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Bacteriological analytical manual

United States.
Washington, 1969.

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BACTERIOLOGICAL ANALYTICAL MANUAL

SECOND EDITION
January 1969

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE / Public Health Service / Consumer Protection and Environmental Health Service / Food and Drug Administration / Division of Microbiology / Washington, D.C. 20204

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INTRODUCTION

The microbiological analytical work of the FDA is done largely in its field district laboratories. Uniformity of methods and of their application in these laboratories is essential. Accordingly, the Bacteriological Analytical Manual (BAM) has been prepared, primarily, in the interest of establishing and maintaining in FDA laboratories uniform analytical procedures for qualitative and quantitative determination of microorganisms in foods, drugs, cosmetics and certain other materials.

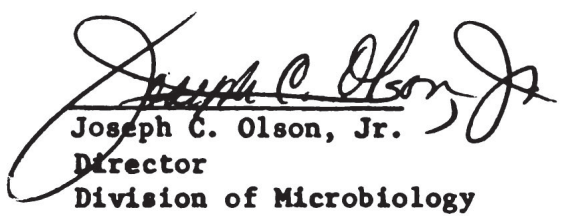
This second edition is a complete revision of the BAM published in 1965 and supersedes that collection of methods. It is of interest to note that the 1965 version was an outgrowth of bacteriological methods which were utilized internally as early as 1940. The aim then, as today, was to insure a uniform application of methods by microbiologists engaged in enforcing the provisions of the FD&C Act.

No claim is made that these methods are perfect, or even that they are superior to others that may be available. They are, however, those currently considered to be the most useful to the FDA. As in the past, revisions will be made as soon as methods or modification of methods better suited to our needs become evident.

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In keeping with established FDA policy, the BAM also provides a mechanism for informing other Government agencies, industry, and others of the bacteriological methods commonly used in FDA district laboratories.

The BAM is the product primarily of those members of the Division of Microbiology most knowledgeable in the subject matter indicated by the respective methods. The Division staff is most appreciative of the many comments and suggestions for revisions that have been received from others; especially valuable were those from our district microbiologists. Also, we wish to express our sincere thanks to those individuals who so generously complied with our request to review certain sections of the Manual. As the need arises in the future, supplements and revisions of various sections of the Manual will be issued. In the interest of making such revisions more responsive to needs we shall welcome any constructive comments and suggestions toward improvement of this Manual.



Joseph C. Olson, Jr.
Director
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1. CANNED FOODS

1.01 Equipment and materials

(a) incubators at 30°C, 35°C, 55°C

(b) pH meter

(c) microscope

(d) soap, water, brush

(e) towels (sterile and non-sterile)

(f) can opener

(g) sterile test tubes

(h) microscope slides and coverslips

(i) examination pans

(j) sterile serological pipets, cotton-plugged

(k) sterile non-tapered pipets, cotton-plugged (8 mm tubing)

(l) 2% iodine in 70% alcohol

(m) wax pencil or felt pen

(n) sterile petri dishes

(o) Bacti-disc cutter [1/]

(p) micro-leak detector

(q) non-foaming wetting agent

(r) micrometer

(s) sterile can punch

[1/] Purchased from Wilkens-Anderson Co., 4525 W. Division Street, Chicago, Ill. 60651 Cat. No. 4768.

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1.02 Media

Bromcresol purple dextrose broth (BCP) (40.04); malt extract broth (40.24); acid broth (40.01); chopped liver broth (40.06) or cooked meat medium (40.07)[2/]; methylene blue stain (41.08)

1.03 Introduction

Can abnormality usually indicates an abnormal product. During progressive spoilage, cans may progress from normal to flipper to springer to soft swell to hard swell. Buckling or denting, closing while cool, overfilling, or prolonged storage may cause flippers or springers. Microbial spoilage or hydrogen from the reaction of acids with the metals of the cans may produce flippers, springers, or swells. Summer temperatures and high altitudes accentuate the degree of swelling. Not all microorganisms that grow in canned foods cause abnormal cans.

Spoilage within the can is caused by leakage or underprocessing. Leakage occurs from can defects, punctures, or rough handling. Contaminated cooling water sometimes leaks to the interior through pinholes or poor seams. The presence of a viable mixed flora of rods and cocci is indicative of leakage and should be confirmed by can examination. Underprocessing may occur from deliberate undercooking to preserve a "fresher" product, from faulty retort operations (including thermometers,

2/ Chopped liver broth is preferred.

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gauges, and controls), from excessive contamination for which normally adequate processes are insufficient, or sometimes from accidental bypassing of the retort operation. When the can contains a spoiled product and no viable microorganisms, spoilage may have occurred before processing, or the microorganisms causing the spoilage may have died off during storage.

1.04 Can examination

Remove labels and transfer the subnumbers to the side of the can as an aid to correlate the findings with the codes.

Separate all cans by code numbers, if they are different, and examine them for any physical defects such as faulty closures, leaks, pinholes, buckling and dents. Classify each can condition as follows:[3/]

(a) Flipper. Only one end of the can is slightly bulged, and by finger pressure the end can be pressed back to remain flat. Or the end bulges when the can is struck sharply on a hard surface, and the bulged end can be pressed flat.

(b) Springer. One end of the can bulges, and when finger pressure is applied to press it flat, the opposite end bulges. Or both ends bulge and one end can be pressed flat.

3/ Cool to room temperature before classifying.

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(c) Swell. Both ends bulge, but neither can be pressed flat. Bulged ends of softswells yield slightly to finger pressure, but those of hard swells do not.

Set aside a reserve portion (702b) consisting of cans representing each condition noted and representative cans for analysis, e.g., if there are two springers, analyze one and place the other in the reserve sample. Record on the worksheet the condition of the cans to be analyzed.

1.05 Can incubation

Analyze all springers and swells immediately (1.07). Do not incubate. Place all normal cans and flippers in the incubator at 35°C. Examine them at frequent intervals for 14 days. When an abnormal can is found during this period, remove immediately and examine (1.07). After 14 days, remove the remaining normal cans and analyze a representative portion of these cans. (It is not necessary to analyze all normal cans.)

Incubation of cans at temperatures above 35°C need not be done unless there is reason to suspect that the product will be held at elevated temperatures in storage or in transit. Many canned foods contain a few thermophilic bacteria which do no harm under normal storage temperatures, but which will grow and spoil the product at 55°C (flat sour and thermophilic anaerobes).

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1.06 Opening the can

Since the canned food method is essentially a test for the sterility of the product, the analysis should be performed in a clean atmosphere (preferably a bacteriological hood) to preclude any possibility of laboratory contamination.

Scrub the entire container with a brush, using warm water and soap on the uncoded end of the can. Rinse and dry with a clean towel. Chill hard swells in the refrigerator before opening them.

Flame the uncoded end by holding over a bunsen burner or similar device. Do not flame hard swells; use 2% iodine in 70% alcohol and wipe away with a sterile towel.

Hydrogen swells sometimes occur in acid canned foods from the action of the acid in the food on the metal in the container. If a test for hydrogen is to be made, hold a sterile test tube near the site of the opening and make a small puncture in the flamed surface, catching some of the escaping gas in the tube. Immediately flip the mouth of the tube to a bunsen burner flame. An explosion indicates the presence of hydrogen in the head space. (Do not flame the hole. Serious explosions have occurred from this technique.) Enlarge the opening in the can, using the Bacti-disc cutter to permit removal of the sample.

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Remove portions of the contents from the center of the can, well removed from the site of the opening, and place in appropriate media (1.07). Sample all products, liquid or solid, using sterile wide-mouth pipets or sterile glass tubing if a larger size opening is desired. Do not use forceps, spoons, or other instruments to sample solid products, since this may introduce outside contamination into the sample. Solid products usually have enough liquid so that a sample may be obtained. Sterile water can be introduced, mixed, and removed to media if the product is too solid. Never pipet by mouth unless the pipet is plugged.

Transfer portions from the contents of representative cans to sterile stoppered test tubes and refrigerate at ca 5°C. Use these for repeat examination, if questionable results are obtained after incubation, and for possible toxicity tests.

1.07 Cultural and direct microscopic tests

[Use either (a) or (b)]

(a) Non-acid foods[4/] (pH above 4.5). From each container inoculate 4 tubes of chopped liver broth or cooked meat medium

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[4/] Non-acid foods are meats, fish, milk, and most vegetables. They generally spoil from spore-forming anaerobes or facultative anaerobes, which may be mesophilic or thermophilic.

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previously heated to 100°C and cooled to room temperature or below.[5/] Also inoculate 4 tubes of bromcresol purple dextrose broth (BCP). Incubate as follows:

Medium	No. of Tubes	Temp, °C	Time of Incubation, hr	To Test For
Chopped liver (cooked meat)	2	35	96	Putrefactive anaerobes
Chopped liver (cooked meat)	2	55	72[6/]	Thermophilic anaerobes
BCP	2	55	48[6/]	Flat sour thermophiles
BCP	2	35	96	Leakage types

(b) Acid foods[7/] (pH below 4.5). Test abnormal cans for hydrogen (1.06). From each container inoculate, with ca 1 ml of product, 4 tubes of acid broth and 2 tubes of malt extract

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5/ Chopped liver broth or cooked meat medium is heated and cooled to remove oxygen inhibitory to the growth of anaerobes.

6/ Also examine at 24 and 48 hr.

7/ Acid foods are tomatoes, fruits and fruit juices, fruit drinks, pickles, jams, jellies, and catsup. Syrups and concentrates may generally be treated as acid foods. Acid foods may spoil from yeasts, molds, or lactobacilli, as well as acid-tolerant sporulating thermophilic or mesophilic anaerobes.

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broth. Incubate as follows:

Medium	No. of Tubes	Temp. °C	Time of Incubation, hr	To Test For
Acid broth	2	55	48	Flat sour thermophiles
Acid broth	2	30	96	Yeasts, molds, lactobacilli
Malt extract broth	2	30	96	Yeasts, molds, lactobacilli

During inoculation (1.07) (a) or (b) note whether the food itself introduces turbidity into the medium. After incubation, do not record such turbidity as positive unless it proves to be growth after transfer to a fresh medium, or by direct microscopic examination.8/

1.08 Direct microscopic examination

Prepare a smear from the contents of each can after subculturing. Dry, fix, and stain with methylene blue stain; then examine under the microscope. If the product is oily, drop xylene over a warm, fixed slide, rinse, and stain. If the product washes off the slide during preparation, examine as a wet mount or hanging drop,

8/ Be certain that the microorganisms seen under the microscope are viable. Dead organisms may sometimes be carried over from the inoculum or the chopped liver.

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or prepare a suspension of the test material in a drop of chopped liver broth before drying. Check the liver broth microscopically before use to be sure it is free of micro-organisms.

1.09 Examination of contents

After culturing:

(a) Determine pH of each subsample with a pH meter.

(b) Cut out most of the end of the can, using the Bacti-disc cutter, and pour contents into examination pans. Examine for odor, color, consistency and texture; examine the can lining for blackening, detinning, and pitting. Do not taste the product.

1.10 Seam evaluation

(a) Micro-leak detection [see Bee and Denny, Ref. (8)]. Remove the uncoded end with a Bacti-disc cutter adjusted to cut out most of the can end, leaving 1/4 inch around the outer edge. Empty and wash the container; add a nonfoaming wetting agent[9/] to a depth of ca one inch.

9/ "Seam Test Type U", Winton Products Co., Inc., Box 3332, Charlotte, North Carolina 28203.

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Place the plastic Plexiglass plate with Plexiglass tubing and the wetted rubber gasket for the particular container size on the open end of the container. Increase the vacuum slowly until the gauge indicates a vacuum normally obtained with the product in question. Swirl the water in the container to dissipate the small bubbles occurring from the application of the vacuum.

Tilt the container slowly to immerse all seam surfaces. Depending on the size of the leak, bubbles will appear as a chain originating from one location or as a large, growing bubble. Release the vacuum by first closing the main vacuum petcock, and then opening the intake petcock.

(b) Tear-down examination. Use appropriate methods such as those described in "Evaluating a Double Seam" by W. R. Grace & Co., Cambridge, Mass.

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2. DETECTION OF PREFORMED CLOSTRIDIUM BOTULINUM TYPES A, B OR E TOXIN IN FOOD SAMPLES

DO NOT START THIS ANALYSIS UNTIL YOU HAVE BEEN PROTECTED BY SUITABLE TOXOIDS.

2.01 Equipment and materials

(a) 14 mice for each sample (32 mice for positives)

(b) types A, B, and E antisera[1/]

(c) Difco trypsin (1:250 activity), 10% solution in distilled water

(d) inoculating syringes (sterile)

(e) Millipore or fritted glass UF filter

(f) 37°C water bath

(g) centrifuge

(h) 70% ethyl alcohol

(i) 1 N HCl

(j) sterile mortar and pestle; sterile sand or mechanical blender

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1/ Obtain antisera directly from National Communicable Disease Center, Atlanta, Ga. 30322 or Division of Microbiology, Food and Drug Administration, Washington, D. C. 20204. If lyophilized, reconstitute with sterile 50% glycerol in water, in accordance with label instructions. Dilute sera 1:10 with sterile saline for mouse injection.

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2.02 Reagents

Saline, sterile, 0.85% (41.10); gel-phospnate diluent (41.06)

2.03 Preparation of food extract

(a) If canned foods are to be tested, scrub the uncoded can end with a brush, using warm water and soap; rinse, and dry with a towel. Number each can on the side, as an aid to correlate findings with code. (Chill hard swells in the refrigerator before opening them.)

Open the can as directed in BAM 1.06. Special care should be taken when opening swollen cans to avoid creating an aerosol.

Record the condition of the can and its content.

(b) Liquid food samples or can washings can be tested for the presence of toxin without further preparation.

(c) Suspected food remnants can be prepared in the same manner as canned solid foods.

Homogenize the sample (either a portion of the can contents or food remnant) with an equal weight of sterile gel-phosphate diluent, using a mechanical blender. With some foods or remnants it may be necessary to grind the sample in a mortar.

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Add an equal weight of sterile gel-phosphate diluent and a small amount of sterile sand (depending on the volume to be ground) and grind with a sterile pestle until a homogeneous suspension is obtained.

2.04 Preparation of filtrates

Centrifuge a portion of the homogenate in the cold at high speed for 30 minutes. Filter through a sterile Millipore or fritted glass filter (not Seitz) to obtain the clear, fat-free liquid. Check the pH. If above 6.5, adjust to 6.0-6.2 with 1 N HCl. Freeze for overnight storage, if necessary.

2.05 Trypsinization and heat treatment

To 2 ml of the filtrate adjusted to pH 6.0-6.2 (2.03) add 0.2 ml of the 10% solution of trypsin. Incubate in a water bath at 37°C for 45 minutes. Cool. Do not store trypsinized extract overnight.

Heat a portion of the sterile filtrate for 10 minutes at 100°C.

2.06 Screening inoculations

Dilute both trypsinized (2.04) and nontrypsinized (2.03) filtrates 1:2, 1:10, and 1:100 in gel-phosphate diluent.

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Inject 0.5 ml of each dilution intraperitoneally into 2 mice (total of 12 mice). In addition, inject 2 mice intraperitoneally with 0.5 ml of the undiluted heated filtrate. Record their condition at frequent intervals during 48 hours. Deaths occurring with either the trypsinized or nontrypsinized unheated filtrate are presumptive evidence of toxin, and should be confirmed with protected mice (2.07).

2.07 Confirmation with protected mice

Dilute a new portion of the nontrypsinized (2.03) or trypsinized (2.04) filtrate (whichever showed the highest titer) to 1:2, 1:10, and 1:100 in gel-phosphate diluent. (Do not store trypsinized material overnight.)

Inject each of 8 mice intraperitoneally with 0.5 ml of a 1:10 saline dilution of type A antiserum, each of 8 mice with 0.5 ml of a 1:10 saline dilution of type B antiserum, and each of 8 mice with 0.5 ml of a 1:10 saline dilution of type E antiserum. Leave 8 mice without this protection. After 30 minutes, inject 0.5 ml of each dilution of the filtrate into each of 2 mice previously protected with either type A, type B or type E antisera and into 2 unprotected mice.

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Record their condition at intervals up to 48 hours. If the unprotected mice die and the group of mice protected with either type A, type B, or type E antisera live, the presence of either type A, type B, or type E toxin is indicated. If all these protected mice die, send the filtrate on dry ice to Division of Microbiology for further tests.

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3. DETECTION OF CLOSTRIDIUM BOTULINUM TYPES A, B AND E IN FOOD SAMPLES[1/]

DO NOT START THIS ANALYSIS UNTIL YOU HAVE BEEN PROTECTED BY SUITABLE TOXOIDS.

3.01 Equipment and materials

(a) 16 (18-24 g) mice for each sample (32 mice for positives)

(b) types A, B, and E antisera[2/]

(c) inoculating syringes (sterile)

(d) sterile inoculating instruments

(e) 35°C incubator

(f) 26°C incubator

(g) centrifuge

(h) sterile centrifuge tubes

(i) Millipore or fritted glass UF filters

[1/] If a product is suspected of being contaminated with botulinum toxin or organisms (i.e., food poisoning), then both methods (BAM 2 and 3) should be used. If the product is being routinely analyzed for botulinum contamination (i.e., no suspected contamination), only the BAM 3 method should be used unless toxin is found; then use the BAM 2 method also.

[2/] Obtain antisera directly from National Communicable Disease Center, Atlanta, Ga. 30322 or Division of Microbiology, Food and Drug Administration, Washington, D. C. 20204. If lyophilized, reconstitute with sterile 50% glycerol in water, in accordance with label instructions. Dilute sera 1:10 with sterile saline for mouse injection.

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3.02 Media and reagents

TPGYT (40.34); chopped liver broth (40.06); gel-phosphate diluent (41.06); saline, sterile, 0.85% (41.10).

3.03 Cultures

Inoculate 3-4 g portions of the sample into each of 3 tubes of freshly boiled and cooled chopped liver broth. At the same time, inoculate 3-4 g portions of the same sample into each of 3 tubes of TPGYT broth. Incubate the inoculated chopped liver tubes at 35°C for 5 days. Incubate the inoculated TPGYT broth tubes at 26°C for 5 days.

3.04 Preparation of filtrates

Following incubation, combine aliquots of the chopped liver broth cultures and separately combine aliquots of the TPGYT cultures. Centrifuge each of the combined aliquots in the cold at high speed for 30 minutes. Filter each supernatant through a sterile Millipore or fritted glass filter (not Seitz) to obtain a clear, fat-free liquid. Freeze for overnight storage, if necessary.

3.05 Heat treatment

Heat a portion of each sterile culture filtrate for 10 minutes at 100°C.

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3.06 Screening inoculations

Dilute each filtrate 1:5, 1:10 and 1:100 in gel-phosphate diluent. Inject 0.5 ml of each dilution of each filtrate intraperitoneally into 2 mice (total of 12 mice). In addition, inject 2 mice intraperitoneally with 0.5 ml of each undiluted heated filtrate. Record their condition at frequent intervals during 48 hours. Deaths occurring with either or both of the unheated filtrates is presumptive evidence of toxin, and should be confirmed with passively protected mice (3.07).

3.07 Confirmation with protected mice

Determine which culture filtrate showed the highest titer in mice. Dilute a new portion of this filtrate 1:5, 1:10, and 1:100 in gel-phosphate diluent.

Inject each of 8 mice intraperitoneally with 0.5 ml of a 1:10 saline dilution of type A antiserum, each of 8 mice with 0.5 ml of a 1:10 saline dilution of type B antiserum, and each of 8 mice with 0.5 ml of a 1:10 saline dilution of type E antiserum. Leave 8 mice without this protection. After 30 minutes, inject 0.5 ml of each dilution of that filtrate which showed the highest titer into each of 2 mice previously protected with either type A, type B or type E antisera and into 2 unprotected mice.

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Record their condition at intervals up to 48 hours. If the unprotected mice die and the group of mice protected with either type A, type B, or type E antisera live, the presence of either type A, type B, or type E toxin is indicated. If all these protected mice die, send the filtrate on dry ice to Division of Microbiology for further tests.

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4. PROCEDURE TO DETERMINE THE PRESENCE OF CLOSTRIDIUM BOTULINUM TYPE E IN SMOKED FISH

DO NOT START THIS ANALYSIS UNTIL YOU HAVE BEEN PROTECTED BY SUITABLE TOXOIDS.

4.01 Equipment and materials

(a) 8 (16-24 g) mice for each subsample (16-24 mice for positives)

(b) types A, B, and E antisera[1/]

(c) inoculating syringes (sterile)

(d) 26°C incubator

(e) Millipore or fritted glass UF filters

(f) centrifuge

(g) water-tight plastic bags

(h) sealing device for sealing plastic bags

4.02 Media and reagents

TPGYT (40.34); gel-phosphate diluent (41.06); saline, sterile, 0.85% (41.10).

1/ Obtain antisera directly from National Communicable Disease Center, Atlanta, Ga. 30322 or Division of Microbiology, Food and Drug Administration, Washington, D. C. 20204. If lyophilized, reconstitute with sterile 50% glycerol in water, in accordance with label instructions. Dilute antisera 1:10 with sterile saline for mouse injection.

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4.03 Incubation

Place each smoked fish subsample (this may consist of 1 or more fish depending on size, and may be either vacuum packed or bulk smoked fish) in a strong, water-tight, plastic bag. Add ca 100 ml of TPGYT broth to the subsample. Squeeze the bag to exclude as much air as possible and have the subsample essentially submerged in the culture medium. Seal the bag with a hot iron or some other sealing device. Incubate at 26°C for 5 days. Precautions should be taken during the incubation period, since the bag may swell and split.

4.04 Cultures

Centrifuge a portion of the TPGYT broth after incubation, and filter through a sterile Millipore or fritted glass filter (not Seitz) to obtain the clear, fat-free liquid.[2/] Freeze for overnight storage, if necessary.

4.05 Screening inoculations

Dilute the sterile culture filtrates (4.04) 1:5, 1:10, 1:100 and 1:1000 in gel-phosphate diluent. Inject 0.5 ml of each dilution intraperitoneally into each of 2 mice (total of 8 mice). Record their condition at frequent intervals during 48 hours. If no deaths occur, no further tests are indicated.

[2/] Chill tubes before centrifuging or use refrigerated centrifuge.

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Deaths are presumptive evidence of toxin, and should be confirmed with protected mice (4.06). Freeze for overnight storage, if necessary.

4.06 Confirmation with protected mice

Dilute a new portion of the sterile culture filtrates (4.04) 1:5, 1:10, 1:100, and 1:1000 in gel-phosphate diluent.

Inject each of 8 mice intraperitoneally with 0.5 ml of a 1:10 saline dilution of type E antiserum. Leave 8 mice without this protection. After 30 minutes, inject 0.5 ml of each dilution into each of 2 mice previously protected with antiserum and into each of 2 mice not so protected.

Record their condition at intervals up to 48 hours. If the unprotected mice die and the protected inoculated mice live, the presence of type E toxin is indicated. If all the protected mice die, repeat serological neutralization tests with mice protected with Clostridium botulinum types A and B antisera.

Inject each of 8 mice intraperitoneally with 0.5 ml of a 1:10 saline dilution of type A antiserum and each of 8 mice with 0.5 ml of a 1:10 saline dilution of type B antiserum. Leave 8 mice without this protection. After 30 minutes, inject 0.5

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ml of each dilution of sterile culture filtrate into each of 2 mice previously protected with types A and B antiserum and into 2 mice not so protected.

Record their condition at intervals up to 48 hours. If the unprotected mice die and the group of mice protected with either type A or type B antisera live, the presence of either type A or type B toxin is indicated. If all these protected mice die, send the filtrate on dry ice to Division of Microbiology for further tests.

Isolate and identify cultures from samples containing toxin of type E (BAM 5).

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5. ISOLATION OF CLOSTRIDIUM BOTULINUM TYPE E IN SMOKED FISH

DO NOT START THIS ANALYSIS UNTIL YOU HAVE BEEN PROTECTED BY SUITABLE TOXOIDS. USE THE SAFETY HOOD FOR HANDLING CULTURES.

5.01 Equipment and materials

(a) sterile inoculating instrument

(b) 35°C incubator

(c) 26°C incubator

(d) sterile pipets

(e) 16 to 40 (18-24 g) mice per positive subsample

(f) anaerobic atmosphere (N_2) or other anaerobic apparatus to create an anaerobic atmosphere

(g) absolute ethyl alcohol

(h) sterile syringes

(i) sterile test tubes

(j) Millipore or fritted glass UF filter

(k) type E antiserum (see footnote under BAM 4.01)

5.02 Media

TPGYT (40.34); anaerobic egg agar (40.02); gel-phosphate diluent (41.06); saline, sterile, 0.85% (41.10).

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5.03 Isolation of pure cultures (To be done only if toxin is confirmed.)

To 1 or 2 ml of the toxic culture (BAM 4.03) (especially the sediment) add an equal volume of absolute ethyl alcohol in a sterile test tube. After 1 hour at room temperature, streak a large loopful of the mixture to anaerobic egg agar, and pipet 0.1 ml into TPGYT broth.

Incubate the plates anaerobically at 35°C for 48 hours and the inoculated TPGYT broth aerobically at 26°C. Look for typical colonies[1/] of type E on the plates after 48 hours of incubation.

5.04 Toxicity of TPGYT cultures

If plates contain apparently pure cultures, let the TPGYT broth inoculated at the time of plate streaking (5.03) incubate for 3 days at 26°C; then filter a portion of the culture through a Millipore or fritted glass filter (not Seitz). Freeze for overnight storage, if necessary.

1/ Colonies of type E are white, flat, and irregular, 1-2 mm in diameter, surrounded by a zone of yellow precipitate ca 2 to 4 mm in diameter. The precipitation zone surrounding the colony is iridescent (pearly-layer) to oblique light. The iridescent precipitation zone is characteristic of several other species of the genus Clostridium, so be careful in the interpretation of results. Streak stock cultures to gain experience.

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5.05 Toxicity tests

Dilute the sterile culture filtrates (5.04) 1:5, 1:10, 1:100, and 1:1000 in gel-phosphate diluent. Inject each of 2 mice intraperitoneally with 0.5 ml of each dilution. Record their condition at frequent intervals up to 48 hours. If animals die, confirm the nature of the toxin (5.06).

5.06 Confirmation of type E toxin in cultures

Dilute a new portion of sterile culture filtrate (5.04) 1:5, 1:10, 1:100, and 1:1000 in gel-phosphate diluent.

Inject each of 8 mice intraperitoneally with 0.5 ml of a 1:10 saline dilution of type E antiserum. Leave 8 mice without this protection. After 30 minutes, inject 0.5 ml of each dilution into each of 2 mice previously protected with antiserum and into each of 2 mice not so protected.

Record their condition at intervals up to 48 hours. If the unprotected mice die and the protected mice live, the presence of type E toxin is indicated.

5.07 Picking colonies

If plates appear contaminated, discard the TPGYT inoculated in parallel with the streaking, and pick at least 10 typical

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isolated colonies[2/] to TPGYT broth. Incubate 3 days at 26°C. Prepare culture filtrates, dilute 1:100 in gel-phosphate diluent, and test for toxin, using protected and unprotected mice (5.04-5.06).

2/ Many colonies will fail to be toxigenic. Pick at least 10 typical colonies to locate a toxin-producing colony.

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6. ISOLATION OF CLOSTRIDIUM PERFRINGENS (WELCHII)[1/]

6.01 Sample collection and storage

Sample the entire portion of food if possible (whole roast, chicken, etc). Transport and examine the sample promptly without freezing. Freeze only if the sample cannot be analyzed within 1 or 2 days.[2/]

6.02 Equipment and material

(a) anaerobic atmosphere (N_2) or other apparatus to create anaerobic atmosphere

(b) sterile mortar and pestle and sterile sand, or a two-speed Waring Blendor

(c) 26°C incubator

(d) 46°C water bath

1/ *Clostridium perfringens* food poisoning may occur when meat or poultry is cooked and held without adequate refrigeration prior to serving. The oxygen level is sufficiently reduced during cooking to permit growth of the clostridia.

Illness occurs 8-15 hours after ingestion, with intense intestinal cramps, gas and diarrhea; nausea and vomiting are rare. Victims usually recover rapidly but serious illness may occur occasionally as a result of dehydration and circulatory failure.

2/ Freezing may destroy 99% or more of the *Clostridium perfringens* vegetative cells; even refrigeration without freezing may kill 90%.

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6.03 Media and reagents

Chopped liver broth (40.06) or cooked meat medium (40.07)[3/]; liver veal egg yolk (LVEY) agar (40.22); indole nitrite medium (40.18) or motility-nitrate medium (40.25); Ellner's sporulation broth (40.11); sulfite-polymyxin-sulfadiazine (SPS) agar (40.32) or tryptone-sulfite-neomycin (TSN) agar (40.36); fluid thioglycollate medium (40.12); iron milk medium (40.19); peptone water diluent (41.11); Gram stain reagents; Reagent I (41.12); Reagent II (41.13)

6.04 Direct microscopic examination

Prepare a smear, Gram stain, and examine for Gram-positive rods.

6.05 Cultural tests

If on direct examination typical cells were few or if the sample was grossly contaminated with aerobic microorganisms, inoculate into fluid thioglycollate and incubate at 46°C (6.06). If there were numerous typical Gram-positive rods and few contaminants, inoculate into chopped liver broth (6.07) or cooked meat medium. Do plate counts for sulfite-reducing clostridia on all samples (6.08).

[3/] Chopped liver broth is preferred.

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6.06 Fluid thioglycollate medium

Inoculate 2 or 3 g portions taken at random[4/] into several tubes of fluid thioglycollate medium. Incubate in a water bath at 46°C for 4-8 hours. Subculture positive tubes in chopped liver broth or cooked meat medium (6.07).

6.07 Chopped liver broth or cooked meat medium

Inoculate 2 or 3 grams of the sample into each of several tubes or chopped liver broth or cooked meat medium previously boiled and cooled.[5/] Incubate aerobically for 24-48 hours. Observe cultures for profuse gas production and examine for characteristic Gram-positive rods. Streak positive tubes to LVEY plates. Incubate plates anaerobically for 24-48 hours at 35°C and examine for colonies producing lecithinase zones.[6/] Confirm as Clostridium perfringens (6.09).

6.08 Plate count of viable clostridia

If the sample is a solid, macerate a portion of it with peptone water in a mortar with sterile sand or blend[7/] with peptone

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4/ The organisms may be unevenly distributed in the sample. Include both surface and deep portions.

5/ Heat all liquid media in boiling water or flowing steam for 10 minutes to expel oxygen just prior to use.

6/ Opalescent zone 2-5 mm in diameter surrounding the colony.

7/ Blend carefully; excessive exposure to oxygen may kill the clostridia.

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water for 1 minute in a Waring Blendor at low speed. Make serial dilutions in peptone water with gentle mixing. Prepare pour plates in SPS agar or TSN agar and, after the agar has solidified, overlay with 5 ml of the same medium. Incubate plates anaerobically at 35°C for 18-24 hours. Count black (sulfite-reducing) colonies. Pick several colonies for confirmation tests (6.09).

6.09 Confirmation as Clostridium perfringens

Pick typical colonies from LVEY (6.07) and SPS (6.08) to chopped liver broth, cooked meat medium, or fluid thioglycollate medium. Incubate 18-24 hours at 35°C. When a pure culture has been obtained, streak 2 plates of LVEY and incubate one anaerobically, the other aerobically. Report which plate supports growth. Inoculate iron milk and incubate 24 hours at 35°C. Inoculate duplicate tubes of indole nitrite or motility-nitrate medium by expelling a column of inoculum into the chilled semisolid medium while withdrawing a 1 ml pipet.

Incubate indole nitrite or motility-nitrate medium 18-24 hours at 26°C. Examine for motility[8/]; then add 5 drops of reagents

[8/] Cl. perfringens is non-motile. Growth should occur only along the line of inoculum and not diffuse throughout the entire medium.

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I and II to tubes showing heavy growth. A red or pink color indicates reduction of nitrate to nitrite. Include a negative control. In iron milk, look for vigorous gas production and early coagulation (stormy fermentation). Report as Clostridium perfringens nonmotile, spore-forming[9/] obligate anaerobes that reduce nitrate to nitrite and produce stormy fermentation in iron milk.

9/ Cl. perfringens does not sporulate readily. To produce spores, use a sporulation broth such as Ellner's medium. Inoculate heavily with a young culture and incubate 24 hours in a water bath at 37°C.

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7. ANALYSIS OF CRABMEAT AND LOBSTERMEAT

7.01 Sample collection, transport, and storage

Sample the entire container, if possible, or if the containers are too large, aseptically sample ca 200 g into a sterile container for each subsample.

It is preferable to examine a minimum of 10 subsamples of each kind of meat or 10 subsamples of the type of meat which would best show the conditions in the plant. As soon as the sample is collected, place the subsamples in an ice chest in wet ice. Transport the sample in wet ice and store the sample in the refrigerator at 3-5°C, and start the analysis within 48 hours after collection. Freezing is undesirable because indicator organisms may be destroyed.

7.02 Preparation of sample

Clean the top and edge of the can and wipe with 70% alcohol. If the can is hermetically sealed, open as directed in BAM 1.06.

7.03 Examination

Determine aerobic plate count, coliform group, Escherichia coli, and coagulase-positive Staphylococcus according to JAOAC 49 (1), 246-250 (1966), and JAOAC 51 (2), 505 (1968).

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8. DRUGS AND DEVICES: STERILITY

8.01 Introduction

The FD&C Act requires that if a drug is described in an official compendium, determinations as to purity shall be made in accordance with the tests set forth in such compendium. This means that USP or NF products must be tested for sterility in accordance with the latest USP or NF. Nothing in what follows should be construed to alter the instructions detailed in these compendia.

Sterility tests are highly exacting and should be conducted only by personnel who have had expert training and experience in rigid aseptic techniques.

The USP describes in detail the equipment and conditions necessary for a proper aseptic filling operation. Although it does not describe the environment required for sterility testing we must be certain that the microorganisms we find in sterility testing come from the product and not from the environment. For this reason, we must be certain that our laboratory testing facilities at least equal the USP specifications for aseptic filling areas. Read Remington's Pharmaceutical Sciences (Martin, 1965). You must be prepared to defend your procedure in court.

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8.02 Equipment and materials

(a) the sterility test area should conform to the requirements for aseptic filling areas (USP).

(b) 2, 5, 20, or 30 ml syringes, Luer-Lok, with 1-1/2 inch #20 needles. (Use smaller or larger needles for some products.)

(c) sterile files, forceps, scissors, scalpels, etc., as required.

(d) an effective disinfectant.

(e) sterile pipets of appropriate sizes.

(f) surgical gown, face mask, and elbow length rubber gloves.

8.03 Media and reagents

Fluid thioglycollate medium (40.12); fluid thioglycollate medium, azolectin-Tween modification (40.13) for products containing quaternary ammonium compounds; fluid Sabouraud medium (see USP). Dispense media to size tubes listed in USP. Butterfield's phosphate diluent (41.03).

8.04 Preparation of media and equipment

Prepare and sterilize your own media and equipment, or supervise the work closely so that you are absolutely sure of its sterility.

(a) Sterilize pipets and other equipment with dry heat at 170°C for 3 hours or overnight at 160°C. This equipment

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should be sterilized the day before use and set aside in a clean, protected place apart from the equipment used for other analysis, to insure against any possibility of recontamination.

(b) Steam sterilize media according to USP directions. Identify sterilizer recorder charts with sample number, type of media, initials, and date, and submit the chart with the sample worksheets. Incubate entire batches of media before use according to USP directions.

(c) Use laminar flow hoods located in a separate room or a clean area which can be easily cleaned and disinfected. Disinfect interior of hood with an effective chemical disinfectant (isopropyl alcohol diluted with water to a concentration of 60-70% is one of the best disinfectants for Gram-negative and Gram-positive organisms, and acid-fast bacilli). Turn the fan on for at least 1 hour (preferably overnight) before analysis is started.

8.05 Bacteriostasis

Using the procedure described in USP, determine the bacteriostasis of the product in fluid thioglycollate medium, using Cl. perfringens, B. subtilis, and E. coli, and the fungistatic activity of the product in Sabouraud medium, using a 1:1000

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dilution of a 24-48 hour culture of C. albicans. Once the bacteriostasis is determined, keep a record for future reference. The record should include brand name, product, formulation, suspected bacteriostat, dilution found effective, inactivating agent found effective, test organisms used, results, and difficulties encountered. USP requires the use of an inactivating agent, if available, and permits dilution beyond bacteriostatic level only as a second choice. Use azolectin-Tween thioglycollate medium for products containing quaternary ammonium compounds.

8.06 Inoculation

Always wear elbow length rubber gloves and sanitize them. Wear sterilized surgical gowns and masks.

(a) Liquids, suspensions, surgical dressings, etc.: Proceed as directed in USP.

(b) Devices (drapes, blankets, transfusion sets, catheters, syringes, and other bulky items): Some of these items are so bulky that suitable containers would be difficult to find. For items that can be sampled by removing pieces from the unit, cut approximately 1 inch square portions from at least 6-10 different locations and place each into suitable quantities of medium.

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For devices in which the sterility of the fluid path is critical, testing should be done by passing a volume of sterile test medium through the fluid path sufficient enough to thoroughly wash all sides of each unit.

Syringes or other small devices which are individually packaged and sterilized should be immersed in the test medium if possible. Be sure the medium gets into the fluid path of the syringe and needle, if attached.

8.07 Controls

Using fluid thioglycollate medium, test a representative number of pipets, syringes, or other equipment being used. Expose agar plates and/or open tubes of sterile media around the working area in the hood.

8.08 Incubation

Incubate inoculated tubes at 30-32°C (thioglycollate) or at 22-25°C (Sabouraud) for times specified in USP (note that incubation times vary with the product).

If the product is a device that was sterilized by irradiation, incubate tubes for 18-20 days before discarding them as negative.

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(a) Examine incubating tubes each workday. When a positive is found, confirm growth as indicated below, and arrange for a check analysis at an approved facility on additional units without waiting until the entire incubation period is over. This may save several days.

(b) Transfer to a solid medium, incubate, and Gram stain; report staining and morphological characteristics.[1/] Transfer a loopful from positive thioglycollate tubes to additional thioglycollate medium and incubate to confirm that cloudiness was caused by microbial growth.

(c) When an ophthalmic solution is found non-sterile, serially dilute a portion of the reserve subsample in Butterfield's phosphate diluent and determine aerobic plate count, incubating plates at 30-32°C for 3 days. If, during the examination of an ophthalmic solution, there is any reason to suspect that contaminating organisms are pathogenic (injury report, or other reasons) identify the organisms, but only to the point where they are eliminated as possible pathogens. Except when pathogens are strongly suspected to be present, do not attempt to identify contaminants.

Consult the latest USP for further details.

[1/] Bacteria from thioglycollate will give improper Gram stain and frequently fail to sporulate, so be careful in reporting the staining characteristics and presence or absence of spores.

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9. FUNGICIDES IN GRAIN[1/]

9.01 Introduction

The method will detect Captan, tetramethylthiuram disulfide, ethyl mercuryphosphate, ethyl mercury-p-toluene sulfonanilide, methyl mercuridicyandiamide, ethyl mercurychloride, Vancide-89, chlorophenyl mercury sulfate, phenylmercuric acetate, phenylmercuric ammonium acetate, and phenylmercuric urea. It does not detect hexachlorobenzene and pentachloronitrobenzene. The method has been successfully used for wheat, corn, barley, and beans. The method is nonspecific, and incapable of identifying or measuring the quantity of the fungicide on the grain.

9.02 Equipment and materials

(a) large heat-resistant glass tray or baking dish, preferably 16 x 24 inches

(b) N-ethylmercury-p-toluene sulfonanilide (Ceresan M), 4 µg/ml in 70% alcohol; store in tightly stoppered bottle

(c) glass plates or other suitable cover for tray

(d) Sarcina lutea, ATCC 9341

(e) vacuum seed sampler, or tweezers and paper template

(f) petri dishes

(g) 35°C incubator

(h) sterile 125 ml Erlenmeyer flask containing glass beads

[1/] Molinas (16).

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9.03 Medium

Penassay seed agar (40.30) in slants and in 100 ml quantities. Saline, sterile, 0.85% (41.10).

9.04 Preparation of suspension

Inoculate the entire surface of 2 or more fresh slants with Sarcina lutea, ATCC 9341. Incubate 24 ± 2 hours at 35°C. Wash the growth from each slant aseptically with 5 ml sterile saline into a sterile 125 ml Erlenmeyer flask containing glass beads. Shake gently and store in the refrigerator. Replace with a fresh preparation each month.

To determine the optimum inoculum add 0.1 ml, 0.2 ml, and 0.5 ml of the Sarcina lutea suspension to each of three 100 ml portions of melted penassay seed agar cooled to 45°C. Pour inoculated agar into sterile petri dishes fo form a layer ca 2 mm deep. When the agar is gelled, but not yet completely hardened, place a circle of filter paper which has been dipped into Ceresan M solution into the surface, and invert. Incubate at 35°C overnight. Choose for an inoculum the level that gives the largest and most distinct zone of inhibition.

9.05 Screening grain for fungicides

Melt penassay seed agar and cool to 45°C. Add the appropriate inoculum of the Sarcina lutea suspension. Shake gently and

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pour into petri dishes or trays to form a layer 2 mm deep. Allow to harden slightly; while agar is still soft, distribute kernels evenly over the surface at ca 15 mm intervals.[2/] Replace the cover and incubate at 35°C overnight. Clear zones (absence of growth) around kernels indicate the presence of a fungicide.

Absence of a fungicide is indicated by growth of the test organism up to the kernel of grain. The sensitivity of the test will vary with the fungicide, the depth of agar, and other factors such as the diffusibility of the fungicide and the quantity present in the portion of the kernel in contact with the agar.

Report number of kernels tested and number positive.

[2/] Place a template under the tray and place kernels with tweezers; or use a vacuum or manual seed counting head.

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10. ISOLATION OF SALMONELLA

10.01 Introduction

Isolating Salmonella from various food products often requires methods different from those used in clinical or public health laboratories. During the processing or storage of food products, the organisms have sometimes been subjected to heat, desiccation, preservatives, freezing, changes in osmotic pressures, and changes in pH. These may weaken the vitality of the organisms. Salmonellae usually comprise a relatively small proportion of the total microbial flora of food products.

In general, pre-enrich all foods that have received a dry heat treatment (desiccated, dehydrated, or powdered) in a non-inhibitory broth to initiate growth of salmonellae. Such foods usually have a reduced microbial population so that salmonellae, if present, are less likely to be overgrown by other organisms. Raw foods and finished products that have been grossly contaminated after processing should be examined by direct enrichment in selective broths to prevent overgrowth by other microorganisms.

It is extremely important to adjust the pH as recommended in order to obtain the best results.

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Somatic O antisera and the polyvalent flagellar H antiserum do not possess antibodies necessary to react with all of the 1,000+ Salmonella types now accepted in the Kauffmann-White schema.

Negative serological results will occur with some Salmonella types when tested with these antisera. The identification of such cultures must be resolved by biochemical tests and further serological analysis.

10.02 Equipment and materials

(a) blender and sterile blender containers with lids

(b) sterile wide-mouth screwcap pint jars

(c) sterile glass rods

(d) sterile spoons

(e) sterile pipets

(f) sterile petri dishes

(g) weighing balance

(h) 35°C hot air incubator

(i) 50°C water bath

10.03 Media and reagents

Distilled water, 200 ml (40.09); distilled water, 1000 ml (40.10); nonfat dry milk solution (40.27); nutrient agar

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(40.28); nutrient broth (40.29); crystal violet solution (41.04); brilliant green solution (41.02); Tergitol Anionic 7 (41.14).

10.04 Dried eggs and dried egg products, prepared powdered mixes (cake, cookie, doughnut, biscuit and bread), infant formula

NOTE: THIS IS THE REFERENCE METHOD.

Examine, using method for "Detection and Identification of Salmonella in Egg Products", JAOAC 50 (1), 231-239 (1967), as revised in JAOAC 51 (2), 505-506 (1968).

10.05 Dyes and coloring substances

(a) Products with a pH of 6.0 or above (10% aqueous suspension): Use Reference Method (10.06)

(b) Examine laked aluminum dyes and dyes with a pH below 6.0 by aseptically weighing 25 g portion into sterile wide-mouth screw cap pint jar. Add 225 ml of brilliant green tetrathionate broth, and mix well. Loosen jar cap approximately 1/4 turn and incubate 24 ± 2 hours at 35°C. Streak selective agar plates according to second paragraph, section 37.X19, and continue examination thru section 37.X28 of Reference Method (10.04).

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10.06 Liquid eggs or frozen egg products (whole egg, egg white or egg yolks)

Thaw frozen product as rapidly as possible to prevent increase in number of microorganisms present and at temperature low enough to prevent destruction of salmonellae (not greater than 45°C for not longer than 15 minutes). Frequent rotary shaking aids in thawing and increases uniformity of product.

(a) For pasteurized liquid eggs or frozen egg products: Use Reference Method (10.04).

(b) For nonpasteurized liquid eggs or frozen egg products: Aseptically weigh duplicate 25 g portions of product into separate sterile wide-mouth screwcap pint jars. Add 225 ml of selenite cystine broth to one jar and add 225 ml of brilliant green tetrathionate broth to the other jar. Shake well. Loosen jar cap approximately 1/4 turn and incubate 24 ± 2 hours at 35°C. Streak selective agar plates according to second paragraph, section 37.X19, and continue examination thru section 37.X28 of Reference Method (10.04).

10.07 Dried yeast (inactive)

Aseptically weigh 50 g into sterile wide-mouth screwcap pint jar. Add 200 ml of sterile distilled water, and mix well. If pH is below 6.6, adjust to 6.8 ± 0.2 with sterile 1 N NaOH.

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Loosen jar cap approximately 1/4 turn and incubate 24 ± 2 hours at 35°C. Continue examination according to sections 37.X19-37.X28 of Reference Method (10.04).

10.08 Coconut

Aseptically weigh 25 g into a sterile wide-mouth screwcap pint jar. Add 225 ml of sterile lactose broth, and shake well. Adjust pH to 6.8 ± 0.2 with sterile 1 N NaOH or HCl. Add 2.2 ml of Tergitol Anionic 7 (sodium heptadecyl sulfate). Mix well. Loosen jar cap ca 1/4 turn and incubate 24 ± 2 hours at 35°C. Continue examination according to sections 37.X19-37.X28 of Reference Method (10.04).

10.09 Egg-containing foods (egg noodles, egg rolls, etc.)

Aseptically weigh 25 g into a sterile blending container. Add 225 ml of sterile lactose broth, and blend for 2 minutes. Aseptically transfer blended mixture to a sterile wide-mouth screwcap pint jar, and shake well. If pH is below 6.6, adjust to 6.8 ± 0.2 with sterile 1 N NaOH. Loosen jar cap ca 1/4 turn and incubate 24 ± 2 hours at 35°C. Continue examination according to sections 37.X19-37.X28 of Reference Method (10.04).

10.10 Dried milk

Aseptically weigh 100 g of dried milk into a sterile 2 L flask

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(or wide-mouth screwcap 2 quart jar). Add 1000 ml of sterile distilled water. If pH is below 6.6, adjust to 6.8 ± 0.2 with sterile 1 N NaOH or HCl. Add 4 ml of 1% aqueous crystal violet or 2 ml of 1% aqueous brilliant green, and mix well. Incubate 24 ± 2 hours at 35°C. Continue examination according to sections 37.X19-37.X28 of Reference Method (10.04).

10.11 Meat, animal feed, animal substances, glandular products or fish meal

(a) For heated, processed or dried product: Aseptically weigh 25 g of product into a sterile blending container. Add 225 ml of sterile lactose broth, and blend for 2 minutes. Aseptically transfer the blended mixture to a sterile, wide-mouth, screwcap pint jar. (If product is a powder, ground or comminuted, blending may be omitted; aseptically weigh 25 g into a sterile jar, and add 225 ml of sterile lactose broth and mix well.) If pH is below 6.6, adjust to 6.8 ± 0.2 with sterile 1 N NaOH. Add 2.2 ml of Tergitol Anionic 7 (sodium heptadecyl sulfate) if product contains a large quantity of fat. Incubate 24 ± 2 hours at 35°C.

Shake incubated product-lactose broth mixture gently, transfer 0.5 ml to 10 ml of selenite cystine broth, and transfer

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an additional 0.5 ml to 10 ml of brilliant green tetrathionate broth. Incubate 24 ± 2 hours at 35°C. Streak incubated selenite cystine broth and brilliant green tetrathionate enrichment broth onto selective agar plates according to second paragraph of section 37.X19 and continue examination through section 37.X28 of Reference Method (10.04).

(b) For raw or highly contaminated meat, animal feed, animal substances or glandular products: Aseptically weigh duplicate 25 g portions into separate sterile blending containers. Add 225 ml of selenite cystine broth to one container and 225 ml of brilliant green tetrathionate broth to the other. Blend each container for 2 minutes. Aseptically transfer blended mixture to separate sterile, wide-mouth, screwcap pint jar. (If product is powder, ground or comminuted, blending may be omitted; aseptically weigh duplicate 25 g portions into separate sterile, wide-mouth, screwcap pint jars. Add 225 ml of selenite cystine broth to one jar and 225 ml of brilliant green tetrathionate broth to the other and mix well.) Incubate 24 ± 2 hours at 35°C. Streak incubated selenite cystine broth and brilliant green tetrathionate enrichment broth onto selective agar plates according to second paragraph of section 37.X19, and continue examination thru section 37.X28 of Reference Method (10.04).

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