Guidelines for Microbiological Evaluation of Meat

Anthony W. Kotula[1]
John C. Ayres[2]
Charles N. Huhtanen[5]
Norman J. Stern[1]
William C. Stringer[3]
R. Bruce Tompkin[4]

Introduction

The microbiology of meat is becoming increasingly important because of the desire by the industry to provide the consumer with meat and meat products of the highest quality. The need for quality assurance programs, involving microbiological assay, is becoming more apparent as the industry and regulatory agencies focus on product losses, product exports and the potential for illness due to improper care of meat. Meat scientists, faced with establishing a program for monitoring microbial contamination of meat and equipment refer to sources such as the Compendium of Methods for the Microbiological Examination of Foods (APHA, 1976), Official Methods of Analysis (AOAC, 1975), Bacteriological Analytical Manual for Foods (Food and Drug Administration, 1976) or the Microbiology Laboratory Guidebook (USDA, 1974). To individuals not accustomed to microbiological determination, an introduction to the terminology, procedures and recognized sources for additional in-depth-understanding of microbiology as it pertains to meat, will provide a minimum base for communicating, understanding, and controlling microorganisms on meat.

The American Meat Science Association Board of Directors in 1976 established a select microbiology committee to a) monitor microbiological guidelines as they are developed by local or state agencies to assure that such standards are reasonable and that AMSA has input where expertise is beneficial, b) assist industry with development of material for use in describing their position with regard to microbiological practices and/or proposed guidelines and c) prepare a document, for subsequent consideration by the membership of AMSA, which could be used as a materials, methods and procedures reference by those involved in meat microbiology research, teaching or extension. These guidelines address the third responsibility of the committee.

The committee members' first attempt at compiling these guidelines provided so much information in such great detail that another reference book was in the process of being written. Adequate reference texts are available, therefore an abbreviated format is provided in these guidelines. The reader will obtain general information about the microorganisms associated with meat and an insight into sampling, enumeration and identification procedures. These guidelines are not "standard methods" which must be adhered to rigidly; rather, they provide a common starting point from which the novice may begin to obtain a familiarity with the microbiological evaluation of meat.

Scope

The guidelines are based on procedures published in the scientific literature. Care was taken to select the simplest available methodology. Evaluation and use of rapid test kits (Cox and Mercuri 1979, Southern 1979) which are used by many for enumeration and identification of bacteria is underway by the scientific community and therefore discussions of such methods are not included in the guidelines.

Whenever possible, specific recommendations for isolation, enumeration and identification of the microorganisms as currently being utilized by meat scientists, are provided. In some instances more than one accepted procedure is described, but in these instances the differences in expected results are explained.

These guidelines apply to the microorganisms associated with meat, meat products and equipment of the meat industry. These guidelines are aimed at the practitioner with interest in applied rather than basic studies. Attention is focussed on sampling procedures, sample transport, sample preparation, enumeration, statistics and a very little on identification. A rough draft of descriptive profiles was prepared for individual spoilage bacteria, indicator organisms, molds, parasites, protozoa, viral agents and yeasts, that may be associated with meat. That section, together with the sections on trouble shooting and glossary of terms could not be subjected to care-

[1]Meat Science Research Laboratory, SEA-AR, USDA, Beltsville, MD.

[2]Food Science Department, University of Georgia, Athens, GA.

[3]Food Science and Nutrition Department, University of Missouri, Columbia, MO.

[4]Research and Development Center, Swift & Company, Oak Brook, IL.

[5]Eastern Regional Research Center, SEA-AR, USDA, Philadelphia, PA.

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ful review by expert microbiologists in time for publication of the RMC proceedings, and therefore do not appear in this paper.

Sampling Procedures

A. *Airborne Microorganisms:*

Some meat plant operations produce aerosols of bacteria and molds that may serve as a potential means of contaminating meat and equipment. Typically for enumerating airborne microorganisms one utilizes a medium selective for the microorganism which is to be enumerated. For example plate count agar incubated at specific temperatures would be appropriate for psychrotrophic and mesotrophic bacteria, violet red bile agar with subsequent overlay for coliforms and potato dextrose agar acidified to pH 3.5 with tartaric acid for yeasts and molds.

1) *Exposure Plates*—The simplest procedure for airborne microorganisms is to remove the cover of a petri dish containing the appropriate sterile media for 15, 30 or 60 min (Kundsin 1960). The procedure presupposes that organisms will drop onto the media. After exposure, the petri dish cover is replaced and the visible colonies are allowed to develop during incubation at the appropriate temperature. The microbial count is reported as the number of organisms per min exposure.

2) *Millipore Filter*—This procedure is not passive because air is forced across a filter by suction. The organisms from the air are trapped on a small filter for subsequent enumeration. The filter can then be placed onto the appropriate media prior to incubation or can be treated as a meat sample and incorporated into diluent prior to serial dilution and incubation. The microbial count is reported as number of organisms per cubic meter of air.

3) *Slit, Single, and Multiple Stage Sieve Samplers*—Several commercial air samplers are available. The slit samplers (Dahlgren 1961) use a revolving culture plate containing media. Air is forced through the slit by vacuum, the bacteria impinge upon the media and can be enumerated after incubation. The single and multiple stage sieve samplers utilize the same principle of moving air but differ because in the sieve samplers the air is forced through 400 holes in a metal plate above the media. Once again the organisms impinge on the media and become visible as colonies after incubation. When the single stage sampler is utilized some microorganisms bounce off the surface of the media and are not enumerated. In the multiple stage sampler the air from the first petri dish cascades over the edge, through another sieve plate covering the second petri dish of media. The air continues through six such sieves in some samplers (Kotula and Kinner, 1964; Sayer et al., 1969). In both the slit and sieve samplers the microbial count is reported as the number of bacteria per cubic meter.

B. *Meat—External Surfaces:*

Intact, healthy, functioning muscle tissue is essentially free of microorganisms because of the scavenging action of the reticulo-endothelial system and the natural barrier membranes of the animal (Gill and Penny, 1979). After slaughter, the surface of the resultant meat becomes inoculated with bacteria from contact with air or other surfaces which contain bacteria. Enumeration of surface bacteria is accomplished by removing as many bacteria as possible from a prescribed area and then allowing the viable bacteria to grow on appropriate media to form visible colonies. Several methods of obtaining an estimate of the surface microbial counts are listed below. They vary depending upon whether or not the surface may be excised.

1. *Contact Plate* (RODAC, Fisher Scientific, 7722 Fenton Street, Silver Spring, MD)—Sterile microbiological medium pressed lightly against the surface to be examined will remove a percentage of the microorganisms present. The media is then incubated to encourage the formation of visible colonies for enumeration. The procedure does not remove all of the surface microorganisms but can be used as an approximation of very high or low surface counts. Some commercial preparations of sterile media are available in tubular form. Once the surface of the medium is used, a thin segment of the medium is sliced off aseptically for incubation and the newly exposed surface is then available for the next sample. The bacteria are counted as number per the surface area of the agar used and reported per cm^2. A commercial adhesive tape is also available (Fung et al., 1980).

2. *Millipore Filter*—A sterile millipore filter pressed against a moist meat surface will remove a percentage of the bacteria present. The filter can then be placed onto an appropriate medium and incubated, or the bacteria can be removed from the filter using one of the methods described in the section entitled Sample Preparation—Solid sample. If the meat surface is not moist, the filter should be moistened in diluent or sterile, distilled water. After incubation in appropriate agar the bacteria are counted on the basis of the surface area of the filter and reported as number of bacteria per cm^2.

3. *Swab*—A sterile cotton tipped swab, moistened in diluent (0.1% peptone or Butterfield diluent) may be utilized to remove surface bacteria. Once moistened the swab should be pressed against the inside neck of the diluent bottle to remove excess diluent. A prescribed area, e.g. 12.3 cm^2 as defined by a sterile Ridak Sanitary Paper Gasket (Booneville Manufacturing Corp., Booneville, NY) is wiped clean of bacteria by placing the swab at about a 20° angle with the surface and swirling the handle of the swab so that the cotton encounters the entire area within the gasket. The swabbing should continue for 15 seconds for each sample area. The swab containing the bacteria is then partially inserted into the neck of the bottle containing 99 ml of diluent and the handle broken to exclude from the diluent the portion of the wooden or plastic handle that was held by the fingers. The dilution bottle then contains the first 1:100 sample for subsequent ten-fold serial dilutions. The dilution bottle should be moved through a 12 in. arc 25 times in 12 sec to remove the bacteria from the swab. After serial dilution and incubation the microorganisms are counted per surface area swabbed and reported as number of bacteria per cm^2. Researchers have demonstrated that swabbing does not remove all of the surface microorganisms. However, the procedure, if utilized without variation, will provide an estimate of the number of surface microorganisms present. The procedure is particularly effective for use in routine microbiological control.

4. *Sponge*—A sterile sponge or gauze may be utilized in-

stead of a swab, particularly if large areas are to be sampled for bacteria, such as salmonellae, which are present in limited numbers, if at all.

5. *Rinse*—Some researchers have developed instruments which would allow a prescribed surface area to be rinsed by diluent, but the method is not commonly being used. Irregular shaped samples such as chicken can be sampled by rinsing. The chicken or part thereof is placed into a plastic bag with an adequate measured amount of diluent to allow the diluent to rinse the sample effectively while the bag is shaken through an arc of about 12 inches. The surface area of the sample can be estimated and the microbial count reported on the basis of surface area or the count can be reported per unit sample. This procedure is effective for determining gross treatment effects.

6. *Cork Borer* (Surface Core Method)—To ensure that all of the surface bacteria are placed in contact with the diluent, a sterile cork borer may be utilized to describe a sample and make it available for excision with a sterile scalpel. The cork bored sample, which is disc shaped, is placed in diluent and agitated by one of several methods described in "sample preparation." The count is reported as microorganisms *per surface area* cut by the cork borer. One should not report the count on the basis of sample weight because we assume the bacteria are a surface phenomenon and weight will vary with depth of the excision. Normally, the thickness of the disc (or depth) is no more than 3 mm.

7. *Free Juice (drippings) From Package*—A 1-ml representative sample of the juices is removed with sterile pipette and the sample is added aseptically to the diluent in preparation for serial dilution. The count is reported as microorganisms per ml of free juices.

C. *Meat—Internal Sample:*

Particularly in processed meats, such as sausages, the internal portions of the meat may need to be sampled. The surface of the sausage is cut and the sausage is then broken in two to expose the center of the sausage. A sample is then excised with a sterile scalpel from the newly exposed center of the sausage, making certain none of the sampling extends to the portion that had been cut with the knife. Subsequent serial dilution, plating, colony development and enumeration allows estimation of bacteria per g of sausage.

D. *Equipment:*

The procedure described for meat surfaces, swab, sponge, contact plate (RODAC) or tape (BIRKO) may be used.

E. *Comminuted Meat and Ingredients Other Than Meat:*

Comminuted raw meat and dry ingredients such as spices are incorporated into diluents on a weight basis. For example a 50-g sample of ground beef can be added to 450 ml of diluent to form a 1:10 dilution. Much smaller samples of spices would be utilized. The count would be reported as numbers of microorganisms per g of sample. Liquid ingredients are added to diluent to form a 1:100 dilution by incorporating a representative 1-ml sample into 99 ml of diluent. The count would be reported as number of bacteria per ml of sample.

Sample Transport

The method of transporting samples for microbiological analysis should be one in which a minimum of microbial growth, injury or death occur. The optimum procedure is to have on-site microbiological capability in the form of a permanent or a mobile laboratory facility. For short distances, less than three hours from sampling to plating, the meat samples or bacteria in diluent can be transported packed in ice. For medium distances, 3 to 18 hours from sampling to plating, samples can be packed in an adequate amount of ice to ensure constant temperature throughout transport. This medium range shipping should not be expected to yield an accurate estimation of the number of bacteria initially present on the sample at time of sampling. The procedure does have merit, however, when absolute numbers of bacteria are not of major concern but rather the emphasis of the study is placed on determination of a treatment effect. For example, what was the difference in effectiveness of different sanitizers on equipment surfaces? Medium range shipping may also be employed to identify the microorganisms present in or on a sample. For long distance storage, in excess of 18 hours, the samples should be frozen and kept frozen until thawed at the laboratory. This sampling procedure does not yield the quantitative or qualitative accuracy obtained by on-the-spot sampling.

Dry ice has been used effectively when samples are transported by car. Precautions to prevent asphyxiation should be taken if the samples in dry ice are transported by car. Airlines usually will accept boxes containing dry ice if they are properly labeled so there is no danger to pets that otherwise may be stored near the box. Commercial gel-type products which can be frozen and packed with the samples can be used effectively to provide the refrigeration needed to protect the sample. Containers such as the Trans Temp (Model 312, Kay Laboratories, Inc., Moberly, MO) which help maintain a constant temperature with styrofoam insulation when used in conjunction with the frozen gel, will provide the following percentage recoveries after 72 hours when compared to dry ice (Kotula, et al. 1979).

	Percent Recovery	
Organism	*Trans Temp*	*Dry Ice*
C. perfringens	<1	16
Coliforms	52	44
E. Coli	53	42
S. aureus	85	96
APC_{20}	62	80

The low recovery of *C perfringens* can be improved by incorporating a 10% glycerol or dimethyl sulfoxide (DMSO—J. T. Baker Chemical Co., Phillipsburg, NJ) solution into a comminuted meat sample before freezing for shipment. The solution is prepared on a weight-to-volume basis with 0.1% phosphate-buffered diluent and adjusted to pH 7.0 ± 0.1. The stock solutions should be autoclaved 15-min at 121°C. Cryoprotectant (25 ml) should be mixed with a 25-g sample of

ground meat into a sterile polyethylene Whirl-Pak bag (NASCO, Inc., Fort Atkinson, WI) and frozen rapidly before shipment. Plastic Whirl-Pak bags usually may be considered sterile.

Swab samples from cleaned equipment may be mailed in individual empty (without diluent) vials without refrigeration. After serial dilution, plating and enumeration, high counts indicate contaminated equipment whereas low counts indicate the equipment may have been cleaned adequately. The counts obtained will not provide absolute quantitative or qualitative microbial values on the equipment at time of sampling. Swab samples from equipment during production or from product surfaces must either be refrigerated or frozen as discussed above.

Sample Preparation

Either solid samples or bacteria on swabs or in diluent will arrive at the laboratory for microbiological evaluation.

Solid Sample:

If the meat samples arrive frozen they may be allowed to thaw overnight in a refrigerator, or may be thawed in waterproof plastic bags under cold running water. Some judgement is necessary to decide the best method. Small samples simply may be tempered at room temperature just before analysis. It is important to avoid long thawing periods which permit microbial growth to occur on the meat surface. Meat shavings may be taken from large frozen samples (e.g., 20 kg blocks) using a pre-sterilized drill bit. Bacteria can be removed from the meat samples to prepare the initial 1:10 dilution by using a) a mechanical blender, b) a stomacher, or c) broken glass chips in a jar (Tompkin 1976).

Several blenders on the market can be used with Ball or Mason type canning jars as a replacement for the container provided with the blender. Adequate blade sets and jars must be autoclaved prior to use. A weighed sample of meat is added to the diluent in a 1 to 10 proportion. For example 25 g of ground beef can be added to 225 ml of diluent, or if a larger sample is desired 50 g of ground beef can be added to 450 ml of diluent to increase the likelihood of finding organisms that are present in low numbers. The blending time should be standardized but must be long enough to macerate the meat sample to ensure removal or dispersion of the bacteria (usually about 2 min). Serial dilutions (Fig. 1) are made using the supernatant as the 1:10 dilution.

A stomacher Model 400 (Cook Laboratory Products, Div. Dynatech Labs, Inc., Alexandria, VA) uses a sterile Cryovac bag (18 cm × 30 cm) to hold the sample and diluent. Two metal paddles in the apparatus beat the sample against the metal door (Sharpe and Jackson 1972). After 2 min of such agitation in the stomacher, samples of the supernatant can be removed for preparing further serial dilutions.

A small pre-sterilized jar (165 ml) containing a tablespoon of broken glass chips can be used for mixing an 11 g meat sample with 99 ml diluent. The jars containing the sample and diluent can be shaken on a mechanical paint shaker for 3 to 5 min to prepare the 1:10 dilution.

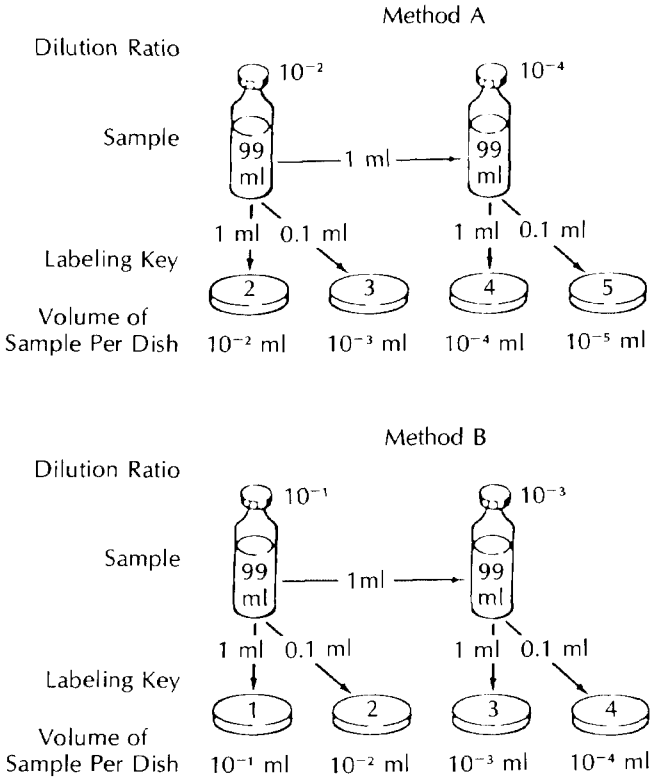

Figure 1. Preparation of serial dilutions. (Courtesy APHA, Speck)

Swab or Liquid Sample:

A swab containing bacteria or 1 ml of liquid sample can be placed into a milk dilution bottle containing 99 ml of diluent or into a screw top test tube containing 9 ml of diluent. The sample in the milk dilution bottle should be mixed by shaking it 25 times through an arc of 1 foot in about 12 sec. This procedure is also utilized for shaking subsequent dilutions. The sample in the test tube can be mixed by shaking as described above or by using a vortex mixer (Scientific Industries, Inc., Queens Village, NY) for 15 sec. Swab samples can also be mixed with the diluent using a stomacher.

Serial Dilution of Samples:

The procedure for preparing serial dilution of the samples is shown in Fig. 1. If a 25 g sample to 225 ml diluent; or 50 g sample to 450 ml diluent; or a swab or 1 ml were added to 9 ml diluent; to produce a 1:10 (10^{-1}) dilution, then procedure A of Figure 1 can be followed. If a swab or 1 ml sample is placed in 99 ml diluent to produce a 1:100 (10^{-2}) dilution, then procedure B of Figure 1 can be followed. A calcium alginate or cotton tipped swab is estimated to retain about 1 ml of diluent when it is moistened prior to swabbing the test surface. Then the swab is considered equivalent to the 1-ml liquid sample for dilution purposes, but the bacterial count is still reported as number of bacteria per cm^2.

The number of dilutions plated is dependent upon the estimated level of bacteria on the sample to be evaluated. The

lowest and highest dilutions plated should be selected to produce colonies too numerous to count (TNTC) and too few to count (TF), respectively. A countable plate for a 100 mm petri dish is 30 to 300 colonies. When cluster plates are used with each well 35 mm in diameter, a countable plate has 15 to 150 colonies after incubation.

Routine testing of fresh meat can be expected to involve aerobic plate count (APC), coliforms, fecal coliforms, molds, yeasts, and perhaps some selected bacteria such as *E. coli, S. aureus, C. perfringens,* salmonellae depending on the reason for testing. Plate count agar is a good general medium for enumerating aerobic bacteria. When vacuum packaged, cured or fermented meats are to be evaluated APT agar has an advantage over plate count agar by aiding lactic acid producing bacteria produce larger colonies. Coliforms are usually enumerated using violet red bile agar with overlay. After the medium has solidified in the petri dish about 5 ml additional medium is layered on the medium surface. After the overlay has solidified the petri dish is inverted and incubated at 35 ± 1°C for 18-24 hours. When the incubation temperature of 45.5°C is used, colonies of fecal coliforms will develop a halo surrounding the colony (Klein and Fung 1976). Coliforms may also be evaluated using three-tube most-probable-number with lauryl sulfate broth (BBL Microbiology Systems) and brilliant green bile broth (2%, Difco Laboratories) as described in the Official Methods of Analysis (AOAC, 1975). Molds and yeasts can be enumerated on potato dextrose agar which has been acidified to pH 3.5 with tartaric acid. Media and incubation temperatures should be reported in manuscripts.

Incubation:

The temperature at which the bacteria on plate count agar are incubated will determine which bacteria will predominate. For example, $APC_{5°C}$, which indicates aerobic plate count at 5°C will require 10 days incubation and will result in colonies consisting mostly of psychrotrophic bacteria. $APC_{35°C}$ will result in mostly mesotrophic bacteria whereas $APC_{20°C}$ will result in a mixture of both psychrotrophic and mesotrophic bacteria. Incubation of $APC_{20°C}$ lasts only 3 days and will yield counts slightly higher than $APC_{5°C}$ or $APC_{35°C}$ (Goepfert, 1976). The normal incubation time for $APC_{35°C}$ is 48 hours.

Enumeration:

A countable plate for a standard size petri dish (100 mm) is 30 to 300 colonies. The counts should decrease by a factor of ten with each successive serial dilution. Sometimes adjacent dilutions have countable plates. When that occurs, the counts for each petri dish should be calculated and averaged.

Statistics

The bacterial counts should be converted to $\log_{10}$ before the data are analyzed statistically. A value of 0.1 should be added to each bacterial count before conversion to $\log_{10}$ to preclude the possibility of dealing with the logarithm of zero values. The need for planning a study having the appropriate statistical design must be considered before the study is initiated. Examples of experimental designs are presented by Cochran and Cox, 1957. Most typically the data can be evaluated by the Analysis of Variance to discern whether the main treatment effects or interactions exhibited a significant effect on the bacterial count (Steel and Torrie, 1960; Snedecor and Cochran, 1968). Once the analysis has shown that a significant difference was present, then a mean separation analysis may be appropriate to determine specifically which segments of the treatment are different. A mean separation analysis such as Duncan (1955) may not be appropriate if the treatment involves a continuum such as time or increasing concentration levels. The fact that a treatment effect is statistically significant may not mean that it is important. The scientist must evaluate whether the magnitude of the difference is large enough to be important. Usually when significant, a difference greater than 1 log will be important. Significance is usually reported when $P < .05$ or $P < .01$. Some scientists report at the $P < .10$ level, but not beyond that. Computer programs are available which calculate the level of significance, wherever it falls.

Identification

Some bacteria, such as *S. aureus,* are identified by plating the serial dilutions directly on selective media. Confirmatory tests (e.g., biochemical, serological, microscopic, staining) are then utilized to ensure proper taxonomic classification of the bacterium. The specific tests for most bacteria that are important to the meat industry are described in the Microbiology Laboratory Handbook (1974).

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