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Salmonella Detection Methods for Food and Food Ingredients

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1. Introduction

Salmonella is the etiologic agent of Salmonellosis in humans causing severe illness in infants, the elderly, and immunocompromised patients (Cross et al. 1989; Tauxe 1991; Smith 1994; Baumler et al. 2000). Salmonellosis symptoms include watery diarrhea, abdominal pain, nausea, fever, headache and occasional constipation with hospitalization required in cases of severe infections. The genus currently contains two species, *Salmonella bongori and Salmonella enterica* (including six subspecies: *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV), and *indica* (VI). However, there are more than 2,500 serovars of *Salmonella* based on the Kauffmann-White antigenic scheme for the classification of Salmonellae (Popoff et al. 1994). *Salmonella* is a gram-negative, non-spore forming rod and facultative anaerobe that can ferment glucose belonging to the family Enterobacteriaceae. Most strains are motile with peritrichous flagella and can reduce nitrate to nitrite (Grimont et al. 2000). The organism is mesophilic with optimum growth temperature in the range of 32 – 37°C but capable of growth within a wide temperature range of 6 – 46°C. *Salmonella* is ubiquitous in the environment originating from the gastrointestinal tracts of domesticated and wild animals and can be present without causing apparent illness. Most infections result from the ingestion of foods of animal origin contaminated with *Salmonella* species such as beef, chicken, turkey, pork, eggs, and milk (D'Aoust 1997; D'Aoust 2000; Olsen et al. 2000). Other vehicles, including non-animal foods such as fresh fruits and vegetables (Mahon et al. 1997), reptiles (Friedman et al. 1998), water (Angulo et al. 1997), and direct person-to-person transmission (Lyons et al. 1980), have also been implicated. However, certain serotypes of *Salmonella* such as *S*. Enteritidis, which can penetrate poultry reproductive organs resulting in the contamination of egg contents has been a prominent cause of human illness for several decades (Gantois et al. 2009). In addition to faecal contamination, cross-contamination of foods by *Salmonella* during food preparation can be an important source of foodborne illness.

Generally, detection methods are based on physiological and biochemical markers of the organism (Williams 1981). Cultural methods are based on nutrient acquisition, biochemical characteristics, and metabolic products unique to *Salmonella* spp. (Ricke et al. 1998). More rapid immunological and molecular screening methods of detection have been devised to detect cell surface markers and nucleic acids, respectively. This chapter will provide an overview of various culture based methods and rapid methods currently available for the detection of *Salmonella* in foods and food ingredients. We will focus our discussion on

advances introduced for the improvement of conventional culture methods, the use of Polymerase Chain Reaction (PCR) technology, immunology-based methods, and bacteriophage based assays. Whenever possible, examples from the academic literature as well as from commercial applications will be considered. The importance of sample preparation will be examined throughout as it relates to its impact on sensitivity and turn-around time for detection. Specific *Salmonella* serovars will be named according to the nomenclature of Leminor and Popoff (2001), e.g. *Salmonella* Enteritidis or *S*. Enteritidis.

2. Culture methods

Culture based methods are still the most widely used detection techniques and remain the gold standard for the detection of *Salmonella* due to their selectivity and sensitivity. For instance, the US Food and Drug Administration (FDA) and the Food Safety and Inspection Service (FSIS) of the US Department of Agriculture (USDA), requires an isolated organism as unambiguous proof of contamination (Alocilja and Radke 2003). Depending on the approach, standard culture methods typically require 5–7 days to obtain a result as they rely on the ability of *Salmonella* to multiply to visible colonies, which can then be characterized by performing additional biochemical and or serological tests. Due to their widespread use, numerous and varied bacteriological media (selective enrichment broths and selective agar plates) are applied to best monitor for *Salmonella* in food and food ingredients. The media may contain inhibitors in order to stop or delay the growth of non-target organisms, or particular substrates that only the target bacteria can degrade, or that confer a particular colour to the growing colonies (Manafi 2000).

Cultural methods typically involve the enrichment of a portion of the food sample to recover sub-lethally injured cells due to heat, cold, acid, or osmotic shock (Sandel et al. 2003; Gracias and McKillip 2004) in a non-selective pre-enrichment media, such as Buffered Peptone Water (BPW), and to increase the number of target cells as these are generally not uniformly distributed in foods, typically occur in low numbers, and may be present in a mixed microbial population. Next, primary enrichment cultures are typically inoculated into secondary selective enrichment broths, such as Selenite Cystine broth (SC), Rappaport Vasiliadis Soy broth (RVS), Tetrathionate Broth (TT), or Muller-Kauffmann Tetrathionate-Novobiocin broth (MKTTn) and incubated at elevated temperatures (37°C or 42°C for 18-24 hours) before being struck onto selective agars such as Xylose Lysine Deoxycholate agar (XLD agar), Bismuth Sulphite agar (BIS), Brilliant Green agar (BG) with or without the addition of sulfadiazine or sulfapyridine (BGS), modified semisolid Rappaport Vasiliadis (MSRV), Salmonella Shigella Agar, or Hektoen Enteric agar. There are several published standard methods utilizing combinations of media such as the current ISO horizontal method, ISO 6579:2002 (updated in 2007) for the detection *Salmonella*, including *Salmonella* Typhi and *Salmonella* Paratyphi applicable to products intended for human consumption and the feeding of animals, and to environmental samples in the area of food production and food handling. Similar standard methods have been published elsewhere, most notably in the FDA Bacteriological Analytical Manual (BAM).

Typical *Salmonella* colonies based on morphology and or indicative biochemical reactions on selective agars are then cultured onto non-selective media prior to confirmatory testing. There are well-established confirmations and identification procedures for *Salmonella*. Preliminary identification is traditionally performed using classical biochemical and serological tests. Key biochemical tests include the fermentation of glucose, negative urease

reaction, lysine decarboxylase, negative indole test, H_2S production, and fermentation of dulcitol. Serological confirmation tests typically utilize polyvalent antisera for flagellar (H) and somatic (O) antigens. Isolates with a typical biochemical profile, which agglutinate with both H and O antisera are identified as *Salmonella* species. Where results are inconclusive, it may be necessary to perform additional biochemical tests. Positive isolates are often sent for further serotyping to identify the serovar using specific antisera as per the Kauffman-White (KW) typing scheme recognizing 46 O antigens, and 119 H antigens, thereby permitting the characterization of 2,541 serotypes (Shipp and Rowe 1980). Serotyping is a useful epidemiological tool in identifying circulating serotypes and to characterize outbreaks. The antigenic formulae of Le Minor and Popoff (2001) is a standard method for naming the serovars. However, serotyping is normally undertaken at reference laboratories and is rarely performed in routine food or clinical laboratories. Reference laboratories are also able to further type isolates using techniques such a phage typing (Anderson and Williams 1956; Callow 1959; Anderson 1964; Anderson et al. 1977), antibiotic susceptibility (Bauer et al. 1966), pulsed-field gel electrophoresis (PFGE), or other emerging genetic typing technologies such Multiple-Locus Variable Number Tandem Repeat Analysis (MLVA) and Multilocus sequence typing (MLST) (Kruy et al. 2011).

Although standard culture methods are excessively time-consuming, there is potential for further improvements, and thus many attempts have been made to maximize their efficiency by introducing new technologies, making reliability of detection more convenient, user friendly, as well as by reducing the costs of materials and labour (de Boer and Beumer 1999; Weenk, 1992). For example, biochemical confirmatory tests may be easily replaced by commercial identification kits such as the API 20E (BioMérieux) or other commercially available bacterial identification kits. The detection of sub-lethally damaged cells is of utmost importance as these may still pose a risk to human health and may lead to false negative results. Strategies for the recovery of injured bacteria are based on overlay methods such as tryptic soy agar (TSA) overlayed on XLD selective agar (Kang and Fung 2000) and other approaches also include the development of single enrichment broths where multiple step enrichments are usually required (Baylis et al. 2000). Other novel approaches include the addition of bacteriophages for the elimination of background microflora that may out-compete the target organism. For example, RapidChek® SELECT™ Salmonella (Strategic Diagnostics Inc.) employs a primary enrichment media supplemented with a bacteriophage cocktail as a selective agent, which reduces the level of background flora in high burden samples allowing *Salmonella* to grow with minimal competition. In addition, there is also the development of enrichment broths for the concurrent enrichment of pathogens thereby reducing laboratory workloads with respect to the preparation of sample homogenates since different enrichment broths would no longer be required, and multiple analyses could be performed from a single universal enrichment culture (Kim and Bhunia 2008). Amendments to media have also been performed such as the addition of novobiocin (Restaino et al. 1977; Devenish et al. 1986), and cycloheximide to decrease fungal overgrowth (Ricke et al. 1998). Lastly, and perhaps the most important advancement is the use of chromogenic or fluorogenic substrates in selective agars, permitting identification to be performed directly on the isolation plate, thereby expediting or eliminating the use of subculture media or additional biochemical tests as these media provide highly specific reactions, and help reduce the workload for unnecessary examination of suspect colonies arising from poor specificity of conventional agars (Manafi 1996; Manafi 2000). A number of selective chromogenic agar media

specifically designed for the differentiation of *Salmonella* colonies are commercially available with varying success of adoption by regulatory agencies such as: Salmonella SMS (AES Chemunex), BBL CHROMagar (CHROMagar), RAPID'Salmonella (Bio-Rad Laboratories, S.A.), chromID Salmonella (BioMerieux), Harlequin Salmonella ABC (Lab M), Oxoid Brilliance Salmonella Agar (Oxoid), and Rambach Agar (Merck), among others.

It is evident that the multitude of options for isolation of *Salmonella* and the lack of inter-laboratory consistency make *Salmonella* isolation one of the most variable procedures in laboratories with new media available every year, promising to be more sensitive, specific, and rapid (Hyatt and Weese 2004). With this myriad of choice, laboratories must chose culture approaches which efficiently and accurately provide timely results via the development of standard methods and participation in proficiency quality assurance programs.

2.1 Immunomagnetic separation

In an attempt to reduce the length of routine microbiological analysis and to minimize the problems associated with rapid detection systems such as interference from foods and food ingredients debris, background micro-organisms, and lack of sensitivity, there has been a lot of interest in the development of separation and concentration techniques prior to detection. Various techniques have been utilized for this purpose including: centrifugation (Basel et al. 1983), filtration (Farber and Sharpe 1984), and lectin-based biosorbents (Payne et al. 1992). However, the most successful of approaches for the separation and concentration of target organisms has been the use of immunomagnetic separation (IMS). The advantages of IMS are that it reduces the total analysis time and improves the sensitivity of detection. IMS is rapid, technically simple, and specific method for the isolation of the target organisms (Shaw et al. 1998). Paramagnetic particles are coated with antibodies specific to the target organism and added to a post enrichment culture. The target organism is captured onto the magnetic particles and the whole complex is then removed from the system by the application of a magnetic field. Target organisms are thus removed from food debris and competing microorganisms, which may otherwise interfere with the detection system. If required, the isolated complex may be re-suspended in an enrichment broth so that cell numbers can be rapidly increased to improve the sensitivity of detection assays. In addition, IMS by design can be used in conjunction with other rapid detection methods, including ELISA, conductance microbiology, electrochemiluminescence, and polymerase chain reaction (PCR) to further increase its analytical sensitivity (Fluit et al. 1993; Cudjoe et al. 1994; Cudjoe et al. 1995; Sapanova et al. 2000). It has been reported that IMS is more sensitive than conventional culture methods and is able to reduce the total culture analysis time by one to two days (Lynch et al. 2004; Ten Bosch et al. 1992).

The most commonly used commercial IMS bead for the recovery of *Salmonella* from food samples is Dynabeads™ anti *Salmonella* (Invitrogen). Similar magnetic beads specific for *Salmonella* are available such as Captivate Salmonella (Lab M), Tecra Salmonella Unique (3M), as wells as for specific serovars such as *S.* Enteritidis, via Rapidchek Confirm *S.* Enteritidis IMS kit (SDIX). IMS can also be automated using automated IMS separators such as the BeadRetriever (Invitrogen) capable of processing up to fifteen 1 mL enrichments volumes per cycle (23 minutes), to larger scale instruments such as the Kingfisher IMS separator (Thermofisher) or Mag Max (Life Technologies) capable of processing up to 100 samples with the capability of re-suspending the IMS target complex in microtitre plates for further testing by PCR, or ELISA. For instance, the VIDAS ICS test (BioMérieux) uses

automated immunoconcentration prior to analysis by an automated ELISA instrument for the detection of *Salmonella* from food and food ingredients. Another IMS variation was also developed by Pathatrix (Matrix MicroScience Ltd) combining IMS and a recirculation step (Flow Through Immunocapture or FTI), to further increase the sensitivity of detection since larger enrichment volumes can be reacted with IMS beads. For example Warren et al. (2007) investigated FTI, using the Pathatrix device, followed by plating on XLD agar (FTI-XLD) or analysis by real-time PCR (FTI-PCR) for the detection of *Salmonella* on smooth tomato surfaces and in potato salad and ground beef. The FTI-XLD method demonstrated the ability to isolate presumptive *Salmonella* colonies up to 48 h faster than did the standard modified BAM *Salmonella* culture method and the FTI-PCR was able to detect *Salmonella* within 8h.

Among the problems associated with IMS is non-target carryover where non-target organisms adhere to the walls of glass test tubes (Meadows 1971). Protamine as well as the use of mild detergents is commonly used to minimize non-target carryover since it adheres to the glass and to the bacteria in the sample reducing the net negative charge to prevent adherence. IMS also suffers in that it requires small sample sizes, organisms may be lost from beads during separation from samples with high fat content, and non specific binding of *Citrobacter freundii* and coliforms with mucoid layers has also been observed (Coleman et al 1995).

3. Immunological based methods

3.1 Rapid agglutination assays

Several rapid latex agglutination assay tests are widely used for the rapid detection of *Salmonella*. These assays however, are primarily used as a confirmation screen for presumptive *Salmonella* colonies after culture isolation from selective agar plates, with further confirmation and identification work carried out on those organisms giving a positive latex reaction. An aliquot of a colony suspension or enrichment broth is simply mixed with the latex reagent and after a few minutes rotation, the results are clearly visible. If the test is negative, the latex remains in smooth suspension and retains its original colour. A positive result is indicated by distinct colour agglutination against an altered background. By reducing the number of samples requiring further confirmatory testing, these tests save time and resources and allow negative results to be reported at least 24 hours earlier than by conventional culture methods. However, depending on the antibodies used they may lack specificity due to non-specific agglutination of some organisms (Cheesbrough and Donnelly, 1996). Some commercial kits include Remel Wellcolex Colour tests for the presumptive identification of *Salmonella* serogroups A, B, C, D, E, and G, and the V_i antigen using just two reagents. Similar tests include Oxoid Salmonella latex test, Microgen Salmonella Latex test, and Denka-Seiken, among others.

3.2 Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) also known as an enzyme immunoassay (EIA), is a biochemical technique used to detect the presence of an antibody or an antigen in a sample. In the context of *Salmonella* detection, a sample with an unknown amount of antigen is immobilized on a solid support (usually a polystyrene microtitre plate) either non-specifically (via adsorption to the surface) or specifically (via capture by another antibody specific to the same antigen, in a "Sandwich" ELISA). After the antigen is immobilized, a detection antibody linked to an enzyme such as Horse Radish Peroxidase (HRP) is added, forming a complex with the antigen. Between each step, the plate is

typically washed with a mild detergent solution to remove any proteins or antibodies that are not specifically bound. After the final wash step, the plate is developed by adding an enzymatic substrate (ABTS or 3,3',5,5'-tetramethylbenzidine) to produce a visible signal (colorimetric or fluorescent product) due to the enzymatic cleavage of the substrate. Colorimetric equipment is used to measure the signal indicating colorimetric equipment indicating the presence of target antigen in the sample.

ELISAs are highly specific, sensitive, rapid, easy to perform, and scalable, allowing laboratories to easily adopt the technology for routine microbiological testing. The ELISA reactivity however, is influenced by various components of the enrichment medium and incubation conditions used. With most ELISA methods, negative results can be obtained within 24 h after an overnight incubation in selective broth. Positive results may still require further cultural isolation and serological and biochemical confirmation depending of regulatory requirements.

Currently, there are numerous ELISA plate based assay systems for the detection on *Salmonella: Salmonella* ELISA (BIO ART SA), TRANSIA® PLATE *Salmonella* Gold (BioControl), and RIDASCREEN® *Salmonella* ELISA (R-Biopharm AG). Some of these tests have the advantage of being able to process numerous samples at once in 96 well microtitre plates, and some such as the Tecra™ *Salmonella* Visual Immunoassay (3M), provide a visual indication of detection without the use of colorimetric equipment. In addition ELISA systems have been automated to facilitate routine laboratory testing such as the EIAFoss (Foss Electronics) and the VitekImmuno Diagnostic Assay System (VIDAS) (BioMerieux). For example, the VIDAS®SLM assay (BioMérieux), is intended for use with the VIDAS as an automated qualitative enzyme-linked fluorescent immunoassay (ELFA) for the detection of *Salmonella* in food and food ingredients. The VIDAS instrument performs all of the assay steps automatically. In contrast to the manual manipulation required for microtitre plate based systems, a pipette tip-like disposable unit (a solid phase receptacle or SPR) serves as the solid phase as well as a pipetter during the process. The SPR is coated with polyclonal anti-*Salmonella* antibodies and reagents for the assay are sealed in reagent strips. An aliquot of the enrichment broth is placed into the reagent strip and the sample and reagents are sequentially cycled in and out of the SPR for a specific length of time until the instrument detects fluorescence.

Nevertheless, ELISA methods are not without disadvantages, some of which include high limits of sensitivity of >10^5 cfu/mL (Cox 1988) variable cell surface antigen production (Peplow et al. 1999); cross reactivity (Westerman et al. 1997), and changes to antigens due to acetylation and changing recognition by assay antibodies (Kim and Slauch, 1999). Newer ELISA-like techniques utilize fluorogenic, electrochemiluminescent, and real-time PCR reporters to create quantifiable signals. However, given that the general principles in these assays are largely similar, they are often grouped in the same category as ELISAs.

3.3 Lateral flow immunoassays

Lateral flow immunoassays typically use a sandwich type ELISA and the majority use polyclonal antibody as a capture antibody and a monoclonal antibody as the detection antibody. The antibodies are fixed on a hydrophobic polyvinylidine difluoride-based membrane. A drop of an enrichment sample is placed in a reaction window and travels by capillary action across the membrane to react with the antibodies and provide a colour change. Results are often available within 24 hours. False positive results may be observed

during the reaction because of denaturation or degradation of the capture antibody and it is likely that detection antibody or enzyme-conjugated antibody may also bind non-specifically to denatured capture antibody. Commercially available lateral flow immunoassays for the detection of *Salmonella* include: DuPont™ Lateral Flow System *Salmonella*, Singlepath *Salmonella* (Merck), Reveal® *Salmonella* lateral flow (Neogen), VIP Gold (BioControl), and RapidChek® SELECT (SDIX). Recently, serotype specific lateral flow immunoassays for the detection of *S.* Enteritidis have also been introduced to serve the egg and poultry industry such as RapidChek® SELECT *S.* Enteritids (SDIX) and Reveal *S.* Enteritidis (Neogen). In general, these types of immunoassays are ideally suited where a low testing throughput is expected. The implementation of these tests is beneficial in that they require low technical expertise, and minimal capital expenditure.

4. Molecular methods

4.1 Polymerase chain reaction (PCR)

Over the past 15 years there has been an important evolution in molecular approaches for the rapid detection of food borne pathogens rather than relying on their biochemical and phenotypic characteristics. Foremost among these tools is the Polymerase Chain Reaction (PCR), a technique based on the specific amplification of a short target DNA sequence (Mullis et al. 1986). Briefly, extracted DNA is first subjected to heat denaturation into single stranded DNA. Next, specific short DNA fragments (primers) are annealed to the single DNA strands, followed by extension of the primers complementary to the single stranded DNA with the aid of a thermostable DNA polymerase, such as Taq polymerase, an enzyme originally isolated from the bacterium *Thermus aquaticus* (Chien et al. 1976). Each new double-stranded DNA is then a targeted during a new thermal cycle and thus the exponential amplification of the specific DNA sequence is achieved. The amplified product is then separated by gel electrophoresis and visualized by staining with fluorescent ethidium bromide. This type of conventional or endpoint PCR, although sensitive and specific under optimized conditions, is time consuming and labour intensive due to post-amplification steps, not sensitive enough to measure the accumulated DNA copies accurately, and can only provide a qualitative result. Nevertheless, PCR techniques have expedited the process of pathogen detection and in some cases, replaced traditional methods for bacterial identification, characterization, and enumeration in foods (McKillip and Drake 2004).

4.2 Real-time PCR

The development of novel chemistries and instrumentation platforms enabling detection of PCR products on a real-time basis has led to widespread adoption of real-time PCR as the method of choice for detection of *Salmonella* (Espy et al. 2006). This method combines amplification and detection stages of the process so that nucleic acid amplification is monitored and recorded continuously hence eliminating the need for post-amplification steps such as gel electrophoresis. The detection of PCR products is accomplished via the generation of a fluorescent signal by any of the commercially available chemistries for real-time PCR: TaqMan® (Applied Biosystems®), Molecular Beacons, Scorpions®, and SYBR® Green (Molecular Probes), among others.

The simplest approach involves the use of the intercalating fluorescent dye SYBR® Green. This fluorogenic dye exhibits little fluorescence when in solution, but emits a strong

fluorescent signal upon binding to double-stranded DNA. Thus, as a PCR product accumulates, fluorescence increases. The advantages of SYBR® Green are that it is inexpensive, simple, and sensitive. The disadvantage is that SYBR® Green will bind to any double-stranded DNA in the reaction, which may result in an overestimation of the target concentration. A second, more accurate and reliable method is to use fluorescent reporter probes (TaqMan®, Molecular Beacons, Scorpions®). These probes depend on Förster Resonance Energy Transfer (FRET) to generate the fluorescence signal via the coupling of a fluorogenic dye molecule and a quencher moiety to the same or different oligonucleotide substrates. The main advantage of TaqMan probes, Molecular Beacons and Scorpions is that they allow for multiplex PCR assays by using spectrally separated fluor/quench moieties for each probe. Multiplex PCR allows internal controls to be co-amplified and permits allele discrimination in single-tube, homogeneous assays. These hybridization probes afford a level of discrimination impossible to obtain with SYBR® Green, since they will only hybridize to true targets in a PCR and not to primer-dimers or other spurious products. However these probes can be expensive to synthesize, with a separate probe needed for each target being analyzed.

Commercial real time PCR assays employ a high degree of automation to reduce the number of operations involved and reduce the risk of contamination. The reaction usually takes place inside a combined thermocycler-fluorescence detection instrument and uses pre-prepared reagents, often in a dehydrated tablet form. The thermo-cycling and detection are controlled by software that also calculates and interprets the results. Total time for an analysis for the detection of *Salmonella* species is normally 20 to 48 hours but can be as little as 12 hours depending on the food matrix, enrichment conditions, and instrument run time. The main advantage of these PCR systems over other methods is in time saving, both in the total time from sampling to result and in the technical time needed to set up and run the assay. In addition many available real time PCR assays have achieved a variety of certifications via AOAC, AFNOR, NORDVAL, and ISO 16140 validation. However, capital costs for automated PCR systems are relatively high and consumable costs are also high by comparison to culture based techniques. There is a clear cost benefit in rapid test results allowing faster HACCP verification and release of finished food products particularly where the prevalence of *Salmonella* is known to be low, thus reducing additional culture confirmation tests or where pooling of samples is permitted. Numerous assays are commercially available using real time PCR for the detection of *Salmonella.* The BAX PCR detection system (DuPont-Qualicon Inc.), a platform adopted by USDA-FSIS as a screening tool offers a detection kit for the detection of *Salmonella* in a variety of food and food ingredients. Other systems offering similar testing capabilities include: ADIAFOOD Rapid Pathogen Detection System (AES Chemunex), the Assurance Genetic Detection System GDS (Biocontrol Inc.) utilizing a post enrichment IMS step followed by real time PCR, iQ-Check™ Salmonella II (BioRad Laboratories, S.A.), and R.A.P.I.D. LT system (Idaho Technology Inc.), among others.

Lastly, real time PCR systems have sufficient flexibility to allow for the rapid development of new assays targeting specific *Salmonella* serovars of clinical significance. More recently in 2010, in order to minimize the potential for foodborne illness from eggs containing *S.* Enteritidis, the FDA implemented new regulations for the egg industry, which included requiring large-scale egg producers to begin SE monitoring programs in their poultry houses and potentially on their products. In response to the industry testing needs, a 27

hour commercial real time PCR assay for the detection of *S.*Enteritidis was developed by Applied Biosystems®, the TaqMan® *Salmonella* Enteritidis Detection Kit.

4.3 Multiplex PCR

In multiplex PCR (mPCR), several specific primer sets are combined into a single PCR assay for the simultaneous amplification of more than one target DNA sequence (Chamberlain et al. 1988). As with conventional or endpoint PCR, the amplified DNA targets are separated by agarose gel electrophoresis and visualized by ethidium bromide staining. Depending on the number of targets, the analysis is carried out by a single amplification reaction of four–to five targets, or could take place via a two-step amplification reaction for five–six targets or more (Settanni and Corsetti 2007). For example, Malorny et al. (2007) developed an assay for the specific detection of *S.* Enteritidis in whole chicken carcass rinses and consumption eggs. The assay used specifically designed primers and a TaqMan probe to target the *Prot6e* gene located on the *S.* Enteritidis 60-kb virulence plasmid. As an internal amplification control to monitor *Salmonella* DNA in the sample, a second primer/TaqMan probe set detected simultaneously the *Salmonella* specific *invA* (invasion protein A) gene. It must be considered however, that the majority of the articles in the scientific literature deal with mPCR methods developed to identify and or characterize *Salmonella* serotypes from pure cultures, or in controlled artificial inoculation experiments, with only a minority of studies providing results from *in situ* detection of pathogens in foods or environmental samples. Soumet et al. (1999) developed a multiplex PCR assay for the simultaneous identification of *Salmonella* species, *S.* Enteritidis and *S.* Typhimurium from environmental swabs of poultry houses. Similarly, O'Regan et al. (2008) developed a real-time multiplex PCR assay for the detection of multiple *Salmonella* serotypes in chicken samples. Poultry-associated serotypes detected in the assay included *S.* Enteritidis, *S.* Gallinarum, *S.* Typhimurium, *S.* Kentucky and *S.* Dublin. Generally, the 16S rRNA gene is the most common target for mPCR as it is routinely used to establish phylogenetic distinctions among bacteria (Rossello-Mora and Amman 2001). However, other target genes are also considered in order to achieve a high specificity. For example, Rajtak et al. (2011) developed a two step real-time mPCR assay for the rapid screening of 19 *Salmonella* serotypes frequently encountered in humans, animals, and animal-associated meat products within the European Union. Specific primers for serotype differentiation were designed to target the genes encoding either phase 1 and 2 flagellar antigens *fliC* and *fljB* or unique serotype-specific loci. In addition, the assay simultaneously screened for the presence of the ampicilin-amoxicillin, chloramphenicol-florfenicol, streptomycin-spectinomycin, sulfanomides, and tetracycline (ACSSuT)-type multidrug resistance pattern, indicated by the *floR* gene, and for the *Salmonella* virulence plasmid encoded by the svp operon in *S.* Typhimurium. The assay represents a more rapid and reliable method for identification of large numbers of serotypes than assays using phenotypic serotyping methods. Multiplex PCR is thus quite versatile and numerous other assays have been published for the rapid detection and characterization of specific *Salmonella* serotypes (Alvarez et al. 2004; Woods et al. 2008; Kim et al. 2006; Chiu et al. 2006) analogous to mPCR approaches used for the differentiation of multiple species belonging to single genera such as gastroenteritis causing themotolerant *Campylobacter* species (Korolik et al. 2001;Klena et al. 2004; Wang et al. 2002; Yamazaki-Matsune et al. 2007) or for the differentiation of the major *L. monocytogenes* serovars (1/2a, 1/2b, 1/2c, and 4b) commonly implicated in food borne listeriosis (Doumith et al. 2004; Zhang and Knabel 2005; Chen and

Knabel 2007). Lastly, and perhaps the largest impact that mPCR may provide in a near future is in the rapid and simultaneous detection of *Salmonella* concurrently with other bacterial pathogens. For instance, Gilbert et al. (2003) established a mPCR assay in order to detect *Salmonella* along with *Campylobacter jejuni*, and *E. coli* O157:H7 in a variety of raw and ready-to-eat food products. The primers amplified a single product from each target bacterium. More recently, Kim et al. (2007) developed a novel mPCR assay for the simultaneous screening of five foodborne pathogenic bacteria including *Salmonella*. Specific primers for mPCR amplification of the Shiga-like toxin gene (*Stx2*), *femA* (cytoplasmic protein), *toxR* (transmembrane DNA binding protein), *iap* (invasive associative protein), and *invA* genes were designed to allow simultaneous detection of *E. coli* O157:H7, *S. aureus*, *Vibrio parahaemolyticus*, *L. monocytogenes*, and *Salmonella* spp., respectively. Furthermore, the detection of all five food borne pathogenic bacteria could be completed in less than 24 h. Similar approaches have been described by others utilizing various primer sets for a variety of pathogens (Li and Mustapha 2004; Park et al. 2006).

4.4 Reverse transcriptase PCR (RT-PCR)

Thus far, there is no correlation between viability and detection as provided by PCR assays. The amplification of genomic DNA by PCR has been shown to be inappropriate for distinguishing viable from non-viable bacteria owing to DNA stability over time (Masters et al. 1994). Furthermore, the detection of pathogens by PCR in food samples often requires additional evidence of viability before risk can be assigned. In an effort to address the issue of viability, many researchers turned to RNA amplification methods using mRNA as a target since it is a molecule with a very short half-life of 0.5 to 2 minutes due to the rapid degradation by endogenous RNases (King et al. 1986). The outcome was the development of an amplification technique for detecting mRNA termed reverse transcriptase PCR (RT-PCR). To date however, due to the variable persistence of nucleic acids in cells post-death, the correlation between the presence of DNA and RNA and viability is still not clear (Cenciarini-Borde et al. 2009). In RT-PCR, an RNA strand is first reverse transcribed into its DNA complement (complementary DNA, or cDNA) using the enzyme reverse transcriptase, and the resulting cDNA is then amplified using conventional, multiplex, or real-time PCR. For example, Rijpens et al. (2002) targeted the housekeeping *rpoD* gene of *Salmonella*. Overall, the assay could not detect viable *Salmonella* in heat or ethanol killed *Salmonella* cells. However, conventional RT-PCR techniques are labour intensive since the amplicon can be visualized only after the amplification ends, requires the rapid extraction of RNA due to its short half-life, suffers from an increased cross-contamination risk of the samples thus requiring DNase treatments, and the target genes must demonstrate abundant transcript expression, expression throughout the growth cycle, and negligible or no transcriptional regulation (Klein and Juneja 1997; Deisingh and Thompson 2004; Yaron and Matthews 2002). Due to these difficulties, the development of RT-PCR applications focusing on the detection of food-borne pathogens, including *Salmonella* in foods and environmental samples has been limited. D'Souza et al. (2009) developed a RT-PCR for the rapid detection of *Salmonella* using *invA* primers. Park et al. (2011) evaluated immunomagnetic beads and a RT-PCR method for the detection of *Salmonella* inoculated into poultry feed demonstrating that the *hilA* gene is a candidate for use in RT-PCR. Techathuvanan and D'Souza (2011) optimized a rapid *Salmonella* detection assay in liquid whole eggs by SYBR® Green based real-time RT-PCR targeting the *invA* gene as described previously for the detection of

Salmonella from jalapeno and serrano peppers, and Pork (Miller et al. 2010; Techatuvanan et al. 2010). To further address the issue of viability of the species detected in a complex matrix such as foods, perhaps the best alternative could be the development and validation of real time and multiplexed PCR assays targeting mRNA, also termed multiplex RT-PCR (Gonzalez-Escalona et al. 2009; Settanni and Corsetti 2007). Thus far however, no commercial PCR assay is available utilizing reverse-transcriptase technology for detecting *Salmonella* in foods.

It is evident that molecular methods offer improved sensitivity and potential reduction in assay time. It has now become possible to rapidly detect and confirm the presence of foodborne *Salmonella* spp. in a wide array of food and environmental samples by commercial amplification detection systems. The primary challenges remaining are to develop more reliable recovery and extraction procedures for routine processing of samples from a wider variety of feed and environmental matrices and apply molecular techniques for further characterizing *Salmonella* spp.

4.5 Nucleic acid hybridization

Endpoint PCR is commonly utilized for the detection of amplified PCR products. However, DNA hybridization has also been described for detection (Chan et al. 1988; Hill and Keasler 1991; Hill and Lampel 1990). Probes directed to specific gene regions of the *Salmonella* genome provide a powerful tool for use in DNA hybridization assays. Such methods of detection have proven to be more sensitive than agarose gel electrophoresis and more specific than culture or immunological based assays (Ten Bosch et al. 1992; Manzano et al. 1998). For example, Maciorowski et al. (1998) was able to detect PCR products from *S.* Typhimurium inoculated animal feeds by hybridization with biotin and fluorescently labeled probes. Such specificity eliminates the need for serological confirmation and incidences of false-positive identification caused by antibody cross-reactivity with other organisms. Also, unlike biochemical differentiations, probe reactions do not rely on enzymatic activities and are therefore unaffected by media interference or the presence of bacteria with similar phenotypes. The majority of DNA based hybridization assays have exploited this specificity for DNA microarray assay targeting multiple genes with few applications related to the detection of *Salmonella* from food and environmental samples. Probes complimentary to amplified gene products have been used for the detection of *Salmonella* in oysters and chicken meat as well as from environmental poultry house drag swabs (Cohen et al. 1994; Doran et al. 1994; Jones et al. 1993; Bej et al. 1996). Commercial hybridization assays for the detection of *Salmonella* include the GeneQuence *Salmonella* assay (Neogen) utilizing probes previously evaluated by D'Aoust et al. (1995). This test employs Salmonella-Specific DNA probes, which are directly labeled with horseradish peroxidase. A colorimetric endpoint is then used for the detection of *Salmonella* spp. in food samples following broth culture enrichment with results available within 24 h.

5. Phage based detection methods

Bacteriophages are viruses infecting bacteria and by definition obligate intracellular parasites lacking their own metabolism, are extremely host-specific, and able only to infect specific species or even strains. Virulent phages with a broad host range within the *Salmonella* genus are ideally suited for detection purposes since they are unable to integrate

into the host genome, with the successful infection always resulting in the death of their host (Hagens and Loessner 2007). Since the first report of the use of phage for detection by Ulitzur and Kuhn (1987), different strategies have been described for the detection of *Salmonella*. Generally, the majority of methods described involve measuring the activity of a reporter gene (generally, the luciferase *lux* genes from *Vibrio fisherii*), cloned into a vector carried by a phage, and expressed only after infection (Kuhn et al. 2002; Thouand et al. 2008). Luciferase genes have the enormous advantage in that background noise or photon emission is absent from food samples and the luminescence, when detected, reflects the presence of viable target bacteria. Other approaches include use of an ice nucleation reporter phage (Wolber and Green 1990); concentration by IMS followed by phage mediated release of adenylate kinase (AK) (Blasco et al. 1998; Wu et al. 2001); fluorescently labelled phage (Jiang et al 2009); and an IMS-bacteriophage plaque formation assay requiring the addition of a virucide to inactivate free phage particles (Fravrin et al. 2001). The usefulness of phage-based cell wall recognition proteins for magnetic capture has also been recently described utilizing cell-wall-binding domains (CBDs) highly specific for recognition and binding to target cells surfaces (Kretzer et al. 2007; Korndoerfer et al. 2006; Loessner et al. 2002). Paramagnetic beads coated with CBD molecules were shown to outperform commercially available antibody-based magnetic beads with respect to sensitivity and percent recovery (Kretzer et al. 2007). An extension to this approach has been the use of phage-tail-associated recognition proteins for the immobilization of gram-negative cells (Galikowska et al. 2011). For example, BioMerieux. has recently introduced *Salmonella* Up, an automated ELISA based VIDAS assay using a phage recombinant protein derived from specific bacteriophage tail fibers for the detection of *Salmonella* in food and food ingredients within 18-24 hours after enrichment in a non-selective broth.

Although at present commercial phage based detection systems are limited, the technology may circumvent the problem of viability presented by PCR, while promising to be more rapid than standard culture methods.

6. Conclusions and future perspectives on *Salmonella* detection methods

A wide range of methods for the detection of *Salmonella* has been developed in the last decade and significant progress has been made in sample preparation techniques for improved isolation and detection of *Salmonella* in foods and food ingredients. The use of immunomagenetic separation technique which separates target organisms from background flora, is now routinely applied in various diagnostic labs for a variety of foodborne pathogens including *Salmonella*. This technique has increased the sensitivity of the detection of *Salmonella* in various types of food and food ingredients as well as environmental samples with high levels of background. Similarly, the application of molecular methods, immunological methods, and bacteriophage detection systems for *Salmonella* is now routine in many diagnostic food microbiology labs. Novel technologies such as the application of biosensors, microarrays, and nanotechnology are currently in the research stage and these are likely to become available for routine testing of food and food ingredients within the next decade.

The application of rapid methods for the detection, identification, and characterization of *Salmonella* provides a useful tool for assessment of the safety of food products when used in conjunction with foodsafety programs such as the Hazard Analysis Critical Control Point (HACCP) program for the assessment of raw materials and food ingredients used in food

processing and production. Further improvements to rapid methods for isolation and detection of *Salmonella* and other microbial pathogens will continue to focus on sample enrichment and preparation procedures to reduce test turn around times and increase the sensitivity of detection, and also on the application of novel technologies such as biosensors, microarrays and nanotechnology for pathogen detection in foods.

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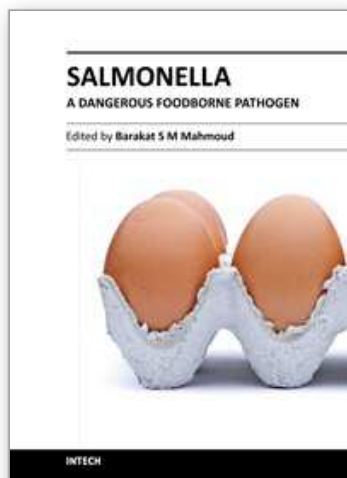
Salmonella - A Dangerous Foodborne Pathogen

Edited by Dr. Dr. Barakat S M Mahmoud

More than 2,500 serotypes of Salmonella exist. However, only some of these serotypes have been frequently associated with food-borne illnesses. Salmonella is the second most dominant bacterial cause of food-borne gastroenteritis worldwide. Often, most people who suffer from Salmonella infections have temporary gastroenteritis, which usually does not require treatment. However, when infection becomes invasive, antimicrobial treatment is mandatory. Symptoms generally occur 8 to 72 hours after ingestion of the pathogen and can last 3 to 5 days. Children, the elderly, and immunocompromised individuals are the most susceptible to salmonellosis infections. The annual economic cost due to food-borne Salmonella infections in the United States alone is estimated at $2.4 billion, with an estimated 1.4 million cases of salmonellosis and more than 500 deaths annually. This book contains nineteen chapters which cover a range of different topics, such as the role of foods in Salmonella infections, food-borne outbreaks caused by Salmonella, biofilm formation, antimicrobial drug resistance of Salmonella isolates, methods for controlling Salmonella in food, and Salmonella isolation and identification methods.

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