
36

MICROBIAL ANALYTICAL METHODOLOGY FOR PROCESSED POULTRY PRODUCTS

OMAR A. OYARZABAL

Department of Biological Sciences, Alabama State University, Montgomery, Alabama

SYEDA K. HUSSAIN

Department of Poultry Science, Auburn University, Auburn, Alabama

Introduction	528
Method evaluation	528
Sample collection	530
Isolation methods	530
Preenrichment	530
Enrichment	531
Agar plates	531
Methods for bacterial counts	532
Pathogenic bacteria	533
<i>Campylobacter</i>	533
<i>Clostridium perfringens</i>	536
<i>Listeria</i>	536
<i>Salmonella</i>	537
Spoilage microorganisms	538
Mesotrophic bacteria	538
Psychrotrophic bacteria	539
Yeasts and molds	539
Application of molecular techniques for identification purposes	540
Future trends in the identification of bacteria	540

INTRODUCTION

Different microbial methods have been described for the isolation and identification of pathogenic and spoilage bacteria present in poultry products. In the case of bacterial pathogens, most methods target the detection of a low number of cells. Therefore, enrichment procedures are used to allow bacterial cells to recover, multiply to large numbers, and be detectable. When using enrichment procedures, the results are *qualitative*: positive or negative. In the case of spoilage bacteria, it is very important to obtain the count of the different bacterial groups at different times during the storage of the product. Consequently, *quantitative* methods that yield numbers or counts per milliliter or gram of product are most commonly used.

The majority of the microbiological methods and most of the accumulated experiences are related to the analysis of commercial broiler meat. Accordingly, in this chapter we review the most successful methods used for the analysis of these products. However, these methods are also used in the microbial analysis of poultry products from other avian species, such as turkeys. The differences stem from the size of the poultry carcasses, and thus the collection of samples may vary from one poultry species to another. For example, turkey carcasses are very large to handle and require additional equipment to perform the initial sample collection (Dickens et al., 1986).

We first review some terms used to evaluate different methods. This topic is relevant to microbiologists trying to incorporate a new method in the laboratory and includes a brief summary from two organizations that are involved in the validation of new methods to assure performance. We then review sample collection methods, which have been discussed extensively in the literature (ICMSF, 1984), and the most commonly used isolation methods, including the most probable number technique and some of the newest developments in enumeration procedures. In the next sections of the chapter we describe techniques used for the detection—and enumeration when necessary—of bacterial pathogens (*Campylobacter* spp., *Clostridium perfringens*, *Listeria monocytogenes*, and *Salmonella* spp.) and spoilage organisms (mesotrophic and psychrotrophic bacteria, and yeast and mold).

At the end of the chapter there is a brief description of the applications of molecular techniques for rapid identification purposes, and a few sentences on chromogenic agars and future trends in the isolation of bacterial pathogens. We believe that food microbiologists have to be aware of the major concepts driving the research in new detection methods, which happen to incorporate newly developed molecular techniques. These new approaches are expanding our understanding of the bacteria that are present in foods.

METHOD EVALUATION

An important decision to make when incorporating a method in a food microbiology laboratory is to determine the most appropriate method for each circumstance.

Although there is a plethora of scientific literature describing specific applications of the various methods, the search can be overwhelming and sometimes frustrating. Good resources are the organizations that evaluate different methods and compare the new methods with established ones. A couple of examples of those organizations are the Association of Official Analytical Chemists (AOAC) International (www.aoac.org) and the Association Française de Normalisation (AFNOR) (www.afnor.org).

Each organization has a series of validation procedures, and the names of the “validated” or “certified” methods are available on their Web sites. For example, the methods validated by AFNOR can be found at www.afnor-validation.com/afnor-validation-food-industry/food-industry.html. AOAC International has a Method Validation Program, which includes the Official Methods of Analysis (OMA) Program, the Peer-Verified Methods, and the Performance Tested (PT) Methods. These methods for comparison provide a range of accuracy that varies from “the highest degree of confidence in performance to generate credible and reproducible results” (OMA Program) to a “validation of performance claims where rapid validation and some degree of confidence is needed” (PT Methods).

For evaluation purposes, several terms are used to compare the efficacy among methods. The most important terms are:

- *Sensitivity*: the percentage of total positive samples (confirmed positive by one or more methods included in the study) that test positive (confirmed) by the test method
- *Specificity*: the percentage of total negative samples (confirmed negative by all methods included in the study) that test negative by the test method
- *Inclusivity*: the ability of the method to detect the target bacterium from a wide range of strains
- *Exclusivity*: the lack of interference on the method from a relevant range of nontarget strains which are potentially cross-reactive
- *Ruggedness*: the ability of the method to withstand perturbations to basic procedural specifications

When performing a comparison between two or more methods, a term that is usually included for the analysis is the *agreement between methods*, defined as the percentage of samples that test the same (confirmed positive or negative) by the two methods. Two other terms that are used to characterize the efficacy of a method are *false negative*, described as the probability that a test sample is a known positive but was classified as negative by the method, and *false positive*, defined as the probability that a test sample is a known negative but was classified as positive by the method.

Microbiologists should know these terms and should be able to compare different values obtained with different methods when performing small-scale evaluations in their laboratories to decide which method to incorporate. These are important terms because they may not correlate directly. For example, when

attempts are made to reduce to a minimum the probability of false negatives in a new method, chances are that the probability of a false positive will increase.

SAMPLE COLLECTION

The collection of samples for microbiological analysis has the goal of obtaining a representative sample of a food lot. The sample should be kept under appropriate conditions—usually, refrigeration—to avoid changes from the time of collection to the start of the analysis. Before the actual microbial analysis takes place, it is important to determine the *sampling plan* to follow, which includes the number of samples and/or the size of the samples to collect, to ensure that the collected samples represent the food lot, and the appropriate method for *sample handling*: transportation and storage. The scope of this chapter does not include a review of the different statistical methods or transportation procedures available for the microbial analysis of foods, and therefore the reader is encouraged to review some relevant literature to incorporate the most appropriate sampling plan for each specific case (ICMSF, 1984; Messer et al., 1992; USDA–FSIS, 1998; FDA, 2000).

The sampling of poultry carcasses offers some difficulties because the whole carcass remains intact until the end of first processing (water cooling in the chiller). Because of the size, broiler carcasses in the United States have traditionally been sampled as a whole in plastic bags using the *carcass rinse method* (Cox and Blankenship, 1975). The amount of rinse used in this method has changed over the years, and with the introduction of hazard analysis and critical control points, the rinse volume has been established at 400 mL (USDA–FSIS, 1996). The rinse most commonly used is Butterfield's phosphate solution (0.00031 M KH_2PO_4 , ca. pH 7.2), although buffered peptone water is preferred when collecting samples for *Salmonella* detection because this is the preferred medium for the preenrichment of the samples.

With other large carcasses the use of surface swabbing or tissue excision and maceration are common sampling procedures. Other sampling procedures include the collection of drip (weep), the spraying or scraping of skin areas, mainly on the breast, and agar contact plates (Cox et al., 1976). Because poultry carcasses are hung by the legs, the breast area has low bacterial counts, while the neck skin is the most contaminated area of the carcass (Barnes et al., 1973).

ISOLATION METHODS

Preenrichment

Preenrichment is the first step in the isolation of the target microorganism after the collection of the samples. There are several reasons to add this important step: (1) the organism of interest is present in low numbers or is distributed irregularly across the sample; (2) the food-processing methods and/or intrinsic

factors of the food may have injured the organism; and (3) competing organisms are present. Hence, the preenrichment step favors multiplication of the organisms present in the samples, without selecting for particular microorganisms. Therefore, preenrichment media are very nutritious and nonselective for the targeted microorganism.

It is important to highlight that all preenrichment and enrichment procedures used in food microbiology are in the liquid form (broths), and they are all intended to help bacterial cells recover from injuries, or sublethal stress, and reproduce to higher, detectable numbers. Traditionally, a ratio of 1 to 9 (where 1 is the amount of sample and 9 is the amount of enrichment) has been used to enrich food samples. In the United States, the suggested amount of food product to sample is 25 g or mL, and therefore the amount of enrichment to add to food samples is 225 mL. But recent studies using simulation and actual sampling programs have shown that the incidence and distribution of pathogens in broiler meat increases in a nonlinear manner as a function of sample size. For example, the incidence of *Salmonella* in 25-g samples has been estimated at 16%, while in 100-g samples the incidence estimated is 51%. We currently do not know the most appropriate sample size for pathogen identification based on risk analysis models. Besides, the ideal sample size may be different according to the targeted pathogen, and thus a linear extrapolation of enumeration results, a common practice in microbial risk assessment, may not appear to be the most appropriate approach (Oscar, 2004).

Enrichment

Enrichment is the step after preenrichment intended to facilitate the growth of the organism of interest to detectable numbers while inhibiting the growth of unwanted competing organisms. Sometimes, the preenrichment is bypassed and samples are only enriched before transferring to agar plates (e.g., for *Campylobacter* isolation). Therefore, enrichment broths contain antimicrobials to selectively isolate the target microorganism. Different types of enrichment broths with different incubation temperatures are employed for the detection of different organisms, or even for the detection of the same organism. For example, tetrathionate (TT) and Rappaport–Vassiliadis (RV) broths are common enrichment media for *Salmonella* isolation, and sometimes both broths are used to maximize *Salmonella* isolation.

Agar Plates

The third step of the isolation procedure includes the transfer of enriched samples to agar plates. Plating media provide a solid surface for growth of the organism and allows for the detection of individual colony-forming units (CFU), which are in most cases representative of the growth of a given bacterial clone. Plate media can also be used for direct isolation and enumeration of the target organism. In this case, the bacteria are in numbers high enough to grow directly on the agar plates and result in a count per milliliter or gram of food.

Methods for Bacterial Counts

As stated in the introduction, the counting of some bacterial groups present in food samples is an important tool to predict the shelf life of a product. Several attempts have been made in the last two decades to replace the *most probable number* (MPN) *technique* for bacterial enumeration. But the reality is that for samples contaminated with less than 1 CFU of the target organism per gram or milliliter of the product, a direct enumeration using plating media is not feasible. It is important to remember that the term *colony-forming unit* refers to the growth seen on agar plates and represents viable bacteria, which may be one or more bacteria growing to produce a single colony.

Some methods, however, concentrate the bacterial cells from a sample and increase the opportunity of detection. An example of these concentration methods is *hydrophobic grid-membrane filtration* (HGMF), which has had a major application for the enumeration of coliforms, *Escherichia coli*, *Salmonella*, and yeasts and molds in food products (Brodsky et al., 1982; Entis et al., 1982; Entis, 1984). Although filter membranes for the concentration of bacteria from water samples have been used for many decades (Goertz and Tsuneshi, 1951), the use of HGMF for counting bacteria in food samples was introduced in 1974 (Sharpe and Michaud, 1974). A specially constructed filter consisting of 1600 wax grids on a single membrane confines the growth of the organism to the square grid cell, restricts the colony size, and reduces the need for extensive dilutions of the sample (Sharpe and Michaud, 1974). Linear counting can result in up to 30,000 CFU per filter and as few as 10 cells/g can be enumerated within 24 h (Sharpe and Michaud, 1975; Sharpe et al., 1983).

When the numbers of bacterial cells in samples are higher than 100 CFU/mL or g, other methods can be used that are not as labor intensive as the MPN method but provide similar results. One of these methods is the *spiral plate method*, which allows for the enumeration of samples with large numbers of bacteria using very few plates and without extensive dilution schemes. A known amount of the sample is deposited on a rotating agar plate and the results are read by a laser eye that calculates the number of CFU based on the number of colonies found on the plate. The sensitivity of the method is higher with samples that have more than 1000 CFU/mL. It is important to obtain discrete colonies on the agar plates to avoid errors during the reading step. This method has cost advantages over the conventional method, which uses a serial dilution with the plating from each dilution for bacterial counts, and it can be done in a shorter time.

Another method is the *slim agar plate*. These agar plates have been developed to count different organisms, and the most common of these systems is Petrifilm (3M, Saint Paul, Mcrrie-sota). There is plenty of information validating the use of Petrifilm as equivalent to the traditional plate count agar method. Petrifilm consists of small, thin paper plates made of a water-soluble gelling agent, nutrients and indicators to facilitate enumeration. These films are cost-efficient and easy to use, and the inoculation and incubation steps are similar to those of agar plates in Petri dishes. An advantage of these plates is that the counting

is simplified by a grid on the film background that helps divide the total inoculated areas in quadrants. They can also be read using a colony counter. During incubation, Petrifilm plates can be stacked, similar to other agar plates, but use much less space. Currently, Petrifilm has been developed to enumerate coliforms, Enterobacteriaceae, *Listeria*, *Staphylococcus*, and total aerobic plate counts.

PATHOGENIC BACTERIA

Although the last few years have unwrapped an entire generation of advanced methods and technologies for pathogen detection and enumeration, conventional enumeration methods are still indispensable. In the case of presumptive positive samples, the pathogen has to be isolated from the food. In addition, these conventional or “traditional” procedures are still cost-efficient for small laboratories.

In this section we review the methods recommended in the *Microbiology Laboratory Guidebook* (MLG) from the U.S. Department of Agriculture’s Food Safety and Inspection Service (USDA–FSIS), which is the federal agency regulating food products that contain 2% of red meat or poultry in their composition (USDA–FSIS, 1998). We also mention the methods recommended by the *Bacteriological Analytical Manual* (BAM) of the U.S. Food and Drug Administration (FDA) (FDA, 2000) and the International Organization for Standardization (ISO) directives in Europe. Within the various protocols we emphasize the methods that have been most accepted in food microbiology laboratories, an acceptability that may be related to the reliability, simplicity, and cost of the method. These more accepted methods may sometimes depart from the methods suggested by government agencies, especially in the isolation and detection of nonregulated bacterial pathogens.

Campylobacter

Campylobacter spp. are the most prevalent bacterial foodborne pathogens isolated from broiler meat in the United States. *C. jejuni* is isolated two to three times more frequently than *C. coli*. The prevalence of *Campylobacter* spp. in commercially processed broiler carcasses is around 80% (Figure 1), with a count averaging 10 or fewer CFU/mL of the rinse (Oyarzabal, 2005), and in retail products is about 60 to 82% (Willis and Murray, 1997; Zhao et al., 2001; Dickins et al., 2002; Oyarzabal et al., 2007), with a count averaging 0.7 CFU/g of product (Oyarzabal et al., 2007).

Campylobacter does not grow below 30°C. For the isolation of *Campylobacter* from poultry meat, 42°C (±1) has been used as the temperature of choice. Yet this temperature allows only for the isolation of *C. jejuni*, *C. coli*, and *C. clari*, which together with *C. upsaliensis* make up the *thermotolerant group*, and which have been the only species isolated from broiler meat until now. With the development of DNA-based methods for the identification of isolates, *C. lari* has not been reported from broiler samples for more than 10 years in the United States, which

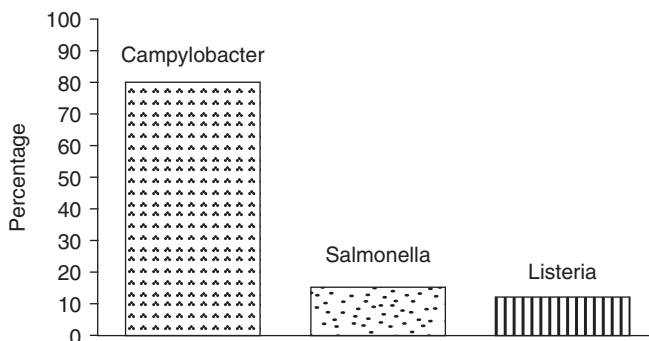


FIGURE 1 Prevalence of *Campylobacter*, *Listeria*, and *Salmonella* in processed broiler carcasses. [Modified from USDA–FSIS (1996) and USDA–FSIS data (www.fsis.usda.gov/Science/Progress_Report_Salmonella_Testing_Tables/index.asp).]

suggests that previous reports may have been misidentifications from biochemical tests (Oyarzabal et al., 1997).

There have been some discussions in the last 10 years on the best methodology for isolation and identification of *Campylobacter* from broiler meat, and although numerous enrichment broths, isolation media, and methods to generate a microaerobic atmosphere have been developed in the past 30 years, few methods have been validated in large-scale studies to guarantee reproducibility of results.

The best enrichment broth for *Campylobacter* spp. isolation from broiler meat is Bolton broth (Bolton and Robertson, 1982), although other broths, such as Preston broth, Park and Sanders (De Boer and Humphrey, 1991), and buffered peptone water (Oyarzabal et al., 2007) may also be used. The addition to the enrichment of 5 to 10% lysed blood appears to increase the likelihood of isolation. The use of cefoperazone (32 mg/L), trimethoprim (10 mg/L), and vancomycin (10 mg/L) appears to be the best combination to inhibit the growth of contaminants without affecting substantially the recovery and growth of *Campylobacter* cells. Bolton broth is also suggested as the enrichment of choice for *Campylobacter* isolation by the USDA–FSIS (1998) and by the protocol for the international standard, ISO 10272 (ISO, 2006), with an incubation of up to 48 h. The ISO protocol suggests an initial incubation at 37°C for 4 to 6 h to allow stressed or injured cells to recover.

It has also been suggested that an initial incubation without antimicrobials, similar to the protocol for the initial isolation of *Listeria monocytogenes* recommended by the FDA, may increase the likelihood of isolation. Yet a larger food sample and a larger incubation time do play a more important role and correlate directly with an increased probability of isolating *Campylobacter*. In addition, recent findings suggest that an enrichment ratio of 1:5 compares similarly to a ratio of 1:10. This lower ratio may allow for an increase in sample size without increasing, substantially, the overall volume during the enrichment step (Oyarzabal et al., 2007).

All the plate media used for isolation of *Campylobacter* spp. from food samples are adaptations, or direct compositions, of media originally designed to isolate *Campylobacter* spp. from clinical, mainly fecal, samples. The plate agar media used for the isolation of *Campylobacter* spp. can be divided into two groups. In one group we have plate media that have blood as a supplement, and in the other group we have plates that have charcoal as a supplement. Both blood and charcoal are added to help reduce the oxygen tension and create the microaerobic environment that allows campylobacters to grow. Although some agar plates have been developed that do not contain blood or charcoal in their composition, the effectiveness of these plates appear to be lower than that of plates with either blood or charcoal.

The most common plate agar used worldwide is the modified charcoal cefoperazone deoxycholate agar (mCCDA) (Bolton and Robertson, 1982; Hutchinson and Bolton, 1984). This plate is available commercially and has been validated in many different studies. The only limitation with this plate is that sometimes *Campylobacter* colonies stick to the surface of the plate and are very difficult to harvest. A plate that has been used extensively in the United States is Campy-Cefex (Stern et al., 1992), a blood-based plate, and its modification (Oyarzabal et al., 2005). Both CCDA and m-Campy-Cefex compare similarly for the direct enumeration of *Campylobacter* spp. in carcass rinses collected from postchilled, processed broiler carcasses. For a review of the most common enrichment broths and plate agars used for isolation of *Campylobacter* from foods, refer to the article by Corry et al. (1995).

The best isolation procedures still rely on the replacement of the air in jars or plastic bags with a microaerobic atmosphere (10% CO₂, 5% O₂, and 85% N₂) to guarantee the survival and reproduction of *Campylobacter* cells. This microaerobic atmosphere can be generated with pouches that are added to the jars or by extracting the air with a vacuum pump and replacing it with a commercial microaerobic mix.

Although *C. jejuni* has usually been found at high, countable numbers in broiler carcasses after processing, the counts have been decreasing in recent years and the current numbers in commercial broiler carcasses are low enough to justify the use of enrichment to detect positive carcasses (Oyarzabal, 2005; Oyarzabal et al., 2005). In retail poultry, the numbers are very low and enrichment is indispensable for isolation (Oyarzabal et al., 2007).

The fact that campylobacters are inert, which means that they do not use or ferment sugars to produce energy, has been a major drawback for the use of biochemical tests for identification. The best identification to the genus level is achieved with latex, enzyme-linked immunosorbent assay (ELISA), or DNA-based tests. But identification to the species level requires several physiological tests that are time consuming and have a low degree of reproducibility. Because few research laboratories perform routine physiological testing of *Campylobacter* isolates, the use of DNA-based methods have become indispensable for species identification in research laboratories working with *Campylobacter* spp.

Clostridium perfringens

Small numbers of *Clostridium perfringens* are present in the alimentary tract skin and in the feathers of a chicken, and therefore fecal contamination during processing is one of the reasons for the appearance of this organism in broiler chicken meat. There is also a high chance of occurrence of *C. perfringens* in meats and poultry that are cooked and not maintained at proper temperatures prior to serving. *C. perfringens* is a spore-forming anaerobic bacterium that produces an enter toxin that results in acute abdominal pain and diarrhea. The typical foodborne associated strains are type A.

When analyzing a food for *C. perfringens*, it is important to analyze the samples promptly to avoid the loss of viability of the *Clostridium* cells present when samples are frozen or refrigerated for lengthy periods. Hence, food samples should be treated with buffered glycerol salt solution (10% glycerol) prior to freezing and shipping.

The USDA–FSIS method for analysis of *C. perfringens* recommends the use of Butterfield’s phosphate diluent, and plating of the dilutions on tryptose sulfite cycloserine (TSC) supplemented with egg yolk and overlaid with egg yolk–free TSC agar. The plates are incubated anaerobically at 35°C for 24 h. Each of the presumptive colonies (black with a halo) is inoculated into thioglycollate broth and incubated at 35°C overnight (USDA–FSIS, 1998).

The FDA method requires that the sample be mixed with peptone dilution fluid and the mix be blended with molten TSC agar without egg yolk and poured on a petri plate (FDA, 2000). Colonies that are black with opaque white zones are inoculated into chopped liver broth and incubated at 35°C for 24 to 48 h. *C. perfringens* is a gram-positive bacterium that will appear as short rods under microscopy. For further confirmation, biochemical tests such as the motility and nitrate reduction tests can be done. *C. perfringens* is nonmotile and can reduce nitrate to nitrite.

Listeria

Listeria monocytogenes is a psychrotrophic foodborne pathogen that can live in microaerobic or even anaerobic environments. *L. monocytogenes* can produce severe diseases in immunocompromized persons and pregnant women. The most recent outbreak of listeriosis in the United States, reported by the Massachusetts Department of Public Health on December 28, 2008, resulted in the death of two infected people and it was traced to pasteurized milk produced locally.

The USDA–FSIS has maintained a zero-tolerance policy on the detection of *L. monocytogenes* in ready-to-eat products (i.e., products that may be consumed without any further cooking or reheating). This policy means that if the product is contaminated with *L. monocytogenes*, the product is considered “adulterated” under the provisions of the Federal Meat Inspection Act and the Poultry Inspection Act, 21 U.S.C. 601(m) or 453 (g), respectively (U.S. Code, 1994). Many media have been formulated for the selective isolation of *Listeria* species, although the success in isolation is highly dependent on identification of the low

numbers of *Listeria* cells present in the food. Experiments have shown that direct plating is not a good method for isolation of *Listeria*; therefore, preenrichment and enrichment steps are employed. The preenrichment broth has fewer amounts of selective agents, permitting the revival of injured cells; the enrichment medium contains acriflavin and nalidixic acid to select for *Listeria*. Modified Oxford (MOX) and PALCAM are selective plating media in widespread use. The agar turns black around the areas where the colonies grow, but this change in color may also occur around non-*Listeria* colonies growing on the plate.

The USDA–FSIS method uses University of Vermont (UVM) broth as the primary enrichment medium and Fraser broth as the secondary enrichment medium. Isolation is carried out on MOX agar plates. The FDA’s BAM method requires use of buffered *Listeria* enrichment broth for nonselective enrichment at 30°C for 4 h and then addition of selective agents with incubation at 30°C for 48 h. The method for detecting *L. monocytogenes* in foods of international standard ISO 11290 (ISO, 1996) includes streaking on a chromogenic agar plate (ALOA) and streaking on a second agar medium from primary enrichment, and transfer followed by to half-strength Fraser broth and secondary enrichment in Fraser broth. Confirmation of *Listeria* spp. is done with biochemical tests, Gram staining, and microscopy to determine motility.

Listeria spp. can be identified using ELISA (polyclonal antibodies) and DNA-based methods. For the identification of species within the genus *Listeria*, several test strips have been developed that differentiate species based on a biochemical test. These test strips have been validated and are reliable for the identification of the species found most commonly in foods.

Salmonella

Even though the numbers of *Salmonella* have been decreasing over the last 10 years, keeping the organism numbers low is still a challenge in broiler processing plants. When small numbers of *Salmonella* are expected, the sampling method has a major influence on the identification of *Salmonella*-positive carcasses (Simmons et al., 2003). Four steps are involved in the isolation and identification of *Salmonella*. They include (1) preenrichment in a nonselective, nutritious medium; (2) selective enrichment; (3) plating on selective agars; and (4) confirmation by biochemical and serological tests. The FDA’s BAM method (FDA, 2000) uses lactose broth as a preenrichment medium; RV broth and TT as selective enrichment; and Hektoen Enteric agar, xylose–lysine–deoxycholate (XLD) agar, and bismuth–sulfite agar as the selective plate media for isolation. The method suggested by the USDA–FSIS (1998) uses BPW for preenrichment, modified RV for selective enrichment, and xylose–lysine–tergitol 4 (XLT4) agar or double-modified lysine–iron agar plates for selective isolation.

The international standard protocol ISO 6579 uses BPW as the preenrichment; RV supplemented with soya and TT supplemented with novobiocin for enrichment; XLD agar for selective isolation (ISO, 2002); and confirmation of presumptive *Salmonella* colonies with suitable biochemical tests. The identification of *Salmonella* to the genus level can be done using test strips that have a

battery of biochemical tests. These tests are very reliable and have been used for several decades. The identification of specific O antigens for most common serotypes can be done with commercial agglutination tests. However, a thorough serotyping scheme requires testing with a large panel of antisera, and few laboratories in the world can do that.

SPOILAGE MICROORGANISMS

Mesotrophic Bacteria

The enumeration of viable bacterial or fungal cells in a food matrix is important and necessary to monitor the microbiological quality and safety of food. The most common plate for enumeration of mesotrophic bacteria is the *aerobic plate count* (APC) agar, a term that refers to agar plates originally composed of trypticase soy agar (containing glucose) (TSA) or tryptic soy agar (without glucose). TSA is highly nutritious because the combination of the soy and casein provides organic nitrogen from amino acids and longer chain peptides, and the sodium chloride provides osmotic balance. This medium can also be used for bacterial enumeration and as a base agar for other media (Forbes et al., 1998). TSA is used in the analysis of water, wastewater, and foods specified in FDA's BAM (Clesceri et al., 1998; FDA, 2000; Downes and Ito, 2001).

Currently, most APCs contain glucose, which is added to avoid undercounts when analyzing meats and using an incubation period of fewer than 4 to 5 days. Other agar plates that have been used for APC analysis in poultry meats include Standard 1 nutrient agar (Vorster et al., 1994), plate count agar (Warburton et al., 1988), tryptone glucose and yeast extract (van der Marel et al., 1988), and Petrifilm (Chain and Fung, 1991).

A plate that has been used for the bacterial count in milk and dairy products, but that can also be used for analysis of poultry products, is the *standard plate count* (SPC) agar. The formulation has been developed by the American Public Health Association and contains the enzymatic digest of casein, which provides amino acid and other complex nitrogenous substances for bacterial growth. The yeast extract supplies vitamins, and the dextrose is the source of carbon to provide energy. The triphenyltetrazolium chloride present in the medium is reduced to the insoluble formazan inside the bacterial cell, and colonies appear in red.

Although APCs have long been used in food microbiology, there is a lack of agreement about the temperature and length of incubation. Most of the current literature on APCs includes psychrotropic and mesotrophic bacteria, which makes a comparison of the different results quite difficult. Since 1985, different temperature–time combinations have been used for APC, ranging from 20°C for 120 h to 37°C for 24 h. Under these different conditions, the isolates grown at one temperature do not grow at other temperatures and reflect different bacterial populations (Jay, 2002).

For direct enumeration of mesotrophic bacteria, various dilutions of the sample are plated and the plates are incubated at 30°C for 48 h to count the numbers of

aerobes. An elaborate procedure for determining the APC of various foods has been developed by the Association of Official Analytical Chemists (AOAC) and the American Public Health Association (APHA, 1984).

Psychrotrophic Bacteria

Psychrotrophic bacteria include a wide variety of bacteria (gram-positive, gram-negative, aerobic, anaerobic, facultative, sporeformers, and non-sporeformers) which grow at temperatures between -5 and 30°C , with an optimum temperature of 21°C . Psychrotrophic bacteria and yeasts and molds make up for the majority of food-spoilage organisms. The enumeration of psychrotrophic bacteria can be done by plate count using trypticase soy agar or Petrifilm. The plates are usually incubated at 7°C for 10 days. A more rapid method called *modified psychrotrophic bacteria count* has been formulated to enumerate bacteria after incubation at 21°C for 25 h (Oliveria and Parmelee, 1976).

Pseudomonas species are some of the common spoilage bacteria of aerobically stored poultry meat (Arnaut-Rollier et al., 1999). Several studies have shown a direct correlation between the starting numbers of *Pseudomonas* and the shelf life of the product at refrigeration temperatures (Barnes et al., 1979). At refrigerated temperatures, spoilage occurs when their numbers reach 10^7 to 10^8 per cm^2 . Other gram-negative bacteria, such as *Acinetobacter* and *Psychrobacter* spp., are also important spoilage organisms, and poultry meat in modified-atmosphere packages develops large numbers of lactic acid bacteria and *Carnobacterium* spp. These bacteria are usually accompanied by pseudomonads, Enterobacteriaceae, and *Brochothrix thermosphacta* (Jones et al., 1982; Mead et al., 1986).

Rapid methods have been developed to overcome the long incubation periods necessary to obtain psychrotrophic counts. Some of these methods are based on quantitative analysis of enzymes, such as catalase and cytochrome *c* oxidase. Psychrotrophic bacteria may become a concern in minimally processed foods if proper processing and handling conditions are not observed. An example is deli-type foods, where low heat and vacuum, instead of sterilization, are used to process the food. This processing results in the survival of cells or spores and leads to food spoilage.

Yeasts and Molds

Yeasts and molds have a wide range of temperature (5 to 35°C) and pH (4 to 6.5) requirements for their growth. The numbers of yeast cells on raw poultry meat can reach up to 10^4 per milliliter of carcass rinse at the end of the shelf life. High numbers of *Candida* spp. (*C. zeylanoides*) and *Yarrowia lipolytica* can be found in the products (Gallo et al., 1988; Ismail et al., 2000; Hinton et al., 2002). There are numerous media, depending on the type of food and the type of fungus being isolated. Acidified media were used traditionally, but better antibiotic media developed recently are prevalent these days. These media prevent unwanted bacterial growth, enhance revival of injured fungi, and minimize food particle precipitation.

The general-purpose media for yeast and mold enumeration are dichloran rose bengal, which restricts excessive mycelial growth, and antibiotic-supplemented plate count agar. This is done by dilution and surface-spread plating methods to expose the cells to atmospheric oxygen and to avoid the heat stress of molten agar when using the pour plate method. Recovery of yeasts and molds from intermediate-moisture foods can be done by soaking the food for some time.

Another widely used medium for cultivation and enumeration of yeasts and molds is potato dextrose agar. Potato starch (potato infusion) and dextrose (corn sugar) provide nutrients for the elaborate growth of fungi. It is essential that the media be adjusted to a pH of 3.5 by the addition of sterile tartaric acid to inhibit the growth of unwanted bacteria. After inoculation, plates have to be incubated for 5 days at 22 to 25°C. The fungal growth can be confirmed by making wet mounts or by Gram staining (yeasts are gram-positive and mold mycelia are gram-negative).

APPLICATION OF MOLECULAR TECHNIQUES FOR IDENTIFICATION PURPOSES

Molecular techniques based on antibody detection of whole bacterial cells and DNA detection of specific DNA markers have been used for several years in food microbiology. The main advantage of these molecular-based methods is shortening of the time necessary to determine if a sample is presumptively positive. In the case of antibodies, monoclonal antibodies have not been very successful for food applications because their high specificity may not detect some bacterial strains and therefore result in false-negative samples. In other words, some strains of the same bacterial group that is targeted by the antibodies may not be detected. Polyclonal antibodies, on the other hand, are more inclusive in their specificity but lack sensitivity to detect bacterial numbers of less than 10^3 CFU/mL. Yet polyclonal-based assays such as ELISA and latex agglutination tests, can be used in some food matrices for a rapid screening of presumptive positives and for the analysis of a larger number of samples (high throughput).

Within the DNA-based methods, the tests more frequently used in food microbiology are based on isothermal hybridization or amplification with the polymerase chain reaction (PCR) technique. The few commercial tests based on PCR assays are very reliable and specific. Yet PCR assays still have a limitation in their sensitivity and cannot detect the target organism if it is present at less than 10^3 CFU/g or mL. In addition, in meat products and other complex food matrices, the presence of organic compounds inhibitory to PCR reactions has been described, which limits the use of PCR in those food samples.

FUTURE TRENDS IN THE IDENTIFICATION OF BACTERIA

In the last 20 years, food microbiologists have seen the appearance of chromogenic agars to facilitate the differentiation of presumptive positives on agar

plates. These media detect the presence of a specific enzyme using suitable substrates, such as fluorogenic or chromogenic enzyme substrates (Manafi, 2000). The enzymes targeted depend on the medium and the manufacturer's selection. Some examples are β -galactosidase, β -glucosidase, β -glucuronidase, and tryptophan deaminase (Edberg and Kontniqué, 1986; Manafi, 2000). There is a change in color around the suspect colonies, and sometimes the colonies have a unique color. These media have brought a more objective way of identifying bacterial colonies on agar plates. For example, one of the first chromogenic media developed to detect *Salmonella* spp. uses a chromogenic substrate for β -galactosidase that in conjunction with propylene glycol generates acids and changes, the color of the media around *Salmonella* colonies (Rambach, 1990). Currently, chromogenic agars have been developed for *Bacillus cereus*, *Clostridium perfringens*, Enterobacteriaceae, enterococci, *Escherichia coli*, *E. coli* O157:H7, *Listeria monocytogenes*, *Salmonella* spp., and *Staphylococcus aureus* (Manafi 2000).

With the advancements in molecular techniques and in our understanding of the genes involved in pathogenicity, there are now more opportunities to develop identification techniques that target specific virulence genes, unique to bacterial pathogens. Although these genetic markers are not present in all clones of the same species, at least the ones that carry them are candidates of concern from a human health standpoint.

Finally, the history of the development of microbiology tests for a given pathogen depends on many variables, such as difficulties of isolation, prevalence in food products, and pathogenicity for humans. An important factor to remember is the effect of established regulations on the development of methodologies. There are pathogens for which regulations have been established. These regulations may be in the form of complete absence after testing (no tolerance), as in the case of *L. monocytogenes* in RTE products or in the allowance of an incidence (prevalence or counts), as for *Salmonella* in process poultry meat. The pathogens that are under regulations have promoted more research, and more methods are available in the market for their testing.

REFERENCES

- APHA (American Public Health Association). 1984. *Compendium of Methods for the Microbiological Examination of Foods*, 2nd ed. Washington, DC: APHA.
- Arnaut-Rollier I, De Zutter L, Van Hoof J. 1999. Identities of the *Pseudomonas* species in flora from chilled chickens. *Int J Food Microbiol* 48:87–89.
- Barnes EM, Impey CS, Parry RT. 1973. The sampling of chicken, turkeys, ducks, and game birds. In: Board RG, Lovelock DW, eds., *Sampling: Microbiological Monitoring of Environments*. Society for Applied Bacteriology Technical Series 7. London: Academic Press, pp. 63–75.
- Barnes EM, Mead GC, Impey CS, Adam BW. 1979. Spoilage organisms of refrigerated poultry meat. In: Russell AD, Fuller R, eds., *Cold Tolerant Microbes in Spoilage and the Environment*. Society for Applied Bacteriology Technical Series 13. London: Academic Press, pp. 101–116.

- Bolton FJ, Robertson L. 1982. A selective medium for isolation *Campylobacter jejuni/coli*. J Clin Pathol 35:462–467.
- Brodsky MH, Entis P, Entis MP, Sharpe AN, Jarvis GA. 1982. Determination of aerobic plate and yeast and mold counts in foods using an automated hydrophobic grid membrane filter technique. J Food Prot 45:301–304.
- Chain VS, Fung DYC. 1991. Comparison of Redigel, Petrifilm™, Spiral Plate System, Isogrid, and aerobic plate count for determining the numbers of aerobic bacteria in selected foods. J Food Prot 54:208–211.
- Clesceri LS, Greenberg AE, Eaton AD. 1998. *Standard Methods for the Examination of Water and Wastewater*, 20th ed. Washington, DC: American Public Health Association.
- Corry JEL, Post DE, Colin P, Laisney MJ. 1995. Culture media for isolation of campylobacters. Int J Food Microbiol 26:43–76.
- Cox NA, Blankenship LC. 1975. Comparison of rinse sampling methods for detection of salmonellae on eviscerated broiler carcasses. J Food Sci 40(6):1333–1334.
- Cox NA, Mercuri AJ, Thompson JE, Chew V. 1976. Swab and excise tissue sampling for total and Enterobacteriaceae counts of fresh and surface-frozen broiler skin. Poult Sci 55:2405–2408.
- De Boer E, Humphrey TJ. 1991. Comparison of methods for the isolation of thermophilic campylobacters from chicken products. Microb Ecol Health Dis 4:S43.
- Dickens JA, Cox NA, Bailey JS. 1986. Evaluation of a mechanical shaker for microbiological rinse sampling of turkey carcasses. Poult Sci 65:1100–1102.
- Dickins MA, Franklin S, Stefanova R, Shutze GE, Eisenach KD, Wesley IV, Cave D. 2002. Diversity of *Campylobacter* isolates from retail poultry carcasses and from humans as demonstrated by pulsed-field gel electrophoresis. J Food Prot 65:957–962.
- Downes and Ito, eds. 2001. *Compendium of Methods for the Microbiological Examination of Foods*, 4th ed. Washington, DC: American Public Health Association.
- Edberg SC, Kontniqum CM. 1986. Comparison of β -glucuronidase based substrate systems for identification of *E. coli*. J Clin Microbiol 24:368–371.
- Entis P. 1984. Enumeration of total coliforms and *Escherichia coli* in foods by hydrophobic grid membrane filter: collaborative study. J Assoc Anal Chem 67:812–823.
- Entis P, Brodsky MH, Sharpe AN, Jarvis GA. 1982. Rapid detection of *Salmonella* spp. in food by use of the iso-grid hydrophobic grid membrane filter. Appl Environ Microbiol 43:261–268.
- FDA (Food and Drug Administration). 2000. *Bacteriological Analytical Manual*. Online edition. <http://www.cfsan.fda.gov/~ebam/bam-mm.html>. Accessed Feb. 25 2008.
- Forbes BA, Sahm DF, Weissfeld AS. 1998. *Bailey & Scott's Diagnostic Microbiology*, 10th ed. St. Louis, MO: Mosby.
- Gallo L, Schmitt RE, Schmidt-Lorenz W. 1988. Microbial spoilage of refrigerated fresh broilers: I. Bacterial flora and growth during storage. Lebensm-Wiss Technol 21:216–223.
- Goertz A, Tsuneshi N. 1951. Applications of molecular filter membranes to the bacteriological analysis of water. J Am Water Works Assoc. 43:943–945.
- Hinton A, Cason JA, Ingram KD. 2002. Enumeration and identification of yeasts associated with commercial poultry processing and spoilage of refrigerated broiler carcasses. J Food Prot 65:993–998.

- Hutchinson DN, Bolton FJ. 1984. An improved blood-free selective medium for isolation of *Campylobacter jejuni* from faecal specimens. *J Clin Pathol* 37:956–995.
- ICMSF (International Commission on Microbiological Specifications for Foods). 1984. Sampling for microbiological analysis: principles and specific applications. In: *Microorganisms in Foods*, 2nd ed. Toronto, Ontario, Canada: University of Toronto Press.
- Ismail SAS, Deak T, Abd. el-Rahman HA, Yassien MAM, Beuchat LR. 2000. Presence and changes in populations of yeast on raw and processed poultry products stored at refrigeration temperature. *Int J Food Microbiol* 62:113–121.
- ISO (International Organization for Standardization). 1996. Microbiology of food and animal feeding stuffs—horizontal method for the detection and enumeration of *Listeria monocytogenes*: 1. Detection method. Added in 2004: Amendment 1: Modification of the isolation media and the haemolysis test, and inclusion of precision data. ISO 11290–1. Geneva, Switzerland: ISO.
- ISO. 2002. Microbiology of food and animal feeding stuffs—horizontal method for the detection of *Salmonella* spp. ISO 6579. Geneva, Switzerland: ISO.
- ISO. 2006. Microbiology of food and animal feeding stuffs—horizontal method for the detection and enumeration of *Campylobacter* spp.: 1. Detection method. ISO 10272. Geneva, Switzerland: ISO.
- Jay JM. 2002. A review of aerobic and psychrotrophic plate count procedures for fresh meat and poultry products. *J Food Prot* 65:1200–1206.
- Jones JM, Mead GC, Griffiths NM, Adams BW. 1982. Influence of packaging on microbiological, chemical and sensory changes in chill-stored turkey portions. *Br Poult Sci* 23:25–40.
- Manafi M. 2000. New developments in chromogenic and fluorogenic media. *Int J Food Microbiol* 60:205–218.
- Mead GC, Griffiths NM, Grey TC, Adams BW. 1986. The keeping quality of chilled duck portions in modified atmosphere packs. *Lebensm-Wiss Technol* 16:142–146.
- Messer JW, Midura TF, Peeler JT. 1992. Sampling plans, sample collection, shipment, and preparation for analysis. In: Vanderzant C, Splitstoeser DF, eds., *Compendium of Methods for the Microbiological Examination of Foods*, 3rd ed. Washington, DC: American Public Health Association, pp. 25–49.
- Oliveria JS, Parmelee CE. 1976. Rapid enumeration of psychrotrophic bacteria in raw and pasteurized milk. *J Milk Food Technol* 39:269.
- Oscar TP. 2004. Predictive simulation model for enumeration of *Salmonella* on chicken as a function of polymerase chain reaction detection time score and sample size. *J Food Prot* 67:1201–1208.
- Oyarzabal OA. 2005. Review: Reduction of *Campylobacter* spp. by commercial antimicrobials applied during the processing of broiler chickens: a review from the United States perspective. *J Food Prot* 68:1752–1760.
- Oyarzabal OA, Wesley IV, Barbaree JM, Lauerma LH, Conner DE. 1997. Specific detection of *Campylobacter lari* by PCR. *J Microbiol Methods* 29:97–102.
- Oyarzabal OA, Macklin KS, Barbaree JM. 2005. Evaluation of agar plates for direct enumeration of *Campylobacter* spp. from poultry carcass rinses. *Appl Environ Microbiol* 71:3351–3354.

- Oyarzabal OA, Backert S, Nagaraj M, Miller RS, Hussain SK, Oyarzabal EA. 2007. Efficacy of supplemented buffered peptone water for the isolation of *Campylobacter jejuni* and *C. coli* from broiler retail products. *J Microbiol Methods* 69:129–136.
- Rambach A. 1990. New plate medium for facilitated differentiation of *Salmonella* spp. from *Proteus* spp. and other enteric bacteria. *Appl Environ Microbiol* 56:301–303.
- Sharpe AN, Michaud GL. 1974. Hydrophobic grid-membrane filters: new approach to microbiological enumeration. *Appl Microbiol* 28:223–225.
- Sharpe AN, Michaud GL. 1975. Enumeration of high numbers of bacteria using hydrophobic grid-membrane filters. *Appl Microbiol* 30:519–524.
- Sharpe AN, Diotte MP, Dudas I, Malcolm S, Peterkin PI. 1983. Colony counting on hydrophobic grid-membrane filters. *Can J Microbiol* 29:797–802.
- Simmons M, Fletcher DL, Berrang ME, Cason JA. 2003. Comparison of sampling methods for the detection of *Salmonella* on whole broiler carcasses purchased from retail outlets. *J Food Prot* 66(10):1768–1770.
- Stern NJ, Wojton B, Kwiatek K. 1992. A differential-selective medium and dry ice-generated atmosphere for recovery of *Campylobacter jejuni*. *J Food Prot* 55:515–517.
- U.S. Code. 1994. *Meat Inspection. Inspection Requirements; Adulteration and Misbranding*. 21 CFR 601. Washington, DC: U.S. Government Printing Office.
- USDA–FSIS (U.S. Department of Agriculture–Food Safety and Inspection Service). 1996. Pathogen reduction; hazard analysis and critical control point (HACCP) systems; final rule. 9 CFR 304, et al. *Fed Reg* 61(144):38806–38943.
- USDA–FSIS. 1998. *Microbiology Laboratory Guidebook*, 3rd ed. Washington, DC: USDA–FSIS. http://www.fsis.usda.gov/Science/Microbiological_Lab_Guidebook/. Accessed Feb. 25 2008.
- van der Marel GM, Van Logtestijn JG, Mossel DAA. 1988. Bacteriological quality of broiler carcasses as affected by in-plant lactic acid decontamination. *Int J Food Microbiol* 6:31–42.
- Vorster SM, Greebe RP, Nortj GL. 1994. Incidence of *Staphylococcus aureus* and *Escherichia coli* in ground beef, broilers and processed meats in Pretoria, South Africa. *J Food Prot* 57:305–310.
- Warburton DW, Weiss KF, Lachapelle G, Dragon D. 1988. The microbiological quality of further processed deboned poultry products sold in Canada. *Can Inst Food Sci Technol* 21:84–89.
- Willis WL, Murray C. 1997. *Campylobacter jejuni* seasonal recovery observations of retail market broilers. *Poult Sci* 76:314–317.
- Zhao C, Ge B, De Villena J, Sudler R, Yeh E, Zhao S, White DG, Wagner D, Meng J. 2001. Prevalence of *Campylobacter* spp., *Escherichia coli*, and *Salmonella* serovars in retail chicken, turkey, pork, and beef from the Greater Washington, D.C., area. *Appl Environ Microbiol* 67:5431–5436.