1000 ml normal saline, add 1.25 ml stock phosphate buffer solution. Adjust pH to 7.2 if required.

c. Phosphate buffer stock solution - pH 7.2:

Dissolve 34 g monobasic potassium phosphate (KH_2PO_4) in 500 ml distilled water. Adjust pH to 7.2 with 1 normal sodium hydroxide (NaOH), (requires approximately 175 ml). Dilute to 1000 ml with distilled water. Store under refrigeration.

d. Agar, 1.0 percent (Oxoid Purified Agar, L28):

To 99 ml buffered saline, add 1.0 g purified agar. Heat with constant stirring until agar is melted. Filter hot agar through glass wool or several thicknesses of cheese cloth.

Dispense into screw cap flasks or tubes and sterilize by autoclaving for 20 minutes at 15 pounds pressure. Cool agar to 49-50°C and add 1.0 ml of stock merthiolate solution (1:100) per 100 ml melted agar, to give a final concentration of 1:10,000. Tighten caps (airtight) and store until needed. Remelt when needed in boiling water bath. (Agar can be stored for extended periods of time if caps are airtight and no desiccation or growth occurs).

e. Tissue extracts from known animal species:

Cut muscle tissue collected from animals (known species) into 10 g portions and freeze until needed. To 10 g of ground or finely diced tissue, add 30 ml normal saline and stomach for specified times as shown in Table 1 Section 21.17. Let stand a minimum of 90 minutes. Decant liquid and filter through Whatman No. 42 filter paper. Use immediately. (note Section Quality Control of key reagents or procedures).

f. Antisera:

Undiluted anti-horse, beef, pork, sheep, chicken and turkey species serum, or others as may be required.

g. Tissue extract-(unknown samples to be confirmed):

Extract unknown tissue(s) as in (e) above, using 25 g tissue and 75 ml normal saline.

h. Staining solution:

Dissolve 2 g acid fuchsin in 500 ml absolute methyl alcohol; add 400 ml distilled water and 100 ml glacial acetic acid.

i. Destaining solution:

To 500 ml absolute methyl alcohol, add 400 ml distilled water and 100 ml glacial acetic acid.

j. Acidified Distilled Water:

To 1000 ml distilled water, add 0.2 ml glacial acetic acid.

k. Mounting fluid:

A commercially available material for mounting cover slips permanently.

21.34 Preparation of Agar-Gel Immunodiffusion Plates

a. Agar Plate Preparation.

Remelt purified agar prepared above and dispense 18-20 ml into the 15 x 100 mm plastic petri dishes. Allow to solidify and refrigerate for a minimum of 30 minutes. Store no more than 2 weeks under refrigeration in a high humidity atmosphere. Do not use plates showing desiccation or microbial growth. (Note: Quality Control Section 21.35)

b. Cutting Pattern of Wells and Troughs.

Remove the plates from refrigeration and cut the desired pattern by one of the two methods described below:

i. Use a gel cutting tool which has the proper well and trough cutting tubes and knife edges permanently

embedded in a fixture such as plexiglas or other solid substance.

Figure 1 illustrates one such tool. Align the tool carefully on the agar surface to obtain a perpendicular cut, then press down firmly to cut the agar.

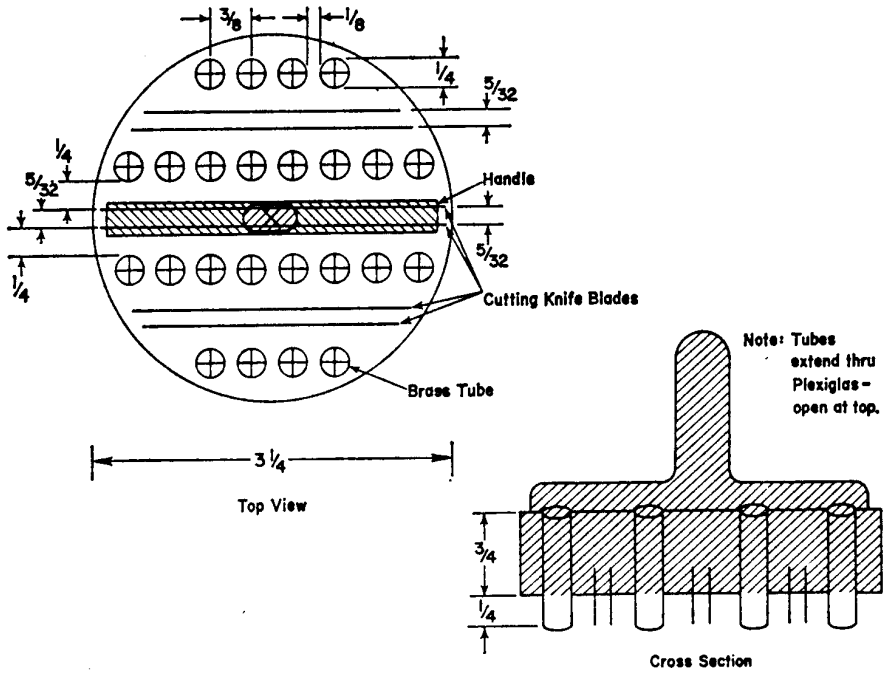

FIG. 1 - Cutting tool used to cut pattern of wells and troughs in agar-gel. (Fugate and Penn, 1971)

ii. Using a pattern of the desired arrangement drawn on graph paper, center the plate over the pattern, agar side up. Press a metal tube of acceptable diameter, connected to a vacuum source by a vacuum tube and side arm flask, through the agar at the indicated places on the pattern. Then cut the troughs with a razor blade or scalpel along the lines of the pattern; or use a tool fashioned with two blades or

knife edges the correct distance apart, and with a downward motion cut the agar.

Remove the agar plugs in the wells with a metal tube connected to a vacuum source. Experience will dictate how to avoid tearing the agar surrounding the wells. Remove the trough plugs with an applicator stick which has one end shaved to present a shovel edge. Gently push the applicator stick to the dish bottom and guide it along the cut, raising the strip of agar as a plow would.

Remove the remaining agar in the wells and troughs with a cotton tipped applicator very carefully so as to not tear the surrounding agar surface.

c. Sealing Wells and Troughs

Hold the plate at a 45^{o} angle and, with a Pasteur pipette, place a thin layer of agar on the floor of each well and trough, sealing the bottom edges of the cut agar to the plate. Do not add an excess of agar. Repair torn wells or troughs in a similar way; if necessary, refill the well or trough and recut it. Caution: An overfilled well will distort the agar and the reaction bands.

d. Preparation of Tissue Extracts: (Protein antigens)

Using the desired known animal species muscle tissue, prepare saline extracts as described in Reagents Section 21.33 e. and g. Do the same for unknown tissue that is to be analyzed. (Note: Quality Control, Section 21.35)

e. Charging the Wells

Mark the outside of the plate to identify the location and contents of each well and trough. Using a Pasteur capillary pipette, partially fill the wells with the known and unknown extracts, maintaining a concave meniscus. Overfilling to form a convex meniscus will interfere with diffusion and may cause wells to overflow. Always place the extract of the unknown between known antigens of two different species. Like antigens will form continuous reactant bands in the agar media, and unlike antigens will form discontinuous bands (See Figure 2).

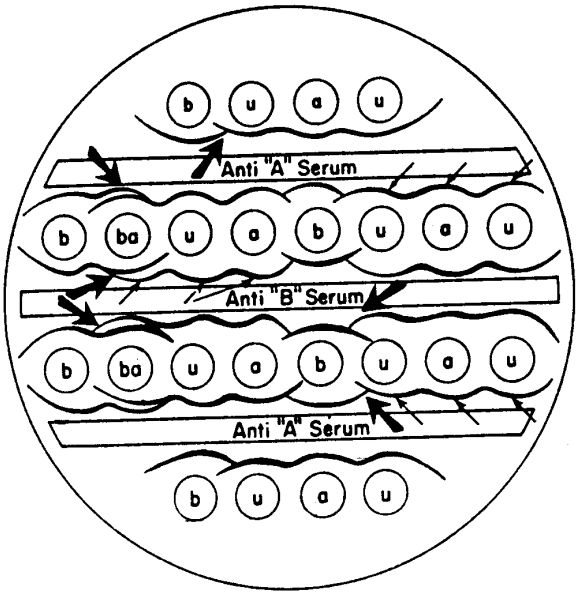

FIG. 2 - Precipitin pattern resulting from heterologous antigen-antisera reactions: a, antigens derived from species A; b, antigens derived from species B; u, antigens derived from unknown; ⇒ , lines of partial identity; → , lines of identity. Although atypical, the above pattern results when all antigens react with antisera used. The identification of unknown antigen u is accomplished by lines of identity formed with antigen a. Both a and u form lines of partial identity with lines formed by antigen b, which is indicated by a spur reaction. It can be concluded that antigen u is derived from species A and is similar but not identical to species B. (Fugate and Penn, 1971)

f. Charging of Troughs:

Fill troughs with the antisera. Use one plate to determine two species only. (e.g., beef and sheep, or beef and horse, etc.). Use the top and bottom troughs for one antiserum, and the center trough for the other. (See Figure 2).

g. Incubation and Observation:

Replace the plate covers and allow the plates to remain at room temperature for 1 1/2 to 2 h. Refill the wells and troughs with the appropriate antigens and antisera. Line the bottom of an airtight chamber with wet filter paper or cotton. Incubate the plates in this high humidity chamber at room temperature for 18 to 24 h. To read the plates, direct a light source parallel to the agar surface, i.e., from the side of the plate, and hold the plate over a dark black background. The reactant bands will appear white on a grey surface. If the bands are not fully developed, refill the wells and troughs, and continue incubation in the chamber for an additional 24 to 48 h under refrigeration.

Following incubation, remove the plates from the humidity chamber, discard the remaining reactants and gently wash the plates under a stream of distilled water. Use a soft cotton applicator to remove any film from the agar surface and precipitated matter from the wells and troughs. Dry the bottom of the petri dish with a soft laboratory tissue and observe the plate for reaction bands. Position the plate in alignment with the worksheet (Figure 3) and draw the reaction bands observed on the plate onto the worksheet.

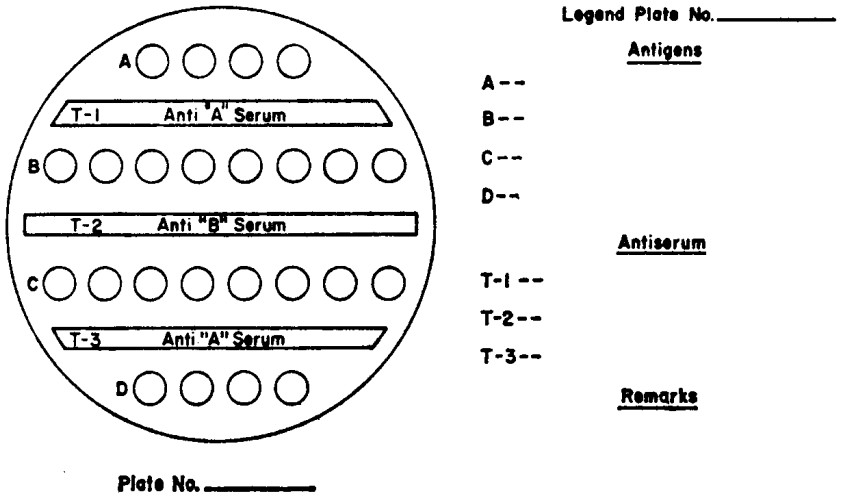

FIG. 3 - Worksheet showing well and trough arrangement and antigen-antisera placement. (Fugate and Penn, 1971)

h. Interpretation of Precipitin Reactions

Interpretation of results depends upon lines formed with known and unknown antigens. Figure 4 (A) illustrates an identity line, i.e., the precipitin line that forms when the antigens are identical. Figure 4 (B) shows partial identity lines, i.e., the lines that form when extracts contain similar but not identical proteins which react with the same antiserum. Figure 2 (page 21-26) illustrates a typical reaction with an unknown and 2 known antigens, showing lines of identity and partial identity. Since unknown antigen u forms a continuous wave pattern with known antigen a, lines of identity form. The lines formed by known antigen b appear as spurs of those formed by antigen a and u, and are typical lines of partial identity.

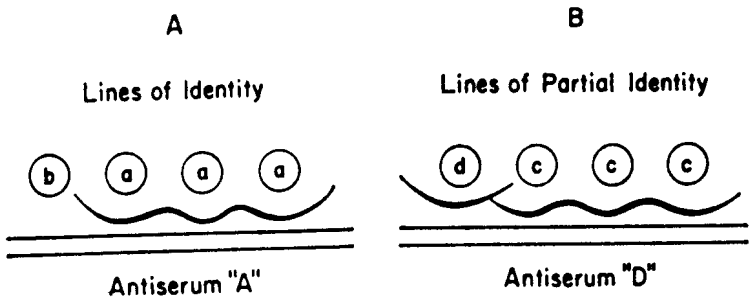

FIG. 4 - Precipitin lines of identity and partial identity. A, lines of identity formed with homologous antigen-antiserum only (antigen a vs. antiserum A); B. lines of partial identity formed when similar antigens react with the same antiserum. Note the typical spur formed, indicating lines of partial identity. (Antigen c and d are similar but not identical). (Fugate and Penn, 1971).

Figure 2 also illustrates the pattern of precipitin lines formed when the sample contains tissue antigens from 2 species (wells ba). In the majority of cases, the antisera will not react with heterologous antigens and lines of partial identity do not form. This occurs when the animal species are closely related (such as bovine and ovine).

Figure 5 illustrates areas containing identical antigen alignment. Four of the 6 areas have antigens reacting with antiserum A and 2 of the 4 areas are in position to react with antiserum B. The 2 remaining areas (2 and 4) are control as well as indicative sites. The mixtures of antigens a and b in wells marked ba are in position to react with both antisera and illustrate precipitin lines that occur when the sample contains tissues from both species.

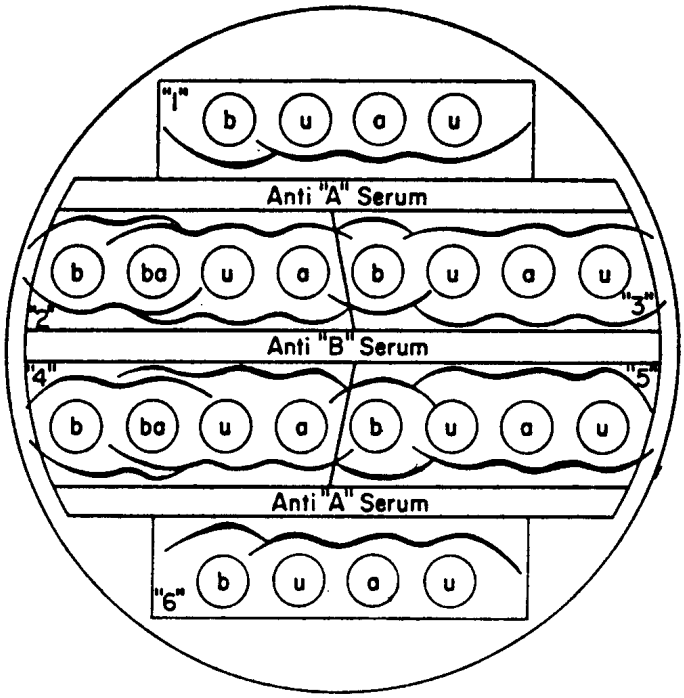

FIG. 5 - Position and reaction sites (6 areas) each consisting of 4 antigen wells. With the exception of areas 2 and 4, antigen placement is identical in each area. Areas 2 and 4 utilize one well each for a mixture of the 2 known antigens (ba), and illustrate precipitin reactions when sample consists of tissues from both species. All areas, except 1 and 6, are positioned to react with both antisera. Interpretation of results from areas 1, 3, 5, and 6 should correlate. Lines enclosing areas indicate portion of plate mounted on slides for preservation. (Fugate and Penn, 1971)

i. Staining Reaction Bands

To keep a permanent record, dialyze to remove free proteins and salts, then dry, stain, and prepare a mount under a cover slip, as follows:

Flood the plate with 500 to 1000 ml pH 7.2 buffered saline in a plastic pan. Replace with fresh buffer twice daily for three days, then once daily for two more days. Finally replace with acidified distilled water and let stand overnight.

Drain off the acidified distilled water, and cut a block of the reaction areas from the agar, and place it onto a 1 x 3 inch (2.54 x 7.62 cm) marked glass slide. Cover the block with a strip of filter paper, and dry in the incubator to a very thin film. Wash gently with a cotton applicator wetted with distilled water to remove adhering bits of the filter paper. Stain the films in acid fuchsin staining solution for 10 minutes. Remove the excess stain and rinse in destaining solution for a period of 15-20 minutes using 2-3 changes, until the agar is clear. Allow the slides to dry, then mount under cover slips with mounting fluid.

j. Photographic Recording of Reaction Bands

One of the easiest methods to obtain a permanent record of the immunodiffusion reaction is to photograph the entire unstained plate. Although there are many ways to achieve this, one of the easiest and quickest is to use a Cordis Immunodiffusion Camera. This is an instrument with preset optics, light source and Polaroid Camera which uses Polaroid Type 084 or 107 black and white film packs. The plate is placed in the instrument, the shutter is tripped, the film tab is pulled from the camera and within 25 seconds an excellent quality black and white print of the immunodiffusion reaction is produced.

21.35 Quality Control Procedures

a. Tissue Extracts from Known Species:

It is extremely important to establish the authenticity of these reference tissues before they are used, since

the basis for the types of immunodiffusion reactions obtained with unknown tissue extracts in the agar gel immunodiffusion test depends upon the use of known species tissue extracts.

b. Prepared Agar Gel Immunodiffusion Plates:

It is usually convenient to prepare a large number of plates at one time for future needs. Care must be taken to prevent deterioration of these plates during storage in the refrigerator. It has been found most useful to stack about 10 plates together in double or triple, air tight, tightly sealed plastic bags. Any plates showing microbial contamination, desiccation, or salt crystal formation should not be used as they will adversely effect the formation of immunoprecipitin lines.

c. The Specific Anti-species Sera: Sera used in the immunodiffusion procedure should always be initially checked for their proper reactivity against known, authentic reference tissues prior to their routine use as a diagnostic reagent.

21.36 Selected References

Fugate, H. G., and S. R. Penn. 1971. Immunodiffusion technique for the identification of animal species. J. Assoc. Off. Anal. Chem. 54:1152-1156.

Hvass, A. 1985. Species differentiation in minced meat products by immunodiffusion, p. 53-64. *In* R. L. S. Patterson (ed.), Biochemical Identification of Meat Species. Elsevier Science Publishing Co., Inc., New York, NY.

Ouchterlony, O. 1968. Handbook of Immunodiffusion and Immunoelectrophoresis. Ann Arbor Science Publishers, Inc., Ann Arbor, MI.

CHAPTER 32. DETECTION AND IDENTIFICATION OF EXTRANEOUS MATERIAL IN MEAT AND POULTRY PRODUCTS.

Mark M. Wheeler and James G. Eye

32.1 General Introduction

Food Safety and Inspection Service (FSIS) is responsible for insuring that the meat and poultry products offered to the consumer are safe, wholesome, unadulterated and truthfully labeled. In fulfilling this responsibility, the Agency's laboratories perform sanitation analyses of the meat and poultry products including investigations for extraneous or foreign materials. According to law, a meat or poultry product is adulterated if it consists in whole or in part of any filthy substance, is for any reason unsound or unwholesome, or if the product was prepared or packed under unsanitary conditions where it may have been contaminated [21 United States Code 601(m)(3)(4), 21 United States Code 453(g)(3)(4)].

Extraneous material is defined as any foreign material found in a food product and associated with objectionable conditions or practices in production, storage, or distribution. Examples of extraneous materials are: filth, metal, glass, sand, wood, paper or plastic. Filth is defined as any objectionable matter contributed by animal contamination of a product such as: rodent, insect or bird matter; or objectionable material contributed by unsanitary conditions. The presence of extraneous material in a food product is not only unappealing but represents a breakdown in good manufacturing practices and could pose a serious health hazard to the consumer. The isolation and identification of extraneous materials sometimes yields evidence that a product was stored or processed under unsanitary conditions and is unfit as human food.

The study of extraneous materials found in food is called Microanalytical Entomology. The U.S. Food and Drug Administration (FDA) and the Association of Official Analytical Chemists (AOAC) have published reference articles, books and methods on this subject. These publications discuss methods of analyses, contaminant identification, and contaminant significance.

This chapter contains the methods developed and used by FSIS Entomology and Extraneous Materials Laboratories (EEML) to isolate and to identify extraneous materials from meat and poultry products. These methods are intended for the stated product and

contaminant. Before using one of these methods on a different product or for a different contaminant, the method must be thoroughly evaluated for that purpose. Aside from the methods developed in our laboratories, FSIS EEMLs use many AOAC methods.

32.2 General Quality Control and Good Laboratory Practices for the Entomology and Extraneous Materials Laboratory

For extraneous materials analyses it is of the utmost importance to maintain a clean and contaminant-free laboratory. All possible action must be taken to prevent the contamination of the sample with insects or extraneous materials. Below are listed general practices and techniques which must be observed in the Entomology and Extraneous Materials Laboratories to insure a quality analysis.

32.21 Equipment and Reagents:

a. Sieves

i. Each analyst should be assigned a sieve. The analyst is responsible for maintaining his/her sieve. The sieve should be cleaned immediately after using it to prevent debris from drying on the sieve.

ii. As specified by the AOAC the #230 sieve should be a plain weave, not a twill weave.

iii. Before beginning an analysis, the sieve should be examined for rips and tears. Small tears can be mended with a drop of solder and will not affect the usefulness of the sieve. Sieves with tears and holes should not be used.

iv. The sieve should be backwashed by spraying water through the bottom of the sieve to remove any debris in the sieve.

v. Annual Cleaning - The sieves should be cleaned once a year (more often if needed) by the following procedures:

(1) Soaked in a 5% aqueous pancreatin suspension, at pH 8.2-8.5 for 4-5 h at 37-40^{o}C.

(2) Soaked in a 10% EDTA (tetrasodium ethylenediaminetetraacetic acid) for 2-3 h at 40-50°C.

(3) Soaked in a 10% NaOH (w/v) for 2-3 h at 80-90°C.

b. Magnetic Stirrers

i. Magnetic stirrers should be stored in a clean plastic container with lid. This should protect the stirrer from picking up metal fragments while not in use. The interior of this container should be kept clean.

ii. Magnetic stirring bars can be cleaned by removing the large particles with forceps and small filings by soaking in a "aqua regia"† solution (a 1:3 mixture of nitric acid and hydrochloric acids).

c. Filter Paper

Filter paper should be stored in a container that will protect it from extraneous materials contamination. A petri dish or a small plastic sandwich container with a tight fitting lid would be ideal. Of course, this precaution is worthless if the analyst does not replace the lid and leaves the filter paper container on the lab bench uncovered for extended periods of time. As with the container for the magnetic stirrers, the container for the filter paper must be kept clean.

d. Laboratory

The entomologists, technicians and aides will routinely:

i. Wipe the lab bench and the work area with a damp sponge before beginning an analysis.

ii. Clean the lab and the microscope room thoroughly at least once a month. This should include wiping down all benches, table tops and tops of any refrigerators or ovens, and cleaning or vacuuming all window sills. DO NOT clean up the laboratory while analyzing samples.

e. Glassware

i. Avoid use of plastic beakers, funnels, graduated cylinders, etc. because insect fragments and hairs adhere to plastics.

ii. After cleaning glassware allow it to dry in an inverted position. Store glassware inverted or cover the opening with aluminum foil. When it is not possible to store the glassware inverted or to cover it, the analyst should rinse the glassware with water prior to use.

f. Trap Rods

Clean the trap rod with soap and water after use.

g. Balances

i. All balances should be inspected and serviced by a trained service technician once a year.

ii. Every month the lab analyst should clean the exterior of the balance, level and check the accuracy of the balance with a 50 g calibration weight.

h. Microscopes

i. All microscopes should be inspected and serviced by a trained service technician on a yearly basis.

ii. Each analyst should be assigned a microscope and will be responsible for the daily maintenance of that instrument. The analyst will clean the exterior surface of the microscope, the eyepieces and the illuminators.

i. Reagents

i. Before mixing reagents, be sure to clean the top of the reagent bottles to prevent contaminants from falling into the solution.

ii. Rinse out carboys before preparing solutions.

iii. Label reagents with the "date prepared" and the "expiration date" (if the later is applicable).

iv. Request the "Certification of Analysis" for chemicals, such as paraffin oils.

j. Sample Handling Procedures

When opening the sample container maintain control of the closure mechanism. Remove rubber bands from bags. Do not cut or otherwise break rubber bands. Remove the staples from bags and paperwork. Do not pull open bags sealed with staples or rubber bands.

32.22 Laboratory Quality Control

a. Air Quality

A petri dish with filter paper wetted with glycerin should be left exposed for 24 h in the laboratory to detect any air borne contaminants. Place these petri dishes on the lab bench, in the fume hood, and near a window. Examine microscopically at 30X. Perform once a week. Record the results of this examination in a bound "Quality Control Notebook".

b. Water Quality

Sample the tap water (hot and cold) by running the water through a #230 sieve for 15 minutes. Wash the trappings from the sieve on to filter paper and examine microscopically at 30X. Perform this analysis once a week. Record the results in a bound "Quality Control Notebook".

c. Hairs and Fibers

The analyst should prepare microscope slide mounts of their head, arm, and eyebrow hair and be able to recognize their own hair from other hair. Include a slide mount with facial hair, if applicable. Analyst should prepare microscope slide mounts of fibers from personal clothes which have a loose knit and could fall into a product. All of these slides should be maintained in the lab as a record.

Part A

32.3 DETECTION OF LIGHT FILTH IN PREPARED INFANT FOODS CONTAINING MEAT AND POULTRY

Mark M. Wheeler and Barbara Bennett

32.31 Introduction

The presence of any objectionable animal material in a food product is defined as filth. Oleophilic filth is defined as light filth. Examples of light filth include insects, insect fragments, hairs, and feather barbules. These adulterants can be detected in a food product by separating them from the food in the oil phase of a oil/aqueous mixture.

The methods described here isolate insect fragments and rodent hairs from prepared baby food containing meat or poultry. Bovine hairs and feathers can also be recovered from the pure meat and poultry. The product is digested in a hydrochloric acid solution and the solubilized material is washed through a #230 sieve. In a pure meat/poultry product, the meat tissue is totally digested and can be washed through the sieve. The material remaining on the sieve can be transferred directly to filter paper. In the baby food dinners, meat products combined with cereals or vegetables, the plant material is not completely digested and thus does not pass through the sieve. In this case, a light filth flotation using paraffin oil is necessary to separate the filth material from the plant material. This flotation step will provide cleaner filter paper, thus easier and more accurate enumeration of the light filth.

32.32 Equipment:

a. Laboratory Balance,
b. Beaker, 2 L
c. Beaker, 600 ml
d. Wildman trap flask, 1 L
e. Hot plate, magnetic stirring
f. Sieve, stainless steel, U.S. Standard No. 230
g. Magnetic stirrer bar, teflon coated (1 X 5 cm)
h. Buchner funnel
i. Vacuum Pump
j. Watch Glass for a 2 L beaker
k. Petri Dish (2), 100 X 10 mm

l. Filter Paper, S&S #8 Ruled
m. Stereoscopic Microscope, 10 - 30X
n. Trap Rod
o. Aerator, local hardware store
p. 1 L graduated cylinder
q. 50 ml graduated cylinder
r. 25 ml graduated cylinder

32.33 Reagents

a. Igepal CO-730 (nonionic detergent, active at low pH) available through GAF Corporation, 140 W. 51st St. NY, NY 10020
b. Concentrated Hydrochloric acid (HCl) †
c. Tergitol #4, Sigma Chemical Co.
d. 40% isopropanol in filtered, distilled water.
e. Paraffin oil (Saybolt viscosity 125/135) Sargent - Welch
f. Glycerin/Ethanol mixture (vol:vol 1:1)
g. Sodium Bicarbonate

32.34 Procedure for Meat and Poultry

a. Preparation - Wash the exterior of the jar, particularly around the lid, to remove any contaminants which may be drawn into the jar upon opening.

b. Quantitatively transfer contents of one jar strained or one jar junior infant food to a two liter beaker with distilled water. Be sure to rinse the inside of the lid into the beaker.

c. Bring volume to around 800 ml with distilled water.

d. Add 5 ml Igepal CO-730 and 45 ml concentrated HCl with stirring. Cover with watch glass.

e. Bring to a boil and boil for 30 minutes.

f. Transfer the hot mixture to a 230 mesh sieve and wash with a forcible stream of hot aerated tap water until washings are clear and acid is removed. Wash the remaining material to one side of sieve. Retain the washings in a pan to neutralize at a later time with sodium bicarbonate.

g. Add 2 ml of "Tergitol #4" to contents of sieve and wash with forcible stream of hot aerated water, until foaming subsides. Wash all the remaining residue to one side of sieve.

h. Wash the contents of the sieve onto lined filter paper in a buchner funnel with isopropyl alcohol. Wash down the sides of the filter paper. Aspirate the paper to near dryness.

i. Add a small amount of glycerine/ethanol (32.33 f) to a petri dish. Using forceps, remove the filter paper from the buchner funnel and place in the petri dish.

j. Examine microscopically at 30X. (See Section 32.36)

32.35 Procedure for Baby Food Dinners

a. As an initial preparation, wash the exterior of the jar, particularly around the lid, to remove any contaminants which may be drawn into the jar upon opening.

b. Quantitatively transfer contents of one jar strained or one jar junior infant food to a two liter beaker with distilled water. Be sure to rinse the inside of the lid into the beaker.

c. Bring volume to around 800 ml with distilled water.

d. Add 5 ml of Igepal CO-730 and 45 ml of concentrated HCl with stirring. Cover with watch glass.

e. Bring to a boil and boil for 30 minutes.

f. Transfer the hot mixture to a 230 mesh sieve and wash with a forcible stream of hot aerated water until washings are clear and acid is removed. Wash the remaining material to one side of sieve. Retain the washings in a pan to neutralize at a later time.

g. Add 2 ml of Tergitol #4 to contents of sieve and wash with a forcible stream of hot water, until foaming subsides. Wash the remaining material to one side of sieve.

h. Transfer contents quantitatively to a 1 L Trap Flask with 40% isopropanol.

i. Bring volume of the liquid in the flask to 500 ml with 40% isopropanol.

j. Bring to a boil and continue simmering boil for 5 minutes with magnetic stirring.

k. Remove from heat and let stand for 1 minute. Insert trap rod into flask. With disc held just below liquid surface slowly add paraffin oil (29.33 e) by pouring it slowly down the trap rod.

l. Stir magnetically for 3 minutes at a speed sufficient to draw a vortex to the stirring bar without splashing and without introducing air into the liquid.

m. Allow the mixture to stand for 1 minute.

n. Fill the flask to the neck with 40% isopropanol by pouring slowly down the trap rod with the disc just below the oil layer.

o. Allow the mixture to stand for 20 minutes. Resuspend the material at the bottom of the flask by turning the flask in a clock-wise or counter clock-wise direction on the bench at 5 and 10 minutes to release any trapped oil, taking care not to disturb the oil layer.

p. Trap off the oil layer into a 600 ml beaker. With 40% isopropanol, rinse the neck of flask and stem of trap rod and pour rinsings into same beaker. Repeat rinsing procedure as necessary.

q. Pour the contents of the above beaker on to lined filter paper in a buchner funnel. Rinse the beaker with 100% isopropanol until all the oil is gone.

r. Wet a petri dish with a small amount of glycerine/ethanol and place the filter paper on this dish.

s. Examine microscopically at 30X. (See Section 32.36)

t. Add 25 ml of paraffin oil to the flask.

u. Slowly push the oil into the aqueous phase with the trap rod. Continue to slowly plunge up and down, about 1/2 the height of the flask, for 1 minute. Be careful not to introduce any air into the liquid.

v. Fill the flask up to the neck with 40% isopropanol and let stand for 15 minutes. Turn the flask at 5 and 10 minute intervals to release any trapped oil drops.

w. Trap off the oil layer into a 600 ml beaker. With 100% isopropanol, rinse the neck of flask and stem of trap rod and pour rinsings into the same beaker. Repeat rinsing procedure as necessary.

x. Continue as in Steps q & r. Examine at 30X. (See Section 32.36)

32.36 Results

The lined filter paper should be examined line by line at 30X magnification. Identify and count any hairs and insect fragments observed. Report the following:

- whole or equivalent insects (adults, pupae, maggots,
- larvae, cast skins)
- insect fragments, identified
- insect fragments, unidentified
- aphids, scale insects, mites, spiders, psocids, thrips,
- etc. and fragments of the above.
- rodent hairs (state the length of the hairs)

32.37 Quality Control

See Section 32.22

32.38 Safety Caution

† Do not dispose of hazardous waste by pouring down sink drains. Collect in separate containers and dispose of this waste according to standard waste management procedures for your laboratory.

Use caution when working with hydrochloric, other acids and strong bases. Wear goggles and gloves to protect eyes and skin when preparing the solution and when moving and wet

sieving the sample. Digest and wet sieve samples under a safety hood.

Part B

32.4 DETECTION AND IDENTIFICATION OF EXTRANEOUS MATERIALS IN NON-MEAT FOOD INGREDIENTS - MACROSCOPIC EXAMINATION

James G. Eye and Mark M. Wheeler

32.41 Introduction

A food is considered adulterated if "it consists in whole or in part" of any filth or decomposed substance or if the food is "otherwise unfit for human food." Extraneous materials detected in the ingredients indicate the product was prepared under unsanitary condition where it may have become contaminated. The presence of extraneous materials in the product ingredients would render the final product adulterated. The purpose of this procedure is to presumptively determine the presence of rodent excreta, insects, insect webbing, mold and other extraneous materials in the dry non-meat food ingredient. This method is intended as a screening procedure. A vast majority of samples analyzed by this procedure will be free of extraneous materials.

This method will allow for prompt examination of samples by all FSIS laboratories and insure that compliant samples are reported promptly to the operating inspectors. This procedure will also reserve the analytical time the analyst has for the smaller number of non-compliant samples that will require more time consuming analyses. This method is recommended only for screening; all positives or apparently violative samples are to be confirmed by AOAC or other accepted microscopic methods.

32.42 Terms and Concepts

The following terms are used in the macroscopic examination and reporting results:

Thrus: Any material going through the sieve.

Overs: Any material remaining on the sieve after sieving.

Animal Contaminated: Any material showing animal excreta or evidence of rodent or other animal chewing or gnawing.

Insect Infested: Any non-meat ingredient that contains live or

dead insects, webbing, excreta, or definite evidence of insect feeding.

Miscellaneous Extraneous Material: Includes stones, dirt, wire, string, non-toxic foreign seeds, etc.

Moldy: A product bearing any evidence of mold.

Rodent Excreta: Excretory pellets of the black rat (*Rattus rattus*), Norway rat (*Rattus norvegicus*) or the house mouse (*Mus musculus*), or pieces/fragments thereof, as determined by the presence of murine rodent hairs in the matrix of the fecal material.

Other Animal Excreta: Any excretory product, other than rodent, as identified by microscopic examination.

Whole Insects: Includes an adult insect, a pupa, a larva, or a major portion thereof.

32.43 Equipment:

a. Jones Riffle Sampler (8261-C10 Arthur H. Thomas)
b. Balance, Top-loading, 1 kg capacity
c. Balance, analytical, 500 g capacity
d. Sieves, U.S. Standard Series (4-881 Fisher Scientific Co.) 3 1/2 through 20
e. Magnifier-Lamp (L6039-2 Scientific Products) (LUXO-LFM2FE)
f. Trays, Cutting, (62686-363, VWR Scientific)

32.44 Procedure

32.441 Examination of Ground Spices

a. Mix sample received by passing through a riffle sampler 4 times, recombining separations before each pass.

b. Separate approximately 200 g of sample and weigh.

NOTE: Retain excess sample for use in confirmatory analysis, if needed.

c. Sift sample portion-wise through a #20 sieve, retain "thrus".

d. Transfer "overs" to a cutting tray and spread evenly so that all material can be observed.

e. If sufficient material is present to preclude spreading, place material on one side of tray and move portion-wise to middle.

f. Examine the middle portion at 3 to 5 magnifications with strong, even light. (A bench-top magnifier-lamp is suitable for this purpose).

g. Note and identify (if possible) all categories of extraneous material observed.

h. If confirmatory analysis is needed, place "thrus" and "overs" into a plastic bag along with the excess sample.

i. Send all portions for confirmatory analysis to an advanced reference laboratory, if available and necessary.

j. A written report of the extraneous material observed should accompany samples submitted for confirmation.

32.442 Examination of Whole Spices, Seeds, and Large Flake Leafy Spices

a. Mix sample received by passing through a riffle sampler 4 times, recombining separations before each pass.

b. Separate approximately (200 g for whole spices and seeds; 50 g for leafy spices and herbs).

c. Sift sample portion wise through a sieve of such size that more of the whole spices are retained as "overs".

NOTE: The sieve should never be of a smaller opening size than "Tyler Standard #8" or USA Standard 2.36 mm".

d. Transfer both "thrus" and "overs" separately on to a cutting tray and spread evenly so that all material can be observed.

e. Examine both portions at 3 to 5X magnification under strong, even light. (A bench-top magnifier lamp is suitable for this purpose).

NOTE: If sufficient material is present to preclude obtaining a single layer, place all material on one side of the tray and move portion-wise to middle and examine.

f. Note and identify (if possible) insects, insect damage, other filth and extraneous material observed.

g. If confirmatory analysis is needed place "thrus" and "overs" into a plastic bag along with the excess sample.

h. Send all sample portions for confirmatory analysis to an advanced reference laboratory, if available and necessary.

i. A written report of the extraneous material observed should accompany samples submitted for confirmation.

32.45 Reporting Results

a. Identify all categories of extraneous materials observed and record the quantity in each category.

b. To report the results as "percent extraneous material by weight", transfer the extraneous material to a tared dish and weigh.

Use the following formula to calculate the percentage:

$$\text{Percent}(\%) = \frac{\text{extraneous material (gm) in category X 100}}{\text{Sample Weight}}$$

32.46 Criteria for Confirmatory Analysis

These criteria are presented as internal guidelines to assist the analyst trained for macroscopic analysis in determining whether or not a sample should be subjected to a more extensive examination. Each type of contamination observed should be considered both on its own sanitation significance and in conjunction with other observations reported by the field or seen by the analyst.

Any sample exhibiting the following characteristics must be confirmed by analysts trained in more sensitive microscopic or chemical analytical techniques:

a. Any sample showing evidence of active or current infestation with insects and/or other animal contamination.

b. Any sample of whole seeds, herbs or other spice material that exhibits evidence of mold and/or insect damage.

c. Any sample that appears to contain in excess of 0.5% by weight of any non-hazardous extraneous material (stones, soil and non-toxic seeds).

d. Any sample appearing to contain animal excreta, including insect excreta identified during macroscopic examination.

32.47 Quality Control and Quality Assurance

See Section 32.22

Part C

32.5 DETECTION OF GLASS AND NON-ALUMINUM METALS IN MEAT AND POULTRY PRODUCTS

Mark M. Wheeler

32.51 Introduction

Meat and poultry products are exposed to a wide variety of materials during processing and packaging. Due to faulty processing, breakage in machinery or improper handling, pieces of the processing equipment or packaging material can be introduced into the finished product. The presence of extraneous materials in a finished product may pose a serious health risk to all consumers.

This method provides a fast, simple, and reliable means for isolating glass or metal contaminants from meat and poultry products. The sample is digested in an alkaline solution. The glass and non-aluminum metals are unaffected by the digestion. These contaminants are separated from other undigested material in a brine solution.

The laboratory equipment used in the analysis will depend on the type of contamination. When the suspected contaminant is glass, use of laboratory glassware in analysis must be avoided. Similarly, when the contaminant is suspected to be a metal, use of metal utensils and containers should be avoided. This will serve to protect the integrity of the sample during analysis.

32.52 Reagents and Material

32.521 Reagents

a. 7% Alcoholic Potassium Hydroxide (KOH) †

Dissolve 7 g of KOH in 100 ml of 95% Ethyl Alcohol

NOTE: KOH pellets can be used.

b. Sodium Chloride (NaCl) Solution

Prepare 2 L of NaCl solution at room temperature by adding 300 g of NaCl/L of distilled water.

c. Tergitol #4

d. Glycerol/Ethanol Mixture (vol:vol 1:1)

32.522 Materials

32.5221 For Metallic Contaminants

a. Heavyweight Plastic Picnic knives and forks or 40 lb.test Monofilament
b. Magnetic Stirring Hot Plate and Magnetic Bar (AOAC XIV 44.002 n)
c. #230 Sieve (AOAC XIV 44.002 r)
d. Filter Paper (AOAC XIV 44.002 i)
e. Hirsch Funnel with Screen (AOAC XIV 44.002 k)
f. 2 L Beaker, glass
g. 600 ml "tall" beaker, i.e. Pyrex #1060
h. 2 L Graduated cylinder
i. Watch Glass for a 2 L beaker

32.5222 For Glass Contaminants

The equipment is the same as above except do not use glassware in analysis and substitute with the following for glass beakers:

a. Stainless Steel Beaker with 2 L capacity
b. Reusable Plastic Beaker with 600 ml capacity (Nalgene Polypropylene #1201)
c. Polypropylene Graduated Cylinder
d. Plastic Basin to cover 2 L beaker, ie. Nalgene #69010040

32.53 Procedure

a. Cut sample to be digested into 1" x 1" pieces to facilitate the digestion process (Use plastic utensils or monofilament if examining for suspected metal contamination).

b. Weigh 225 g sample into a 2 L beaker.

c. Add 1.5 L 7% Alcoholic KOH. (see Section 32.56, Safety Caution)

d. Cover with a watch glass. Heat to a boil while stirring on a stirring hot plate until the sample is completely

digested. (Approximately 1 h). Initially, it will be difficult to stir the sample magnetically but after 10 min at medium to low heat the sample will be sufficiently digested to permit magnetic stirring.

e. Transfer sample to a No. 230 sieve. Apply a moderately forceful stream of hot water to push digested residues through sieve. Retain washings in a pan for hazardous chemical disposal.

f. If there is only a little residue present, transfer this directly to filter paper and examine microscopically.

g. If large amounts of undigested material remain, add 2 ml of Tergitol to help solubilize remaining residues. Repeat washing until suds subside. Transfer sample to a "tall" 600 ml beaker with distilled water.

h. Add 400 ml NaCl solution.

i. Wait 30 seconds, then pour off suspended material. Be careful not to disturb or pour off material on bottom.

j. Repeat steps h and i.

k. Wash the material remaining on bottom of beaker onto ruled filter paper with distilled water and examine microscopically.

NOTE: Check the magnetic stirring bar for metal contamination.

32.531 Procedure for Index Sample

a. If an index sample of the contaminant is available, put a portion of the index sample in the 7% Alcoholic KOH Solution. (see Section 32.56 Safety Caution)

b. Bring the solution to a boil and examine the index sample noting any chemical reaction it may have undergone.

c. Repeat the boiling and examine again. If the sample reacts with the solution, do not use an alkaline digestion. Use an acid or enzymatic digestion instead.

32.54 Result

The lined filter paper should be examined line by line at 30X magnification. Report the following:

a. Metal

- **Count the number of pieces of metal recovered.**
- **Record the size or the number of contaminants within a size range.**
- **Provide a general description of the contaminants recovered. Note the shape, thickness, color or discoloration, magnetism, and surface markings.**

b. Glass

- **Count the number of fragments recovered.**
- **Record the size or the number of contaminants within a size range.**
- **Provide a general description of the contaminants. Note the presence or absence of the following characteristics: very thin, cube shaped, mold markings, rounded edges, smooth curved surfaces, color.**
- **Examine suspected fragments under polarized light to determine if they are isotropic.**

32.55 Quality Control

See Section 32.22

a. In step 32.53 e, be sure to wash the heavy contaminants from the bottom of the beaker to the sieve. Heavy contaminants settle quickly to the bottom of the beaker and an ample stream of water is needed to wash them from the beaker. The beaker should be inverted over the sieve and the material in the beaker should be washed into the sieve with a gentle stream of water.

b. Check the magnetic stirring bar for small magnetic filings before beginning analysis.

32.56 Safety Caution

† Do not dispose of hazardous waste by pouring down sink drains. Collect in separate containers and dispose of as

hazardous waste as per standard waste management procedures for your laboratory.

Use caution when working with potassium hydroxide. Wear goggles and gloves to protect eyes and skin when preparing the solution and when moving and wet sieving the sample. Digest and wet sieve samples under a safety hood.

Part D

32.6 METHOD FOR THE ISOLATION OF GLASS FROM PREPARED MEAT AND POULTRY BABY FOODS

Mark M. Wheeler

32.61 Introduction

The recommended procedure for isolating glass from a food product is the heavy sediment procedure for that product. There is no heavy sediment procedure for meat/poultry baby food products. The method outlined below was developed in response to the need for a standard procedure for isolating glass from meat/poultry baby food products.

Bottled food can become contaminated with glass in a number of ways. The container may already be contaminated when it arrives at the food processors. The finished food product may become contaminated by glass breakage during processing. Containers can break during storage, shipping, retail, and consumer handling and fragments from broken containers can contaminate the exterior of other containers. If these exterior contaminants are in or around the jar opening, they could contaminate the product when the jar is opened.

This method is quick and easy. The sample is washed in a #60 sieve. The bulk of the sample is washed through the sieve. The remaining material is transferred to a beaker and mixed with a brine solution. In a brine solution the heavy contaminants, such as glass, settle to the bottom of the beaker. The brine solution and the suspended food material are poured off and discarded. These two steps, adding and pouring off the brine solution, are repeated three times. The heavy contaminants remaining on the bottom of the beaker are washed on to a filter paper which is examined microscopically. This isolation procedure takes less than 15 minutes. Suspect particles must be tested to confirm that they are in fact glass.

To protect the integrity of the sample, no glass should be used in any part of this method. After adding the brine solution to the plastic beaker containing the sample, any glass fragments will settle to the bottom within seconds. The longer the settling step, the more food material settles to the bottom which creates dirty

plates. Ten seconds is plenty of time for glass fragments to settle to the bottom.

32.62 Reagents

a. Sodium Chloride

32.63 Equipment

NOTE: DO NOT USE ANY GLASS APPARATUS DURING THE ANALYSIS.

a. Plastic Beaker w/ 600 ml capacity, ie. Nalgene #1201
b. Plastic Beaker w/ 2 L capacity, ie. Nalgene #1201
c. Plastic Basin to cover 2 L beaker, ie. Nalgene #69010040
d. Plastic Graduated Cylinder
e. #60 Sieve (AOAC 16.1.01(B)(r) 16th Ed.)
f. #230 Sieve (AOAC 16.1.01(B)(r) 16th Ed.)
g. Magnetic Stirrer and Bar (AOAC 16.1.01(B)(n) 16th Ed.)
h. Ruled Filter Paper (AOAC 16.1.01(B)(i) 16th Ed.)
i. Disposable Petri Dish (100 X 10 mm)
j. Lab Spatula
k. Hirsch Funnel
l. Side arm trap flask connected to vacuum pump
m. Laboratory Balance, 1 kg capacity
n. Compound Microscope with polarizer
o. Aerator, Water (AOAC 16.1.01(B)(a) 16th Ed.)

32.64 Reagent Preparation

a. Sodium Chloride Solution (300 gm/L)

Add 2 L of distilled water to 2 L plastic beaker. Add 600 g of NaCl while magnetically stirring. The above recommended plastic beaker will accommodate 2 L of salt solution. Cover beaker with plastic basin and continue stirring until NaCl is completely dissolved.

32.65 Procedure

32.651 Cleaning of Exterior of Sample Container

a. Thoroughly rinse exterior of jar and around lid on to a #230 sieve using hot water. Use the spatula to clean food residues from lid and jar threads. This step is included to be sure no glass is on exterior of the jar.

b. Wet filter paper with water and center it in Hirsch funnel. Turn on vacuum and fit filter paper to sides of funnel.

c. Transfer material on the sieve to filter paper with distilled water. Do not aspirate the paper to dryness otherwise the glass fragments will "pop" off the paper. If needed, wet the paper with a drop or two of water. The paper should be moist enough so that it adheres to the petri dish but it should not be soaked. If the paper is too wet, the water will hide small, flat pieces of glass.

d. Transfer filter paper to petri dish and examine paper microscopically for glass fragments.

e. Confirm any suspect particles using a compound microscope with polarized light.

f. Count, measure and describe all glass fragments found on the exterior of container. Report any particles of glass as contaminants found on the exterior of the sample container. Report number of fragments found within a size range. Fragments less than 1 mm can be reported as " Less than 1 mm." An excessive number of fragments can be reported as "Too Numerous To Count."

32.652 Sample Analysis

a. Quantitatively transfer contents of jar to #60 sieve. A spatula can be used to remove the bulk of the sample. Use water from a squirt bottle to thoroughly rinse interior of jar. Retain jar for further examination at step l.

b. Thoroughly wash sample in sieve with hot aerated water. When no more material passes thru sieve, wash remaining material to one side of sieve.

c. Quantitatively transfer contents of sieve to a plastic beaker w/ distilled water. Use no more than 200 ml of water.

d. Dilute to 400 ml with NaCl solution.

e. Let material settle 10 seconds, then pour off suspended material. More than 10 seconds is not needed. Glass will settle to bottom in 10 seconds. Waiting longer than 10 seconds allows more food material to settle to the bottom. Be careful not to disturb or pour off residues on bottom.

f. Repeat Steps d & e to remove excess plant material, usually twice more.

g. Wet filter paper with water and center it in Hirsch funnel. Turn on vacuum and fit filter paper to sides of funnel.

h. Wash residues remaining on bottom of beaker to ruled filter paper. Do not aspirate the paper to dryness otherwise the glass fragment will "pop" off the paper. If needed, wet the paper with a drop or two of water. The paper should be moist enough so that the paper adheres to the petri dish but the paper should not be soaked. If the paper is too wet, the water will hide small, flat pieces of glass.

i. Transfer paper to petri dish and examine microscopically.

j. Count, measure, and describe all glass fragments found in the food product. Report number of fragments found within a size range. Fragments less than 1 mm can be reported as " Less than 1 mm." An excessive number of fragments can be reported as "Too Numerous To Count."

k. Confirm any suspected particles using a compound microscope and polarized light. Glass is an isotropic compound and will not transmit crossed polar light. Sand or quartz are birefringent, thus will transmit crossed polar light.

l. Examine all retail sample jars for chips, fractures, or other defects if any glass fragments are found within product.

m. Maintain in reserve all glass fragments and all jars from which glass fragments were removed.

32.66 Characterization of Contaminants

If classification or comparison of glass contaminants is needed to identify a possible source, determine the refractive index of the glass contaminants.

32.67 Quality Control

See Section 32.22

a. Do not use any glassware in this analysis.

b. Before beginning an analysis, wipe down or wash the entire work area.

c. Rinse the beakers and graduated cylinder before using them.

d. Backwash the sieve by spraying water through the bottom to remove any debris in the sieve before using it.

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CHAPTER 33. DETECTION OF ANTIMICROBIAL RESIDUES IN MEAT AND POULTRY TISSUE BY SCREEN TESTS

B. P. Dey, Clarence A. White, Richard H. Reamer and Nitin H. Thaker

33.1 Introduction

Rapid microbiological screen tests are used in slaughter establishments to detect the presence of antimicrobial residues in food animal tissues. The Swab Test on Premises (STOP) is used for all red meat species except bob veal calves, where the Calf Antibiotic and Sulfa Test (CAST) is used. The Fast Antimicrobial Screen Test (FAST) developed recently and tested on bovine tissue, has been found to have greater sensitivity than STOP and CAST. The test is being conducted in bovine slaughter establishments on a limited basis. The FAST procedure is presently being tested in swine.

These microbial inhibition tests are simple to perform, cost effective and allow routine testing and release of large numbers of food animal carcasses in the shortest possible time. Use of these screen tests permit FSIS to analyze only those carcasses which were found to contain antimicrobial compounds by in-plant tests.

PART A

33.2 DETECTION OF ANTIMICROBIAL RESIDUES BY SWAB TEST ON PREMISES (STOP)

Clarence A. White, B. P. Dey and Richard H. Reamer

33.21 Background

The Swab Test on Premises (STOP) was developed for tentative detection of antimicrobial residues in carcasses. It is performed by inserting a sterile cotton swab into the kidney sample of a carcass. After 30 minutes, the tissue fluid soaked top, one-fourth portion of the swab is transferred to an agar plate seeded with *Bacillus subtilis* spores. After incubation for 16-18 h at 29°C, plates are examined for a zone of inhibition (ZI) around the swab. If no inhibition is seen, the carcass is free of antimicrobial residue at detectable levels. In case of inhibition, presence of antimicrobial residues is suspected and muscle, liver and kidney tissues from the suspect carcass are collected and submitted for confirmation and identification at FSIS laboratories.
In 1980, a modified version of the original test was introduced in slaughter establishments. The agar plates and vials of spores are

separately supplied. In the modified version, prior to performing the test, the plates are surface streaked with the spore suspension by sterile swabs. The rest of the procedure is similar to the original test. The supplies are now commercially available and are stable for 6 months when stored at either room or refrigerated temperatures.

Initially when the test was developed, it used tissues from kidney, liver, muscle, and injection site. However, at present kidney is the target tissue. The sensitivity of the STOP test for sulfonamide detection is unsuitable for regulatory purposes.

33.22 Equipment, Reagents and Supplies

33.221 Equipment

a. Laminar Flow Hood or equivalent clean room
b. Sorvall RC5C Refrigerated Centrifuge, Sorvall Rotor SS-34, and Sorvall Swinging Bucket Rotor HB-4 or equivalent. Centrifuge must be able to operate at 20,000 x G at a constant 5°C. It should also operate with a swinging bucket rotor at 1,500 x G at room temperature or equivalent.
c. Virtis homogenizer, Model 60K or equivalent
d. Sterile Virtis jars
e. Vortex mixer or equivalent
f. Incubators, one maintaining 37°C and the other 29°C
g. Precision water bath (48 ± 1°C) with cover (Model 183) or equivalent
h. Quebec Colony Counter or equivalent
i. Fisher-Lilly Antibiotic Zone Reader (Fisher Scientific, Cat. No. 07-906) or equivalent

33.222 Reagents

a. Distilled water:

The distilled water must be prepared using an all glass still (Corning Megapure 6L or equivalent) and stored in a glass or any acceptable reservoir which is not a part of the system. All spore lots must be prepared using glass distilled water.

* Do not use deionized water.

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* Resins of some systems produce quaternary ammonium compounds which interfere with the analysis.

b. Phosphate buffer (3 M, pH 7.1)

Dissolve 306.9 g of K_2HPO_4 and 168.6 g KH_2PO_4 per liter of

distilled water. If necessary, adjust pH by dropwise addition of either 0.1 N HCl or NaOH depending upon pH reading. Sterilize by autoclaving at 121°C for 15 minutes or filtering through a 0.2 μm filter.

c. Ethyl alcohol (USP grade, 200 proof)

Dehydrated Alcohol, USP, Ethyl Alcohol, 200 Proof Punctilious®, (Ethyl Alcohol [Ethanol] CAS #64-17-5, Warner-Graham Company, 160 Church Lane, Cockeysville, MD 21030). For a 50% solution, mix 1 part of ethyl alcohol with 1 part glass distilled water. Prior to use, filter sterilize through a 0.2 μm filter.

d. Polyethylene glycol, Mol. Wt. 4000 (Baker Chemicals) Sterilize in a covered beaker by autoclaving prior to use.

e. Butterfield's Phosphate Buffer, sterile

33.223 Supplies

a. Sterile Roux bottles
b. Sterile glass beads, 4 mm diameter
c. Sterile 100 ml graduated glass stoppered cylinders or volumetric flasks
d. Sterile centrifuge tubes, 40 ml (Nalgene 3118 or equivalent)
e. Sterile pipettes, 10 ml and 1 ml graduated to the tip
f. Sterile, clear glass vials 51 x 15 mm with deep seated screw caps
g. Pressure sensitive labels not to exceed 2" x 1/2"
h. Acetate shrink-wrap material for sealing 51 x 15 mm glass vials or equivalent closure material
i. Forceps
j. Permanent marking pen
k. Antimicrobial sensitivity discs containing 5 mcg of the antibiotic neomycin (N5)
l. Sterile cotton swabs on hollow plastic tubes
m. Sterile, plastic 60 mm diameter X 15 mm petri plates (Falcon Cat. # 1007 or equivalent)

33.23 Media

a. Brain Heart Infusion broth (BBL or equivalent); reconstitute according to manufacturer's directions, dispense 10 ml/tube and sterilize (121°C for 15 minutes)
b. Blood agar plates (Columbia Blood Agar Base, 5% HRBC)
c. Antibiotic Agar No. 5 (Streptomycin Assay Agar)
d. Mueller-Hinton Agar

e. A-K Sporulating Agar No. 2

i. Agar slants - reconstitute A-K Sporulating Agar No. 2 according to manufacturer's directions with an extra 0.5% Purified Agar (Difco or equivalent), sterilize by autoclaving at 121°C for 15 minutes and prepare as slants.

ii. Roux bottles - add 300 ml reconstituted A-K Sporulating Agar No. 2 with an extra 0.5% Purified Agar. Sterilize (121°C for 15 minutes) and allow medium to harden in Roux bottles placed in a horizontal position.

33.24 Test Organism

Bacillus subtilis ATCC 6633 (American Type Culture Collection, Rockville, MD)

33.241 Purity and Biochemical Properties of *Bacillus subtilis*

a. Reconstitute a lyophilized culture in Brain Heart Infusion broth and incubate at 37°C for 18 h. Streak blood agar plates with the broth culture, incubate at 37°C for 18 h and check for culture purity.

b. For isolation, streak the culture onto two Columbia Agar plates with 5% defibrinated horse blood. Incubate at 37°C for 18 h.

c. Prepare a Gram stain of three well isolated colonies. All cultures should be Gram positive.

d. Stain a drop of the spore suspension with malachite green and counterstain with carbol-fuchsin solution. The spores will appear green, whereas the vegetative cells will appear red or pink.

e. Use one Columbia Agar plate with 5% defibrinated horse blood from the culture to test for the presence of catalase. *Bacillus* sp. are catalase positive.

f. Use colonies from the other plate to check biochemical characteristics of the culture by inoculating O-F glucose, Voges-Proskauer, and mannitol broths. Incubate at 35°C for 18 h. The biochemical patterns of *B. subtilis* should agree with the following chart:

Catalase	Gram stain	Spore forming	O-F glucose	Voges-Proskauer	Mannitol
+	+	+	O	+	V

(+) = positive; (-) = negative; (F) = fermentative; (O) = oxidative; (A) = acid; (V) = variable.

g. If the organism does not meet all the above criteria, replace with a new ATCC culture of the organism.

33.242 Preparation of *B. subtilis* spores

a. After the culture meets all biochemical criteria, pick several well isolated colonies from the plates and streak A-K Sporulating Agar No. 2 slants (one per Roux bottle) and incubate the slants at 37°C for 18 h.

b. To each agar slant, add 4-6 sterile glass beads and 2-3 ml sterile distilled water and gently shake for 2 minutes to dislodge bacterial growth.

c. Aseptically transfer the slant suspensions to a Roux bottle containing A-K Sporulating Agar No. 2 and spread with the help of the glass beads. Multiple cultures may be prepared and pooled for transferring.

d. Incubate the Roux bottles horizontally for 18-24 h at 37°C and then at room temperature for the remainder of 1 week (6 days).

e. Harvest the growth from the Roux bottles by adding 20-30 sterile glass beads and approximately 25 ml of sterile distilled water per bottle. Gently agitate bottles to dislodge bacterial growth. (Care must be taken not to break the agar during harvesting).

f. Aseptically transfer the bacterial suspension into sterile centrifuge tubes (40 ml volume) and heat the tubes in boiling water (100°C) for 10 minutes.

g. Wash the heated suspension three times with sterile distilled water by centrifuging and decanting in the following manner:

   i. Centrifuge at 5°C for 20 minutes at 20,000 x G.
   ii. Pour off supernatant.
   iii. Resuspend the pellet in 20 ml sterile distilled water.
   iv. Repeat Steps i, ii and iii two more times.

h. Wash and coat a Virtis jar with a mixture of sterile phosphate buffer and sterile polyethylene glycol in the following manner:

   Mix 34.1 ml of sterile phosphate buffer and 11.8 g of sterile polyethylene glycol in a 100 ml sterile glass stoppered volumetric flask and shake vigorously. Bring to volume with sterile distilled water. Pour the mixture into a Virtis jar and place the jar on the homogenizer. Blend for 5 minutes at 5,000 RPM. Discard the mixture. Repeat the process.

i. Prepare a fresh solution of sterile buffered polyethylene glycol (34.1 ml of phosphate buffer and 11.8 g of polyethylene glycol) in a 100 ml glass stoppered sterile volumetric flask. Add 25 ml of the washed spore mixture and bring to volume with distilled water. Shake vigorously. Pour the mixture into a coated Virtis jar and homogenize for 5 minutes at 5,000 RPM.

j. Dispense the mixture equally into four sterile centrifuge tubes and centrifuge in a swinging bucket rotor at 1,500 x G (3,000 RPM in H-4 Rotor in Sorvall RC5C) for 2 minutes at room temperature.

k. A two-phase system with an interface will be formed in the centrifuge tube. Being careful not to disturb or disperse the interface layer, transfer the spore containing, upper phase using a 10 ml pipette to a second set of sterile centrifuge tubes.

l. Centrifuge the tubes at 20,000 x G for 20 minutes at 5°C. Pour off the supernatant. Resuspend the pellet in each tube with 20 ml sterile distilled water and pool the contents of all tubes into a sterile container.

m. Pipette 25 ml aliquots of spore suspension into each sterile centrifuge tube. Centrifuge tubes at 20,000 x G for 20 minutes at 5°C. Repeat the process five times after decanting the supernatant and re-suspending the

pellet in 20 ml of sterile distilled water.

n. After the last wash step, resuspend each spore pellet in 20 ml 50% ethyl alcohol. Pool all spore suspensions into a sterile bottle containing 15-20 sterile glass beads. Store the stock suspension at 35-40°F (2-4.4°C). (Properly preserved stock spore suspension may be used indefinitely).

33.243 Enumeration of *B. subtilis* Spores in Working Suspension

a. To determine the number of spores/ml in each new spore stock suspension, prepare tenfold serial dilutions (10^{-2}-10^{-10}) of the suspension using Butterfield's Phosphate Buffer. (Pipet 1.0 ml of well mixed spore stock suspension (use vortex mixer) into 9 ml buffer and then make serial dilutions up to 10^{-10}.).

b. Using separate pipettes, pipette 1.0 ml of each dilution into triplicate 100 x 15 mm plates.

c. Pipette 15 ml molten Plate Count Agar (cooled to 50 ± 1°C) into each plate. Mix by swirling or tilting plates to evenly disperse the inoculum throughout the medium. Incubate for 48 h at 37 ± 1°C.

d. Count colonies (30-300) in triplicate plates on a Quebec Colony Counter. Record and average the number of colonies/ml in each dilution. Determine the number of colony forming units (cfu)/ml of the stock solution.

e. To prepare the final spore suspension at a concentration of 1 x 10^6 cfu/ml in 50% ethyl alcohol from the stock spore suspension, use the following formula:

$$\text{Concentration of stock spore suspension (cfu/ml)} = \text{Dilution factor} \times \text{Desired concentration of working spore suspension (cfu/ml)}$$

Example:

Stock spore suspension = 1 x 10^9 spores/ml
Desired concentration of working spore suspension = 1 x 10^6 spores/ml:

$$(1 \times 10^9 \text{ cfu/ml}) = (x)\ (1 \times 10^6 \text{ cfu/ml})$$

$$\frac{(1 \times 10^9 \text{ cfu/ml})}{(1 \times 10^6 \text{ cfu/ml})} = x$$

x = 1000

In this example, the stock spore suspension must be diluted 1:1000 (1 part stock spore suspension plus 999 parts diluent) in 50% ethyl alcohol to prepare the 1 x 10^6 spore/ml concentration.

33.244 *Packaging of *B. subtilis* Spore Suspension (for Field Use)

a. Dispense 4.0 ml of the final (working) spore suspension (1 x 10^6 cfu/ml in 50% ethyl alcohol) into sterile 51 x 15 mm clear, glass vials with deep seated, leak-proof screw caps.

b. After securely capping spore vials, seal with shrink-seal, or equivalent closure material, to prevent leakage or dehydration.

c. Label the vials with the following information on a transparent mylar pressure sensitive label, or equivalent:

i. "STOP spores"
ii. *B. subtilis* ATCC 6633
iii. Lot Number
iv. Packaging Date

NOTE: *B. subtilis* spores (1 x 10^6 or 1 x 10^7 cfu/ml) can also be obtained from EDITEK, Burlington, NC, by special order.

33.25 *Preparation of STOP Plates (for Field Use)

a. Add 25.5 g of Antibiotic Agar No. 5 (Streptomycin Assay Agar) powder into 1 L of glass distilled water. Heat while stirring and bring to a boil. Sterilize at 121°C for 15 min. Cool and mix the medium thoroughly in a 48°C water bath. Continue mixing during cooling and dispensing.

b. Aseptically add 6.0 ml of the agar to each 60 x 15 mm plate and distribute evenly. Place plates on a flat level surface and allow agar to harden.

*NOTE: Under FSIS contract, STOP spores (1 x 10^6 cfu/ml) and plates are now produced commercially and are routinely available for use. After they meet all quality control specifications they are used in

slaughter plants.

c. Label the lid of each plate with the following information:

i. "STOP PLATE"
ii. Lot Number
iii. Expiration Date

d. Refrigerate plates in sealed double plastic bags to prevent moisture evaporation. These plates can be used for a period of 90 days.

33.251 Preparation of STOP Plates (Used in Laboratory)

a. Add 25.5 g of Antibiotic Agar No. 5 (Streptomycin Assay Agar) powder to 1 L of distilled water. Heat while stirring and bring to a boil. Sterilize at 121°C for 15 minutes. Cool and mix the medium thoroughly in a 48°C water bath.

b. Aseptically add 1 ml of 1 x 10^7 cfu/ml *B. subtilis* spore suspension per 100 ml of the agar. Mix thoroughly. Pipette 8 ml of the agar into each 100 x 15 mm plate and tilt plates to insure even distribution. Allow the plates to harden on a flat, level surface.

c. Label the lid of each plate with the following information:

i. "STOP PLATE"
ii. Date

d. Refrigerate plates in sealed double plastic bags to prevent moisture evaporation. These plates can be used for a period of 10 working days.

33.26 Performing the STOP Test

33.261 Sample Condition

a. Assure that the samples are cold, 4°C or below.

b. Identify samples according to standard operating procedures.

NOTE: Presently STOP is used only on kidney tissue of all classes of animals, i.e., bovine, swine, sheep/goat, and horses with the exception of bob veal calves.

33.262 Procedure

a. Allow frozen samples to thaw completely at room temperature for a sufficient period of time such that ice crystals are no longer present within the sample.

b. Open a sterile cotton swab pack, remove one swab, and insert the sharp end of the swab shaft about 1/2" to 3/4" into each kidney tissue.

c. Move the swab shaft back and forth several times to macerate the tissue, disrupting tissue cells and releasing tissue fluid. Remove the swab shaft.

d. Reverse the swab and insert the cotton tip into the tissue opening, twisting to make sure that the cotton tip is in good contact with the macerated tissue.

e. Allow swabs to remain in the tissue for a minimum of 30 minutes.

f. Allow refrigerated plates to warm to room temperature for about 10 minutes before streaking. Check each plate for absence of contamination, cracking of agar or dryness.

g. Lift the plate cover slightly and mark an "X" reference mark on the outer side wall of the plate. Place the covered plate bottom side down on the work place surface with the reference mark at the 12 o' clock position. With a fine-tip permanent marking pen, start at the "x" and draw a line across the bottom of the plate dividing it into two equal sections.

h. Check for seal integrity of vials containing spores.

i. Shake the ***B. subtilis*** spore vial (1×10^6 cfu/ml) and dip a sterile swab in the solution. Gently touch the swab to the side of the vial to remove excess fluid. Replace the screw cap on the vial.

j. Streak the surface of the agar plates with the swab from a point marked on the side of the plate moving up and down and from left to right. Turn the plate 1/4 turn and streak again.

k. Repeat this streaking process 2 more times. Finally turn the plate 1/2 turn and streak. (Use a separate swab for each plate)

NOTE: Above applies only for plates used in the plant. The plates used in laboratories are seeded at a different

concentration level and therefore should not be surface streaked.

l. Place a neomycin 5 μg disc on the agar surface near the vertical line on a plate.

m. Remove the swab from the tissue, break the shaft approximately two inches from the swab end.

NOTE: If the swabs appear dry, reinsert them in the tissue and squeeze the tissue around the swab to absorb tissue fluids. For small portions of dry muscle tissue, moisten swab with distilled water prior to inserting.

n. Gently place the swab on the surface of the plate with the broken end of the shaft near the neomycin 5 μg disc making sure not to break the agar surface. Make sure the swab has uniform contact with the agar.

NOTE: Swabs from two kidney tissues from two different carcasses can be placed on each plate provided they are properly identified on the plate.

If two tissue swabs are used per plate, place cotton tips in "rabbit ears" configuration (Fig. 1)

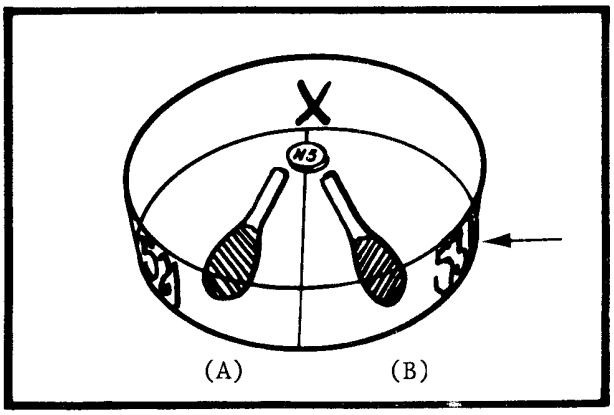

Figure 1. Swab placement on plate

o. Incubate the plates upright at 29 ± 1°C for 16-18 h.

p. Store samples under refrigeration until the test is completed.

33.27 Results and Interpretation

a. Remove the incubated plates from incubator and remove swabs.

b. Measure the ZI by the N5 disc with a mm ruler or with an antibiotic zone reader. The zone should be 20-26 mm wide. If the zone is not 20-26 mm in width, the test is inconclusive and should be repeated.

c. Observe the plates for inhibition of *B. subtilis* growth surrounding the swabs.

i. If a zone of inhibition is observed, the test is positive. Measure the length and the width of the zone and record results.

ii. If no zone of inhibition is observed, the test is negative. Record the result.

33.28 Selected References

Johnston, R. W., R. H. Reamer, E. W. Harris, H. G. Fugate, and B. Schwab. 1981. A new screening method for the detection of antibiotic residues in meat and poultry tissues. J. Food Prot. 44:828-831.

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PART B

33.3 DETECTION OF ANTIMICROBIAL RESIDUE IN CALVES BY CALF ANTIBIOTIC AND SULFONAMIDE TEST (CAST)

Clarence A. White, B. P. Dey and Richard H. Reamer

33.31 Introduction

The Calf Antibiotic and Sulfa Test (CAST) is a modified form of the Sulfa Swab Technique (SST). Sulfonamides are frequently used in bob veal calves, a class of animals weighing under 150 pounds and less than three weeks old. This test is used to detect antibiotic and sulfonamide residues in bob veal calves at slaughter.

The inspectors performing the test at slaughter plants are supplied with agar plates and vials containing an alcohol suspension of spores. To perform the test, a sterile cotton tipped applicator (swab) is inserted into the kidney sample of a bob veal calf and left for 30 minutes to absorb tissue fluids. The agar plates are surface streaked by sterile swabs with the supplied *Bacillus megaterium* spore suspension. The swab is removed from the kidney, broken as close to the cotton tip as possible, and placed on to the agar plate streaked with spores. After 16-18 h incubation at 44°C, plates are examined for a zone of inhibition (ZI) around the swab. If no inhibition is seen, the carcass is free of antimicrobial residues at a detectable level. All carcasses presenting inhibition are subjected to laboratory confirmation.

33.32 Equipment, Reagents and supplies

33.321 Equipment

a. Laminar Flow Hood or equivalent clean room
b. Sorvall RC5C Refrigerated Centrifuge, Sorvall Rotor SS-34, and Sorvall Swinging Bucket Rotor HB-4 or equivalent. Centrifuge must be able to operate at 20,000 x G at a constant 5°C. It should also operate with a swinging bucket rotor at 1,500 x G at room temperature.
c. Virtis homogenizer, Model 60K or equivalent
d. Sterile Virtis jars
e. Vortex mixer or equivalent
f. Incubators 37°C and 44 + 1°C
g. Precision water bath (temperature 48 ± 1°C) with cover (Model 183) or equivalent
h. Quebec Colony Counter or equivalent
i. Fisher-Lilly Antibiotic Zone Reader (Fisher Scientific, Cat. No. 07-906) or equivalent

33.322 Reagents

a. **Distilled water:**

The distilled water must be prepared using an all glass still (Corning Megapure 6L or equivalent) and stored in a glass or any acceptable reservoir which is not a part of the system. All spore lots must be prepared using glass distilled water. * Do not use deionized water.

b. **Phosphate buffer (3 M, pH 7.1)**

Dissolve 306.9 g of K_2HPO_4 and 168.6 g KH_2PO_4 in 1 L distilled water. If necessary, adjust pH by dropwise addition of either 0.1 N HCl or NaOH depending on pH reading. Sterilize at 121°C for 15 minutes or filtering through a 0.2 μm filter.

c. **Ethyl alcohol (USP grade, 200 proof)**

Dehydrated Alcohol, USP, Ethyl Alcohol, 200 Proof Punctilious®, (Ethyl Alcohol [Ethanol] CAS #64-17-5, Warner-Graham Company, 160 Church Lane, Cockeysville, MD 21030). For a 50% solution, mix 1 part of ethyl alcohol with 1 part glass distilled water. Prior to use, filter sterilize through a 0.2 μm filter.

d. **Polyethylene glycol, Mol. Wt. 4000 (Baker Chemicals). Sterilize (121°C for 5 minutes) in a covered beaker prior to use.**

e. **Butterfield's Phosphate Buffer, sterile**

33.323 Supplies

a. **Sterile Roux bottles**
b. **Sterile glass beads, 4 mm diameter**
c. **Sterile 100 ml graduated glass stoppered cylinders or volumetric flasks**
d. **Sterile centrifuge tubes, 40 ml (Nalgene 3118 or equivalent)**
e. **Sterile pipettes, 10 ml and 1 ml graduated to the tip**
f. **Sterile, clear glass vials 51 x 15 mm with deep seated screw caps**
g. **Pressure sensitive labels not to exceed 2" x 1/2"**
h. **Acetate shrink-wrap material for sealing 15 x 51 mm glass vials or equivalent closure material**

*** Resins of some systems produce quaternary ammonium compounds**

which interfere with the analysis.

i. Forceps
j. Permanent marking pen
k. Antibiotic discs: Neomycin - 5 μg
l. Sterile cotton swabs on hollow plastic tubes
m. Sterile, plastic 60 X 15 mm petri plates (Falcon Cat. No. 1007 or equivalent)

33.33 Media

a. Brain Heart Infusion broth (BBL or equivalent); reconstitute according to manufacturer's directions, dispense 10 ml/tube and sterilize at 121°C for 15 minutes.

b. Blood agar plates (Columbia Blood Agar Base, 5% HRBC).

c. A-K Sporulating agar No. 2.

i. Agar slants - reconstitute A-K Sporulating Agar No. 2 according to manufacturer's directions with extra 0.5% Purified Agar (Difco or equivalent), sterilize by autoclaving at 121°C for 15 minutes and prepare slants.

ii. Roux bottles - add 300 ml reconstituted A-K Sporulating Agar No. 2 with extra 0.5% purified Agar. Sterilize (121°C for 15 minutes) and allow medium to harden in Roux bottles placed in a horizontal position.

d. Mueller-Hinton Agar (Acumedia Manufacturers Inc., Baltimore, MD); reconstitute according to manufacturer's directions, dispense 100 ml/flask and sterilize (121°C for 15 minutes).

33.34 Test Organism

Bacillus megaterium ATCC 9885 (American Type Culture Collection, Rockville, MD)

33.341 Purity and Biochemical Properties of *Bacillus megaterium*

a. Reconstitute a lyophilized culture in Brain Heart Infusion broth and incubate at 37°C for 18 h. Streak blood agar plates with the broth culture, incubate at 37°C for 18 h and check for culture purity.
b. Streak the culture for isolation onto two Columbia Agar plates with 5% defibrinated horse blood. Incubate at 37°C

for 18 h.

c. Prepare a Gram stain of three well isolated colonies. All cultures should be Gram positive.

d. Stain a drop of the spore suspension with malachite green and counterstain with carbol-fuchsin solution. The spores will appear green, whereas the vegetative cells will appear red or pink.

e. Use one Columbia Agar plate with 5% defibrinated horse blood from the culture to test for presence of catalase. *Bacillus* are catalase positive.

f. Use colonies from the other plate to check biochemical characteristics of the culture by inoculating O-F glucose, Voges-Proskauer, and mannitol broths. Incubate at 35°C for 18 h.

The biochemical patterns of *B. megaterium* should agree with the following chart:

Catalase	Gram stain	Spore forming	O-F glucose	Voges-Proskauer	Mannitol
+	+	+	O	-	A

(+) = positive; (-) = negative; (F) = fermentative; (O) = oxidative; (A) = acid.

g. If the test organism does not meet all the above criteria, replace with a new ATCC culture of the test organism.

33.342 Preparation of *Bacillus megaterium* Spore Suspension

a. After the culture meets all biochemical criteria, pick several well isolated colonies from the plates and streak A-K Sporulating Agar No. 2 slants (one per Roux bottle) and incubate the slants at 37°C for 18 h.

b. After incubation, put 4-6 sterile glass beads and 2-3 ml sterile distilled water into each tube and gently shake for 2 minutes to dislodge organisms from agar slants.

c. Aseptically transfer the suspension from slants to a Roux bottle containing A-K Sporulating Agar No. 2 and spread with the help of glass beads. (Multiple cultures may be prepared and pooled for transfer to Roux

bottles).

d. Incubate the Roux bottles horizontally for 18-24 h at 37°C and then at room temperature for the remainder of 1 week (6 days).

e. Harvest the growth from the Roux bottles by the use of 20-30 sterile glass beads and approximately 25 ml of sterile distilled water per bottle. Gently agitate bottles to dislodge bacterial growth. (While harvesting care must be taken not to break the agar).

f. Aseptically transfer the bacterial suspension into sterile centrifuge tubes (40 ml volume) and heat the tubes in boiling water (100°C) for 10 minutes.

g. Wash the heated suspension three times with sterile distilled water by centrifuging and decanting in the following manner:

i. Centrifuge at 5°C for 20 minutes at 20,000 x G.
ii. Pour off supernatant.
iii. Resuspend the pellet in 20 ml sterile distilled water.
iv. Repeat Steps i, ii and iii two more times.

h. Wash and coat a Virtis jar with a mixture of sterile phosphate buffer and sterile polyethylene glycol in the following manner:

Mix 34.1 ml of sterile phosphate buffer and sterile 11.8 g of polyethylene glycol in a 100 ml glass stoppered volumetric flask and shake vigorously. Bring to volume with sterile distilled water. Pour the mixture into a Virtis jar and place the jar on the homogenizer. Blend for 5 minutes at 5,000 RPM. Discard the mixture. Repeat the process.

i. Prepare a fresh solution of sterile buffered polyethylene glycol (34.1 ml of phosphate buffer and 11.8 g of polyethylene glycol) in a 100 ml glass stoppered sterile volumetric flask. Add 25 ml of the washed spore mixture and bring to volume with sterile distilled water. Shake vigorously. Pour the mixture into a coated Virtis jar and homogenize for 5 minutes at 5,000 RPM.

j. Dispense the mixture equally into four sterile centrifuge tubes and centrifuge in a swinging bucket rotor at 1,500 x G (3,000 RPM in H-4 Rotor in Sorvall

RC5C) for 2 minutes at room temperature.

k. A two-phase system with an interface will be formed in the centrifuge tube. Being careful not to disturb or disperse the interface layer, transfer the spore containing, upper phase using a 10 ml pipette to a second set of sterile centrifuge tubes.

l. Centrifuge the tubes at 20,000 x G for 20 minutes at 5°C. Pour off the supernatant. Resuspend the pellet in each tube with 20 ml sterile distilled water and pool the contents of all tubes into a sterile container.

m. Pipette 25 ml aliquots of spore suspension into each sterile centrifuge tube. Centrifuge tubes at 20,000 x G for 20 minutes at 5°C. Repeat the process five times after decanting the supernatant and re-suspending the pellet in 20 ml of sterile distilled water.

n. After the last wash step, resuspend each spore pellet in 20 ml 50% ethyl alcohol. Pool all spore suspensions into a sterile bottle containing 15-20 sterile glass beads. Store the stock suspension at 35-40°F (2-4.4°C). (Properly preserved stock spore suspension may be used indefinitely).

33.343 Preparation of Working Spore Suspension of *B. megaterium*

a. To determine the number of spores/ml in each new spore stock suspension, prepare tenfold serial dilutions (10^{-2}-10^{-10}) of the suspension using Butterfield's Phosphate Buffer. (Pipet 1.0 ml of well mixed spore stock suspension (use vortex mixer) into 9 ml buffer and then make serial dilutions up to 10^{-10}.).

b. Using separate pipettes, pipette 1.0 ml of each dilution into triplicate 100 x 15 mm plates.

c. Pipette 15 ml molten Plate Count Agar (cooled to 48 $\pm$ 1°C) into each plate. Mix by swirling or tilting plates to disperse the inoculum evenly throughout the agar. Incubate for 48 h at 37 $\pm$ 1°C.

d. Count colonies (30-300) in triplicate plates on a Quebec Colony Counter. Record and average the number of colonies/ml for each dilution. Determine the number of colony forming units (cfu)/ml of the stock solution.

e. To prepare the final spore suspension at a concentration of 1 x 10^6 cfu/ml in 50% ethyl alcohol from the stock

spore suspension, use the following formula:

Concentration of stock spore suspension (cfu/ml)	=	Dilution factor	X	Desired concentration of working spore suspension (cfu/ml)

Example:

Stock spore suspension = 1×10^9 spores/ml
Desired concentration of working spore suspension = 1×10^6 spores/ml:

$(1 \times 10^9 \text{ cfu/ml}) = (x)\ (1 \times 10^6 \text{ cfu/ml})$

$$\frac{(1 \times 10^9 \text{ cfu/ml})}{(1 \times 10^6 \text{ cfu/ml})} = x$$

$x = 1000$

In this example, the stock spore suspension must be diluted 1:1000 (1 part stock spore suspension plus 999 parts diluent) in 50% ethyl alcohol to prepare the 1 x 10^6 spore/ml concentration.

33.344 Packaging of *B. megaterium* Spore Suspension

a. Dispense 4.0 ml of the working spore suspension (1 x 10^6 cfu/ml in 50% ethyl alcohol) into each (51 x 15 mm) clear glass vial with leak-proof screw caps.

b. After capping the vials, seal with shrink-seal, or equivalent material to prevent leakage or dehydration.

c. Label the vials with the following information on a transparent mylar pressure sensitive label, or equivalent:

i. "CAST Spores"
ii. *B. megaterium* ATCC 9885
iii. Lot Number
iv. Packaging Date

NOTE: Under FSIS contract, CAST spores are produced commercially. After these spores meet all quality control specifications they are used in slaughter plants.

33.35 Preparation of CAST Plates

a. Weigh and add 38 g of Mueller-Hinton Agar powder to 1 L distilled water. Heat while stirring and bring to a boil. Sterilize the medium at 121°C for 15 minutes and then mix it thoroughly. Allow agar to cool to 48°C in a water bath. Continue mixing during cooling and dispensing.

b. Using a sterile agar delivery system, deliver 6.0 ml of the agar to each 60 x 15 mm plate. Distribute the agar evenly to cover entire surface of the plate. Allow the agar to harden on a flat, level surface.

c. Label the lid of each plate using a label containing the following information:

   i. "CAST PLATE"
   ii. Lot Number
   iii. Expiration Date

d. Refrigerate plates in sealed double plastic bags to prevent moisture evaporation. These plates can be used for a period of 90 days.

NOTE: Under FSIS contract, CAST plates are produced commercially. After these plates meet all quality control specifications they are used in slaughter plants.

33.36 Performing the CAST Test

33.361 Sample Condition

a. Assure that the samples are received at a temperature of 4°C or below.

b. Identify samples according to standard operating procedures.

NOTE: CAST test should only be used on kidney tissue of bob veal calves.

33.362 Procedure

a. Allow frozen samples to thaw completely at room temperature for a sufficient period of time such that ice crystals are no longer present within the sample.

b. Open a sterile cotton swab pack, remove one swab, and

insert the sharp end of the swab about 1/2" to 3/4" into the kidney tissue.

c. Move the swab shaft back and forth several times to macerate the tissue, disrupting tissue cells and releasing tissue fluid. Remove the swab shaft.

d. Reverse the swab and insert the cotton tip into the tissue opening, twisting to make sure that the cotton tip is in good contact with the macerated tissue.

e. Allow swabs to remain in the tissues for a minimum of 30 minutes.

f. Allow refrigerated plates to warm to room temperature for about 10 minutes before streaking. Check each plate for absence of contamination, cracking of agar or dryness.

g. Lift the plate cover slightly and mark an "X" reference mark on the outer side wall of the plate. Place the covered plate bottom side down on the work place surface with the reference mark at 12 o' clock position. With a fine-tip permanent marking pen, start at the "x" and draw a line across the bottom of the plate dividing it into two equal sections.

h. Check for seal integrity of vials containing spores.

i. Shake the *B. megaterium* spore vial and dip a sterile swab in the solution. Gently touch the swab to the side of the vial to remove excess fluid. Replace the screw cap on the vial.

j. Streak the surface of the agar plates with the swab from a point marked on the side of the plate moving up and down and from left to right. Turn the plate 1/4 turn and streak again.

k. Repeat this streaking process 2 more times. Finally turn the plate 1/2 turn and streak. (Use a separate swab for each plate)

l. Place a neomycin 5 μg disc on the agar surface near the vertical line on a plate.

m. Remove the swab from the tissue, break the shaft approximately two inches from the swab end.

NOTE: If the swabs appear dry, reinsert them in the tissue

and squeeze the tissue around the swab to absorb tissue fluids. For small portions of dry muscle tissue, moisten swab with distilled water prior to insertion.

n. Gently place the swab on the surface of the plate with the broken end of the shaft near the neomycin 5 μg disc making sure not to break the agar surface. Make sure the swab has uniform contact with the agar.

NOTE: Swabs from two different tissues or carcasses may be placed on each plate provided they are properly identified as to location on the plate.

If two tissue swabs are used per plate, place cotton tips in a "rabbit ears" configuration (Fig. 2).

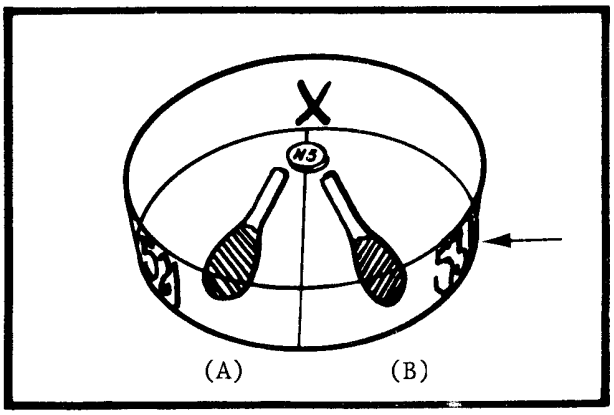

Figure 2. Swab placement on plate

o. Incubate the plates upright at 44 ± 1°C for 16-18 h.

p. Store samples under refrigeration until the test is completed.

33.363 Results and Interpretation

a. Remove plates from the incubator and remove the swabs.

b. Measure the zone of inhibition around the N5 disc with a mm ruler. The ZI should be 24-29 mm wide. If the ZI is not 24-29 mm in width, the test is inconclusive and

should be repeated.

c. Observe the plates for inhibition of *B. megaterium* growth surrounding the swabs.

i. If a zone of inhibition is observed, the test is positive. Measure the width of the zone and record results.

ii. If no zone of inhibition is observed, the test is negative. Record the result.

33.37 Selected References

Johnston, R. W., R. H. Reamer, E. W. Harris, H. G. Fugate, and B. Schwab. 1981. A new screening method for the detection of antibiotic residues in meat and poultry tissues. J. Food Prot. 44:828-831.

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PART C

33.4 TENTATIVE CONFIRMATION OF CAST RESULTS FOR SULFONAMIDE RESIDUES IN MEAT AND POULTRY TISSUE

B. P. Dey, Sandra L. Kamosa and Clarence A. White

33.41 Background

The Calf Antibiotic and Sulfa Test (CAST) is presently being used for detecting sulfonamide residues in bob veal calves. The test as performed by inspectors is as follows: a sterile cotton tipped applicator (swab) is inserted into the kidney sample of an animal and left for 30 minutes to absorb tissue fluids. A *Bacillus megaterium* spore suspension is applied to CAST agar plates by a sterile swab. The swab from the kidney is then placed on the agar plate and incubated at 44°C for 16-24 h. The plate is then examined for a zone of inhibition (ZI) around the swab. In the case of an 18 mm or greater zone of inhibition, the carcass is subjected to further laboratory analysis. The muscle, liver and kidney tissues from the suspect carcass are sent to the laboratories for analysis. This procedure describes a modified CAST method with sensitivity equal or better than commercial CAST for verifying field results in 5-6 h with inclusion of another plate for confirming the presence of sulfonamide residues in suspected samples at the same time.

33.42 Equipment, Reagents and supplies

33.421 Equipment

a. Laminar Flow Hood or equivalent clean room
b. Sorvall RC5C Refrigerated Centrifuge, Sorvall Rotor SS-34 and Sorvall Swinging Bucket Rotor HB-4 or equivalent. Must operate at 20,000 x G at a constant 5°C and also with a swinging bucket rotor at 1,500 x G at room temperature or equivalent.
c. Virtis homogenizer, Model 60K or equivalent
d. Sterile Virtis jars
e. Vortex mixer or equivalent
f. Incubators: one capable of maintaining a constant 37°C and the other 44 ± 1°C
g. Precision water bath (48 ± 1°C) with cover (Model 183) or equivalent
h. Quebec Colony Counter or equivalent
i. Fisher-Lilly Antibiotic Zone Reader (Fisher Scientific, Cat. No. 07-906)

33.422 Reagents

a. **Distilled water:**

The distilled water must be prepared using an all glass still (Corning Megapure 6L or equivalent) and stored in a glass or any acceptable reservoir which is not a part of the system. All spore lots must be prepared using glass distilled water. * Do not use deionized water.

b. **Phosphate buffer (3 M, pH 7.1)**

Dissolve 306.9 g of K_2HPO_4 and 168.6 g KH_2PO_4 in 1 L distilled water. If necessary, adjust pH by dropwise addition of 0.1 N HCl or NaOH. Sterilize at 121°C for 15 minutes or filter through a 0.2 μm filter.

c. **Ethyl alcohol (USP grade, 200 proof)**

Dehydrated Alcohol, USP, Ethyl Alcohol, 200 Proof Punctilious®, (Ethyl Alcohol [Ethanol] CAS #64-17-5, Warner-Graham Company, 160 Church Lane, Cockeysville, MD 21030). For a 50% solution, mix 1 part of ethyl alcohol with 1 part glass distilled water. Prior to use, filter sterilize through a 0.2 μm filter.

d. **Polyethylene glycol, Mol. Wt. 4000 (Baker Chemicals). Sterilize (121°C for 5 minutes) in a covered beaker prior to use.**

e. **Bromcresol Purple (0.04%) solution. Dissolve 0.1 g Bromcresol Purple dye with 18.5 ml of 0.01 N sodium hydroxide, add 231.5 ml of distilled water.**

f. **Bacto-Dextrose (Difco, Detroit, MI; Cat. No. 0156-17-4)**

g. **p-aminobenzoic acid (Fisher Scientific Co. NJ; Cat. No A-41-70522)**

h. **Butterfield's Phosphate Buffer, sterile**

33.423 Supplies

a. **Sterile Roux bottles**
b. **Sterile glass beads, 4 mm diameter**
c. **Sterile 100 ml graduated glass stoppered cylinders or volumetric flasks**

*

Resins of some systems produce quaternary ammonium compounds which interfere with the analysis.

d. Sterile centrifuge tubes, 40 ml (Nalgene 3118 or equivalent)
e. Sterile pipettes graduated to the tip, 10 and 1 ml
f. Sterile, clear glass vials 51 x 15 mm with deep seated screw caps
g. Pressure sensitive labels not to exceed 2" x 1/2"
h. Acetate shrink-wrap material for sealing 51 x 15 mm glass vials or equivalent closure material
i. Forceps
j. Permanent marking pen
k. Antibiotic discs: Neomycin - 5 μg
l. Sterile cotton swabs on hollow plastic tubes
m. Sterile, plastic 60 X 15 mm plates (Falcon Cat. # 1007 or equivalent)

33.43 Media

Proceed exactly as that described in Section 33.33.

33.44 Test Organism

Bacillus megaterium ATCC 9885 (American Type Culture Collection, Rockville, MD)

33.441 Purity and Biochemical Properties of *Bacillus megaterium*

Proceed exactly as that described in Section 33.341.

33.442 Preparation of *B. megaterium* Spore Suspension

Proceed exactly as that described in Section 33.342

33.443 Enumeration of *B. megaterium* Spores in Working Suspension

Proceed exactly as that described in Section 33.343 except prepare the final spore suspension such that it contains 1 x 10^7 cfu/ml.

33.444 Packaging of *B. megaterium* Spore Suspension

a. Dispense 4.0 ml of the working spore suspension (1 x 10^7 cfu/ml in 50% ethyl alcohol) into sterile 51 x 15 mm clear, glass vials with deep seated, leak-proof screw caps.

b. After securely capping spore vials, seal with shrink-seal, or equivalent closure material, to prevent leakage or dehydration.

c. Label the vials with the following information on a transparent mylar pressure sensitive label:

i. "CAST Spores"
ii. *B. megaterium* ATCC 9885
iii. Date

NOTE: *B. megaterium* spores (1×10^7 cfu/ml) can be obtained from EDITEK, Burlington, NC, by special order.

33.45 Preparation of Plates

33.451 Preparation of Modified CAST (M-CAST) Plates

a. Weigh and add 38 g of Mueller-Hinton Agar (Acumedia) powder to each liter of glass distilled water. Weigh and add 8 g dextrose to the mixture. Add 70 ml Bromcresol Purple solution (0.04%) to the mixture. Heat while stirring and bring to boil.

b. Cool to 48°C and adjust the pH to 7.2 ± 0.1. Sterilize at 121°C for 15 minutes and mix thoroughly. Allow the agar medium to cool to 48°C in a water bath.

c. Continue mixing during cooling.

d. Add 1 ml of *B. megaterium* spore suspension (1×10^7 cfu/ml) to every 100 ml of the medium and mix thoroughly.

e. Aseptically dispense 8 ml of the seeded agar to each 100 x 15 mm plate.

f. Distribute the agar evenly to cover entire surface of the plate. Place plates on a flat, level surface and allow the agar to harden.

g. Label the side of each plate with a marker with the following information:

i. "M-CAST PLATE"
ii. Date

h. Refrigerate plates in sealed double plastic bags to prevent moisture evaporation. These plates can be used for a period of 15 working days.

33.452 Preparation of Modified CAST Plus (M-CAST+) Plates

a. Weigh and add 38 g of Mueller-Hinton Agar (Acumedia) powder to 1 L glass distilled water. Weigh and add 8 g dextrose to the mixture. Add 70 milliliters of Bromcresol Purple solution (0.04%) to the mixture. Heat while stirring and bring to a boil.

b. Add 200 mg of p-aminobenzoic acid to the medium.

c. Cool to 48°C and adjust pH to 7.2 ± 0.1. Sterilize at 121°C for 15 minutes and mix thoroughly. Cool the medium in a 48°C water bath.

d. Continue mixing during cooling.

e. Add 1 ml of *B. megaterium* spore suspension (1 x 10^7/ml) to every 100 ml of the medium and mix thoroughly.

f. Aseptically dispense 8 ml of the seeded agar to each 100 x 15 mm plate.

g. Distribute the agar evenly to cover entire surface of the plate. Place plates on a flat surface and allow the agar to harden.

h. Label the side of each plate with a marker with the following information:

   i. "M-CAST+ PLATE"
   ii. Date

i. Refrigerate plates in sealed plastic (Ziplock®) bags to prevent moisture evaporation. These plates can be used for a period of 15 working days.

33.46 Performing the Test

33.461 Sample Condition

a. Assure that the samples are received at a temperature of 4°C or below.

b. Identify samples according to standard operating procedures.

33.462 Procedure

a. Allow frozen samples to thaw completely at room temperature for a sufficient period of time such that

ice crystals are no longer present within the sample.

b. Open a sterile cotton swab pack, remove both swabs, and insert the sharp end of the swabs shaft 1/2" to 3/4" into the kidney tissue.

c. Move the swab shafts back and forth several times to macerate the tissue, disrupting tissue cells and releasing tissue fluid. Remove the swab shafts.

d. Reverse the swabs and insert the cotton tips into the tissue opening, twisting to make sure that the cotton tip is in good contact with the macerated tissue.

e. Allow swabs to remain in the tissue for a minimum of 30 minutes.

f. Leave refrigerated plates (M-CAST and M-CAST+) at room temperature for about 20-30 minutes to warm up. Discard plates which are contaminated, dried or cracked.

g. Place a neomycin 5 μg (N5) disc and a sulfamethazine 2 μg (S2) disc on separate M-CAST and M-CAST+ plates (control plates) in use each day the test is performed. Make sure that the distance between the two discs is 35-40 mm.

h. Remove the swabs from the tissue, break the shafts approximately two inches from the swab end.

NOTE: If the swabs appear dry, reinsert them in the tissue and squeeze the tissue around the swab to absorb tissue fluids. For small portions of dry muscle tissue, moisten swab with distilled water prior to insertion.

i. Gently place one of the swabs on an M-CAST plate and the other swab on an M-CAST+ plate making sure not to break the agar surface. Make sure the swab has uniform contact with the agar.

NOTE: Properly identified, four (4) swabs from 4 samples can be placed on each plate provided the cotton tip end of one lies next to the shaft of another as shown in Fig. 1.

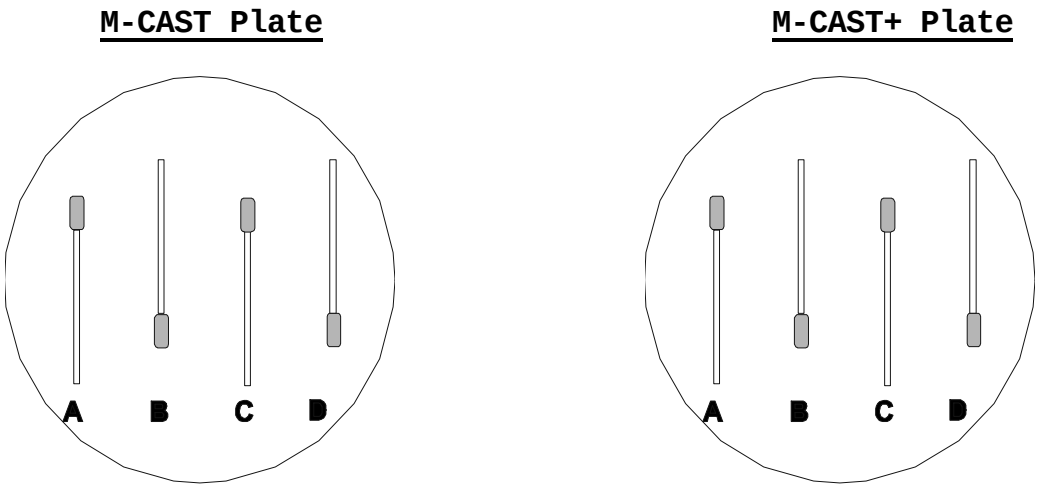


Fig. 1 . Placement of swabs on M-CAST and M-CAST+ plate

j. Incubate plates with sample swabs and the control discs (N5 and S2) upright at 44 ± 1°C for 5-6 h.

k. Refrigerate sample until the test is complete.

33.47 Results and Interpretation

a. Remove the plates from incubator and remove swabs.

b. Measure the ZI around the N5 and S2 discs on the control plates with a mm ruler or by a zone reader. The N5 zone should measure between 20-26 mm on both M-CAST and M-CAST+ plates. There should be a 16-19 mm zone by the S2 disc on the M-CAST plate only, where as there will be no zone by the S2 disc on the M-CAST+ plate. If the observed ZI are not in agreement with the above, repeat the test.

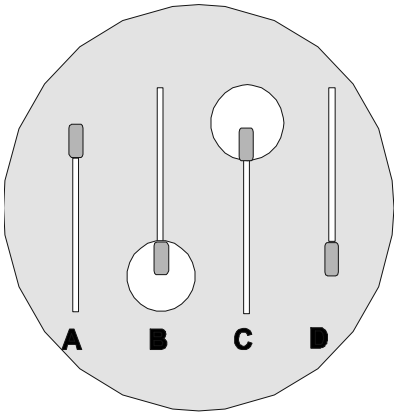
c. Measure the zone of inhibition surrounding each swab corresponding to a sample on each plate (from right to left).

NOTE: It is essential to read test results within 6 h. As the inhibitory effect by bacteriostatic drugs such as sulfonamides diminishes, organisms temporarily inhibited recover over time causing reduction in the zone of inhibition as incubation time increases.

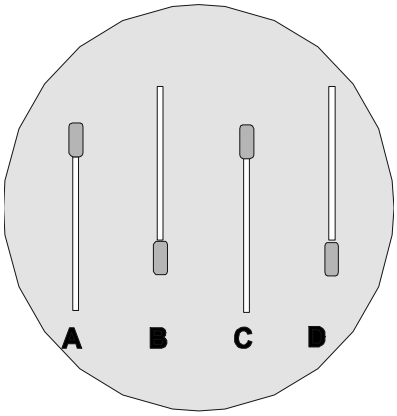
d. i. Samples with sulfonamide residue appear as illustrated below:

M-CAST plate: Zone of inhibition (Samples B and C)
M-CAST+ plate: No zone of Inhibition (Samples B and C)

M-CAST Plate



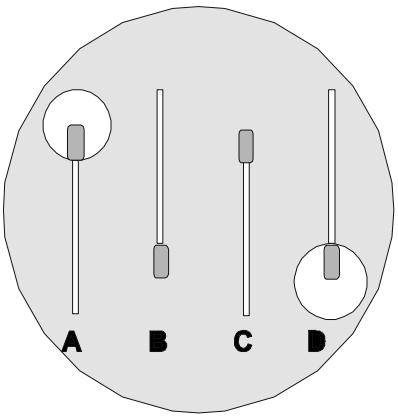
M-CAST+ Plate



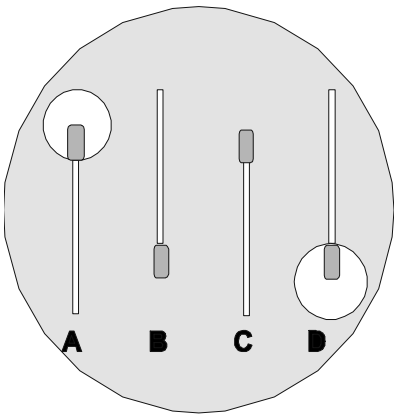
ii. Samples free of sulfonamide residue, but containing antibiotics, appear as illustrated below:

M-CAST plate: Zone of inhibition (Samples A and D)
M-CAST+ plate: Zone of Inhibition (Samples A and D)

M-CAST Plate



M-CAST+ Plate



33.48 Quality Control

a. Test organism must be evaluated for purity and proper biochemical patterns.

b. Freshly prepared plates must be tested with the N5 and S2 discs to assure proper performance.

c. Plates must not be used for more than 15 working days past preparation.

d. Extreme caution should be taken in adding para-amino benzoic acid because the chemical at a higher concentration than the recommended level is toxic to the test organism.

e. New chemicals/reagents and agar should be checked to assure quality.

33.49 Selected References

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PART D

33.5 DETECTION OF ANTIMICROBIAL RESIDUE BY FAST ANTIMICROBIAL SCREEN TEST (FAST)

B. P. Dey, Clarence A. White and Nitin H. Thaker

33.51 Introduction

The Fast Antimicrobial Screen Test (FAST), an in-plant screen test, was developed in 1989 to improve the capability of the Antibiotic Residue Detection Program. FAST has higher sensitivity and can detect a wider range of antibiotics and sulfonamides than STOP and CAST. The test has been introduced in 50 bovine slaughter establishments. It is also being evaluated in swine species. If found suitable in both bovine and swine, it may be used in all species of food animals for detecting antimicrobial residues. Besides improving efficiency, this test would be used uniformly for detecting antibiotic and sulfonamide residues in food animal carcasses.

The test as performed by inspectors is as follows: a sterile cotton tipped applicator (swab) is inserted into the kidney sample of an animal and left for 30 minutes to absorb tissue fluids. The agar plates are surface streaked with *Bacillus megaterium* spore suspension on a sterile cotton swab. The swab from the kidney is removed, broken as close to the cotton tip as possible, and placed onto the agar plate and incubated at 44°C. The plate is examined for a zone of inhibition (ZI) around the swab at 6 and 18 h. In the case of inhibition at 6 h, the plate is further examined at 18 h for confirmation. If there is clear inhibition, muscle, liver and kidney tissues from the suspect carcass are collected and further analyzed for confirmation at an FSIS laboratory. When no inhibition is seen at 6 h, the carcass is free of antimicrobial residues at detectable levels. The test allows screening and releasing a large number of residue free carcasses within a work shift.

33.52 Equipment, Reagents and Supplies

33.521 Equipment

a. Laminar Flow Hood or equivalent clean room
b. Sorvall RC5C Refrigerated Centrifuge, Sorvall Rotor SS-34 and Sorvall Swinging Bucket Rotor HB-4 or equivalent. Must operate at 20,000 x G at a constant 5°C and also with a swinging bucket rotor at 1,500 x G at room temperature or equivalent.
c. Virtis homogenizer, Model 60K or equivalent

d. Sterile Virtis jars
e. Vortex mixer or equivalent
f. Incubators: one capable of maintaining a constant 35°C and the other at 44 ± 0.5°C
g. Precision water bath (with cover (Model 183) or equivalent
h. Quebec Colony Counter or equivalent
i. Fisher-Lilly Antibiotic Zone Reader (Fisher Cat.# 07-906)

33.522 Reagents

a. Distilled water:

The distilled water must be prepared using an all glass still (Corning Megapure 6L or equivalent) and stored in a glass or any acceptable reservoir which is not a part of the system. All spore lots must be prepared using glass distilled water. * Do not use deionized water.

b. Phosphate buffer (3 M, pH 7.1)

Dissolve 306.9 g of K_2HPO_4 and 168.6 g KH_2PO_4 in 1 L distilled water. If necessary, adjust pH by dropwise addition of 0.1 N HCl or NaOH. Sterilize at 121°C for 15 minutes or filtering through a 0.2 μm filter.

c. Ethyl alcohol (USP grade, 200 proof)

Dehydrated Alcohol, USP, Ethyl Alcohol, 200 Proof Punctilious®, (Ethyl Alcohol [Ethanol] CAS #64-17-5, Warner-Graham Company, 160 Church Lane, Cockeysville, MD 21030). For a 50% solution, mix 1 part of ethyl alcohol with 1 part glass distilled water. Prior to use, filter sterilize through a 0.2 μm filter.

d. Polyethylene glycol, Mol. Wt. 4000 (Baker Chemicals). Sterilize (121°C for 5 minutes) in a covered beaker prior to use.

e. Bromcresol Purple (0.04%) solution. Dissolve 0.1 g Bromcresol Purple dye with 18.5 ml of 0.01 N sodium hydroxide, add 231.5 ml of distilled water.

f. Bacto-Dextrose (Difco, Detroit, MI; Cat. # 0156-17-4) or equivalent

g. Butterfield's Phosphate Buffer, sterile

* Resins of some systems produce quaternary ammonium compounds which interfere with the analysis.

33.523 Supplies

a. Sterile Roux bottles
b. Sterile glass beads, 4 mm diameter
c. Sterile 100 ml graduated glass stoppered cylinders or volumetric flasks
d. Sterile centrifuge tubes, 40 ml (Nalgene 3118 or equivalent)
e. Sterile pipettes, 10 ml and 1 ml graduated to the tip
f. Sterile, clear glass vials 51 x 15 mm with deep seated screw caps
g. Pressure sensitive labels not to exceed 2" x 1/2"
h. Acetate shrink-wrap material for sealing 15 x 51 mm glass vials or equivalent closure material
i. Forceps
j. Permanent marking pen
k. Antibiotic discs: Neomycin - 5 μg
l. Sterile cotton swabs on hollow plastic tubes
m. Sterile, plastic 60 X 15 mm plates (Falcon Cat. No. 1007 or equivalent)

33.524 Media

a. Brain Heart Infusion broth (BBL or equivalent); reconstitute according to manufacturer's directions, dispense 10 ml/tube and sterilize (121°C for 15 min).

b. Blood agar plates (Columbia Blood Agar Base, 5% HRBC).

c. A-K Sporulating Agar No. 2.

i. Agar slants - reconstitute A-K Sporulating Agar No. 2 according to manufacturer's directions with extra 0.5% purified Agar (Difco or equivalent), sterilize by autoclaving at 121°C for 15 minutes and prepare slants.

ii. Roux bottles - add 300 ml reconstituted A-K Sporulating Agar No. 2 with extra 0.5% purified agar. Sterilize (121°C for 15 minutes) and allow medium to harden in Roux bottles placed in a horizontal position.

d. Mueller-Hinton Agar (Acumedia Manufacturers Inc., Baltimore, MD); reconstitute according to manufacturer's

directions, dispense as desired and sterilize (121°C for 15 minutes).

33.53 Test Organism

Bacillus megaterium ATCC 9885 (American Type Culture Collection, Rockville, MD)

33.531 Purity and Biochemical Properties of *Bacillus megaterium*

a. Reconstitute a lyophilized culture in Brain Heart Infusion broth and incubate at 37°C for 18 h. Streak blood agar plates with the broth culture and incubate plates at 37°C for 18 h. After incubation check for culture purity.

b. Streak the culture for isolation onto two Columbia Agar plates with 5% defibrinated horse blood. Incubate at 37°C for 18 h.

c. Prepare a Gram stain of three well isolated colonies. All cultures should be Gram positive.

d. Stain a drop of the spore suspension with malachite green and counterstain with carbol-fuchsin solution. The spores will appear green, whereas the vegetative cells will appear red or pink.

e. Use one Columbia Agar plate with 5% defibrinated horse blood from the culture to test for presence of catalase. *Bacillus* are catalase positive.

f. Use the other plate to check biochemical characteristics of the culture by inoculating O-F glucose, Voges-Proskauer, and mannitol broths. Incubate at 35°C for 18 h.

The biochemical patterns of *B. megaterium* should agree with the following chart:

Catalase	Gram stain	Spore forming	O-F glucose	Voges-Proskauer	Mannitol
+	+	+	O	-	A

(+) = positive; (-) = negative; (F) = fermentative; (O) = oxidative; (A) = acid.

g. If the organism does not meet all the above criteria, replace with a new ATCC culture of the test organism.

33.532 Preparation of *Bacillus megaterium* Spore Suspension

a. After the culture meets all biochemical criteria, pick several well isolated colonies from the plates and streak A-K Sporulating Agar No. 2 slants (one per Roux bottle) and incubate the slants at 37°C for 18 h.

b. Add 4-6 sterile glass beads and 2-3 ml sterile distilled water to each slant and gently shake for 2 minutes to dislodge organisms.

c. Aseptically transfer the slant suspensions to a Roux bottle containing A-K Sporulating Agar No. 2 and spread with the help of sterile glass beads. Multiple cultures may be prepared and pooled.

d. Incubate the Roux bottles horizontally for 18 h at 37°C and then at room temperature for the remainder of 1 week (6 days).

e. Harvest the growth from the Roux bottles by adding 20-30 sterile glass beads and approximately 25 ml of sterile distilled water per bottle. Gently agitate each bottle to dislodge bacterial growth. (Care must be taken not to break the agar during harvesting).

f. Aseptically transfer the bacterial suspension into sterile centrifuge tubes (40 ml volume) and heat the tubes in boiling water (100°C) for 10 min.

g. Wash the heated suspension three times with sterile distilled water by centrifuging and decanting in the following manner:

   i. Centrifuge at 5°C for 20 minutes at 20,000 x G.
   ii. Pour off supernatant.
   iii. Resuspend the pellet in 20 ml sterile distilled water.
   iv. Repeat Steps i, ii and iii two more times.

h. Wash and coat a Virtis jar with a mixture of sterile phosphate buffer and sterile polyethylene glycol in the following manner:

   Mix 34.1 ml of sterile phosphate buffer and 11.8 g of polyethylene glycol in a 100 ml glass stoppered sterile volumetric flask and shake vigorously. Bring to volume with sterile distilled water. Pour the mixture into a

Virtis jar and place the jar on the homogenizer. Blend for 5 minutes at 5,000 RPM. Discard the mixture. Repeat the process.

i. Prepare a fresh solution of sterile buffered polyethylene glycol (34.1 ml of phosphate buffer and 11.8 g of polyethylene glycol) in a 100 ml glass stoppered sterile volumetric flask. Add 25 ml of the washed spore mixture and bring to volume with distilled water. Shake vigorously. Pour the mixture into a coated Virtis jar and homogenize for 5 minutes at 5,000 RPM.

j. Dispense the mixture equally into four sterile centrifuge tubes and centrifuge in a swinging bucket rotor at 1,500 x G (3,000 RPM in H-4 Rotor in Sorvall RC5C) for 2 minutes at room temperature.

k. A two-phase system with an interface will be formed in the centrifuge tube. Being careful not to disturb or disperse the interface layer, transfer the spore containing, upper phase using a 10 ml pipette to a second set of sterile centrifuge tubes.

l. Centrifuge the tubes at 20,000 x G for 20 minutes at 5°C. Pour off the supernatant. Resuspend the pellet in each tube with 20 ml sterile distilled water and pool the contents of all tubes into a sterile container.

m. Pipette 25 ml aliquots of spore suspension into each sterile centrifuge tube. Centrifuge tubes at 20,000 x G for 20 minutes at 5°C. Repeat the process five times after decanting the supernatant and re-suspending the pellet in 20 ml of distilled water.

n. After the last wash step, resuspend each spore pellet in 20 ml 50% ethyl alcohol. Pool all spore suspensions into a sterile bottle containing 15-20 sterile glass beads. Store the stock suspension at 35-40°F (2-4.4°C). (Properly preserved stock spore suspension may be used indefinitely).

33.533 Enumeration of *B. megaterium* Spores in Stock Suspension

a. To determine the number of spores/ml in each new spore stock suspension, prepare tenfold serial dilutions (10^{-2}-10^{-10}) of the suspension using Butterfield's Phosphate Buffer. (Pipet 1.0 ml of well mixed spore stock suspension (use vortex mixer) into 9 ml buffer and then make serial dilutions up to 10^{-10}.).

b. Using separate pipettes, pipette 1.0 ml of each dilution into triplicate 100 x 15 mm plates.

c. Pipette 15 ml molten Plate Count Agar (cooled to 48 ± 1°C) to each plate. Mix by swirling and tilting plates for even dispersal of the inoculum. Incubate the plates at 37 ± 1°C for 48 h.

d. Count colonies (30-300) of triplicate plates on a Quebec Colony Counter. Record and average the number of colonies/ml in each dilution. Determine the number of colony forming units (cfu)/ml of the stock solution.

e. To prepare the final spore suspension at a concentration of 1 x 10^6 cfu/ml in 50% ethyl alcohol from the stock spore suspension, use the following formula:

Concentration of stock suspension (cfu/ml) = Dilution factor X Desired concentration of working spore suspension (cfu/ml)

Example:

Stock spore suspension = 1 x 10^9 spores/ml

Desired concentration of spore suspension = 1 x 10^6 spores/ml:

(1 x 10^9 cfu/ml) = (x) (1 x 10^6 cfu/ml)

$$\frac{(1 \times 10^9 \text{ cfu/ml})}{(1 \times 10^6 \text{ cfu/ml})} = x$$

x = 1000

In this example, the stock spore suspension must be diluted 1:1000 (1 part stock spore suspension plus 999 parts diluent) in 50% ethyl alcohol to prepare the 1 x 10^6 spore/ml concentration.

33.534 Packaging of *B. megaterium* Spore Suspension (Field Use)

a. Dispense 4.0 ml of the working spore suspension (1 x 10^6 cfu/ml in 50% ethyl alcohol) into sterile (15 mm diameter x 51 mm height) clear, glass vials with deep seated, leak-proof screw caps.

NOTE: Under FSIS contract, FAST spores are produced commercially. After they meet all quality control specifications they are used in slaughter plants.

b. After securely capping spore vials, seal with shrink-seal, or equivalent closure material, to prevent leakage or dehydration.

c. Label the vials with the following information on a transparent mylar pressure sensitive label:

i. "FAST Spores"
ii. *B. megaterium* ATCC 9885
iii. Date

33.54 Preparation FAST Plates (Used in the Plant)

a. Weigh and add 38 g Mueller-Hinton Agar (Acumedia) powder to 1 L glass distilled water. Add 7 g dextrose to the mixture. Add 70 ml Bromcresol Purple solution (0.04%) to the mixture. Heat while stirring and bring to boil. After sterilizing at 121°C for 15 minutes, mix the medium thoroughly, and cool it in a 48°C water bath. Continue mixing during cooling and dispensing.

b. Using a sterile agar delivery system, deliver 6.0 ml agar to each 60 x 15 mm plate. Distribute the agar evenly to cover entire surface of the plate. Place plates on flat level surface and allow the agar to harden.

c. Label the lid of each plate using a label, containing the following information:

i. "FAST PLATE"
ii. Lot Number
iii. Expiration Date

d. Refrigerate plates in sealed double plastic bags to prevent moisture evaporation. These plates can be used for a period of 90 days.

NOTE: Under FSIS contract, FAST plates are produced commercially. After these plates meet all quality control specifications they are used in slaughter plants.

33.55 Performing the FAST Test

33.551 Tissue Sample and Conditions

a. The kidney is the target tissue for FAST

b. The kidney and other tissue samples should be received at 4°C or below and identified properly.

33.552 Procedure

a. Allow frozen samples to thaw completely at room temperature for a sufficient period of time such that ice crystals are no longer present within the sample.

b. Open a sterile cotton swab pack, remove one swab, and insert the sharp end of the swab shaft about 1/2" to 3/4" into the kidney tissue.

c. Move the swab shaft back and forth several times to macerate the tissue, disrupting tissue cells and releasing tissue fluid. Remove the swab shaft.

d. Reverse the swab, insert the cotton tip into the tissue opening and twist to make sure that the cotton tip is in contact with the macerated tissue.

e. *Allow swabs to remain in the tissues for a minimum of 30 minutes.

f. Allow refrigerated plates to warm to room temperature for about 10 minutes. Check plates for contamination, cracking or dryness of agar.

g. Lift the plate cover slightly and mark an "X" reference mark on the outer side wall of the plate. Place the covered plate bottom side down on the work place surface with the reference mark at 12 o' clock position. With a fine-tip permanent marking pen, start at the "x" and draw a line across the bottom of the plate dividing it into two equal sections.

h. Shake the *B. megaterium* spore vial and dip a sterile swab in the solution. Gently touch the swab to the side of the vial to remove excess fluid. Replace the screw cap on the vial.

i. Streak the surface of the agar plates with the swab from a point marked on the side of the plate moving up and down and from left to right. Turn the plate 1/4 turn and streak again.

j. Repeat this streaking process 2 more times. Finally turn

the plate 1/2 turn and streak. (Use a separate swab for each plate)

*NOTE: If the swabs appear dry, reinsert them in the tissue and squeeze the tissue around the swab to absorb tissue fluids. For small portions of dry muscle tissue, moisten swab with distilled water prior to insertion.

k. As a control, place a neomycin (N5) 5 μg disc one half inch from the edge of the plate on the agar surface.

l. Remove swab from the tissue and break the shaft approximately two inches from the swab end.

m. Gently place the swab on the agar without breaking the surface. Make sure that swab has uniform contact with the surface.

NOTE: Two (2) swabs from two samples can be placed on one plate as illustrated below in (Figure 1).

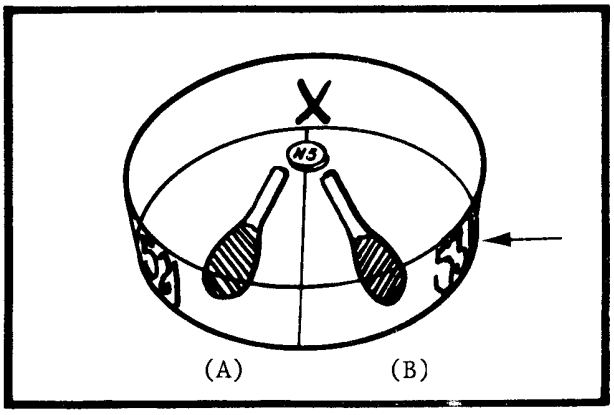

Figure 1. Swab placement on plate

n. Incubate the plates upright at 44 ± 0.5°C for 6 h, up to a maximum of 16-18 h.

o. Store samples in refrigerator until the test is completed.

33.56 Results and Interpretation

a. Remove plates from the incubator and remove the swabs.

b. Measure the ZI around the N5 disc with a mm ruler or a zone reader. The zone should be 20-26 mm wide. If not, the test must be repeated.

c. Observe the plates for inhibition of *B. megaterium* growth surrounding the swabs (Figure 2).

i. Samples with Antimicrobial Chemical Residue

Zone of inhibition around swab "A": Sample A may contain antimicrobial residue, and must be subjected to confirmatory testing procedures.

ii. Samples without Antimicrobial Chemical Residue

No Zone of Inhibition around swab "B" : Sample B is free of antimicrobial residue.

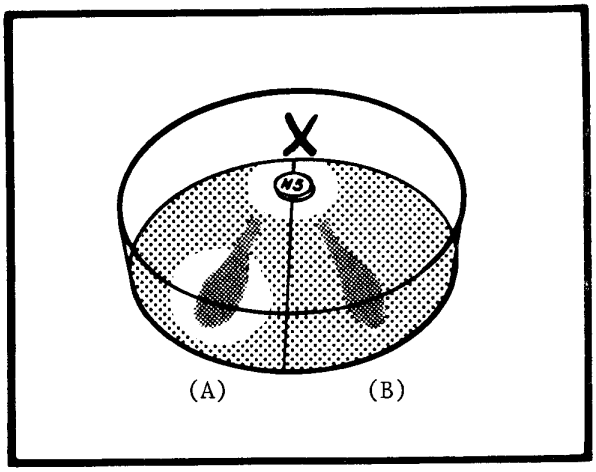

Figure 2. Inhibition of microorganism by swab

33.57 Quality Assurance

a. The FAST plates can be stored at room temperature protected from extremes of heat, cold and moisture.

b. Store spore suspensions under refrigeration condition with cap tightly closed.

c. Store neomycin disc vial in a plastic bag in refrigerator.

d. Do not use outdated plates, spores or N5 discs.
e. Shake the spore vial for even dispersal of spores.

f. Check plates before use for contamination, cracking or drying of agar.

g. Do not to break the agar surface while placing the neomycin disc and the swab.

h. Allow swabs to remain in the tissues for 30 minutes.

i. Read plates any time after 6 h of incubation, up to a maximum of 18 h.

j. Make sure that the incubator temperature is 44 ± 0.5°C.

33.58 Selected References

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PART E

33.6 EVALUATION OF ANTIMICROBIAL RESIDUES IN MEAT AND POULTRY TISSUE BY A MODIFIED FAST ANTIMICROBIAL SCREEN TEST (M-FAST)

B. P. Dey, Richard H. Reamer and Sandra L. Kamosa

33.61 Introduction

The Fast Antimicrobial Screen Test (FAST) developed in 1989, is presently being used in selected bovine slaughter plants. It is expected that the test will be used universally in plants for the detection antimicrobial residue in all species of food animal carcasses. The test as performed by inspectors is as follows: a sterile cotton tipped applicator (swab) is inserted into the kidney sample of an animal and left for 30 minutes to absorb tissue fluids. The agar plates are surface streaked with *Bacillus megaterium* spore suspension using a sterile cotton swab. The swab from the kidney is removed, broken as close to the cotton tip as possible, and placed onto the agar plate and incubated at 44°C. The plate is examined for a zone of inhibition (ZI) around the swab at 6 and 18 h. In the case of inhibition at 6 h, the plate is further examined at 18 h for confirmation. If there is no inhibition at 6 h, the carcasses is released. The test allows screening and releasing a large number of residue free carcasses within a work shift. If there is clear zone of inhibition, muscle, liver and kidney tissues from the suspect carcass are collected and further analyzed for confirmation at an FSIS laboratory. The method described here is a modified FAST procedure for verifying field test results in 6 h with sensitivity equal to the commercial FAST at comparable incubation times.

33.62 Equipment, Reagents and Supplies

33.622 Equipment

a. Laminar Flow Hood or equivalent clean room
b. Sorvall RC5C Refrigerated Centrifuge, Sorvall Rotor SS-34 and Sorvall Swinging Bucket Rotor HB-4 or equivalent. Must operate at 20,000 x G at a constant 5°C and also with a swinging bucket rotor at 1,500 x G at room temperature or equivalent.
c. Virtis homogenizer, Model 60K or equivalent
d. Sterile Virtis jars
e. Vortex mixer or equivalent
f. Incubators: one capable of maintaining a constant 37°C and the other 44 ± 0.5°C
g. Precision water bath (48 ± 1°C) with cover (Model 183) or

equivalent
h. Quebec Colony Counter or equivalent
i. Fisher-Lilly Antibiotic Zone Reader (Fisher Scientific, Cat. No. 07-906)

33.623 Reagents

a. Distilled water:

The distilled water must be prepared using an all glass still (Corning Megapure 6L or equivalent) and stored in a glass or any acceptable reservoir which is not a part of the system. All spore lots must be prepared using glass distilled water. * Do not use deionized water.

b. Phosphate buffer (3 M, pH 7.1)

Dissolve 306.9 g of K_2HPO_4 and 168.6 g KH_2PO_4 in 1 L distilled water. If necessary, adjust pH by dropwise addition of 0.1 N HCl or NaOH. Sterilize at 121°C for 15 minutes or filtering through a 0.2 μm filter.

c. Ethyl alcohol (USP grade, 200 proof)

Dehydrated Alcohol, USP, Ethyl Alcohol, 200 Proof Punctilious®, (Ethyl Alcohol [Ethanol] CAS #64-17-5, Warner-Graham Company, 160 Church Lane, Cockeysville, MD 21030). For a 50% solution, mix 1 part of ethyl alcohol with 1 part glass distilled water. Prior to use, filter sterilize through a 0.2 μm filter.

d. Polyethylene glycol, Mol. Wt. 4000 (Baker Chemicals). Sterilize (121°C for 5 minutes) in a covered beaker prior to use.

e. Bromcresol Purple (0.04%) solution. Dissolve 0.1 g Bromcresol Purple dye with 18.5 ml of 0.01 N sodium hydroxide, add 231.5 ml of distilled water.

f. Dextrose (Bacto Dextrose-Difco, Detroit, MI; Cat. No. 0156-17-4) or equivalent.

g. Butterfield's Phosphate Buffer, sterile

33.624 Supplies

a. Sterile Roux bottles.
b. Sterile glass beads, 4 mm diameter

*

Resins of some systems produce quaternary ammonium compounds which interfere with the analysis.

c. Sterile 100 ml graduated glass stoppered cylinders or volumetric flasks
d. Sterile centrifuge tubes, 40 ml (Nalgene 3118 or equivalent)
e. Sterile pipettes graduated to the tip, 10 and 1 ml.
f. Sterile, clear glass vials 51 x 15 mm with deep seated screw caps
g. Pressure sensitive labels not to exceed 2" x 1/2"
h. Acetate shrink-wrap material for sealing 15 x 51 mm glass vials or equivalent closure material
i. Forceps
j. Permanent marking pen
k. Antibiotic discs: Neomycin (N5)- 5 μg
l. Sterile cotton swabs on hollow plastic tubes
m. Sterile, plastic 60 X 15 mm plates (Falcon Cat. No. 1007 or equivalent)

33.63 Media

Proceed exactly as that described in Section 33.524.

33.64 Test Organism

Bacillus megaterium ATCC 9885 (American Type Culture Collection, Rockville, MD)

33.641 Purity and Biochemical Properties of *Bacillus megaterium*

Proceed exactly as that described in Section 33.531.

33.642 Preparation of *B. megaterium* Spore Suspension

Proceed exactly as that described in Section 33.532.

33.643 Enumeration of *B. megaterium* Spores in Stock Suspension

Proceed exactly as that described in Section 33.533 except prepare the final spore suspension such that it contains 1 x 10^7 cfu/ml.

33.644 Packaging of *B. megaterium* Spore Suspension

a. Dispense 4.0 ml of the working spore suspension (1 x 10^7 cfu/ml in 50% ethyl alcohol) into sterile (51 x 15 mm) clear, glass vials with deep seated, leak-proof screw caps.

b. After securely capping spore vials, seal with shrink-seal, or equivalent closure material, to prevent leakage or dehydration.

NOTE: *B. megaterium* spore (1×10^7 cfu/ml) can be obtained by special order from EDITEK, Burlington, N.C.

c. Label the vials with the following information on a transparent mylar pressure sensitive label:

i. "FAST Spores"
ii. *B. megaterium* ATCC 9885
iii. Date

33.65 Preparation of Plates

a. Weigh and add 38 g Mueller-Hinton Agar (Acumedia) powder to 1 L glass distilled water. Add 8 g dextrose to the mixture. Add 70 ml Bromcresol Purple solution (0.4%) to the mixture. Heat while stirring and bring to boil. Cool (48°C waterbath). Adjust the pH to 7.2 ± 1. After the medium has been sterilized at 121°C for 15 minutes, mix the medium thoroughly.

b. Keep mixing the medium while cooling in a 48°C water bath.

c. Add 1 ml of *B. megaterium* spore suspension (1×10^7/ml) to every 100 ml of the medium and mix thoroughly.

d. Aseptically dispense 8 ml of the seeded agar to each 100 x 15 mm plate.

e. Distribute the agar evenly over the entire plate. Place plate on a flat, level surface and allow agar to harden.

f. Label the lid of each plate using a label containing the following information:

i. "M-FAST PLATE"
ii. Expiration Date

g. Refrigerate plates in sealed double plastic bags to prevent moisture evaporation. These plates can be used for a period of up to 15 working days.

33.66 Performing the Test

33.661 Sample Condition

a. Assure that the samples are received at a temperature of

4°C or below.

b. Identify samples according to standard operating procedures.

NOTE: FAST test is used on the kidney tissue of all bovine species where implemented.

33.662 Procedure

a. Allow frozen samples to thaw completely at room temperature for a sufficient period of time such that ice crystals are no longer present within the sample.

b. Open a sterile cotton swab pack, remove one swab, and insert the sharp end of the swab shaft about 1/2" to 3/4" into kidney tissue.

c. Move the swab shaft back and forth several times to macerate the tissue, disrupting tissue cells and releasing tissue fluid. Remove the swab shaft.

d. Reverse the swab, insert the cotton tip into the tissue opening and twist to make sure that the cotton tip is in contact with the macerated tissue.

e. Leave the swab in the tissues for a minimum of 30 minutes.

f. Allow the plates to warm at room temperature for about 20 minutes. Check plates for contamination, cracking and dryness of agar.

g. As a positive control place a neomycin 5 μg (N5) disc in the center of a plate from the same batch used in the analysis.

h. Remove the swab from the tissues, break the shaft approximately two inches from the swab end.

NOTE: If a swab appears dry, reinsert and squeeze the tissue around the swab to absorb fluid. For a dry muscle tissue, moisten the swab with distilled water prior to insertion.

i. Place the swab on the agar surface gently with uniform contact with the surface.

NOTE: Properly identified, four (4) swabs from 4 samples can

be placed on each plate provided the cotton tip end of one lies next to the shaft of another as shown in Fig. 1.

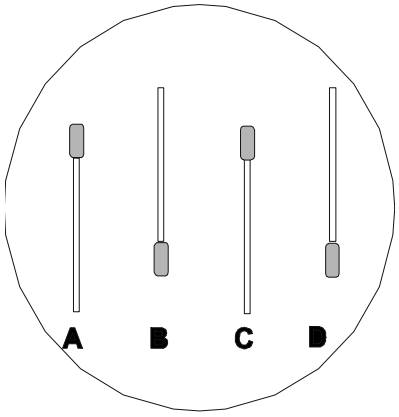

Figure 1. Swab placement on plate.

j. Incubate the plates upright at 44 ± 0.5°C for 6 hours, up to a maximum of 16-18 h.

k. Store samples in refrigerator until the test is completed.

33.663 Results and Interpretation

a. Remove the control and test plates with swabs from incubator and remove swabs.

b. Measure the ZI around the N5 disc on the control plate with a mm ruler or a zone reader. The zone should be 20-26 mm wide. If not, the test should be repeated.

c. Observe the plates for inhibition of *B. megaterium* growth surrounding the swabs (Figure 2).

i. Samples Free of Antimicrobial Chemical Residue

If no zone of inhibition is observed around a swab, the test is negative, .i.e. the samples (A, B, C and D) do not contain an antimicrobial residue (Fig. 2).

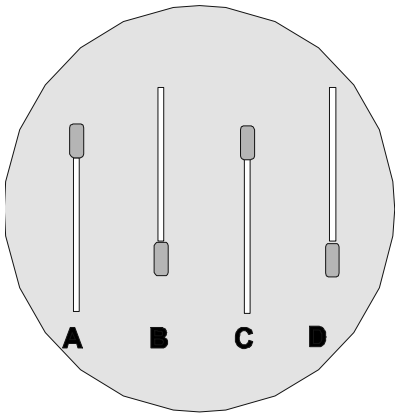

Figure 2. Swabs with no zone of inhibition.

ii. Samples with Antimicrobial Residue

If a zone of inhibition is observed around a swab, the test is positive, i.e. the samples (A and D) may have an antimicrobial residue. Measure the width of the zone.

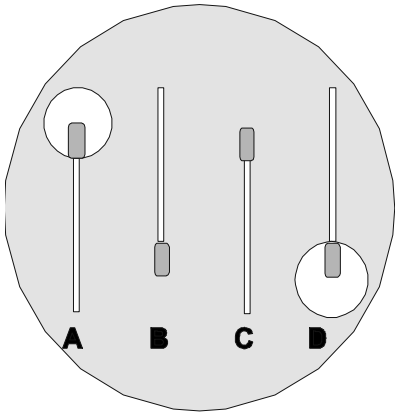

Figure. 3. Positive samples illustrating zone of inhibition around the swabs (samples) A and D.

d. Record and compare result with the field result. Positive samples must be subjected to confirmatory testing.

33.67 Quality Assurance

a. The M-FAST plates wrapped in plastic bags should be stored at refrigerator temperature (4-8°C).

b. Spore suspensions in tightly closed container should be stored at refrigerator temperature (4-8°C).

c. Neomycin disc vial wrapped in a plastic bag should be stored at refrigerator temperature (4-8°C).

d. Observe expiration date of plates. More than 2 week old plates should be discarded.

e. The spore vial should be shaken thoroughly before use.

f. Incubate 1 plate each day at 44°C as control.

g. Check plates before use for contamination, drying or cracking of agar.

h. Allow enough room for each swab placed on a plate.

i. Be careful not to break the agar surface while placing the neomycin disc and the swabs.

j. Leave swabs in the tissues for a minimum of 30 minutes.

k. Read plates any time after 6 h of incubation, up to a maximum of 18 h.

l. Stabilize the incubator temperature at 44 ± 0.5°C.

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Dey, B. P., Richard Reamer, and S. Kamosa. 1995. Evaluation of antimicrobial residues in meat and poultry tissues by a modified Fast Antimicrobial Screen Test (M-FAST). Laboratory Communication No. 80. USDA, Food Safety and Inspection Service, S&T, Microbiology Division, Washington, D.C.

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CHAPTER 35. DETECTION OF ANTIBIOTIC AND SULFONAMIDE RESIDUES IN MEAT TISSUE BY COMMERCIALLY AVAILABLE IMMUNOASSAY KITS

Anne M. Dulin, Clarence A. White and Nitin H. Thaker

35.1 Introduction

In recent years, the application of immunochemical methods for detecting veterinary drug residues in animal tissue has increased. These methods are based on highly specific antigen-antibody reactions on a solid phase matrix involving conjugation of an enzyme to the drug analyte and specific antibody directed against the analyte. Advantages of enzyme immunoassays include sensitivity (usually in the ng/ml range), simplicity of test performance, stability of reagents, lack of radioisotope use and associated hazards, potential for automation, and relatively inexpensive equipment.

In direct competitive enzyme immunoassays, enzyme labeled drug antigen and unlabeled drug antigen (sample analyte) compete for limited antibody binding sites. Specific antibody is generally bound to a solid phase support. The amount of enzyme labeled drug that binds to antibody is inversely proportional to the amount of unlabeled drug antigen (analyte) present in the tissue sample, which also competitively binds to the same antibody. Upon addition of substrate to the reaction mixture, to provide a visible indication of the test reaction, the amount of colored end product produced is inversely proportional to the amount of unlabeled drug analyte bound to the antibody. Thus, positive reactions indicating the drug's presence in the sample are generally indicated by no color change, while negative reactions indicating absence of the drug analyte are usually colored products. The exact color of the end product depends upon the specific substrate - chromogen system used in the particular assay. The applications of direct competitive enzyme-linked immunosorbent assays (ELISA) have provided additional support to the FSIS regulatory programs by enabling the detection of drug residues in food animal tissues at appropriate levels.

Presently, there are a number of screen test kits commercially available for detecting the presence of antibiotic and sulfonamide residues. However, regulatory action cannot be based on screen test results alone, since they are not quantitative, do not relate to biological activity of the detected drug and they are not considered to be absolutely definitive and confirmatory in nature. The presence of antibiotic residues, therefore, must be confirmed by bioassay and/or chemical methods, when a chemical method exists.

35.2 Commercially Available Test Kits

Currently there are commercially available, immunoassay, screen test kits, in several different formats, for such antimicrobial agents as:

penicillin (β-lactams)
ceftiofur (cephalosporin)
chloramphenicol
gentamicin
sulfonamides
tetracyclines
neomycin

Many of these test kits, however, were originally developed for specific application in bulk milk tank testing for antimicrobial residues. Before any of these kits can be used in an FSIS Laboratory for testing meat tissue extracts for antimicrobial residues, they must first be thoroughly evaluated to determine their suitability and applicability with regard to appropriate performance characteristics. They must perform in a manner to meet minimum sensitivity detection levels for the drug in question relative to that drug's established tolerance level, be specific, show excellent lot-to-lot reproducibility, have stability over the reported shelf life of the kit, and also have very low (0-5%) false positive and false negative reaction rates. Non-government users of this Guidebook must assume individual responsibility for evaluating commercial test kits for their applicable suitability with regards to the above performance parameters.

35.3 Equipment

This generic list applies to all test kits. Depending on the exact kit used, other supplies might be required.

a. Tekmar stomacher®, Model 400 (Tekmar Company, Cincinnati, OH)
b. Eppendorf centrifuge, Model 5412 (Thomas Scientific Co., Swedesboro, NJ)
c. Timer
d. Tekmar strainer bags, 18 oz capacity. (Tekmar Company, Cincinnati, OH)
e. Micro centrifuge tubes, 1.5 ml volume. (Thomas Scientific Co., Swedesboro, NJ)

35.4 Reagents

a. 0.1 M phosphate buffer, pH 8.0 (± 0.1). Dissolve 16.73 g dibasic potassium phosphate and 0.523 g monobasic potassium phosphate in distilled water and

dilute to 1 liter with distilled water. Check pH of the nonsterile buffer before autoclaving. If necessary, adjust by the dropwise addition of 0.1 N HCl or NaOH. Autoclave for 15 minutes at 121°C and 15 lbs pressure.

b. 0.1 M phosphate buffer, pH 4.5 (± 0.1). Dissolve 13.6 g monobasic potassium phosphate in distilled water and dilute to l liter with distilled water. For pH adjustment, proceed as in (a) above.

c. 0.1 M phosphate buffer, pH 6.0 (± 0.1). Dissolve 11.2 g monobasic potassium phosphate and 2.8 g dibasic potassium phosphate in distilled water and dilute to l liter with distilled water. For pH adjustment, proceed as in (a) above.

d. U. S. Pharmacopeia (USP) antibiotic and sulfonamide standard reference materials

35.5 Tissue Extraction Procedure

This procedure generally applies to all test kits:

a. Weigh out 10 g of sample (muscle, kidney, or liver tissue) into a sterile container.

b. Place the sample into a labeled Tekmar strainer bag.

c. Add 40 ml of appropriate phosphate buffer for the antibiotic or sulfonamide residue under evaluation.

d. Place strainer bag in a Tekmar stomacher® and stomach for 30 seconds for kidney or liver and 60 seconds for muscle tissue.

e. Allow the extract to settle for 45 minutes.

f. Place 1.5 ml of the settled extract into a labeled micro centrifuge tube.

g. Centrifuge for 10 minutes in an Eppendorf micro centrifuge at maximum speed.

h. Pipette supernatant fluid into another labeled test tube avoiding any fat and debris.

i. The 1:5 extracts prepared for bioassay analysis (Chapter 34) can be used instead of performing steps a through e.

35.6 Performing the Assay

Perform the assay procedure according to the specific test kit manufacturer's directions if no modifications were found to be necessary, or according to any specific instructions provided by the Microbiology Division, OPHS, for a particular test kit, when extensive protocol modifications were necessary.

35.7 Reporting and Confirmation of Screen Test Results

All positive screen test results should initially be reported as a presumptive positive finding. All samples presenting a positive screen test result must be subjected to confirmatory testing by the bioassay procedure or an appropriate chemical analysis procedure, if available for that particular drug, to confirm the drug's identity and determine it's quantitation. All sulfonamides must be confirmed by appropriate chemical methods. Final violative result reports must be based on confirmed drug quantitative levels present above that of established tolerance levels for that drug in a specific animal slaughter class.

35.8 Quality Assurance Procedures

a. Maintain a written log of all kits purchased, used and appropriate dates.

b. Test kits must be stored under refrigeration (4-8°C). Do not freeze.

c. Upon receipt of new test kits, perform positive and negative control testing at appropriate drug concentration levels.

d. Do not mix reagents and test components from kits with different serial numbers or from different manufacturers kits that detect the same analyte.

e. Do not use kits past their expiration date.

f. Use a separate pipet and test device for each sample.

g. Before performing the test, allow all reagents to reach room temperature. If the room temperature is not within the range of 18-29°C (65-85°F), perform test in another area within the proper temperature range.

h. Observe all test time intervals accurately by using a timer.

i. Two weeks before test kit expiration, perform positive

and negative control tests at appropriate drug levels to assure proper kit performance as expiration approaches.

j. U.S. Pharmacopeia (USP) standards of antibiotics and sulfonamides at appropriate quantitative levels should be used.

k. Record the results of all positive and negative control tests in a log book.

35.9 Selected References

Agarwal, V. K. 1992. Analysis of Antibiotic/Drug Residues in Food Products of Animal Origin. Plenum Press, New York, NY 10013.

Boison, J. O., and J. D. MacNeil. 1995. New test kit technology, p. 77-119. In H. Oka, H. Nakazawa, K. Harada and J. D. MacNeil (ed.), Chemical Analysis for Antibiotics Used in Agriculture. AOAC International, Gaithersburg, MD 20877.

CHAPTER 36. Equipment Calibration, Maintenance, and Performance Verification. Revision 1, 7/11/00

Steven T. Benson, Terry Dutko, Leon Ilnicki, Michael Lankford, Cathy Pentz, Joel Salinsky, Bashir Teirab and Wayne Ziemer

36.1 Introduction

The guidelines for equipment (i.e. maintenance, calibration, and performance verification) covered in this chapter include the criteria for testing equipment and analytical instruments. Also included is guidance for monitoring and controlling environmental conditions including sanitation, safety, and discard procedures for hazardous material. The quality control parameters that are used in the analysis of a food product for specific microorganisms, species, and residues are included with the method.

The Microbiologist-in-Charge or Branch Chief ensures that all quality assurance and quality control procedures are consistently followed by everyone in the laboratory operation. Compliance with these procedures is verified by the internal audits conducted yearly by the Quality Assurance Manager.

Any deviation from an expected quality control result (nonconformance) is documented and verified by the individual responsible for the analysis. The unit supervisor must be informed. The nonconformance is recorded in the appropriate log along with any corrective action taken. It is the unit supervisor's responsibility to review all logs weekly. Daily verification means normal work days. If a piece of equipment is not in service it is so labeled and records so indicate. Non-working days (i.e., weekend, holidays) are noted.

The following are taken from the ISO/IEC 17025, Food Microbiology ALACC standards, or manufacturer requirements that have been tailored and/or expanded to meet the specific needs of the laboratory.

36.11 Equipment Manuals

a. Master copies of all available equipment manuals are stored and filed in a manner that allows easy retrieval.

b. For all testing equipment not in this chapter a working copy of the appropriate manual(s) containing the operating procedures, care, and maintenance is located near each piece of equipment.

36.12 Equipment Logs/Records

a. A log is maintained for and near each piece of equipment. The log includes:
- the name of the equipment
- the manufacturer's name, the serial number, or other unique identification
- the date received and placed in service (if available)
- the current location, where appropriate
- the condition when received (e.g. new, used, reconditioned)
- a copy of or the location of the manufacturer's instructions
- a copy of or the location of the dates and results of calibrations and/or verifications and the date of the next calibration and/or verification
- details of maintenance performed to date and planned for the future
- the history of any damage, malfunction, modification, or repair.

b. Each event relative to a piece of equipment is recorded in the log, showing the date, the event, any corrective action taken, the name or initials of the person making the entry.

c. All equipment records and maintenance logs are maintained for 3 years past last entry.

36.2 Temperature Control Equipment

36.21 Autoclaves

36.211 Temperature Calibration and Verification

a. All autoclaves are calibrated at installation and annually using a certified/traceable thermometer to assure stability of temperature.

b. To verify autoclave performance, a biological indicator spore vial or strip is added to each fully loaded autoclave once per week. Manufacturer's instructions for followed. (An unautoclaved vial or strip, incubated as a positive control, should show growth, and the autoclaved item should not.)

c. To verify autoclave performance daily each autoclave is equipped with an automatic temperature recorder. This chart/record is used to demonstrate proper time and temperature of each load.

d. Each chart is identified with the autoclave number, date, product, run number, time into autoclave, time at desired temperature, and time out of autoclave.

e. Each chart is reviewed at the end of a run and initialed by the operator to make sure that the temperature and time used conform to the directions for sterilizing that product or material. Any problems are noted in the autoclave log along with the corrective action taken.

36.212 Operation

a. A copy of the operating manual, a protocol for each type of material being processed, and an equipment log is located near each autoclave.

b. Temperature sensitive autoclave tape, or equivalent, is placed on all autoclaved containers to validate that the load was processed.

c. Insulated autoclave gloves, or equivalent, are kept near the autoclaves at all times.

36.213 Maintenance

a. Each autoclave will be serviced at 6-month intervals by a qualified contractor. In addition, each autoclave must have an annual temperature validation against a certified thermometer and a temperature uniformity check.

b. The "strainer" in the steam exhaust line of the autoclave is checked and cleaned weekly.

c. The autoclave is kept clean and free of debris to provide maximum heat transfer.

d. A log is maintained for each autoclave documenting all services performed and temperature validations.

e. The supervisor examines and initials each log weekly to ensure that it is correct and complete.

36.22 Incubators

36.221 Temperature Calibration and Verification

a. Each incubator will be calibrated for stability and uniformity of temperature at installation.

b. To verify performance the incubator temperature is recorded AM and PM using a certified/traceable device (i.e. thermometer, temptale®, thermocouple, etc).

c. Any nonconforming temperature is noted in the incubator log along with the cause, if identified, and any corrective action taken.

36.222 Operation

a. Incubators should be located where ambient temperature variation is minimal.

b. The temperature of a cabinet type incubator should not vary more than ± 1°C. A walk-in incubator may be hard to control closer than ± 2°C.

36.223 Maintenance

a. Incubators are cleaned and sanitized biannually to prevent the accumulation of mold or other microorganisms.

b. The over all condition of the incubator (i.e. door gaskets, blower fan, etc.) is checked annually. A record of all maintenance, repairs, etc. is kept in the incubator log.

c. If a container of water has to be added to an incubator to maintain humidity, a non-volatile microbial inhibitor can be added to prevent build-up of microorganisms. The container is cleaned and sanitized monthly.

36.23 Water Baths and Heating Blocks

36.231 Temperature Calibration and Verification

a. All water baths and heating blocks will be calibrated for stability and uniformity of temperature at installation.

b. To verify temperature performance a certified/traceable thermometer is placed in the bath or block and the temperature is recorded at the time of use.

c. Water baths used as close tolerance incubators should have a built-in water circulation system and a cover. The temperature is maintained within a temperature range of ± 0.5ºC. The temperature is checked at each use.

d. Most block heaters used in microbiology have a built-in thermostat that can be adjusted from ambient to approximately 115 ± 0.5ºC. The temperature of block heaters will be checked and recorded daily.

36.232 Operation

a. Operate the water bath or heating block according to the manufacturer's instructions.

b. For the most accurate temperature reading make sure the recording thermometer is not contacting the sides of the equipment.

36.233 Maintenance

a. All water baths are emptied, cleaned, and sanitized at least monthly.

b. Records of all maintenance, performance deviations, and corrective actions are maintained.

36.24 Refrigerators and Freezers

36.241 Temperature Calibration and Verification

a. All refrigerators and freezers are calibrated for stability and uniformity of temperature at installation.

b. To verify temperature performance the analyst checks and records the temperature daily using a certified/traceable device.

36.242 Operation

a. Freezer temperatures are maintained at or below –10ºC.

b. Ultra low freezer temperatures are maintained at or below –70 or –90ºC.

b. Refrigerators are maintained at a temperature within a range of 2-8ºC.

36.243 Maintenance

a. The overall condition of each freezer and refrigerator (i.e. door gaskets, blower fan, etc.) is checked annually. A log is kept for each refrigerator and freezer. The log contains the dates of all scheduled maintenance, any problems encountered, and any corrective action taken.

b. Where applicable, freezers and refrigerators are defrosted, cleaned, and sanitized at least once a year. (e.g. Neither self defrosting units nor cascade units generally need defrosting.)

c. The following procedures are applicable to all the ultra-low freezers in the laboratories. These do not preclude the addition of other cleaning/maintenance steps that may be specified for individual freezers

1. Air filters/coils shall be checked and cleaned quarterly.

2. Ice build-up inside door gaskets and seals shall be removed promptly. Any seals that allow significant ice build-up over a thirty-day period shall be replaced.

3. Defrosting and cleaning of the interior of the box need only be performed when the freezer is down for repairs.

36.25 Hot Air Ovens

36.251Temperature Calibration and Verification

a. All hot air ovens will be calibrated for stability and uniformity of temperature at installation.

b. To verify temperature performance the analyst records the temperature daily or at the time of use with a certified/traceable device.

36.252 Operation

a. The materials placed in the oven to dry are well separated to allow heat penetration.

b. Follow manufacturer's instructions for operation.

c. Keep at least 1 pair of insulated autoclave gloves, or equivalent, near the oven at all times.

36.253 Maintenance

a. Ovens are cleaned and sanitized at least annually.

b. The over all condition of the oven (i.e. door gaskets, door latches, burners, etc.) are checked annually. All maintenance observations, performance deviations, and corrective actions taken are recorded in the oven log.

36.3 Measuring Equipment

36.31 Laboratory Balances Calibration and Verification

a. All balances will be calibrated using certified/traceable weights annually.

b. To verify performance, a mass measurement is recorded daily using a single weight in the desired range.

36.311 Operation

a. Balances are placed on solid surfaces to guard against drafts and vibrations.

b. Balances and any associated weighing equipment and supplies are located in clean, dry areas. These criteria are especially important for analytical balances.

c. Boats or special papers can be used for weighing. Avoid spills and creation of aerosols.

d. All laboratory balances, top loading and analytical, are appropriately sensitive for their intended purpose.

e. If a balance is equipped with a leveling device care is taken to ensure that the balance is level before use.

36.312 Maintenance

a. All balances are professionally cleaned and calibrated annually using certified/traceable weights.

b. Balances are cleaned after each use.

c. A log is maintained for each balance showing the daily checks, all cleaning, maintenance, performance deviations, and any corrective actions taken.

36.32 pH Meter Calibration and Verification

a. To verify the performance of a pH meter a calibration is recorded daily using standard buffers. If pH readings are going to be taken intermittently throughout the day the pH meter is re-calibrated with fresh portion of buffers before each use.

b. Calibrate the instrument using two standard buffers that bracket the desired pH value of the test material (e.g. pH 4.0 and 7.0 or 7.0 and 10.0).

c. Ensure that the acceptance criteria for calibration, usually found in the manufacturer's instruction manual, have been met prior to use, and record all the calibration information.

36.321 Operation

a. The buffer aliquot used for the calibration is discarded after each use.

b. The calibration temperature should approximate that of the test solution. The most desirable temperature range for determining pH is 20ºC to 30ºC. It is preferable to use a temperature compensating probe, otherwise temperature corrections shall be made according to the manufacturer's instructions.

c. Reference buffers are labeled with identification/number, date received, and expiration date.

36.322 Maintenance

a. A professional will service all pH meters annually. A certificate of calibration/service is required.

b. Electrodes are cleaned after each use. Electrodes are stored as recommended by the manufacturer. Electrodes should never be allowed to dry out.

c. A log is maintained for each pH meter. Dated entries are made each time the pH meter is used, the buffers or electrodes are changed, and the instrument is serviced. Observed performance deviations are noted along with corrective actions taken.

36.33 Water Activity (a_w) Calibration and Verification

Follow the instructions in the manufacturer's operating manual for calibration, maintenance, and test procedure.

36.331 Operation

a. The test method and operation of this instrument is discussed in Chapter 2 of the MLG.

b. Temperature is very important when determining a_w. A small change in temperature can produce a large change in vapor pressure. Therefore the instrument, the reference salts, and the sample should be at the same temperature.

c. A sample should not be left in the instrument after a reading has been taken. When a sample is loaded, avoid tipping or moving the instrument.

d. To ensure a correct reading, fill the disposable cup no more than half full

e. Wipe any excess sample from the top rim of the cup before placing it in the unit to prevent contamination of the unit. If a spill occurs, the unit must be cleaned and re-calibrated.

36.332 Maintenance

a. The Hydrodynamics Instrument shall have the sensors checked at least once a year following the instruction manual. At any time, if the data of a salt standard deviates significantly from the expected results, check the suspect sensor and if found to be defective discard or return it to the manufacturer for re-calibration.

b. Maintain a log for the instrument documenting the date used, all repairs, readings of standard salt solutions, all performance deviations, and any corrective actions taken.

36.34 Micropipettor Calibration and Verification

a. Delivery volumes are verified monthly using a mass/volume measurement near mass/volume used. The following is an example of how to meet the mass/volume criteria.

Single volume pipette
5 reps/mg at set volume (ul)

Multivolume pipette
5 reps at low, mid, and high volumes = 20, 50 and 100% maximum volume. Record the average at each setting.

Multichannel pipette
Conduct a visual inspection of the draw, and take cumulative readings. Again 5 reps (at 20, 50, and 100% of max, if adjustable).

b. If performance verification fails re-calibrate following manufacturer instructions or return to the manufacturer for re-calibration.

36.341 Operation

a. This is a precision instrument that must be maintained and used with care.

b. Follow the manufacturer's instructions for use.

c. Select an appropriate pipette and tip combination.

36.342 Maintenance

a. Keep the pipettes clean and store them according to manufacturer's instructions.

b. Keep a record of all maintenance, service, calibration, and verification measurements.

36.35 Automated Pumps/Washing Equipment/Vial Fillers Calibration and Verification

a. If the equipment is used to dispense a designated volume, it is calibrated using a mass/volume measurement (see section 36.34 a) at installation.

b. If the delivery volume is constant then the performance verification is met by the daily calibration. If the volume is changed then the performance must be verified for each volume used.

36.351 Operation

a. When using sterile media, use aseptic technique at all times prior to and during a filling operation.

c. Aluminum foil or equivalent autoclave material may be used to wrap equipment for sterilization.

36.352 Maintenance

a. All equipment is cleaned and sanitized after each use.

b. The over all condition of the equipment (i.e. switches, spindles, hoses, etc.) is checked annually. All maintenance observations, performance deviations, and corrective actions taken are recorded in a log.

36.4 Microscope Calibration and Verification

a. All microscopes will have the stage micrometer calibrated at installation.

36.41 Operation

The manufacturer's instructions will be followed when using and adjusting any microscope.

36.42 Maintenance

a. Each microscope is professionally serviced annually.

b. The eyepiece and objective lens is cleaned after each use.

36.5 Automated Equipment

a. Unattended operation increases the importance of strict adherence to instrument operation, maintenance, and calibration instructions.

b. A standard operating procedure is followed for each instrument to ensure the maintenance and calibration is adequate for its intended use.

c. Quality control requirements for certain instrument components (e.g. ovens, incubators, and refrigerators) are included in Section 36.2 of this chapter.

36.51 Spiral Platers Calibration and Verification

a. Spiral platers will be calibrated for use by comparing to conventional plating method at the time of installation.

b. To verify the performance of a spiral plater check the siphon condition daily (see manufacture's instructions), volume dispersal monthly, and compare with conventional plating method annually.

36.511 Operation

Follow manufacturer's instruction for proper operation.

36.512 Maintenance

Spiral platers are cleaned and sanitized after each use by following the manufacturer's instructions.

36.52 Spectrophotometer Calibration and Verification

a. All spectrophotometers will have the wavelength calibrated by the manufacturer at installation.

b. To verify the performance of a spectrophotometer a blank reading will be recorded daily.

36.521 Maintenance

Spectrophotometers are cleaned according to manufacturer's recommendation.

36.53 Hydrometer Calibration

Calibrate to chemical compound annually.

36.6 Laminar Flow Hood/Biohazard Cabinet/Safety Cabinet Calibration and Verification

a. Safety cabinet and laminar flow hoods are services at installation and annually.

b. To verify performance, with each use check the sterility of the hood/cabinet using an open media control. In addition, check the airflow monthly using an appropriate monitor.

36.61 Maintenance

a. Hoods/cabinets are serviced annually.

b. Hoods/cabinets are cleaned and sanitized after each use.

36.7 Centrifuge Maintenance

a. A professional will service all centrifuge equipment on an annual basis. The laboratory will clean and sanitize each centrifuge monthly.

b. Rotors on ultra high centrifuge are maintained annually. The usage of rotor is maintained.

36.8 Measurement Traceability and Calibration of Reference Standards.

Equipment	Requirement	Frequency
Calibrated thermometer*	Calibration Standard reverification	every 5 years
Reference thermocouples	boiling water & ice point	annually
Working thermometers (Including Infra Red)	Calibration Standard traceable calibration	annually
Working thermocouples	Calibration Standard traceable calibration or ref. Thermocouple	annually
Weights*	Recertification to Calibration Standard weights	every 5 years
Balances	Calibration Standard traceable calibration	annually
Timers	national time standard	annually
Volumetric glassware (non class A)	mass, traceable to Calibration Standard weights	annually
Autoclaves	Calibration Standard traceable thermometers or thermocouples	annually

*All thermometers and weights must be calibrated and traceable to national and/or international calibration standards, such as NIST or SI units, etc.

36.9 Microbiology Supplies

36.91 Consumables

Laboratory consumables consist of those items used during the test method and then disposed of after use. These items would include but are not limited to disposable pipettes, petri dishes, scalpels, weigh boats, stomacher/whirlpak bags, or any other item consumed during the course of the test.

These items must be shown to be clean, sterilized, and accurate. The laboratory can satisfy this requirement by having a manufacturer's certificate for each lot to demonstrate performance. The mass/volume delivery of each lot of pipettes shall be verified. The laboratory will maintain the certificates.

36.92 Re-Usables

Laboratory re-usables consist of those items that are used during the analysis and are then cleaned, sterilized, and used again. These items include, but are not limited to, glass pipettes, hockey sticks, test tubes, glassware (non class A), plastic ware, stainless instruments, blenders, knives, or other reusable materials.

Items that have been cleaned and sterilized shall be clearly labeled (e.g. autoclave tape). Cutting utensils can be washed, flamed, and cooled just prior to use.

36.93 Reference Culture/Material

Certified reference cultures (CRC) must be traceable to a nationally or internationally recognized type culture collection (e.g. ATCC). Reference cultures (RC) from laboratory sources must be identified relative to standard reference sources.

These reference cultures must be handled to maintain their biochemical reaction and physiological characteristic integrity. All RC and CRC must not be transferred more than 5 times from the original source. After the fifth transfer the laboratory may purchase another culture from a type culture collection or re-identify the culture for key biochemical and physiological characteristics using nationally or internationally recognized reference sources. Alternatively, the type culture may be grown, then freeze dried or stored frozen and then used periodically, thus, extending the length of time required before repurchase or re-identification.

Stock cultures must be maintained as indicated in the specific chapters of this guidebook. Working stocks are used for quality control and cannot be sub-cultured more than five times. Commercially prepared lyophilized cultures traceable to ATCC can also be used. Records shall clearly show the cross-reference between the identification of each lot of media and the samples analyzed with that media.

36.94 Water Still/DI/RO Units (Laboratory Grade Water)

a. Only water that has been treated to be free from traces of dissolved metal, bactericidal, and inhibitory compounds should be used to prepare culture media, reagents, and dilution blanks. Inhibitor free water is referred to as microbiologically suitable (MS) water. The following tests are performed on the water source to ensure that the water is inhibitor free. Records of the following parameters will be kept.

Weekly testing (or prior to use):
- >1.0 megohms-cm resistance at 25^{o} C.

Monthly testing:
- Total Residual Chlorine must be < 0.01 mg/l
- Aerobic Plate Count must be < 1,000 colony forming unit (cfu)/ml

Annual testing:
- Heavy Metals (Cd, Cr, Cu, Ni, Pb, and Zn-single) must be < 0.05 mg/L
- Heavy Metals (total) must be < 10 mg/L

The suitability of water for microbiological analyses must pass the test for toxicity annually.

b. The DI/RO system-cartridge is replaced as recommended by the manufacturer.

c. Stills are cleaned as recommended by manufacturer.

36.10 Laboratory Maintenance Requirements

36.101 Work Surfaces

a. Prior to processing a sample or initiating culture work, the area must be thoroughly cleaned and sanitized with a suitable EPA registered disinfectant. The area must be thoroughly cleaned and sanitized again at the end of a work segment (e.g. sample preparation, plating, transfers, etc.) and/or the end of the day.

b. When working with pathogenic materials use a solution of 70% ethyl alcohol, 70-90% isopropyl alcohol, or an EPA registered commercial disinfectant (i.e. Lysol, hypochlorite, etc.) prepared at the manufacturer's recommended concentration. If there is a potential for contamination by *Clostridium botulinum* toxin, the 70% ethanol or the hypochlorite solution is adjusted to pH 11.0.

c. Only ethyl alcohol is used in areas where antibiotic residue testing is being done to avoid chance contamination with the phenolic or the hypochlorite solutions.

36.102 Biohazard Material

a. Immediately after use all items are placed in a suitable container with a disinfectant solution prepared at the manufacturer's recommended concentration or directly into a biohazard bag. All items are terminally sterilized at 121ºC for at least 45 minutes.

c. Remove and discard all implements after sterilization. Follow local regulations for final disposal.

36.11 Nonconforming Equipment (Defective)

All equipment shall be properly maintained. Any item of the equipment that has been subjected to overloading or mishandling, or which gives suspect results, or has been shown to be defective, shall be taken out of service. The equipment will be clearly identified and wherever possible, stored at a specific location until it has been repaired and shown by calibration, verification or test to perform satisfactorily. The laboratory shall examine the effect of this defect on previous test results.

36.12 Selected References

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O'Leary, W. M. (ed.). 1977. Practical Handbook of Microbiology. 2nd Edition. CRC Press, 2000 Corporate Blvd., Boca Raton, Fl 33431.

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AOAC International, AOAC INTERNATIONAL Accreditation Criteria for Laboratories Performing Food Microbiological Testing (ALACC)., 1999.

ISO/IEC 17025:1999 GENERAL REQUIREMENTS FOR THE COMPETENCE OF TESTING AND CALIBRATION LABORATORIES

ISO 7218, Microbiology of food and animal feeding stuffs-General rules for microbiological examinations, second edition, 1996-02-15.

Nordic Committee on Food Analysis, Quality Assurance Guidelines for microbiological laboratories, Report no. 5, 2nd edition, 1994.

United States Department of Agriculture
Food Safety and Inspection Service
Office of Public Health and Science
Laboratory QA/QC Division
950 College Station Road
Athens, GA 30605

Microbiology Laboratory Guidebook
Notice of Change

Chapter new, **revised**, or archived: MLG 4.02

Title: Isolation and Identification of *Salmonella* from Meat, Poultry, and Egg Products

Effective Date: 10/25/02

Description and purpose of change(s):

The Microbiology Laboratory Guidebook (MLG) chapters are currently under revision. The formatting is being changed to meet the requirements of the laboratory's document control system. Additional content is being added to meet the requirements of ISO 17025. The chapter has been revised to include a statement of the method detection limits, a section on safety precautions and a revised section on quality control practices. Previous pen-and-ink changes are also incorporated into the revision.

United States Department of Agriculture
Food Safety And Inspection Service, Office of Public Health and Science

MLG 4.02		Page 1 of 16
Title: Isolation And Identification of *Salmonella* From Meat, Poultry And Egg Products		
Revision:02	Replaces: MLG Chapter 4 Revision 1	Effective: 10/25/02

Procedure Outline

4.1 Introduction
- 4.1.1 General
- 4.1.2 Limits of Detection

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- 4.4.1 Equipment
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4.6 Examination of and Picking Colonies from Plating Media
- 4.6.1 Picking colonies
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4.7 Biochemical Procedures

4.8 Serological Tests
- 4.8.1 Somatic (O) Antigen Agglutination Tests
- 4.8.2 Flagellar (H) Antigen Agglutination Tests

4.9 Storage of Cultures

4.10 References

4.1 Introduction

4.1.1 General

This method describes the analysis of various meats, meat products, sponge and rinse samples, eggs, and egg products for *Salmonella*. It is not intended for the isolation and identification of *Salmonella* Typhi.

Success in isolating *Salmonella* from any food can be related to a number of factors including food preparation procedures, the number of organisms present, sample handling after collection, etc. With raw meat samples the competitive flora may be the most important factor. It varies from sample to sample and from one kind of meat to another.

Another consideration is whether the examination is for routine monitoring or epidemiological purposes. The analyst may choose to augment the method for epidemiological purposes with additional enrichment procedures and culture media, two temperatures of incubation, intensified picking of colonies from plates, and/or rapid screening methods.

All isolates must be identified as *Salmonella* biochemically and serologically.

Unless otherwise stated all measurements cited in this method have a tolerance range of ± 2%.

4.1.2 Limits of Detection

The *Salmonella* detection limit for this method has been determined to be less than 1 colony forming unit (cfu)/g in a 25 g sample.

4.2 Safety Precautions

Salmonella are generally categorized as BioSafety Level 2 pathogens. CDC guidelines for manipulating Biosafety Level 2 pathogens should be followed whenever live cultures of *Salmonella* are used. A Class II laminar flow biosafety cabinet is recommended for procedures in which infectious aerosols or splashes may be created. All available Material Safety Data Sheets (MSDS) must be obtained from the manufacturer for the media, chemicals, reagents and microorganisms used in the analysis. The personnel who will handle the material should read all MSDS sheets.

4.3 Quality Control Procedures

4.3.1 Method Controls

Include at least three method controls in all analyses. These controls must include a *Salmonella* spp. H_2S-negative culture, a *Salmonella* spp. H_2S-positive culture and an uninoculated media control. To facilitate identification of control isolates, the laboratory may use strains of uncommonly found serogroups. S. Abaetetuba, serogroup F, is suggested as a readily available, H_2S-positive culture that is not commonly found in meats or meat products. *Salmonella* serotype Choleraesuis is typically negative for H_2S production. These cultures may be obtained from ATCC. Other serotypes may be found that have aberrant H_2S-negative strains. The inoculum level for the positive controls should approximate 30 to 1000 cfu per sample. A 1 microliter loopful of a suspension of a fresh culture equivalent to a 0.5 McFarland Standard may be used for this purpose. Alternatively, commercially prepared bacterial pellets containing concentrations of 100 to 1000 cfu/pellet may be used according to the manufacturer's instructions. The control cultures should be inoculated into either a meat matrix or the matrix that is being analyzed. Incubate the controls along with the samples, and analyze them in the same manner as the samples. Confirm at least one isolate from each positive control sample. In the absence of a positive test sample, control cultures may be terminated at the same point as the sample analyses.

4.3.2 Specific Procedure Controls

The biochemical and serological tests used for confirmation of the sample isolates require the use of appropriate controls to verify that the results are valid. *Salmonella* 'O' antisera should be tested with QC control sera before initial use, and with a saline control for each test. Biochemical kit and rapid test manufacturers may specify control cultures for use with their products. If not specified, quality control procedures for biochemical tests and test media should include cultures that will demonstrate pertinent characteristics of the product.

4.4 Equipment, Reagents, Media and Test Kits

All of the materials listed below may not be needed. Media and reagents specific to the biochemical test method that is used will be needed in addition to the materials listed below. See Section 4.7.

4.4.1 Equipment

a. Sterile tablespoons, scissors, forceps, knives, glass stirring rods, pipettes, petri dishes, test tubes, bent glass rods ("hockey sticks") as needed
b. Blending/mixing equipment: Sterile Osterizer-type blender with sterilized cutting assemblies, and blender jars or Mason jars and adapters for use with Mason jars; *or* a Stomacher™ (Tekmar or equivalent) with sterile Stomacher bags
c. Sterile Stomacher™ 3500 bags, plain, clear polypropylene autoclave bags (ca. 24" x 30 - 36"), or Whirl-Pak™ bags (or equivalent)
d. Incubator, 35 ± 1°C
e. Incubator or water bath, 42 ± 0.5°C
f. Water bath, 48-50°C
g. Glass slides, glass plate marked off in one-inch squares or agglutination ring slides
h. Balance, 2000 g capacity, sensitivity of 0.1 g
i. Inoculating needles and loops
j. Vortex mixer

4.4.2 Reagents

a) Crystal violet dye, 1% aqueous solution, steamed
b) Butterfield's phosphate diluent
c) Saline, 0.85%
d) Saline, 0.85% with 0.6% formalin for flagellar antigen tests
e) Calcium carbonate, sterile
f) *Salmonella* polyvalent O antiserum
g) *Salmonella* polyvalent H antiserum
h) *Salmonella* individual O grouping sera for groups A-I (antisera for further O groups are optional)
i) (Optional) Oxoid *Salmonella* Latex Test (Unipath Company, Oxoid Division, Ogdensburg, NY) or equivalent
j) Additional reagents as needed for biochemical tests

4.4.3 Media

a) Buffered peptone water (BPW)
b) TT broth (Hajna)

c) Modified Rappaport Vassiliadis (mRV) broth, Rappaport-Vassiliadis R10 broth, or Rappaport-Vassiliadis Soya Peptone Broth (RVS)
d) Brilliant green sulfa agar (BGS; contains 0.1% sodium sulfapyridine)
e) Xylose lysine Tergitol™ 4 agar (XLT4) or Double modified lysine iron agar (DMLIA)
f) Triple sugar iron agar (TSI)
g) Lysine iron agar (LIA)
h) Trypticase soy broth (TSB) or Tryptose broth
i) Trypticase soy agar (TSA)
j) Nutrient agar slants
k) Nutrient broth, semi-solid
l) Tryptic soy agar with 5% sheep blood agar
m) Additional media as needed for biochemical tests

4.4.4 Cultures

At least one H_2S-positive strain of *Salmonella* and one H_2S-negative strain of *Salmonella* are required for method controls.

4.4.5 Commercially Available Test Kits (optional)

Any screening method under consideration for *Salmonella* testing must meet or exceed the following performance characteristics: sensitivity ≥ 97%, specificity ≥ 90%, false-negative rate ≤ 3%, and false-positive rate ≤ 10%.

4.5 Isolation Procedures

4.5.1 Sample Pooling

NOTE: Follow sample pooling instructions in specific program protocols. Otherwise, do not use sample pooling.

When examining products that are expected to be *Salmonella*-free, sample pooling can save valuable time, labor and materials. There are several ways this can be done. Pooling at the non-selective enrichment step is appropriate when the likelihood of finding *Salmonella* is unlikely or when, if a positive is found, it is not important to know which particular sample contained the organism.

a. Those samples that are to be examined only for *Salmonella* may be pooled in the blenders. Up to 30 samples may be combined, depending on the capacity of the blenders and culture flasks. The proportions of sample volume to BPW must be maintained at the 1 to 10 ratio (1 part sample to 9 parts BPW). The BPW should be pre-warmed to the temperature of incubation.

b. When food homogenates (Section 4.5.9) are pooled, the culture flask should be warmed in a water bath to bring the contents up to incubation temperature before placing it in the incubator.

c. Incubation of large pools should be prolonged to two days if growth is not apparent in one day. Subculture 5 ± 0.5 ml of the incubated non-selective broth pool into 100 ±1 ml of TT (Hajna), 1 ± 0.1 ml into 100 ± 1 ml of mRV, and proceed as usual.

In cases in which it is important to identify particular samples that may contain *Salmonellae*, it is still possible to take advantage of labor-saving by pooling. In such cases, the samples may be started in the usual way in non-selective broth. After incubation, up to ten of these cultures may be pooled in selective enrichment broth. Maintain the 0.5 to 10 ratio for inoculation of TT broth and the 0.1 to 10 ratio for inoculation of mRV broth. The remaining non-selective broths (or portions of them) are refrigerated. The total volume of selective enrichment broth used will be the same, but the number of plates to be streaked is reduced. If a positive pool is found, all the pooled samples are started individually in selective enrichment broth by going back to the refrigerated non-selective broths.

4.5.2 Breading Mixes, Dehydrated Sauces and Dried Milk

For dehydrated sauces, dried milk, and breading mixes add BPW as described for powdered egg in Section 4.5.8.

4.5.3 Ready-to-Eat Foods

Follow program requirements for preparing sample and sub-sample composites. Outbreak investigation requirements may differ, in which case, follow the client specifications for those samples.

a. Weigh 325 g of the composite sample into a Stomacher bag (or sterile blender jar if required by the client or sample type).

b. Add approximately one third to half of 2925 ml of ambient temperature sterile buffered peptone water. Blend or stomach approximately 2 minutes, and then add the remainder of the 2925 ml of BPW.

c. Incubate at 35 ± 1°C for 20-24 h.

d. Transfer 0.5 ± 0.05 ml of incubated broth into 10 ml TT and 0.1 ± 0.02 ml into 10 ml of mRV broth.

e. Incubate the enrichment broths at 42 ± 0.5°C for 22-24 h, or in a water bath at 42 ± 0.5°C for 18-24 h.

f. Streak above enrichments on BGS and either DMLIA or XLT4 agar plates. Use one 10-microliter loopful for each plate. Do not subdivide plates for streaking multiple samples; streak the entire agar plate with a single sample enrichment.

g. Incubate at 35 ± 1°C.

h. Examine in 18-24 h. Select colonies. Refer to Section 4.6 et seq.

i. Re-incubate all plates for an additional 18-24 h. Reexamine initially negative plates and pick colonies as above. Reserve, under refrigeration, all plates from which colonies were picked. If suspect *Salmonella* colonies do not confirm, reexamine the plates from which they were picked, and if appropriate, re-pick colonies for confirmation. See Section 4.6.1.b.

4.5.4 Fermented Products

Follow the procedure for ready-to-eat foods (Section 4.5.3) except:

a. Blend/stomach the sample with 10 g of sterilized calcium carbonate.

b. Use buffered peptone water that contains 1 ml of a 1% aqueous solution of crystal violet per liter.

4.5.5 Raw Meat

If the sample is not already ground, in some cases it may be best to mince it with scissors or leave it whole (e.g. chicken wings) to avoid jamming blender blades with skin or connective tissue. Whirl-Pak™ bags can be used in culturing these samples.

a. Weigh 25 ± 0.5g of meat into a sterile blender jar, other sterile jar or a Whirl-Pak™ or Stomacher™ bag. HACCP program samples collected using a sampling ring are allowed a weight range of 25 ± 2.5 g.

b. Add 225 ml of BPW. Stomach or blend, as required, for approximately two minutes or shake thoroughly.

c. Incubate at 35 ± 1°C for 20-24 h.

d. Transfer 0.5 ± 0.05 ml into 10 ml TT broth and 0.1 ± 0.02 ml into 10 ml mRV broth.

e. Incubate at 42 ± 0.5°C for 22-24 h.

f. Streak on DMLIA or XLT4 and BGS agar plates. Use one loopful of inoculum for each plate. Do not subdivide plates for streaking multiple samples; streak the entire agar plate with a single sample enrichment.

g. Incubate at 35 ± 1°C.

h. Examine in 18-24 h. Select colonies. See Section 4.6 et seq.

i. Re-incubate all plates for an additional 18-24 h. Reexamine initially negative plates and pick colonies as above. Reserve, under refrigeration, all plates from which colonies were picked. If suspect *Salmonella* colonies do not confirm, reexamine the plates from which they were picked, and if appropriate, re-pick colonies for confirmation. See Section 4.6.1.b.

4.5.6 Carcass Sponge Samples

a. Add 50 ml of BPW to the sample bag containing the moistened sponge to bring the total volume to 60 ml. Mix well.

b. Incubate at 35 ± 1°C for 20-24 h.

c. Follow the procedures in Section 4.5.5, d-i.

4.5.7 Whole Bird Rinses

Due to differences between sample types/sizes (e.g. chicken vs. turkey carcasses), follow instructions given in the specific program protocol. For chicken carcasses, aseptically drain excess fluid from the carcass and transfer the carcass to a sterile Stomacher™ 3500 bag, a plain, clear polypropylene bag (ca. 24" x 30-36"), or equivalent. Pour 400 ml (or other volume specified in program protocol) of Buffered Peptone Water (BPW) into the cavity of the carcass contained in the bag. Rinse the bird inside and out with a rocking motion for one minute (ca. 35 RPM). This is done by grasping the broiler carcass in the bag with one hand and the closed top of the bag with the other. Rock with a reciprocal motion in about an 18-24 inch arc, assuring that all surfaces (interior and exterior of the carcass) are rinsed. Transfer the sample rinse fluid to a sterile container.

Use 30 ml of the sample rinse fluid obtained above for *Salmonella* analysis. Add 30 ml of sterile BPW, and mix well. Incubate at 35 ± 1°C for 20-24 h, and then proceed according to 4.5.5 (d-i).

NOTE: If analyses other than *Salmonella* are to be performed, the carcass may be rinsed in Butterfield's Phosphate Diluent instead of BPW. In this case, add 30 ml of 2X BPW to 30 ml of carcass-rinse fluid, mix well, and continue as above.

4.5.8 Liquid, Frozen, Cooked or Dried Egg Samples

a. Mix the sample with a sterile spoon, spatula, or by shaking.

b. Aseptically weigh a minimum of 100 g of egg sample into a sterile blender jar, other sterile jar, or a Whirl-Pak™ or Stomacher™ bag containing 900 ml of sterile BPW. If a special sample or specification requires a sample size other than 100 g, the ratio of egg sample to BPW is to be maintained at 1:10.

c. Mix the inoculated BPW well by shaking, stomaching, or blending.

d. With dried egg samples, gradually add BPW to the sample. Add a small portion of sterile BPW and mix to obtain a homogeneous suspension. Add the remainder of the BPW. Mix until a lump-free suspension is obtained.

e. Incubate at 35 ± 1°C for 20-24 h, and then proceed according to 4.5.5 (d-i).

4.5.9 Sanitation Series Food Homogenates (optional)

To isolate salmonellae from food samples homogenized as outlined in MLG 3 Section 3.3.1, as part of a sanitation test series, use the 10^{-1} food homogenate dilution (See also this chapter, Section 4.5.1 Sample Pooling).

a. Weigh 250 g of food homogenate into a sterile jar (this contains 25 g of product).

b. Add 25 ml of 10x BPW (broth made to ten time's normal strength).

c. Incubate 24-26 h at 35 ± 1°C.

d. Transfer 0.5 ± 0.05 ml into 10 ml of TT broth and 0.1 ± 0.02 ml into 10 ml of mRV broth.

e. Incubate at 42 ± 0.5°C for 22-24 h.

f. Streak on DMLIA or XLT4 and BGS agar plates. Use one loopful for each plate. Do not subdivide plates for streaking multiple samples; streak the entire agar plate with a single sample enrichment.

g. Incubate 18-24 h at 35 ± 1°C.

h. Select colonies. See Section 4.6 et seq.

i. Re-incubate all plates for an additional 18-24 h. Reexamine initially negative plates and pick colonies per Section 4.6. Reserve all plates from which colonies were picked. If suspect *Salmonella* colonies do not confirm, reexamine the plates from which they were picked, and if appropriate, re-pick colonies for confirmation. See Section 4.6.1.b.

4.5.10 Most Probable Numbers (MPN) Determination

Due to differences between sample types (e.g. whole chicken rinse vs. ground beef) and sample sizes (e.g. 25 g vs. 325 g) follow MPN instructions given in the specific program protocol. See also MLG Appendix 2, Most Probable Number Tables.

4.6 Examination of and Picking Colonies from Plating Media

4.6.1 Picking Colonies

a. After the recommended incubation interval, examine the selective-differential agar plates for the presence of colonies meeting the description for suspect *Salmonella* colonies. Pick well-isolated colonies.

- BGS. Select colonies that are pink and opaque with a smooth appearance and entire edge surrounded by a red color in the medium. On very crowded plates, look for colonies that give a tan appearance against a green background.
- XLT4. Select black colonies or red colonies with or without black centers. The rim of the colony may still be yellow in 24 h; later it should turn red.
- DMLIA. Select purple colonies with or without black centers. Since salmonellae typically decarboxylate lysine and ferment neither lactose nor sucrose, the color of the medium reverts to purple.

b. Pick up to three colonies from each plate, if available. (NOTE: Before any sample is reported as *Salmonella*-negative, a total of three typical colonies, if available, from each selective agar plate must be examined). Pick only from the surface and center of the colony. Avoid touching the agar because these highly selective media suppress growth of many organisms that may be viable.

If there are typical colonies on a plate, that are not well isolated, pick from the typical colonies and re-streak directly to selective agar plates. Alternatively, place a loopful of growth into a tube of TT or mRV broth and incubate overnight, then re-streak to selective agars.

4.6.2 Screening Media

a. Inoculate TSI and LIA slants in tandem with a single pick from a colony by stabbing the butts and streaking the slants in one operation. If screw cap tubes are used, the caps must be loosened. Incubate at 35 ± 1°C for 24 ± 2 h.

b. Examine TSI and LIA slants as sets. Note the colors of butts and slants, blackening of the media and presence of gas as indicated by gas pockets or cracking of the agar. Note also the appearance of the growth on the slants along the line of streak. Discard, or re-streak for isolation, any sets that show "swarming" from the original site of inoculation. Discard sets that show a reddish slant in lysine iron agar. Isolates giving typical *Salmonella* spp. reactions and isolates which are suggestive, but not typical of *Salmonella* spp. should be confirmed by a combination of biochemical and serological procedures. Refer to Table 1 for a summary of TSI-LIA reactions. The motility testing in the last column of the table is optional.

c. (Optional: for some biochemical test kits) Streak a TSA + 5% sheep blood agar plate from either the TSI or LIA slant. Incubate 18-24 h at 35 ± 1°C.

4.7 Biochemical Procedures

Commercially available biochemical test kits, including automated systems may be used for biochemical identification. If the VITEK® test kit is used, the cytochrome oxidase and gram stain tests are optional. Alternatively, use traditional methods of biochemical identification. Refer to AOAC Official Method 967.27 or "Edwards and Ewing's Identification of Enterobacteriaceae", 4th Edition, for biochemical reactions of *Enterobacteriaceae* and for fermentation media and test procedures.

Table 1.

Triple Sugar Iron Agar			Lysine Iron Agar		Polyvalent Sera		Disposal
Butt	Slant	H_2S	Butt	H_2S	O	H	
Y	R	+	P	+	+	+	B. & M. T.
Y	R	+	P	+	+	-	B. & M. T.
Y	R	-	P	-			B. & M. T.
Y	R	-	Y	-	+	+	* B. & M. T.
Y	R	-	Y	-	-	-	Discard
Y	R	+	Y	+/-			B. & M. T.
Y	Y	-	Y or P	-			Discard
Y	Y	+	P	+			** B. & M. T.
NC	NC						Discard

Y = Yellow; R = Red; P = Purple; B. & M. T. = Biochemical and motility tests; NC = No change in color from uninoculated medium.

* *Salmonella* Typhisuis (found seldom in swine in U.S.)
** *Salmonella enterica* subsp. *arizonae* or *S. enterica* subsp. *diarizonae*

4.8 Serological Tests

4.8.1 Somatic (O) Antigen Agglutination Tests

At a minimum, isolates should be tested with polyvalent O antiserum reactive with serogroups A through I. Following a positive reaction with polyvalent O antiserum, it is necessary to type the isolate using individual *Salmonella* antisera for O groups A through I.

Testing for O groups A through I should encompass the majority of the *Salmonella* serotypes commonly recovered from meat and poultry products. Occasionally, however, an isolate will be recovered which is typical of *Salmonella* biochemically and is serologically poly H-positive, but is non-reactive with antisera to groups A through I. Such an isolate should be reported as "*Salmonella* non A-I" or "*Salmonella* O group beyond I".

Use growth from either the TSI or LIA slant. Test first with polyvalent O antiserum. Include a saline control with each isolate. If there is agglutination with the saline control alone (autoagglutination), identify such a culture by biochemical reactions only. If the saline control does not agglutinate and the polyvalent serum does, test the culture with *Salmonella* O grouping antisera. Record positive results and proceed to H agglutination tests.

4.8.2 Flagellar (H) Antigen Agglutination Tests

Inoculate trypticase soy broth or tryptose broth. Incubate at 35 ± 1°C overnight or until growth has an approximate density of three on the McFarland scale. Add an equal amount of saline containing 0.6% formalin and let sit one hour. Remove one ml to each of two 13 x 100 mm test tubes. To one of the tubes, add *Salmonella* polyvalent H serum in an amount indicated by the serum titer or according to the manufacturer's instructions. The other tube serves as an autoagglutination control. Incubate both tubes at 48-50°C in a water bath for up to 1 h. Record presence or absence of agglutination.

If desired, use Spicer-Edwards pooled serum or H typing serum. Find details in "Edwards and Ewing's Identification of Enterobacteriaceae" (Ewing, 1986).

The Oxoid *Salmonella* Latex Test, or equivalent, may be used as an optional method for H antigen agglutination testing. Follow the manufacturer's instructions. If a suspect *Salmonella* isolate is negative by the latex test, perform the poly H tube agglutination test described above.

4.9 Storage of Cultures

Do not store cultures on TSI agar because this tends to cause roughness of O antigens. For short-term (2-3 months) storage, inoculate a nutrient agar slant, incubate at 35 ± 1°C overnight and then store at 4-8°C.

Store "working" *Salmonella* stock cultures on nutrient agar slants. Transfer stocks monthly onto duplicate nutrient agar slants, incubate overnight at 35 ± 1°C, and then store them at 4-8°C. Use one of the slants as the working culture. Use the other slant for sub-culturing to reduce the opportunity for contamination. Cultures may be subcultured up to 5 times. After this period the culture must be re-confirmed biochemically or a new culture initiated.

For long term storage freeze cultures using cryo-beads, i.e. Cryostor™ or equivalent, lyophilize or use the procedure that follows. Subculture *Salmonella* isolates by picking a colony with an inoculating needle and stabbing it into semi-solid nutrient broth (0.75% agar). Incubate at 35 ± 1°C overnight, and then seal with hot, paraffin-soaked corks. Household wax is better than embedding paraffin because it stays relatively soft at room temperature making the corks easy to remove. Store the cultures in the dark at room temperature. Such cultures will remain viable for several years.

4.10 Selected References

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United States Department of Agriculture
Food Safety and Inspection Service
Office of Public Health and Science
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Microbiology Laboratory Guidebook
Notice of Change

Chapter new, **revised**, or archived: MLG 5.03

Title: Detection, Isolation, and Identification of *Escherichia coli* O157:H7 and O157:NM (Nonmotile) from Meat Products

Effective Date: 10/25/02

Description and purpose of change(s):

The Microbiology Laboratory Guidebook method chapters are currently under revision. The formatting is being changed to meet the requirements of the laboratory's document control system. Additional content is being added, i.e. Section 5.1.2. Limits of Detection, to meet the requirements of ISO 17025. Safety Precautions are also included in the revised chapters.

United States Department of Agriculture
Food Safety And Inspection Service, Office of Public Health and Science

MLG 5.03		Page 1 of 13
Title: Detection, Isolation and Identification of *Escherichia coli* O157:H7 and O157:NM (Nonmotile) from Meat Products		
Revision: 03	Replaces: MLG Chapter 5 Revision 2	Effective 10/25/02

Procedure Outline

5.1 Introduction
- 5.1.1 General
- 5.1.2 Limits of Detection

5.2 Safety
5.3 Quality Control Practices
5.4 Equipment, Materials, Media, Reagents and Test Kits
- 5.4.1 Equipment
- 5.4.2 Media, Reagents and Cultures
- 5.4.3 Test Kits

5.5 Detection Procedure
5.6 Isolation Procedure
5.7 Identification and Confirmation
5.8 Storage of Cultures
5.9 Selected References

5.1 Introduction

5.1.1 General

The following method is used for the analysis of raw and ready-to-eat meat products for *Escherichia coli* O157:H7 and O157:NM (O157:H7/NM). The method is based on enrichment in a selective broth medium, application of a rapid screening test, immunomagnetic separation (IMS) in paramagnetic columns, and plating on a highly selective medium.

The following definitions are used for reporting purposes. A potential positive sample causes a positive reaction on the screen test kit. A presumptive positive sample has typical colonies, observed on Rainbow Agar, and reacts specifically with O157 antiserum. A sample is a confirmed positive sample for E.coli O157:H7 or E. coli O157:NM when the isolate is confirmed biochemically and serologically, and the presence of Shiga toxin(s) or Shiga toxin gene(s) is demonstrated.

Unless otherwise stated all measurements cited in this method have a tolerance of ± 2%.

5.1.2 Limits of Detection

This test has been shown to consistently detect less than 1 colony forming unit (cfu)/g in a 65 g sample.

5.2 Safety

E. coli O157:H7/NM is a human pathogen with a low infectious dose. (Ingestion of 100 cells can cause disease.) The use of gloves and eye protection is mandatory and all work surfaces must be disinfected prior to and immediately after use. Laboratory personnel must abide by CDC guidelines for manipulating Biosafety Class II pathogens. A Class II laminar flow biosafety cabinet is recommended for activities with potential for producing aerosols of pathogens. All available Material Safety Data Sheets (MSDS) should be obtained from the manufacturer for the media, chemicals, reagents and microorganisms used in the analysis. The personnel who will handle the materials should read all MSDS sheets.

5.3 Quality Control Practices

a. Rainbow® Agar plates have a shelf life of 2 weeks.

b. All media and E-Buffer must be pre-warmed to 18-35ºC prior to use.

c. The recommended fluorescent strain of *E. coli* O157:H7 must be used in this procedure to monitor for cross contamination. The protocol for the use of fluorescent strains of *E.coli* O157:H7 as positive controls follows:

   Wild-type strains of *E. coli* O157:H7 transformed with pGFP produce a green fluorescent protein. As a result of this transformation, fluorescent strains of *E. coli* O157:H7 possess the unique property of expressing bright green fluorescence visible in the dark when illuminated by long-wave UV light. This property, which sets them apart from typical *E. coli* O157:H7, makes them useful positive controls for analyses of meat samples for *E. coli* O157:H7/NM. At different steps in the procedure, both test samples and (fluorescent) positive controls can be tested for the bright green fluorescence as a Quality Control measure to make sure that positive sample isolates actually came from the test sample and not from accidental contamination by the positive control cultures.

Results of studies done at the FSIS Beltsville Microbial Pathogens Laboratory showed that these fluorescent cultures can be subjected to *E. coli* O157:H7/NM isolation and identification procedures without losing their fluorescent properties. These strains retain their fluorescent properties when grown in SOB media with added ampicillin (SOB + A). These cultures must be transferred every 7 days to fresh SOB + A media, according to the protocol outlined below. The fluorescent colonies are ready to be used as positive controls on day 3 of the following protocol, and for the next 6 consecutive days without losing their fluorescent properties. If these cultures are not needed on a continuous basis, they can be stored at refrigeration temperatures on SOB + A agar plates in zip-lock bags or sealed with parafilm® for 1 month and then transferred, or started up again 2 days before needed. *Strict adherence* to the protocol described below is essential, in order to ensure that the fluorescent strains do not lose their ability to express green fluorescence.

i. Test the fluorescent *E. coli* O157:H7 strain (FSIS culture # EC 465-97 or the currently designated control strain) on SOB + A agar plate for fluorescence by illuminating colonies under long-wave UV light in the dark.

ii. Select only fluorescing colonies and inoculate into 10 ml of SOB + A broth in a tube. Incubate at 35 ± 2°C overnight.

iii. Streak the culture from the SOB + A broth onto a SOB + A agar plate. Incubate at 35 ± 2°C overnight.

iv. Examine colonies on the plate for fluorescence. The fluorescent colonies are ready to be inoculated into modified EC broth + novobiocin (mEC+n) at this stage. These cultures on SOB + A agar plates can be stored refrigerated and be used as positive controls for 6 more days. Incubate the inoculated mEC+n positive control culture at 35 ± 2°C overnight, along with the test samples.

v. Continue analysis per Sections 5.5-5.7 and test the Blood Agar Plates of the fluorescent positive controls and any positive sample cultures for fluorescence.

5.4 Equipment, Materials, Media, Reagents and Test Kits

5.4.1 Equipment

a. Balance, sensitivity of 0.1 g
b. Stomacher™ 400 or 3500 with appropriate sizes of sterile Stomacher™ bags, with or without mesh. (Tekmar Co., Cincinnati, Ohio), or equivalent
c. Incubator, static 35 ± 2°C
d. Micropipettors to deliver 15-1000 µl with sterile disposable filtered micropipet tips
e. Mechanical Pipettor with 1.0 ml, 5.0 ml, 10.0 ml sterile pipettes
f. Inoculating loops, "hockey sticks" or spreaders, and needles
g. UV light (long-wave, e.g. VWR # 36553-124, or equivalent)
h. Filter unit, 0.2 µm, nylon, sterile
i. Infrared thermometer
j. LabQuake® Agitator (or equivalent) with clips to hold microcentrifuge tubes
k. Sterile disposable 12 x 75 mm polypropylene tubes (e.g. Fisher # 14-956-1B, or equivalent)
l. Microcentrifuge and sterile 1.5 ml microcentrifuge tubes
m. Sterile 50 ml conical tubes (e.g. Falcon® # 2070, or equivalent) or sterile bottles
n. Sterile 40 µm Cell Strainer (Falcon® # 2340, or equivalent)
o. MACS® Large Cell Separation Columns (Miltenyi Biotec # 422-02, or equivalent)
p. OctoMACS® Separation Magnet (Miltenyi Biotec # 421-09, or equivalent)
q. Multistand to support OctoMACS® Separation Magnet (Miltenyi Biotec # 423-03, or equivalent)
r. Tray, autoclavable, approximately 130 mm x 83 mm (e.g. VWR # 62663-222, or equivalent) for use with the OctoMACS®

5.4.2 Media, Reagents and Cultures

a. Modified EC broth with novobiocin (mEC+n) (or equivalent)
b. Rainbow® Agar O157 (Biolog Inc., Hayward California, 94545) containing 10 mg/L novobiocin plus 0.8 mg/L potassium tellurite, or equivalent selective medium
c. Tryptic soy agar with 5% sheep blood

d. SOB + A Medium
e. E Buffer, approximately 7 ml per sample [Buffered Peptone Water, Bovine Albumin Sigma # 7906 (or equivalent) , and Tween-20®, or equivalent]
f. Disinfectant (Lysol® I. C., 2.0%, or equivalent)
g. Dynal® # 710.04 anti-*E. coli* O157 antibody-coated paramagnetic beads (Dynal Inc., Lake Success, NY 11042), or equivalent
h. *E. coli* O157:H7 strain 465-97 (positive control used throughout method)
i. *E. coli* ATCC strain 25922 (negative control for bead capture and screen tests)

5.4.3 Test Kits

a. The screening test for the detection of *E. coli* O157:H7/NM should meet or exceed the following performance characteristics:

Sensitivity	≥98%
Specificity	≥90%
False Negative Rate	≤ 2%
False Positive Rate	≤10%

b. *E. coli* O157:H7 latex agglutination test kit (RIM® *E. coli* O157:H7 Latex Test Kit, REMEL, 12076 Santa Fe Drive, Lenexa, KS 66215, or equivalent)

c. Biochemical test kits and systems [Vitek® GNI and GNI Plus cards (bioMerieux Vitek, Inc., 595 Anglum Drive, Hazelwood, MO 63042-2395), or equivalent]

d. Shiga Toxin test kit [Premier® EHEC, cat. # 608096 (Meridian Diagnostics, Inc., 3471 River Hills Dr., Cincinnati, OH, 45244), or equivalent

5.5 Detection Procedure

a. Sample Preparation

i. Raw ground beef microbiological testing programs.
Randomly collect five 65 ± 2 g sub-samples (total of 325 ± 10 g) that are representative of the entire sample. Place each 65 ± 2 g sub-sample in a

sterile Strainer Stomacher™ bag. Add 585 ml mEC+n broth and pummel for two minutes in a Stomacher™.

ii. Cooked meat patties and semi-dry and dry fermented sausages. Randomly prepare five 65 ± 2 g sub-samples (total of 325 ± 10 g) that are representative of the entire sample. When appropriate, sample representative portions from both the outer surface (shell) and inner section (core) of RTE products, especially semi-dry and dry fermented sausages. Place each 65 ± 2 g sub-sample in a sterile Strainer Stomacher™ bag. Add 585 ml mEC+n broth and pummel for two minutes in a Stomacher™.

iii. Outbreak-related samples. Randomly collect thirteen 25 ± 1 g sub-samples (total of 325 ± 13 g) that are representative of the entire sample. Place each 25 ± 1 g sub-sample in a sterile Strainer Stomacher™ bag and add 225 ml of mEC+n broth. Pummel for 2 minutes in a Stomacher™.

b. Incubate all bags (static) with their contents for 20 to 24 h at 35 ± 2°C. Include a positive, negative, and uninoculated medium control for each group of samples tested. Use the fluorescent *E. coli* O157:H7 strain (FSIS culture # EC 465-97) as a positive control and *E. coli* ATCC strain 25922 as the negative control.

c. From the enrichment cultures in the Stomacher™ bags, perform the screening test for *E. coli* O157:H7/NM following the manufacturer's instructions. The enrichment culture may be analyzed immediately upon removal from the incubator without waiting for tempering to room temperature. To prevent clogging the pipette tip, be sure to collect the appropriate size sample from the enrichment culture outside the inner strainer bag.

d. Samples negative by the screening test can be reported as negative for *E. coli* O157:H7/NM and discarded.

e. Samples positive by the screening test should be reported as potential positives. Begin isolation procedures from the enrichment culture in the Stomacher™ bag.

5.6 Isolation Procedure

Note: Steps a.-l. may be performed in a sequence that is convenient to the laboratory personnel.

a. Prepare E Buffer by mixing 0.5 g Bovine Albumin and 50 µl Tween-20® into 100 ml Buffered Peptone Water (BPW). Filter sterilize (0.2 µm) and store at 2-8°C.

b. Remove Rainbow® Agar plates from 2-8°C storage, allowing 3 plates for each screen-positive culture and each control. Be sure that plates have no visible surface moisture at the time of use. If necessary, dry plates (e.g. for up to 30 minutes in a laminar flow hood with the lids removed) prior to use. Dried plates that are not used should be labeled "dried", placed in bags and returned to 2-8°C.

c. Remove a bottle of E Buffer from 2-8°C storage. Decant 7 ml of E Buffer for each culture and each control into a sterile tube or bottle and allow it to warm to at least 18°C. (Return the stock E Buffer to 2-8°C.)

d. For each positive control, negative control and screen-positive culture to be analyzed, order and label 50 ml conical centrifuge tubes so that the positive control is first, followed by the negative control, then all cultures. Maintain this order for subsequent steps.

e. For each positive control, negative control, and screen-positive culture, label two sterile 1.5 ml microcentrifuge tubes (for step g and step s), one 50 ml conical centrifuge tube (for step h.) and two 12 x 75 mm capped tubes (one for step p.). For each pair of 12 x 75 mm tubes, label one tube and add 0.9 ml E Buffer (for step q.).

f. Prepare the Dynal #710.04 *E. coli* O157:H7 immunomagnetic bead suspension by following Table 1 below. Be sure to include the positive and negative controls in the total number of cultures. Use the bead suspension immediately (step g), or hold at 2-8°C. Return the stock vial of Dynal #710.04 *E. coli* O157:H7 immunomagnetic beads to 2-8°C.

g. Vortex the bead solution briefly (2-3 seconds), then add 50 µl to a labeled

microcentrifuge tube (from step e), one for each control and screen-positive culture. Use immediately or hold these tubes at 2-8°C.

h. Place a 40 µm Cell Strainer on a labeled 50-ml conical centrifuge tube (from step e.). Pipet 5 ± 1 ml of each control and enrichment culture into the respective Cell Strainer and collect at least 1.0 ml of filtrate.

i. Do not proceed with more than the number of tubes that the OctoMacs® magnet(s) will hold. Transfer 1.0 ml of a filtrate (step h.) to the corresponding microcentrifuge tube containing the immunomagnetic bead suspension (step g.) and place in the clips of the LabQuake® tube agitator. Rotate the tubes for 10-15 min at 18-30°C.

j. Attach the OctoMACS® Magnet to the Multistand.

k. Position a tray on the base of the Multistand so that it will collect the filtrate passing through the columns. Add approximately 300 ml of 2% Lysol® I. C. (or equivalent) disinfectant to cover the bottom of the tray.

l. Label and place the appropriate number of Large Cell Separation columns on the OctoMACS® Magnet. Insert columns from the front making sure the column tips do not touch any surfaces. Leave the plungers in the bags at this time to maintain sterility.

m. Transfer at least 0.5ml E Buffer to the top of each column and let the buffer run through.

n. Resuspend, then transfer each culture and control from step i. to its corresponding column.

o. After a culture or control has drained through, wash the column by applying 1.0 ml of E Buffer to each column and allow to drain. Repeat 3 more times for a total of 4 washes.

p. After the last wash has drained, remove the column from the OctoMACS® Magnet and insert the tip into an empty labeled 12 x 75 mm tube (from step e.).

Apply 1.0 ml of E Buffer to the column, and using the plunger supplied with the column, *immediately* flush out the beads into the tube. Use a smooth, steady motion to avoid splattering. Cap the tubes. Repeat this for each column. If the OctoMACS® magnet is to be used for a second set of cultures, it must be decontaminated as described in step u, below. Repeat steps j.-s. for the additional cultures.

q. Vortex the tubes from step p. briefly to resuspend the beads. Make a 1:10 dilution of each treated bead suspension by adding 0.1 ml of the bead suspension to a 12 x 75 mm labeled tube containing 0.9 ml E Buffer (from step e.).

r. Vortex briefly to maintain beads in suspension and plate 0.1 ml from each tube (from step p. and step q.) onto a labeled Rainbow® Agar plate. Use a hockey stick or spreader to spread plate the beads, being careful not to spread the beads against the edge of the plate.

s. Vortex the tubes containing undiluted beads (from step p.) and transfer to a labeled microfuge tube (from step e.) and centrifuge at least one minute using a bench-top microcentrifuge to concentrate the beads. Withdraw and discard the supernatant without disturbing the beads. Add 0.1 ml of E Buffer to the beads, resuspend the beads and transfer the beads to a labeled Rainbow® Agar plate. Spread plate the beads as described in step r.

t. As soon as there is no visible moisture on the agar surface, invert plates and incubate for 24-26 h at 35 ± 2°C.

u. Decontaminate the OctoMACS® Magnet by applying 2% Lysol® I. C. (or equivalent) disinfectant directly to the surface. After approximately ten minutes, rinse with deionized or tap water. Allow the unit to air-dry or use absorbent paper towels to dry the unit.

Table 1.

# of Cultures	*ul of Beads**	*ul of E-Buffer*	*# of Cultures*	*ul of Beads**	*ul of E-Buffer*
1	15	135	26	145	1305
2	20	180	27	150	1350
3	25	225	28	155	1395
4	30	270	29	160	1440
5	35	315	30	165	1485
6	40	360	31	175	1575
7	45	405	32	180	1620
8	50	450	33	185	1665
9	55	495	34	190	1710
10	60	540	35	195	1755
11	65	585	36	200	1800
12	70	630	37	205	1845
13	75	675	38	210	1890
14	80	720	39	215	1935
15	85	765	40	220	1980
16	90	810	41	230	2070
17	95	855	42	235	2115
18	100	900	43	240	2160
19	105	945	44	245	2205
20	110	990	45	250	2250
21	120	1080	46	255	2295
22	125	1125	47	260	2340
23	130	1170	48	265	2385
24	135	1215	49	270	2430
25	140	1260	50	275	2475

* Dynal® anti-*E. coli* O157:H7 antibody-coated paramagnetic beads (vortex briefly before use)

5.7 Identification and Confirmation

a. After incubation, *E. coli* O157:H7 colonies have black or gray coloration on Rainbow® Agar. When *E. coli* O157:H7 colonies are surrounded by pink or magenta colonies, they may have a bluish hue. Mark colonies typical of *E. coli* O157:H7 and perform latex agglutination assays for O157, following manufacturer's instructions. Streak all latex positive colonies, up to a total of five from each sample (one per sub-sample, if possible) onto Blood Agar plates. Incubate Blood Agar plates for 16-24 h at 35 ± 2°C.

Note: If no typical colonies are present, hold the original Rainbow® plates at 20-24°C for an additional 6-24 h then re-examine for typical colonies.

b. After incubation, examine the Blood Agar plates for purity under visible light, and evidence of cross contamination with the positive control by using long wave UV light. Only the positive control culture, *E. coli* O157:H7 strain 465-97, should fluoresce. If the Blood Agar plates appear pure and uncontaminated, perform the following confirmatory tests:

i. Biochemical confirmation.
Inoculate Vitek-GNI or GNI Plus cards or use an equivalent biochemical identification testing system. The cytochrome oxidase and gram stain tests are optional.

ii. Serological confirmation.
To confirm the absence or presence of O157 and H7 antigens, use an *E. coli* O157:H7 latex test agglutination kit (RIM® *E. coli* O157:H7 Latex Test Kit, or equivalent). Use growth from the Blood Agar plate (from step b).

iii. Shiga toxin/toxin genes confirmation.
The presence of Shiga toxin(s) in a culture isolate should be confirmed by the use of a toxin assay, e.g., Meridian Premier® EHEC Kit, or equivalent. When Shiga toxin(s) is (are) not demonstrated, detection of one or more toxin genes by PCR should be used for confirmation. The positive control culture, *E. coli* O157:H7, is toxin-negative.

c. If the isolate confirms as an *E. coli* O157:H7, or *E. coli* O157:NM (or H-indeterminate) and the Shiga toxin(s) and/or one or more toxin genes are present, the sample will be treated as positive for *E. coli* O157:H7, and regulatory action will be taken. The cultures will also be tested by pulsed-field gel electrophoresis (PFGE) for potential epidemiological association.

5.8 Storage of Cultures

For storage requirements of the fluorescent *E. coli* O157:H7 strain (FSIS culture # EC 465-97 or the currently designated control strain), refer to Section 5.3.c. of this chapter.

Store other "working" *E. coli* stock cultures on nutrient agar slants. Transfer stocks monthly onto duplicate nutrient agar slants, incubate overnight at 35 ± 1°C, and then store them at 4-8°C. Use one of the slants as the working culture. Use the other slant for sub-culturing to reduce the opportunity for contamination. Cultures may be subcultured up to 5 times. After this period the culture must be re-confirmed biochemically or a new culture initiated.

For long term storage freeze cultures using cryo-beads i.e. Cryostor™ or lyophilize.

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Microbiology Laboratory Guidebook
Notice of Change

Chapter **new**, revised, or archived: MLG 8A.00

Title: FSIS Procedure for the Use of *Listeria monocytogenes* BAX® Screening Test

Effective Date: 4/29/02

Description and purpose of change(s):

The use of a rapid screening procedure potentially reduces report-out time for true negative samples by 24 hours. FSIS has validated use of this commercial PCR based screening procedure for processed meat and poultry products. All samples identified as presumptively positive for *Listeria monocytogenes* by these tests are subject to cultural confirmation as described in this chapter and MLG 8 Isolation and Identification of Listeria monocytogenes from Red Meat, Poultry, Egg, and Environmental Samples.

United States Department of Agriculture
Food Safety And Inspection Service, Office of Public Health and Science

MLG 8A.00		Page 1 of 4
Title: FSIS Procedure For the Use of *Listeria monocytogenes* BAX® Screening Test		
Revision: Original	Replaces: NA	Effective: 4/29/02

Procedure Outline

8A.1 Introduction
- 8A.1.1 General
- 8A.1.2 Limits of Detection

8A.2 Safety Precautions

8A.3 Quality Control Procedures
- 8A.3.1 Culture Controls
- 8A.3.2 Sterility Control

8A.4 Equipment, Reagents, and Media

8A.5 Sample Preparation and Primary Enrichment

8A.6 Secondary Enrichment and Direct Plating

8A.7 The BAX® System for Screening L. monocytogenes Test Procedure

8A.8 Cultural Confirmation

8A.9 Interpretation of Results

8A.10 Completion of Testing if BAX® Unavailable

8A.11 Selected References

8A.1 Introduction

8A.1.1 General

This method describes the use of a commercial PCR based screening procedure as described in MLG 8 Section 8.4.5. to screen-test processed meat and poultry products for the presence of *Listeria monocytogenes.* All samples identified as presumptively positive for *Listeria monocytogenes* by these tests are subject to cultural confirmation.

8A.1.2 Limits of Detection

For this method, *L. monocytogenes* detection limits are determined to be better than 1 cfu/g in a 25g sample.

8A.2 Safety Precautions

CDC guidelines for the handling of BioSafety Level 2 organisms should be followed whenever live cultures of *Listeria monocytogenes* are used. All available Material Safety Data Sheets (MSDS) must be obtained from the manufacturer for the media, chemicals, reagents, and microorganisms

used in the analysis. The personnel who will handle the material should read all MSDS sheets, and all MSDS requirements should be followed.

Pregnant women and potentially immunocompromised individuals must be prohibited from laboratory rooms or areas where *L. monocytogenes* isolation or identification procedures are in progress. Although a properly sanitized laboratory area should not harbor *L. monocytogenes* or other pathogens, supervisors should use their own discretion in allowing high-risk individuals into these areas when not in use for these activities.

8A.3 Quality Control Procedures

8A.3.1 Culture Controls

a. At least one *L. monocytogenes* positive control strain is required. Appropriate cultures include ATCC 19111, NCTC 7973 or other *L. monocytogenes* cultures validated to perform in an equivalent manner.

b. At least one *L. innocua* negative control culture is required. Appropriate cultures include L. *innocua* strain ATCC 33090 or other *L. innocua* strains validated to perform in an equivalent manner.

8A.3.2 Sterility Control

Additionally, always prepare one "blank" (incubated but un-inoculated pre-enrichment/ enrichment broth) to provide a sterility control for the process.

8A.4 Equipment, Reagents, and Media

In addition to equipment, reagents, and media used in analysis of samples as described in MLG 8, the following materials will be needed.

a. PCR tube holder (Qualicon)
b. Cell Lysis Tube Cooling Block (Qualicon) held at 4 ± 2°C
c. Techne DB-2A Heating block set at 55 ± 2°C
d. Techne DB-2A Heating block set at 95 ± 2°C
e. Eppendorf Repeater Plus Pipettor (or equivalent) set at 200:l µl, and tips
f. Corning Lambda 20 Pipettor (or equivalent) set at 5 ± l µl, and tips
g. Corning Lambda 200 Pipettor (or equivalent) set at 150 ± l µl, and tips

h. 12 X 75 mm (Falcon 352063, or equivalent) tubes
i. Cell Lysis Tubes and Caps, Cell Lysis Tube Rack and box (Genemate 8 strip tubes, ISC Bioexpress, T-3120-5)
j. Pipettor and 5 ml pipettes
k. BAX® Assay for Screening *L. monocytogenes* (Qualicon # 17710609) held at 4 ± 2°C
l. MOPS-BLEB medium
BBL Listeria enrichment broth (BBL #12333, or equivalent)
MOPS free acid (Sigma #1254, or equivalent)
MOPS sodium salt (Sigma #M9381, or equivalent)

8A.5 Sample Preparation and Primary Enrichment

Perform sample preparation and pre-enrichment in as described in MLG 8, Section 8.5.1 and 8.5.2.

8A.6 Secondary Enrichment and Direct Plating

a. Transfer 0.1 ± 0.02 ml of the UVM enrichment to 10 ± 0.5 ml of MOPS-BLEB. Incubate inoculated MOPS-BLEB tubes at 35 ± 2°C for 18-24 h.

b. Streak a MOX plate. Streak a loopful or a drop approximating 0.1 ml of the UVM over the surface of the plate. Alternatively, dip a sterile cotton-tipped applicator or equivalent into the UVM and swab 25-50% of the surface of a MOX plate. Use a loop to streak for isolation from the swabbed area onto the remainder of the plate. Incubate the MOX at 35 ± 2°C for 26 ± 2 h.

8A.7 The BAX® System for Screening L. monocytogenes Test Procedure

Follow the current BAX® User's Guide for preparing reagents, performing the test, and reading the results. The equipment must be set up, and operated, and all records must be documented, according to laboratory work instructions.

8A.8 Cultural Confirmation

a. Streak a MOX plate using a loopful of the MOPS-BLEB, or by streaking a drop approximating 0.1 ml or aseptically dip a sterile cotton-tipped applicator or equivalent into the MOPS-BLEB and swab 25-50% of the surface of a MOX plate. Use a loop

to streak for isolation from the swabbed area onto the remainder of the plate. Incubate the MOX at 35 ± 2°C for a minimum of 24 h.

b. Proceed with all isolation and purification procedures as per MLG 8, Sections 8.5.4.a, 8.5.6, and 8.6.

8A.9 Interpretation of Results

a. Samples that test BAX®-negative will be reported as negative if the concurrent 24 h Direct Plating is also negative. Cultural analysis will continue on samples that are BAX®-negative but have typical colonies on the 24 h Direct Plating MOX plates, or have a BAX®-positive, BAX®-indeterminate or BAX® signal-error result.

b. In analytical runs where the positive control tests negative, either the reserve samples will be retested or the laboratory shall complete the cultural method by streaking all samples and controls from MOPS-BLEB medium onto MOX plates. Proceed with all isolation and purification procedures as per MLG 8, Sections 8.5.6 and 8.6.

8A.10 Completion of Testing if BAX® Unavailable

If circumstances (e.g. a power outage or equipment failure) do not allow testing using the BAX® system, the laboratory shall complete the cultural method by streaking all samples and controls from MOPS-BLEB medium onto MOX plates. Proceed with all isolation and purification procedures as per MLG 8, Sections 8.5.6 and 8.6.

8A.11 Selected References

Centers for Disease Control and Prevention and National Institutes of Health (CDC/NIH). 1999. BioSafety in Microbiological and Biomedical Laboratories, 4th ed. U.S. Government Printing Office, Washington, D.C. (also found on the internet at:

http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm)

BAX® System PCR Automated Detection for Bacterial Screening User Guide, Dupont Qualicon.