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

Cunniff, P. (ed.). 1995. Official Methods of Analysis of AOAC International, 16th Edition. AOAC International Inc., Gaithersburg, MD 20877.

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