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A review of culture-dependent and molecular methods for detection of *Salmonella* in food safety

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Abstract

Salmonella is the well-recognized foodborne pathogen leading the most research and surveillance attention especially from government agencies as well as in food industry. In Malaysia, *Salmonella* is one of the main bacteria which monitored by the National Laboratory Surveillance System. Previously, standard culture methods have always been employed by many laboratories for *Salmonella* detection in Food Surveillance Programs. However, more advanced detection methods will be needed to improve the sensitivity and specificity of *Salmonella* identification. In this review, *Salmonella* detection methods including conventional and recent advances in molecular-based methods will be discussed.

1. Introduction

Foodborne infectious disease caused by bacterial pathogens is a major health problem worldwide. Among the foodborne pathogens, *Salmonella* is the most common bacteria associated with outbreaks known as salmonellosis, and is contracted through consumption of contaminated animal products, which always results either from infected animals used in food production or from contamination of the carcasses (Cheung and Kam, 2012; Park *et al.,* 2014). Based on surveillance studies, the main vehicles of *Salmonella* infection include raw meat, eggs and poultry products (de Freitas *et al.,* 2010; Sallam *et al.,* 2014). Of greater consequence is that fruits and vegetables have gained notoriety in recent years as vehicles of human salmonellosis (Mritunjay and Kumar, 2015). The prevailing hygienic conditions especially in

tropical and subtropical countries during the production, harvesting and distribution of fresh and dehydrated fruits and vegetables do not always meet minimum standards and may facilitate product contamination. For example, *Salmonella* spp. and *Salmonella enterica* serotype Typhimurium were detected in sliced fruits, like papaya, mango, jackfruit, watermelon, sapodilla, honeydew and dragon fruit (Pui *et al.,* 2011), and local salad known as *ulam* (e.g., *selom, pegaga nyonya, kacang panjang* and *kacang botol*) (Najwa *et al.,* 2015).

Rapid and reliable detection methods such as standard culture methods coupled with molecular techniques are necessary to control the safety of food. Typically, rapid methods have become increasingly popular among food testing laboratories and some of them have been accepted by international authorities as

standard methods after thorough evaluation and validation. Therefore, different aspects of *Salmonella* detection and identification methodologies will be discussed in this review article.

2. Detection and enumeration of *Salmonella*

Detection and enumeration of *Salmonella* in foods and on surfaces that come into contact with food are an important component for ensuring food safety. For instance, conventional detection methods which provide both qualitative and quantitative information are used to isolate and enumerate viable bacterial cells in foods (de Boer and Beumer, 1999). Recently, considerable efforts have been made to improve the detection of *Salmonella* in different food ingredients by employing molecular detection method (Cheung and Kam, 2012). Hence, microbiological analysis comprises methods for quantitative detection is often used by both government authorities and food companies to monitor the state of contamination as well as to detect emerging risks (Park *et al.,* 2014).

2.1 Conventional methods

Conventional culture methods acquired good reputation as standard techniques due to their selectivity and sensitivity (Taskila *et al.,* 2012). Moreover, conventional test methods are simple, very practical and easily adaptable. Based on the standard ISO 6579:2002, detection process was involved in four stages: (a) non-selective pre-enrichment, (b) selective enrichment, (c) plating on selective media, and (d) biochemical and serological tests.

Prior to the enrichment cultivation, *Salmonella* could be injured during handling procedures and/or food processing, such as freezing, refrigeration, irradiation, drying, thermal treatment, low water activity, acidity, and starvation (Gracias and McKillip, 2004; Shintani, 2006). Thus, the first-line recovery of injured cells needs to be performed in a non-selective pre-enrichment step during the initial analysis. Buffered peptone water (BPW) contains sodium chloride and phosphates for the purpose of ensuring buffering capacity and maintaining osmotic balance, respectively. Due to low amounts of the target bacterial cells present in mixed microbial population, BPW often used to increase it (Taskila *et al.*, 2012). Since non-selective media contain peptones (main sources of nitrogen and carbon), it might support or enhance other microbes to grow. Therefore, variety selective enrichment broths such as selenite cystine broth (SC), tetrathionate broth (TT), Rappaport Vassiliadis soy broth (RVS) and others have been developed for *Salmonella* isolation purposes (Taskila *et al.,* 2012). RVS was reported as the best selective enrichment broth

for *Salmonella* cultivation where it contains high concentration of malachite green and magnesium chloride which could be used to inhibit the growth of other microbial (Schönenbrücher *et al.,* 2008).

Selective agar media such as brilliant green (BG) agar, bismuth sulphite (BS) agar and xylose lysine desoxycholate (XLD) agar are commonly used to differentiate and to receive presumptive *Salmonella* colonies (Sallam *et al.,* 2014). Previously, chromogenic media such as CHROMagar *Salmonella*, chromogenic *Salmonella* esterase (CSE) agar, and alpha-beta chromogenic medium (ABC medium) have been developed to improve the selectivity (Perry and Freydière, 2007). In another study, researchers found that several chromogenic plating media such as Miller-Mallinson agar (MM), AES *Salmonella* agar plate (ASAP) and oxoid *Salmonella* chromogen media (OSCM) did not show any detection problems for false-positive results in contrast to BG, XLD and xylose lysine tergitol 4 (XLT4) agar (Schönenbrücher *et al.,* 2008).

Preliminary identification of *Salmonella* is often performed by biochemical tests, which may include the negative urease reaction, H_2S production, fermentation of dulcitol and glucose (Mirmomeni *et al.,* 2009), whereas confirmation of the *Salmonella* genus is determined by serological tests using somatic (O), flagellar (H) and capsular (Vi) antigens and their corresponding antibodies (Sallam *et al.,* 2014). For instance, identification via biochemically and serologically was reported on various *Salmonella* serovars such as Agona, Arizonae, Bardo, Cerro, Dublin, Hadar, Gallinarum, Kentucky, Paratyphi A, Enteritidis, Typhimurium, Heidelberg and Virginia (Moussa *et al.,* 2012).

On the other hand, the most probable number (MPN) method is widely applied to enumerate low concentration of microorganisms with <10-100 MPN/g (Malorny *et al.,* 2008). It appears particularly interesting as it provides quantitative data, ideally for estimating the numbers of viable bacteria which are distributed randomly within the sample. For example, researchers had employed MPN method to enumerate *S. enterica* serovar Typhimurium and *S. enterica* serovar Typhi in sliced fruits with estimated quantity ranging from 0 to 19 MPN/g (Pui *et al.,* 2011). Similarly, MPN method was used to determine the density of *S. enterica* serovar Enteritidis and *S. enterica* serovar Typhimurium concentration in retail beef (Thung *et al.,* 2018) and chicken meat (Thung *et al.,* 2016). Recently, Tan *et al.* (2019) have also described the use of MPN method in the enumeration of *Salmonella* spp. in raw beef from wet market and hypermarket.

2.2 Molecular detection methods

The advent of nucleic acid-based technologies, which referred to as rapid method, has improved sensitivity and specificity for detecting foodborne *Salmonella* with enhanced analytical performance and testing efficiency (Cheung and Kam, 2012). Molecular techniques are able to detect the target pathogens until defining subtle genetic differences at the strain level although the pathogens present in complex biological environments (Park *et al.,* 2014). Therefore, these rapid detection methods could be of high value to the food industry.

2.2.1 Polymerase chain reaction (PCR)

Since the 1990s, molecular-based PCR assays are commonly used for the detection of *Salmonella* pure isolates from different samples (Soumet *et al.,* 1999; Abouzeed *et al.,* 2000). For instance, PCR assays have been used to detect selected virulence or antibiotic resistance genes along with genus-, species-, and/or serotype-specific genes of *Salmonella* in poultry and poultry products (Maciorowski *et al.,* 2000; Oliveira *et al.,* 2002). PCR methods employ several primer pairs which specific to *Salmonella* spp. genes, resulting in high sensitivity for the detection of *Salmonella* (Table 1).

The essential for detecting low levels of *Salmonella* and the non-even distribution of the bacterial cells in the samples, an enrichment step is usually required prior to PCR assay. It has been proposed that the use of culture enrichment prior to PCR analysis serves the following purposes, (i) multiplication of the target bacterial cells to provide detectable concentrations, (ii) dilution of dead cells, (iii) dilution of PCR-inhibitory substances such as detergents, organic and inorganic chemicals, polysaccharides, fats and denatured proteins present in the sample matrix, and (iv) the possibility of isolating the target bacterial cells for complementary tests (Cheung and Kam, 2012). For instance, PCR assay failed to detect positive samples when no pre-enrichment step was carried out for the detection of *Salmonella* in raw poultry tissue samples of naturally contaminated (Myint *et al.,* 2006), but *Salmonella* was successfully detected after the enrichment cultivation step. In another study, a PCR assay incorporated with several DNA extraction methods was used for *Salmonella* detection in poultry samples (Soumet *et al.,* 1994). They found that the presence of *Salmonella* was detected using PCR associated with an enrichment cultivation step for 10 hrs. Besides, detection of *Salmonella* at different enrichment times in poultry dietary samples using PCR was also investigated by Maciorowski *et al.* (2000). Noteworthy, one of the major factors affecting the efficiency of PCR is depending on the quality of the target DNA. However, the presence of contaminated DNA had resulted in poor efficiency of the PCR (Wegener *et al.,* 2003). Therefore, developments of other sample preparation methods based on the sample matrix type are indispensable to improve PCR detection.

2.2.2 Multiplex PCR (mPCR)

The mPCR is a simultaneous detection for multiple targets DNA sequence occurs within a single reaction. It has been widely used for simultaneous detection of different pathogens or virulence factors, as well as applied in nucleic acid diagnostics field, which includes gene deletion and mutation analysis (Xu *et al.,* 2013).

Table 1. Sequences of oligonucleotide primers used for detection of *Salmonella*

Target gene	Primer	Sequence (5'-3')	Size (bp)	Reference
Random sequence	ST11	GCCAACCATTGCTAAATTGGCGCA	429	Soumet *et al.* (1999)
	ST15	GGTAGAAATTCCCAGCGGGTACTGG		
fliC	Fli15	CGGTGTTGCCCAGGT TGGTAAT	620	Oliveira *et al.* (2002)
	Typ04	ACTGGTAAAGATGGCT		
SdfI	ENTF	TGTGTTTTATCTGATGCAAGAGG	304	de Freitas *et al.* (2010)
	ENTR	TGAACTACGTTCGTTCTTCTGG		
Spy	TyphF	TTGTTCACTTTTTACCCCTGAA	401	de Freitas *et al.* (2010)
	TyphR	CCCTGACAGCCGTTAGATATT		
ViaB	ViaBF	CACGCACCATCATTTCACCG	738	de Freitas *et al.* (2010)
	ViaBR	AACAGGCTGTAGCGATTTAGG		
23S rRNA	Sty-1	TGCCGGAAACGAATCT	300	Pui *et al.* (2011)
	Sty-2	GGTTGTCATGCCAATGCACT		
floR	floRF	GAGATCGGATTCAGCTTTGC	198	Deekshit *et al.* (2013)
	floRR	CGGTAGGATGAAGGTGAGGA		
invA	invAF	GTGAAATTATCGCCACGTTCGGGCAA	284	Deekshit *et al.* (2013)
	invAR	TCATCGCACCGTCAAAGGAACC		
ssaT (SPI-2)	ssaTF	ATGCGCAAATAATGGTTGAT	780	Deekshit *et al.* (2013)
	ssaTR	TCAGGCGCGTTAACAGGACG		
spvC	spvCF	CGGAAATACCATCAAATA	669	Thung *et al.* (2018)
	spvCR	CCCAAACCCATACTTACTCTG		
ompC	ompCF	ATCGCTGACTTATGCAATCG	204	Tan *et al.* (2019)
	ompCR	CGGGTTGCGTTATAGGTCTG		

Also, mPCR was used for screening of single nucleotide polymorphism (SNP) (Hayden *et al.,* 2008). High compatibility of the primers and the reaction mixture is of great significance for mPCR, especially in the detection of *Salmonella* simultaneously with other pathogens in food samples. For example, four sets of specific primers were used in mPCR to detect *Escherichia coli* O157: H7, *Salmonella* spp., *Staphylococcus aureus*, and *Listeria monocytogenes* in kimchi products (Park *et al.,* 2006). Similarly, the detection of *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella* spp. in meat and meat products, *Vibrio parahaemolyticus* and *Vibrio cholerae* in seafood and seafood products, and *Staphylococcus aureus* in ready-to-eat foods was also performed by using six pairs of primers in the mPCR assay (Lei *et al.,* 2008). On the other hand, mPCR assay appears to be a more reliable method for identification of specific *Salmonella* serotypes, as compared to other assays by using phenotypic serotyping methods. In the study of Moussa *et al.* (2012), mPCR was used for molecular typing of *Salmonella enterica* serovars, especially in detecting *S. enterica* serovar Enteritidis and *S. enterica* serovar Typhimurium. It has also been widely used for differentiation of the Enteritidis and Typhimurium serovars in meat samples by other researchers. For example, mPCR was successfully adapted to identify *S. enterica* serovar Enteritidis, *S. enterica* serovar Typhi and *S. enterica* serovar Typhimurium in chicken meat with the amplification of *SdfI* gene, *ViaB* gene and Spy gene, respectively (de Freitas *et al.,* 2010). Meanwhile, Deekshit *et al.* (2013) had employed mPCR for the detection of pathogenicity island 2 (SPI-2) genes in *Salmonella* from seafood.

3.   Whole genome sequencing (WGS) of *Salmonella*

To date, WGS technology is used to evaluate and identify new species based on the entire DNA sequence of a bacterium thereby making it as a good surveillance tool (Ricke *et al.,* 2018). WGS can be used to characterize individual microbes which include the full complement of resistance determinants, providing definite genotype information (McDermott *et al.,* 2016). For example, WGS (Illumina HiSeq 2000 high throughput sequencing system) was used to analyze *S. enterica* serovar Weltevreden strains which isolated from contaminated food samples and human stool, where antimicrobial resistance (AMR) genes linked to resistance to ampicillin, ciprofloxacin or tetracycline was identified (Li *et al.,* 2018). Previously, Wang *et al.* (2017) performed a WGS (PacBioRS II system) study analyzing *Salmonella* Indiana strain D90 isolated from the chicken carcass in China. They found that 24 multi-drug resistance (MDR) genes, located on 4 plasmids,

were identified, including the *mcr-1* gene related to colistin resistance. Concurrently, a WGS approach was used to study cefoxitin-resistant *Salmonella enterica* serovar Heidelberg isolates from retail poultry, abattoir poultry and humans (Edirmanasinghe *et al.,* 2017). They found that transmission of a common antimicrobial resistance plasmid (CMY-2) which linked to microbial resistance against β-lactamic antibiotics might occur among *Salmonella* Heidelberg strains with different genetic backgrounds. While the application of WGS coupled with specific computer programs might improve the interpretation of the heterogeneity typically in *Salmonella* genome classification. For instance, a software program Panseq was developed for the analyses of genomes as well as to identify bacterial strain (species -, subspecies- and serovar) using a SuperPhy platform (Laing *et al.,* 2017).

Conflict of Interest
The authors declare no conflict of interest.

Acknowledgments

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