

## REVIEW

# Accurate and Rapid Methods for Detecting *Salmonella* spp. Using Polymerase Chain Reaction and Aptamer Assay from Dairy Products: A Review

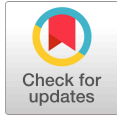
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## Abstract

*Salmonella* spp. is the most common cause of gastrointestinal food poisoning worldwide, and human salmonellosis is mostly caused by the consumption of contaminated food. Therefore, the development of rapid detection methods for *Salmonella* spp. and rapid identification of the source of infection by subtyping are important for the surveillance and monitoring of food-borne salmonellosis. Therefore, this review introduces (1) History and nomenclature of *Salmonella* spp., (2) Epidemiology of *Salmonella* spp., (3) Detection methods for *Salmonella* spp. – conventional culture method, genetic detection method, molecular detection methods, and aptamer, and (4) Subtyping methods for *Salmonella* spp. – pulsed-field gel electrophoresis and repetitive sequence-based polymerase chain reaction (PCR).

## Keywords

*Salmonella* spp., dairy products, culture method, genetic- or molecular-based detection, aptamer, repetitive-sequence-based polymerase chain reaction (PCR)

## Introduction

The World Health Organization (WHO) defines foodborne illnesses as diseases, usually either infectious or toxic in nature, caused by agents that enter the body through the ingestion of food [1]. It has been reported that in 2005 alone 1.8 million people died from diarrhoeal diseases and a great proportion of these cases can be attributed to contamination of food and drinking water [1]. For example, in the USA, it has been estimated that foodborne disease may cause up to 76 million cases, 325,000 hospitalizations, and 5,000 deaths occur each year [2]. Though viruses, bacteria, parasites, and a variety of chemicals are causes of foodborne disease, the leading known causes are bacterial. The widely cited USA estimate by Mead et al. [3] at the Centers for Disease Control and Prevention (CDC) is that non-typhoidal *Salmonella*, *Campylobacter*, enterohaemorrhagic *Escherichia coli*, and *Listeria monocytogenes* account for the vast majority of bacterial food-borne disease. In addition, a recent national surveillance study in Korea reported that non-typhoidal *Salmonella* is the most important bacterial cause of sporadic cases of foodborne-illness, and followed by *Staphylococcus aureus* and pathogenic *E. coli* [4]. *Salmonella* spp. is the most common cause of gastrointestinal

food poisoning in the world [5]. Human salmonellosis is most often caused by the consumption of contaminated food [1-5]. Raw and processed meat products are the principal reservoir of *Salmonella*, and agricultural products such as fruits and vegetables are also vehicles of human salmonellosis [6]. Therefore, the surveillance and monitoring of food safety are keys to prevent food borne salmonellosis. Conventional culture methods have been known as the most reliable and accurate techniques for foodborne pathogen detection, and they are found to be standard microbiological methods to detect the single bacteria [7]. Many standard culture methods for detection of food-borne bacteria such as coliforms, *E. coli*, *L. monocytogenes*, *S. aureus*, *Salmonella*, *Campylobacter jejuni*, *Vibrio parahaemolyticus*, and *Yersinia enterocolitica* have been published [7]. The major drawbacks of microbiological methods are their labor-intensiveness and time-consuming as it can require up to 7-10 days for confirmation [7]. This is an obvious inconvenience in many food industrial applications. In addition, viable bacterial strains in the environment can enter a dormancy state where they become viable-but non-culturable (VBNC) which can subsequently lead to a failure to isolate a pathogen from a contaminated sample [8]. Therefore, as a means of improving the detection of these pathogens, these methods are often combined together with or replaced with other detection methods like a nucleotide-based, immune-based or biochemical-based method to yield more robust results [7]. The polymerase chain reaction (PCR) first described by Kary Mullis in the mid-1980s is a three-step cyclic *in vitro* procedure based on the ability of the DNA polymerase to copy a strand of DNA [9]. The PCR is sufficiently sensitive so that, in theory, the presence of even one copy of the template within the reaction mixture can be detected within a couple of hours [10]. The real-time PCR assay introduced in the early 1990s is based on an increase in fluorescence from a dsDNA-specific dye or hybridization probe that is monitored during the amplification of a target gene [11]. This technology could potentially save time and effort in the laboratory and thus may lower testing-related costs incurred by the food industry [12]. In addition, multiplex real-time PCR assays have been applied to simultaneously detect more than two gene sequences of each bacterial strain targeted in a single reaction by using spectrally distinct dye-labeled probes [12]. However, major difficulties with PCR are the presence of compounds that inhibit the PCR reaction and possibility of detection of dead bacteria [13]. Immunological detection is based on the highly specific binding reaction between antibodies and antigens. The immunology-based methods provide very powerful analytical tools for bacteria detection [14]. While the immunology-based method is not much specific and sensitive than nucleic acid-based detection, but it is faster and has the ability to detect biotoxins that may not be expressed in the organism's genome that cannot be detected by PCR assays [15]. The limitations of immunological detection are that the targets should be immunogenic to produce antibody in animals or cell lines [16]. In addition, the activity of antibody is not stable in high temperature and chemical modifications and restricted in physiological condition [7]. Therefore, recently, new detection tools such as aptamers have been researched to overcome the limits of immunology-based methods [17]. Aptamers are artificial specific single stranded oligonucleotides, DNA or RNA, with the capability of binding specifically to



non-nucleic acid target molecules, such as peptides, proteins, drugs, organic and inorganic molecules, and whole cells [18]. The aptamers have unique characteristics over more antibodies used for immunology-based methods [17]. In addition to detection of pathogen, identifying sources of infection and pathways of transmission by subtyping is also important for surveillance and understanding the epidemiology of sporadic cases acquired from various sources [19]. Pulse-field gel electrophoresis (PFGE) is a common subtyping method and has been considered the “gold standard” method for subtyping all of the major foodborne pathogens [20]. However, the major disadvantages of PFGE are the time needed (ca. 2 to 4 days), variations in analysis, interpretation of the data and the requirement of specialized equipment required [21]. Because of the limitations of PFGE, PCR-based subtyping methods such as repetitive-sequence-based PCR methods (repPCR) are attractive because many laboratories already have the necessary reagents, equipment, and trained personnel, and these methods are also more rapid, simpler to perform, and less costly than PFGE [22].

## History and Nomenclature for *Salmonella*

The Genus *Salmonella* is named after an American bacteriologist, D. E. Salmon, who first isolated from porcine intestine in 1884 [5,23]. The organism was originally called “*Bacillus choleraesuis*,” which was subsequently changed to “*Salmonella choleraesuis*” in 1900 [23]. *Salmonella* was differentiated based on the serologic identification of O (somatic) and H (flagellar) antigens, and Kauffmann proposed that each serovar be considered a separate species [24]. In 1973, Crosa et al. [25] demonstrated that all *Salmonella* strains belonged in a single species on the basis of DNA-DNA hybridization experiments. In 2002, the Judicial Commission issued an opinion (the Judicial Opinion 80) which finally approved that from January 2005, “*Salmonella enterica*” would replace “*Salmonella choleraesuis*” to become the type species of the genus *Salmonella* [26]. The antigenic classification system of various *Salmonella* serovars used today has established by Kauffman and White almost a century ago and listed in the Kauffmann-White scheme [27]. The WHO Collaborating Centre for Reference and Research on *Salmonella* at the Pasteur Institute is responsible for updating the scheme [23]. Currently, according to the nomenclature system used at the CDC based on recommendations from the WHO Collaborating Centre, the genus *Salmonella* contains two species, *S. enterica* and *S. bongori* [23,27]. *S. enterica* is divided into six subspecies which are referred to a Roman numeral and a name (I, *S. enterica* subsp. *enterica*; II, *S. enterica* subsp. *salamae*; IIIa, *S. enterica* subsp. *arizonae*; IIIb, *S. enterica* subsp. *diarizonae*; IV, *S. enterica* subsp. *houtenae*; and VI, *S. enterica* subsp. *indica*) by biochemically and genomic relatedness [27]. *S. bongori* was formerly subspecies V of *Salmonella enterica*, and it was separated to distinct species by DNA-DNA hybridization in 1989 [28]. Currently, a third species “*Salmonella subterranea*” was recognized in 2005, and the CDC may incorporate it in their system in the near future [23]. Before 1966 all serotypes in all subspecies except subspecies IIIa and IIIb were given names. In subspecies I, serovars were named by a name usually indicative of associated diseases, animal and geographic origins, or usual

habitats. In the remaining subspecies, as well as those of *S. bongori*, antigenic formulae determined by the Kauffmann-White scheme are used for unnamed serovars [23,27]. In 1966, the WHO Collaborating Centre began naming serotypes only in subspecies I and dropped all existing serotype names in subspecies II, IV, and VI and *S. bongori* from the Kauffmann-White scheme [29]. Serotype names designated by antigenic formulae include the following: (i) subspecies designation (subspecies I through VI), (ii) O (somatic) antigens H (flagellar) antigens (phase 1) H antigens (phase 2, if present) followed by a colon, for example, *Salmonella* serotype IV 45:g,z51:2. For serotypes in *S. bongori* (which previously belonged to subgenus V), V is still used for consistency, e.g., *S. V* 61:z35:2. In the latest report published in 2004, there were a total of 2,541 serovars in the genus *Salmonella* [23]. To avoid confusion between serovars and species, the serovar name is not italicized and the first letter is capitalized. At the first citation of a serotype the genus name is given followed by the word “serotype” (or the abbreviation “ser.”) and then the serotype name, e.g., *Salmonella enterica* serotype or ser. Enteritidis. Subsequently, the name may be written with the genus name followed directly by the serotype name (for example, *Salmonella* Enteritidis or *S. Enteritidis*) [30].

## Epidemiology of *Salmonella*

*Salmonella* causes approximately 1.4 million human infections each year in the USA, resulting in 116,000 hospitalizations and 600 deaths [1,2,5]. Nontyphoidal *Salmonella* is the most commonly identified cause of foodborne illness in the U.S., and salmonellosis accounts for >30% of deaths resulting from foodborne illnesses in the United States [5,31]. The peak occurrence of salmonellosis is during the summer months as with most foodborne illnesses [1,2,5,31]. According to preliminary FoodNet data on the incidence of infection with pathogens transmitted commonly through food in US in 2005, the most common serotypes identified are *Salmonella enterica* serotype or ser. Typhimurium, *S. Enteritidis*, and *S. Newport*, in descending order [31]. Most human cases of nontyphoidal *Salmonella* involve ingestion of a contaminated food item, and waterborne infections also can occur less commonly [5,31]. Most infections are acquired by eating contaminated poultry, eggs or dairy products. The *Salmonella* spreads easily from raw or undercooked meat product to innocent vegetables, fruit or other foods via contaminated hands, knives, countertops or cutting boards [32]. Recent outbreaks of *Salmonella* infection have been linked to eggs, cheese, dry cereal, ice cream premix, a variety of fresh sprouts, juice, cantaloupes, and other fresh vegetables [5,31]. In addition, contact with an animal carrying *Salmonella* such as reptiles, cats, dogs, and rodents may result in human infection [5]. Clinical manifestations of nontyphoidal salmonellosis include gastroenteritis, bacteremia, endovascular infections, and localized infections [31]. The most common presentation is gastroenteritis which manifests as abdominal pain, nonbloody diarrhoea, mild fever, chills, headache, nausea and vomiting, occurring 12–72 hours (but occasionally as long as 7 days) after infection [32]. Symptoms are similar with other GI pathogens such as *Campylobacter* or *Yersinia* and generally last a few days [31]. Most *Salmonella* infection is limited to gastroenteritis and usually self-limited so



that rarely requires antimicrobial treatment [23]. However, there is a risk of development of complications of extra-intestinal salmonellosis such as bacteremia, meningitis, and joint inflammation include patients at the two age extremes and those with immune suppression, or accompanying severe infections [5]. In addition, the development of infectious endarteritis (infectious aortitis or mycotic aneurysm) is a serious complication of *Salmonella* bacteremia in adults [23]. These severe *Salmonella* infections can be fatal and antimicrobial treatment is essential in these circumstances [5]. The serotypes most commonly associated with invasive salmonellosis in humans are *S. Typhi*, *S. Paratyphi*, *S. Choleraesuis* and *S. Dublin*, and *S. Typhimurium*, *S. Enteritidis* and *S. Heidelberg* are also associated with a relatively low proportion of invasive infections [23]. Among the invasive non-typhoid *Salmonella* serotypes, *S. Choleraesuis* is particularly prevalent in Asian countries, including Taiwan, but *S. Dublin* in western countries.

## Detection methods for *Salmonella*

### 1. Conventional culture method

Culture methods is the oldest bacterial detection technique and remain the most reliable and accurate method for isolating *Salmonella* spp. from foods [33]. US Food and Drug Administration (FDA) / American Association of Official Analytical Chemists (AOAC) standard culture method for detection of *Salmonella* in foods includes pre-enrichment, selective enrichment, plating on selective media and serological and biochemical identification of suspected colonies [34]. The pre-enrichment (16–20 h) allows the resuscitation and multiplication of sub-lethally damaged cells [35]. The pre-enriched samples enriched in selective enrichment media that contain inhibitory substances to suppress competing organisms for 18–48 h, and plating on selective/differential agar media (24–48 h) to allow the differentiation of *Salmonella* spp. from other Enterobacteria [35]. Biochemical and serological confirmation (4–48 h) follows for confirmation of presumptive-positive colonies. Culture methods provide a theoretical level of sensitivity of one *Salmonella* cell per 25 g of food [35]. According to BAM (Bacteriological Analytical Manual) of FDA, the 25 g of sample is pre-enriched in different broth such as lactose broth and buffered peptone water depending on sample types at 37°C for 24 h followed by selective enrichment in Rappaport-Vassiliadis (RV) and tetrathionate (TT) broth at 42°C and 37°C for 24 h, respectively [35]. The enriched cultures should be streaked onto bismuth sulphite (BS), xylose lysine desoxycholate (XLD), and hektoen enteric (HE) agars for isolation, and presumptive positive colonies are stabinoculated into triple sugar iron (TSI) and lysine iron agar (LI) followed by biochemical and serological tests [5]. To reduce the workload for unnecessary examination of suspect colonies, amendments to media and development of new chromogenic and fluorogenic media that make the *Salmonella* diagnostic easier faster have been investigated [36]. The addition of novobiocin to HE, XLD, TSA and brilliant green agars (BG) enhanced the isolation of *Salmonella* from food and fecal samples, and nitrofurantoin has been used to isolate *S. Enteritidis* [5]. New selective media have been developed based on biochemical characteristic of *Salmonella* such as  $\alpha$ -galactosidase activity in the absence

of b-galactosidase activity, C8-esterase activity, catabolism of glucuronate, glycerol and propylene glycol, hydrolysis of X-5-Gal, and H<sub>2</sub>S production. e.g., SMID agar (BioNerieux, France), Rambach agar (Merck, Germany), Rainbow *Salmonella* agar (Biolog, USA), CHROMagar *Salmonella* (CHROM agar, France), chromogenic *Salmonella* esterase agar (PPR Diagnostics, UK), Compass *Salmonella* agar (Biokar diagnostics, France), and chromogenic ABC medium (Lab M., UK) [5,36]. The time to detection and the performance convenience of the method has been improved by modifications. For example, the semisolid medium [MSRV, SMS® (AES Chemunex) or SESAME *Salmonella* test (Biokar Diagnostics)] for the selective enrichment of *Salmonella* spp. allow differentiation between motile and non-motile micro-organisms by the incorporation of selective agent in the agar [37]. The Rapid' *Salmonella* Short Protocol is available from BioRad where one single enrichment broth is used (BPW + *Salmonella* capsule) to shorten and to facilitate the enrichment step [37]. Culture methods are labor intensive and time consuming when handling many samples. In addition, detection can be prevented by the presence of other competing microorganisms during cultural enrichment, and the selective agar media have a very poor specificity creating an abundance of false positives (such as *Citrobacter*, *Proteus*) [36]. Therefore, there is a need for *Salmonella* detection methods that provide results more rapidly with sensitivity similar to or greater than, the conventional methods.

## 2. Genetic-based detection method

Immunology-based methods are based on the highly specific binding reaction between antibodies and antigens [37]. The immunology-based methods provide very powerful analytical tools for a wide range of targets, but any positive result for pathogens is considered as presumptive and requires further confirmation [37]. Several types of immunoassays are available in food diagnostics of Enzyme Linked Immunosorbent Assays (ELISAs) and enzyme linked fluorescent assays (ELFA) and Immunomagnetic separation (IMS) and concentration. ELISA and ELFA are the most established biochemical techniques that couple an immunoassay with an enzyme assay and rely on chromogenic substrates and fluorescence for end-point detection, respectively [5,37]. The ELISA detection is known to approximately 10<sup>4</sup>-10<sup>5</sup> CFU/mL of detection limit, depending upon the type and affinity of antibody, and only takes 2-3 h [37]. A sandwich ELISA is the most common kind, and many ELISA tests are automated to reduce the hands-on time and to improve the reproducibility and standardization of each step of the assay [37]. The two monoclonal antibodies were developed for detection *Salmonella*: an IgA mouse myeloma protein MOPC 467 which reacts with structural peptides of *Salmonella* flagellin and an IgG2b hybridoma antibody (6H4) recognizing a nonflagella antigen of *Salmonella* [5]. The combination of two monoclonals was used in ELISA, *Salmonella*-Tek™ ELISA Test System, with the capture antibodies bound to the well of a microtitre plate [5]. The BacTrace™ Microwell ELISA (Kirkegaard and Perry Laboratories, USA) employs a polyclonal antibody that reacts with numerous common structural antigens of *Salmonella* [35]. The 'I'ECRA™ *Salmonella* Visual Immunoassay (Bioenterprises Pty, Australia) uses high-affinity antibodies against *Salmonella*, and the Assurance *Salmonella*



Enzyme Immunoassay (BioControl Systems, USA) is a colorimetric assay that uses polyclonal antibodies and adoption as an AOAC official first action method [35]. In order to reduce the time and complexity of the ELISA for *Salmonella*, a dipstick-based assay (LUMAC®PATH-STIK Rapid *Salmonella* Test (LUMAC B.V., The Netherlands)) and a lateral flow devices (Reveal® for *Salmonella* (RSS) test system (Neogen, USA) has been developed [35,38]. The dipstick-based assay took 20 min to perform, had a sensitivity of  $10^6$  CFU/mL of *Salmonella* in milk powder after an overnight enrichment [35]. The RSS test system is a presumptive qualitative test that detects *Salmonella* organisms in foods within 21 h including enrichment [38]. The RSS test system has a limit of detection of 5-10 CFU/25 g in many food types and showed an 81% overall agreement with the traditional procedure of the U.S. Department of Agriculture's Food Safety Inspection Service [38]. An automated ELISA has been developed to be rapid and less labour-intensive and to handle large numbers of samples. The automated ELISA systems that are commercially available such as EIAFoss (Foss Electronics, Denmark) and Vitek Immunodiagnostic Assay System (VIDAS, BioMérieux, USA) are currently used in the meat and poultry processing industries [5]. Several validation studies have been reported that the detection rate of VIDAS systems were comparable to that of culture method and real-time PCR for detecting of *Salmonella* in food [5]. The EIAFoss *Salmonella* Method showed better performance compared with the RV cultural procedure in raw and processed foods [39]. Five commercially available screening methods were compared to the culture methods in naturally contaminated feed samples. The conventional culture method detected 17 (80.9%), MSRV 19 (90.5%), SALMOSYST-Rambach 8 (38.1%), *Salmonella*-Tek 19 (90.5%), Dynabeads anti-*Salmonella* 7 (33.3%) and EIAFoss 21 (100%) of the 21 total *Salmonella* contaminated samples [40]. IMS assay is sample preparation tool based on superparamagnetic particles coated with antibodies [5]. This assay has been developed to reduce the time for the enrichment step prior to detection by capture and isolation intact cells specifically and directly from a complex sample suspension [5,37]. The custom derivatized magnetic beads is "Dynabeads", monosized superparamagnetic polymer particles commercialized by Invitrogen-Dynal [37]. Pathatrix, an automated system, is a patented re-circulating IMS technology and approved by AOAC International [37]. Cudjoe et al. applied Dynabeads Anti-*Salmonella* to IMS followed by plating (IMS-Plating) and immunomagnetic particle based ELISA (IMPELISA) [5,38]. The sensitivity of IMS-Plating was superior to the conventional ISO *Salmonella* method and the modified semi-solid Rappaport-Vassiliadis (MSRV) method [5,38]. The detection rate of IMP-ELISA was comparable to ISO, IMS-Plating and *Salmonella*-Tek™ ELISA (Organon Teknika, USA) [5]. The immune-based detection assay has some limits in sensitivity and specificity for the detection of *Salmonella* spp. in foods. The minimum sensitivity of the assay requires enrichment for production of cell surface antigen and detection [5]. Crossreactivity is also a difficulty. The VIDAS assay was reported that false-positive resulted from some *Citrobacter freundii* strains competing microflora [5]. Irwin et al. [41] reported that the background organisms in ground chicken which adhered to varying degrees to Dynal anti-*Salmonella* and anti-*E. coli* O157, were *Pseudomonas oleovorans*, *Acinetobacter lwoffii*, *Serratia* spp., and one *Rahnella* spp.

### 3. Molecular-based detection methods

The genetic material of each living organism possesses sequences of its nucleotide that are uniquely and specifically present only in its own species [32]. Various nucleic acid-based techniques have been developed, including direct DNA probes, PCR, amplification of the hybridizing, and transcription based amplification [32]. DNA-hybridization and the PCR have been common techniques for detection of pathogenic bacteria [37]. The essential principle of these methods is the specific formation of double stranded nucleic acid from two complementary and single stranded molecules under defined physical and chemical conditions, and this is termed hybridization when performed *in vitro* [5]. In diagnostic assays, one of the strands to serve as a probe or primer is produced in the laboratory, and it is the determinant factor for the specificity of these molecular methods [5,37].

#### 1) DNA-hybridization

Hybridization assay is the most commonly applied technique among the non-PCR-based molecular techniques [37]. It results in positive if hybrids are formed between the labelled probe and the target nucleic acids [5]. The isolation of specific DNA probes for *Salmonella* has been approached by selection and test randomly cloned chromosomal fragments [5]. Between 1983 and 1992, 6 different DNA probes have been published, five of which are cryptic DNA fragments. In 1983, Fitts et al. [42] demonstrated that random chromosomal fragments could be used for specific detection of *Salmonella* directly from non-selective enrichment broths, and radio labeled probes were released commercially by the company Gene-Trak (GeneTrak Systems, USA). Based on collaborative studies of 11 laboratories, the assay generally performed equally to or better than the standard culture method for detection of *Salmonella* in food [5]. The assay was approved for *Salmonella* detection in foods in 1988 by the AOAC [5]. The second-generation Gene-Trak assay uses a sandwich hybridization format and enzyme-mediated colorimetric detection, and target to adjacent sequences of *Salmonella* ribosomal RNA (rRNA) [5]. The next development with the Gene-Trak assay used a direct-labeled probe format (DLP), and microtiter format assays have been developed using this system was named GeneQuence™ [5]. This *Salmonella* assay received AOAC PTM certification after a validation study demonstrating that performance of the method was comparable to that of the BAM/AOAC culture method as well as to the earlier Gene-Trak assay [5]. Numerous probes for DNA hybridization assay specific *Salmonella* spp. including Gene-Trak and GeneQuence™ targeted rRNA. Namimatsu et al. [43] developed a microwell format sandwich hybridization assay for *Salmonella* similar to Gene-Trak assay. Fang et al. [44] described FISH (fluorescent in situ hybridization) targeting rRNA, and development of rRNA-based probes has also been reported. The rRNA based oligonucleotide probe assay must be considered the best evaluated DNA probe assay for *Salmonella* detection [5]. The sensitivity and specificity of the assay is close to the culture method.





## 2) Polymerase chain reaction / Real-time PCR

The PCR technique, first described by Kary Mullis in the mid-1980s is a three-step cyclic *in vitro* procedure based on the ability of the thermo stable DNA polymerase to copy a strand of DNA [5]. Widjoatmodjo et al. [45] developed a PCR assay using primers isolated from replication genes and used specific antibody-coated magnetic particles to concentrate *Salmonella* bacteria from pure and mixed cultures. On spiked samples of chicken meat the detection limit of direct PCR was  $10^7$  CFU/g, while a detection limit of  $10^5$  CFU/g was obtained when the magnetic immune polymerase chain reaction assay (MIPA) technique was used directly on the spiked samples. When pre-enrichment in 24 hours before MIPA was employed, detection limits of 1 CFU/g frozen product and 0.1 CFU/g meat was obtained [5]. With the advent of PCR, there was initial excitement at the prospect of being able to detect pathogens directly from food samples without enrichment [5]. However, there is no practical PCR method at present to recover 1 cell from a 25 g sample without enrichment step [5]. Stevens and Jaykus [46] detected approximately 10 CFU/g *Salmonella enterica* serotype or ser. Enteritidis (*S. Enteritidis*) in yogurt and cheddar cheese following polyethylene glycol concentration and DNA extraction. The first-generation BAX assay, gel-based PCR assay developed in kit form, has been subjected to extensive validation studies [5]. Result of an AOAC PTM validation study reported the test claims of 98% sensitivity [5]. Shear et al. [47] reported that the sensitivity of BAX system was equivalent to that of the BAM/AOAC culture method in testing of fresh produce inoculated with *S. Enteritidis*. In the early 1990s, the second-generation of PCR technologies was introduced by the use of fluorescent double-stranded DNA dyes or DNA probes [37]. Real-time PCR enables both the detection and quantification of a nucleotide signal by continuously measuring fluorescence from a dsDNA-specific dye or hybridization probe that is monitored during the amplification of a target gene [5]. Chen et al. [48] evaluated the TaqMan system for the detection of *Salmonella* that utilizes primers and probes developed from a novel target sequence (*invA*). The detection limit was below 3 CFU/25 g or 25 mL when raw milk, ground beef and ground pork inoculated with *Salmonella* were pre-enriched overnight. Malorny et al. [49] used specifically designed primers and a probe target within the *ttrRSBCA* locus, and included internal amplification control, which is coamplified with the same primers as the *Salmonella* DNA in the assay. The diagnostic accuracy was shown to be 100% compared to the traditional culture method when 110 various food samples (chicken rinses, minced meat, fish, and raw milk) were investigated for *Salmonella* by real-time PCR including a pre-enrichment step in buffered peptone water. The Taqman system that was designed primers and a probe target within the *sefA* has also been used successfully for detection of *Salmonella* Enteritidis [5]. Various types of real-time PCR methods were developed. Many real-time PCR methods based on fluorescent signal generated by binding of intercalating dyes such as SYBR Green I have been reported [5]. The second-generation BAX *Salmonella* test is based on dye binding and melting curve analysis [5]. The sensitivity of BAX methods was statistically equivalent to those of the BAM/AOAC culture method for 5 foods and superior for mozzarella cheese [5]. Another category of real-time PCR involves the use of molecular beacons

that are single hybridization probe labeled at one end with a fluorophore and at the other end with a quencher [5]. A commercial PCR kit using molecular beacons, iQ-Check™ (BioRad Laboratories), evaluated for detection of *Salmonella* in meat and poultry samples [5]. It was demonstrated that the sensitivity of the iQ-Check assay in food was only 87% because diluted DNA was used for PCR reaction to avoid inhibition problem. In Liming and Bhagwat's study [50], and culture-positive samples were positive by iQ-Check assay four types of produce. Another real-time PCR procedure is using fluorescent-labeled probes for detection of amplification products such as fluorescence resonance energy transfer (FRET) between two probes [5]. Commercially available LightCycler *Salmonella* Detection Kit (Roche Diagnostics, Swiss) was evaluated in raw chicken and fried rice after 18 h enrichment in BPW, false negative results were observed at low inoculation levels and internal PCR control reactions were valid in most cases [5]. To rapidly detect multiple pathogens in a single reaction, simultaneous amplification of more than one locus is required [5]. This method is referred to as multiplex PCR which uses numerous primers within a single reaction tube in order to amplify nucleic acid fragments from different targets [32]. Recently, multiplex real-time PCR assays have been applied to detect more than two gene sequences in a single reaction by using spectrally distinct dye-labeled probes (TaqMan system) [5]. There have been numerous studies of multiplex PCR assays followed by discrimination of specific amplification products by colorimetric hybridization, sandwich hybridization assay, and gel electrophoresis [5]. These include assays for 3 *Vibrio* spp., *Escherichia coli*, and *Salmonella*; 3 *Vibrio* spp. and *Salmonella*; *Salmonella* and shiga-like toxin-producing *E. coli*; *Salmonella*, *E. coli* O157:H7 and *Shigella* spp.; *Salmonella*, *E. coli* O157:H7 and *Listeria monocytogenes*; and *Salmonella*, *L. monocytogenes* and *Staphylococcus aureus* [5]. Multiplex real-time PCR have also been a number of formats such as TaqMan system, intercalating dyes, and sandwich hybridization assay [5]. Sharma and Carlson [51] reported a multiplex real-time PCR assay for *Salmonella* and *E. coli* O157:H7 using the TaqMan systems. Wang et al. [52] reported rapid and simultaneous quantitation of *E. coli* O157:H7, *Salmonella*, and *Shigella* in ground beef by multiplex real-time PCR and IMS. *Salmonella* and *L. monocytogenes* were detected using the LightCycler platform on the basis of meting curve analysis [5]. The LabMAP system™ (Luminex, USA) has been used in unique demonstration of multiplex detection of *Salmonella*, *E. coli*, and *L. monocytogenes* by sandwich hybridization using microspheres labeled with florescent dyes [5]. PCR methods (particularly real-time PCR) potentially offer faster detection time and increased accuracy compared with traditional culture method, but some problems still exist with nucleic acid diagnosis [32]. There is always a risk that dead/nonviable cells are detected in PCR assays, so in case of positive PCR results it should be confirm the positive result by the culture-based method [32]. However, PCR methods are generally more sensitive than culture methods, and so pathogens detected by PCR but not culture (defined here as false positives) may be true positives [32]. PCR may fail to detect in samples where the presence of unusually high concentrations of inhibitory compounds such as blood constituents, fats, proteins, bile slats, heavy metals, and others [32]. Rossen et al. [53] reported partial or total inhibition by selective media,



chemical compounds, and substances inherited by different natural food samples. The presence of PCR inhibitors should be monitored by the use of an appropriate internal amplification control in each reaction, and it referred in criteria for standardized PCR-based method for the detection of foodborne pathogens [5]. The inclusion of an internal control is recommended to highlight inhibition of the PCR reaction [5].

#### 4. Aptamer

Recently, there has been an increased interest in overcoming the limitations of immunoassays or nucleic acids-based techniques by using alternative recognition species which could assure the same sensitivity and selectivity and present a higher stability and facility of production [54]. Aptamers are artificial specific single stranded oligonucleotides, DNA or RNA, with the capability of binding specifically to non-nucleic acid target molecules, such as peptides, proteins, drugs, organic and inorganic molecules, and whole cells [5,54]. The aptamers have unique characteristics over more antibodies used for immunology-based methods [5]. The main advantage is that the aptamers can be easily achieved by chemical synthesis and the overcoming of the use of animals or cell lines for the production [54]. Therefore, the various molecules could be target even non-immunogenic molecules. The aptamers are stable in high temperature and chemical modifications and not restricted in physiological condition to selection process [5]. In addition, the aptamers have advantages to overcome drawbacks of nucleic acid-based methods. The nucleic acid-based method is sensitive and specific method but detect only amplified nucleic acid not proteins [5]. In addition, PCR analysis should contain DNA or RNA sample preparation steps, and could generate false-negative results by compounds contaminated the DNA templates that inhibit PCR reaction and PCR reaction error [5]. The application of aptamers to detection method of pathogen takes advantage of the high affinity and tuneable properties of aptamers [5]. The aptamers are also reusable and their small size and versatility allow efficient immobilizations, labelling and high-density monolayers in miniaturized systems such as biosensors [54]. Aptamers offer very interesting applications in therapeutics, diagnostics and bioanalytical applications. However, to date only limited studies have reported the possibility of using aptamers in assays for the analysis of environmental or food samples. The primary limitation on applicability of aptamers in bioanalytical methods has been stability and nuclease sensitivity particularly with reference to RNA rather than DNA aptamers [54]. The hydroxyl group at the 2'-position of the pyrimidine moiety attacks the neighbouring phosphodiester bond, then produces a cyclic 2', 3'-phosphate and break the nucleic acid backbone [5]. This reaction is catalyzed by neutral pH, many transition metal ions, and a range of ribonucleases present in biological samples [5]. However, various routes to stabilize the aptamers have been explored, and the stability of such molecules can be improved by chemical modification of either the oligonucleotide backbone or of the 2'-position of the pyrimidine moiety [5]. Various modifications of nucleotides were introduced either at the phosphate/ribose backbone or at the nucleobases [5]. The most prominent modification of aptamers is the derivatization of the 2"-ribose, and 2"-fluoro- and 2"-amino-2"-deoxy pyrimidine triphosphates have frequently been used

for the direct selection of nuclease stabilized RNA aptamers [5,55]. In addition, the replacement of the DNA phosphate backbone by a phosphorothioate has enhanced the stability against nucleases and the cellular availability of such molecules [5]. With use of  $\alpha$ -thio-substituted deoxynucleoside triphosphates, “thio-RNA” aptamers were successfully screened for aptamers [5]. A different approach to stabilize aptamers comes from chemical synthesis of spiegelmers. Spiegelmers is the mirror-image aptamer that is substitute form of the natural D-ribose with L-ribose, and bind specifically to a target but are not recognized by ribonucleases [5]. Several spiegelmers that bind to various target molecules have been developed, such as n-octanoyl ghrelin, a potent stimulant of growth hormone release, calcitonin gene-related peptide, staphylococcal enterotoxin B and the neuropeptide nociceptin [5]. The spiegelmers are ideal candidates for *in vitro* and *in vivo* diagnostics and are being produced and commercialized by the German company Noxxon [56]. Systemic Evolution of Ligands by Exponential Enrichment (SELEX) is a relatively new and revolutionary method that involves the progressive selection of aptamers by repeated rounds from a random oligonucleic acid library [5]. There have been reported various type of SELEX procedure such as nitrocellulose filtration using affinity surface, affinity tags, magnetic bead, column matrices, and so on [5]. To develop aptamer for pathogenic microorganisms, whole-cell and non-whole-cell targeting approaches have been applied in microbial SELEX [5]. In most previous studies, non-whole-cell targeting approaches were utilized to develop aptamer for microorganisms, a structural protein PilS of the type IVB pili of *Salmonella* Typhi, and surface membrane proteins of *C. jejuni* [5]. However, recently, Cao et al. [57] developed ssDNA aptamers specific against *S.aureus* by whole-cell targeting SELEX, and Chen et al. [58] found ssDNA aptamers with potential therapeutic application for *Mycobacterium tuberculosis* using whole-cell targeting SELEX. Pan et al. [59] focused on *S. Typhi* which is an important pathogen for humans and causes typhoid or enteric fever. They reported for the first time the direct selection of aptamers for type IVB pilus which is important in the pathogenic process, bacterial invasion. The aptamer have a high affinity with a Kd value around 8 nM and to inhibit the bacterium action with a reduction of cell invasion. In another study focused on aptamer against *Salmonella* spp. DNA aptamers were selected and evaluated for the capture and detection of outer membrane proteins (OMPs) of *S. Typhimurium* [5]. Two candidates showed low-end detection limits of 10-40 CFU, and one of them was bound to magnetic beads and used for the capture of 10-10<sup>2</sup> CFU of *S. Typhimurium* in 9 mL of whole carcass chicken rinse samples. Another aptamer for pathogens has been developed for MgCl<sub>2</sub>-extracted surface proteins from *C. jejuni* by a magnetic bead [5]. The selected DNA aptamer can detect both heat-killed and live *C. jejuni* as low as an average of 2.5 CFU in buffer and 10-250 CFU in various food matrices and exhibits low cross-reactivity *C. coli* and *C. lari*. In Cao et al.’s study [57], five ssDNA aptamers demonstrated high specificity and affinity to *S. aureus* individually, but combining these five aptamers had a much better effect than the individual aptamer in the recognition of different *S. aureus* strains. A number of studies have been reported aptamer application in various areas, and most attention has been found in the fields of reporter-linked aptamer assay, affinity chromatography, and



biosensors, although several reports on their use in affinity PCR and ligation assays have also appeared [5,55]. However, compared to therapeutic field, the field of analytical assay is still under the supremacy of immunoassays, but deep analytical studies are now demonstrating that some of the limitations of these conventional assays can be circumvented by alternative recognition reagents such as aptamers.

## Subtyping Methods for *Salmonella*

Typing technologies are essential for bacterial source tracking and to determine the distribution of pathogens isolated from ill people [60]. Traditional typing methods based on their phenotypic traits, such as biotyping, antibiotic susceptibility profiles, and serotyping and phage typing of isolates provide insufficient information for epidemiological purposes [60,61]. Molecular subtyping methods have revolutionized the fingerprinting of microbial strains, but most of them have not been internationally standardized [61]. The subtyping methods have been developed based on three main mechanisms of discrimination: restriction analysis of the bacterial DNA, PCR amplification of particular genetic targets, and the identification of DNA sequence polymorphism at specific loci in the genome [60]. It is difficult to select the one most applicable for epidemiological investigations. Ideally, the typing method should be inexpensive, easy to use and interpret, and have high typeability, reproducibility and discriminatory power [61].

### 1. Pulsed-field gel electrophoresis

PFGE is a form of restriction fragment length polymorphism (RFLP) analysis typing, and restriction patterns of whole bacterial genomes are analyzed and compared [60,61]. The bacterial chromosome is digested by a selected rare cutting enzyme to yield a moderate a smaller number of DNA fragments of a wide range of sizes [60,61]. Differences in the restriction profiles that can be visualized using specialized electrophoresis techniques are used to carry out genetic comparisons among isolates [60,61]. Currently, PFGE is often considered the gold standard of molecular typing methods for bacterial foodborne pathogens, such as *Salmonella*, *E. coli*, *Campylobacter*, *Listeria*, *Yersinia*, and *Vibrio* [60,61]. It is used by the PulseNet program, a molecular subtyping network for foodborne bacterial disease surveillance that is maintained at the CDC, to identify widespread outbreaks of bacterial foodborne illness [5]. In general, the bacteria are immobilized by mixing the bacterial suspension with melted agarose prior to cell lysis to protect the chromosomal DNA from mechanical breakage that can occur with the manipulation of free DNA [60,61]. The embedded cells are lysed and the released DNA is immobilized in the agarose plugs [60]. The purified DNA from the agarose plugs is digested with a rare cutting restriction enzyme [60,61]. The plugs containing the immobilized DNA are then added to an agarose gel to electrophoresis [60,61]. PFGE has been successful in typing *Salmonella* from foods, food animal sources, and human patients [60,61]. The choice of restriction enzyme is usually depend on experience of scientists, but the most commonly used enzymes in *Salmonella* have been *Xba*I, *Spe*I and *Not*I [5]. Nayak and Stewart-King [62] successfully performed tracking the bacterial

horizontal transmission pathways of *Salmonella enterica serovars* in a preharvest turkey production environment by XbaI-digested pulsed-field gel electrophoresis (PFGE) macrorestriction profiles. Foley et al. [63] evaluated a number of typing methods to distinguish among *S. Typhimurium* isolates of cattle, pigs, chickens, and turkeys or derived food products and found that both PFGE and MLST provide good discriminatory power to differentiate isolates. A particular disadvantage of the PFGE method has previously been that it is time consuming, often taking three days to complete, labour intensive, and equipment costs can approach \$20,000 for the gel system alone with costs increasing significantly when computer imaging and data analysis systems are included [61]. In addition, some strains are not typeable since the DNA of these strains is degraded during electrophoresis [61]. Some serotypes of *Salmonella* with certain distinct phage types can be so genetically homogeneous that multiple genotypic techniques fail to discriminate outbreak from non-outbreak strains [5]. Ahmed et al. [64] evaluated PFGE to differentiate *S. Enteritidis* DT8 strains that developed during a Canada-wide outbreak of gastroenteritis. Phage typing and PFGE linked the clinical and cheese isolates of *S. Enteritidis* but failed to differentiate outbreak from non-outbreak strains [64]. Successful discrimination was only achieved with a combination of intensive epidemiological, and bacteriological typing methods [64].

## 2. Repetitive sequence-based PCR

Repetitive sequence-based PCR is amplification-based method, which utilizes the fact that many bacterial species have repeated DNA sequence elements distributed throughout their genomes [60]. Rep-PCR uses primers for noncoding repetitive sequences and produces DNA fragments that can be separated by electrophoresis [5]. Banding pattern diversity can result from differences in the number of repetitive elements due to their positions within the bacterial genome and compared to one another to determine genetic relatedness [5,60]. Multiple repeat sequences including enterobacterial repetitive intergenic consensus (ERIC), repetitive extragenic palindromic (REP) and BOX sequences have been targeted for rep-PCR [38]. The REP element, ERIC sequence and BOX A elements have been identified in *Salmonella* [5]. Johnson et al. [65] reported that rep-PCR was quite effective at serotype discrimination within isolates of analyze *Salmonella* Infantis causing a hospital cafeteria associated outbreak of gastroenteritis. In Weigel et al.'s study [66], repPCR and PFGE analyses have been compared, and the rep-PCR method provided highly reproducible fragmentation patterns and greater discriminative power for closely related *Salmonella* isolates. In addition, Kilic et al. [67] investigated a large food-borne outbreak associated with eggs contaminated by *S. Enteritidis* in a military unit using PFGE and the rep-PCR employing the DiversiLab system. PFGE and repPCR results were concordant for *S. Enteritidis* isolates from patients and food samples associated with a foodborne outbreak. Not all serotypes of *Salmonella* appear to be separable by rep-PCR [5]. Wise et al. [68] created a library of rep-PCR patterns from 14 *Salmonella* serotypes and successfully determined the serotype of unknown *Salmonella* isolates by comparison with the library. Milleman et al. [69] discovered a common ERIC PCR pattern shared by *S. Typhimurium* and *S. Enteritidis*



strains. Rep-PCR appears to be a screening tool and surrogate serotyping method to analyze a specific outbreak setting since ribotype or PFGE analyses often are better able to discriminate isolates to a subserotype level [69]. Therefore, combinations of many subtyping techniques with or without traditional phenotypic methods are frequently superior to the use of a single technique [5]. As with many of the amplification-based typing methods, rep-PCR can be performed in a relatively short amount of time and require a minimum amount of DNA for typing than other methods [60]. Rep-PCR can have some problems with reproducibility and intra-laboratory variability if there is variability in reagents, thermal cycling and gel electrophoresis [60]. The development of a commercially available, semi-automated rep-PCR assay system, the DiversiLab System, offers advances in standardization and reproducibility over manual, gel-based rep-PCR [5,67-69].

## Conflict of Interest

The authors declare no potential conflict of interest.

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