Chapter 5

Current and Emerging Innovations for Detection of Food-Borne *Salmonella*

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Abstract

Salmonella is one of the leading causes of food-borne illnesses worldwide, and one of the main contributors to salmonellosis is the consumption of contaminated egg, poultry, pork, beef, and milk products. Since deleterious effects of *Salmonella* on public health and the economy continue to occur, improving safety of food products by early detection of food-borne pathogens would be considered an important component for limiting exposure to *Salmonella* contamination. Therefore, there is an ongoing need to develop more advanced detection methods that can identify *Salmonella* accurately and rapidly in foods before they reach consumers. In the past three decades, there have been increasing efforts toward developing and improving rapid pathogen detection and characterization methodologies for application to food products. In this chapter, we discuss molecular methods for detection, identification, and genetic characterization of *Salmonella* in food. In addition, the advantages and disadvantages of the established and emerging rapid detection methods are addressed here. The methods with potential application to the industry are highlighted in this chapter.

Keywords: *Salmonella*, food-borne pathogens, rapid detection, molecular methods, aptamer, antibody

1. Introduction

Food-borne disease is one of the major public health problems for the food industry, especially in developing countries [1]. Failure to detect food-borne pathogens may lead to a dreadful effect. The World Health Organization (WHO) reported that in 2010 alone 1.8 million people died from diarrheal diseases, a great proportion of these cases can be attributed to contaminated food and drinking water [2]. The Centers for Disease Control and Prevention

(CDC) have estimated that 48 million cases of food-borne illnesses occur in the United States (US) annually, approximately 128,000 cases require hospitalization, and 3,000 cases result in death [3]. The CDC reported that viruses, bacteria, and parasites are major causative agents for food-borne illnesses. Among these, bacterial agents including *Salmonella*, *Listeria monocytogenes*, and *Escherichia coli* are associated with these cases, being responsible for most of the hospitalizations (63.9%) and deaths (63.7%). Especially, *Salmonella* species were considered as the leading cause for these more severe cases resulting in 35% of the hospitalizations and 28% of the deaths [4]. *Salmonella*, belonging to the family of *Enterobacteriaceae*, are Gram-negative, facultative anaerobic, and nonspore-forming bacilli. The genus *Salmonella* is consisted of two species, *enterica* and *bongori*, with six subspecies of *S. enterica*. The different serotypes are divided based on the specific surface molecules O-antigen (O-Ag) and H-antigen (H-Ag) [5]. Collectively, there are over 2500 serotypes of salmonellae capable of causing disease in humans. Most serotypes of the salmonellae could cause gastroenteritis, while a few serotypes of salmonellae would cause severe disease enteric fever, which was characterized as the onset of high fever accompanied with abdominal pain and malaise without diarrhea or vomiting [6]. Commonly, salmonellosis is self-limiting, resolving in about a week. Occasionally, however, the infection becomes systemic, a much more severe disease requiring antibiotic interventions [7]. The dose of *Salmonella* causing infection in humans indicated a wide range for the number of cells required to cause disease, ranged from 10^5 to 10^{10} cells. In contrast, enumeration of food products indicate much lower numbers of organisms, as low as ten cells, were present to cause illness [8, 9].

Most human salmonellosis cases are associated with consumption of contaminated egg, poultry, pork, beef, and milk products, which are considered one of the most important reservoirs from which *Salmonella* is passed through the food chain and ultimately transmitted to humans [10]. With increasing consumption of these food products, the number of associated salmonellosis continues to be a public health issue all around the world. It is estimated that 95% of *Salmonella* infections are due to the consumption of contaminated foodstuffs, which suggest that salmonellae may be present at low levels in food but still capable of causing a significant number of infections [11]. Yearly, in the United States, it is estimated that *Salmonella* is responsible for over a million illnesses, 19,000 hospitalizations, and almost 400 deaths. This is in part due to their marked ability to persist in a wide range of varying environmental conditions [12]. For example, *Salmonella* strains can grow in foods stored at low (2–4°C) and high (54°C) temperatures [13].

Since *Salmonella* is a major causative agent for food-associated food-borne illnesses, improving safety of poultry products by early detection of food-borne pathogens would be considered an important component for limiting exposure to *Salmonella* contamination. In order to safeguard the food supply and ensure public health, it is essential to establish rapid, reliable, and sensitive method for *Salmonella* detection. In the past two decades, there has been a thrust to develop rapid methods for identifying and detecting *Salmonella* specifically in foodstuffs [14–17]. This chapter will focus on the current culture-dependent and culture-independent methods for the rapid, accurate detection, identification, and subtyping of salmonellae in foodstuffs.

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2. Methodologies for detection of *Salmonella*

2.1. Culture-dependent methods

Current testing of food samples for the presence of salmonellae can be divided into three steps: (1) detection of pathogen by plate culture, (2) identification of the isolate and its specific serovar designation, and (3) subtyping of the isolate for association with salmonellosis [18, 19]. These methods rely on traditional bacterial culture procedures that apply serial enrichments with increasing selectivity culminating in the isolation of *Salmonella* on selective differential agar plates (**Figure 1**). It always takes up to 5 days to obtain a presumptive positive result. Then traditional biochemical testing of nutrient utilization medium is needed for confirmation, another few days to complete [20]. Although innovative technologies have been applied to subtype salmonellae isolation, at least 24 h is needed for a confirmation of *Salmonella* in multiple analytes. DNA fingerprinting techniques are based on DNA size differences on an agarose gel. The digested genomic DNA of target bacteria is separated on an agarose gel and then hybridized with complementary sequences for identifying the banding pattern. A database of fingerprint species, serovar, and strain identifications is used for comparison [21–23]. The fingerprinting methods include pulsed-field gel electrophoresis (PFGE), ribotyping, and intergenic sequence (IGS) ribotyping. The use of PFGE has greatly increased the ability of track and trace back illness clusters and outbreaks. However, PFGE still requires a pure isolate and a minimum of 3 days to complete [24, 25].

Due to its sensitivity, with a limit of detection of 1 cfu, this analytical schema is considered as the "gold standard" of regulatory agencies (**Figure 1**). The disadvantages of this method are as follows. First, it is time-consuming, taking at least a week for isolation and few more days for serotyping and subtyping. The long time frame hampers its application in many food commodities, especially fresh products, before they are consumed or on hold in warehouses while awaiting test results before they spoil. Second, the operation is tedious; the amount of media and numerous plates are required for each sample. The procedures are labor-consuming and necessitate large areas of space, particularly in many sample detections. Finally, the complex ingredients in foodstuffs, such as indigenous microbiota and antimicrobials, make it notably difficult for traditional microbiological methods [11, 26–29].

2.2. Culture-independent methods

Recent advances in technology have made the detection of food-borne pathogens more rapid and convenient, while achieving improved sensitivity and specificity in comparison to conventional methods. These methods employing newer technologies are generally referred as "rapid methods," which include nucleic acid-based or antibody-based assays that are modified or improved compared to conventional methods [30–35]. These rapid detection methods can be of high value to the food industry by providing several key advantages such as speed, specificity, sensitivity, cost-efficiency, and labor efficiency.

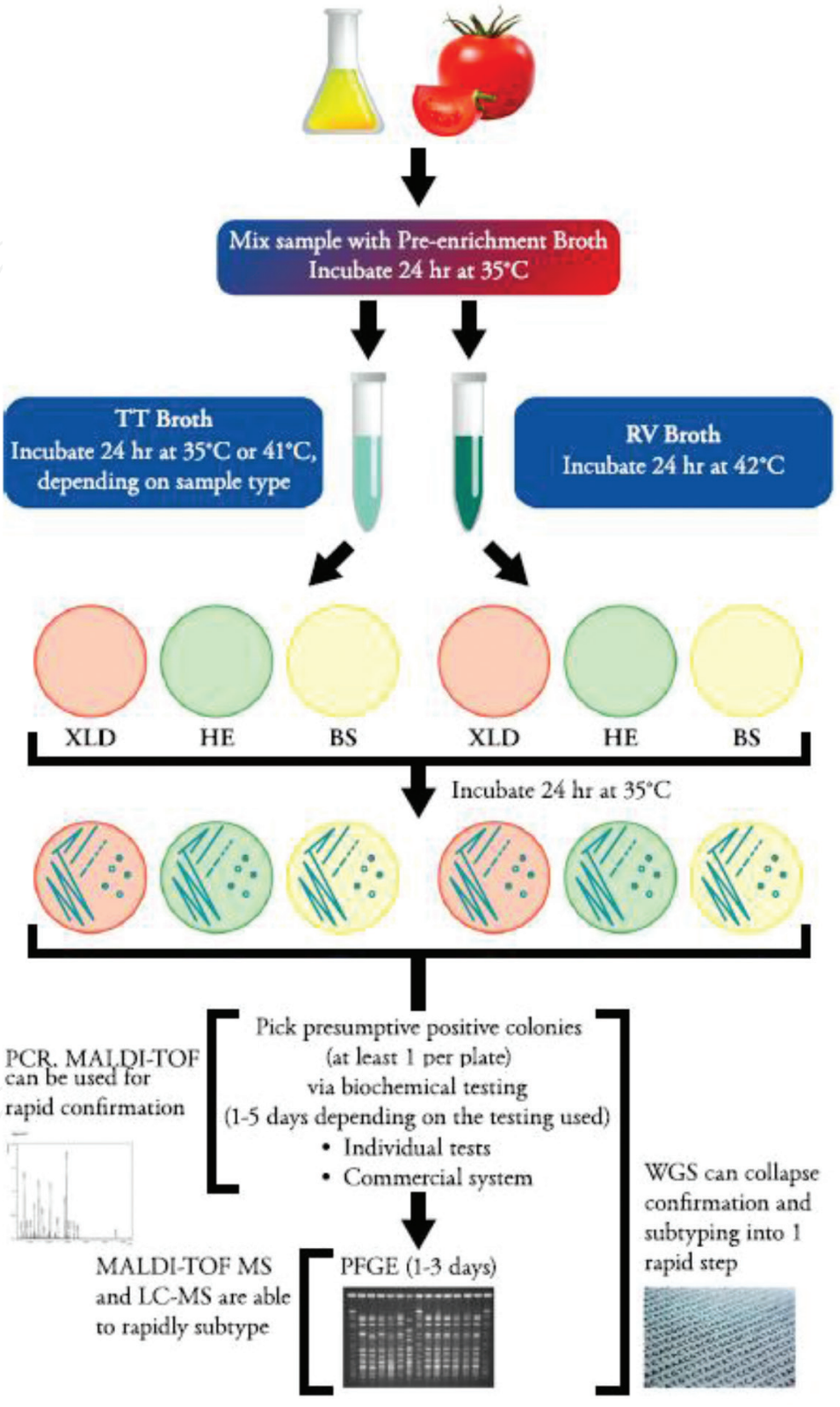

Figure 1. Overview of Bacteriological Analytical Manual (FDA-BAM) workflow for the detection, isolation, and subtyping of *Salmonella*. It takes 5 days for the detection and isolation of *Salmonella*, and a week more for subsequent confirmation and subtyping recent molecular methods, such as MS, WGS, and PCR/qPCR, may shorten the result time [36].

2.2.1. Polymerase chain reaction (PCR)

The largest advance toward faster detection of salmonellae has been in the realm of molecular biology, where polymerase chain reaction (PCR) and quantitative PCR (qPCR) are predominantly being applied as the methods of choice for the detection. Different protocols targeting different specific genes or gene regions specific to salmonellae have been published. Numerous studies have been conducted to detect and characterize *Salmonella* in poultry, poultry products, and feeds using PCR assays to target selected antibiotic resistance or virulence genes along with genus-, species-, and serotype-specific genes [16, 37–40].

Over the past years, PCR-based methods have advanced to provide high sensitivity for *Salmonella* detection and identification. Aabo et al. used PCR assay for *Salmonella* detection in minced meat and compared this method to a culture-based methodology. The sensitivity of the PCR was 89% (85 out of 96 samples), which was much higher than that of the culture method (50%, 48 out of 96 samples) [41]. Rychlik et al. established nested PCR with high sensitivity, which has a higher annealing temperature than the primers used in the first PCR, to detect *Salmonella* in chicken feces [42].

As we all know, the quality and quantity of target DNA, PCR template, are important factors during the design of a PCR assay. Although well-designed PCR primer and good PCR template can bring high specificity of the target detection, it is still not sufficient to overcome the side effects of PCR inhibitors in samples, such as denatured proteins, organic chemicals, and sucrose. Moreover, the presence of DNA and cells other than those from the targeted organism can affect the efficiency of the PCR methods. To overcome this, an enrichment step is commonly performed to enhance assay sensitivity by ensuring the detection of viable pathogens before PCR reaction. Ferretti et al. reported that PCR with a 6 h nonselective enrichment could detect various *Salmonella* serotypes in salami stuffs as low as 1 cfu in 100 ml of food homogenate [43, 44]. Myint et al. reported a PCR method for *Salmonella* detection in contaminated poultry tissue samples, and false negative results were obtained without enrichment. However, a positive rate of 90% was observed after enrichment. Generally, culture enrichment is recommended in order to distinguish live cells from dead cells before PCR [45]. Maciorowski et al. investigated different enrichment times to detect indigenous *Salmonella* in poultry dietary samples using PCR. It was found that it could not be detectable for *Salmonella* with 7 h enrichment, and the sensitivity for detection was 25 and 50% with 13 h enrichment and 24 h enrichment, respectively [46].

Improvements have also been made on the basic PCR technology as well. In particular, two primary PCR-based methods have emerged over the past several years, such as multiplex PCR and real-time quantitative PCR [47, 48]. The current status of the optimization and development of these PCR applications is summarized in the following.

Multiplex PCR is a modified PCR method that allows for multiple sequence targets to be simultaneously detected within a single reaction. This method has proven useful for the rapid identification of multiple pathogens simultaneously in a given sample. Generally, multiplex PCR amplifies the target samples using multiple primers in a reaction, which can detect and identify several target sequences in *Salmonella*. Sharma employed a multiplex fluorogenic

PCR assay for simultaneous detection of *Salmonella* and *E. coli* O157:H7, which was capable of detecting as low as 10 cfu/g in meat [49]. Similarly, Kawasaki detected multiple *Salmonella* serotypes, *L. monocytogenes*, and *E. coli* O157:H7 simultaneously in enriched meat samples using multiplex PCR [48]. Cortez et al. identified *Salmonella* from chicken abattoirs by multiplex PCR. In this paper, 29 out of 288 (~10%) samples were found to be positive for *Salmonella* spp., and 16 (~5.6%) and 7 (~2.4%) samples were characterized as *Salmonella Typhimurium* and *Salmonella enteritidis*, respectively [50]. Kim differentiated the 30 most prevalent *Salmonella* serotypes in the United States by using two five-plex PCR assays. In this study, primer pairs targeting six genetic loci from *S. Typhimurium* and four from *S. Typhi* were designed to evaluate various *Salmonella* serotypes [51]. More recently, Salemis et al. also established two five-plex assays for the detection of the most common *Salmonella* in Tunisia as well [52]. Although multiplex PCR can simultaneously detect several targets, the primary difficulties are uncommitted, in which reaction conditions are needed optimized as high amounts of DNA in the reaction mixture compared to single PCR-based assays. The complex conditions and ingredients in the reaction still increase the difficulty in discrimination between prominent PCR product sizes on traditional agarose gel electrophoresis. In practice, cross-reactivity of primer pairs and sensitivity limitations associated with the procedure make it still quite challenging to routinely use multiplex PCR for reliable simultaneous *Salmonella* serovar detection [53].

With the appearance of fluorescence technology that endows increased sensitivity (e.g., intercalating dyes such as SYBR Green or labeled probes), the limitations of conventional PCR can be overcome, such as the errors associated with end-point analyses and lack of quantification. The "real-time" aspect of real-time PCR, also referred to as qPCR, technology is linked to its ability to label and cumulatively quantify the generated PCR products at each cycle throughout the ongoing amplification process. The qPCR has been widely used to quantify *Salmonella* [54–56]. Daum screened nine foodstuffs associated with a *Salmonella* outbreak in Texas using qPCR. It was reported that only one food item was positive for *Salmonella* [57]. Wang et al. reported a qPCR method to detect *Salmonella* in raw sausage meat with detection limit of 4 cfu/g [58]. He also used this method to quantify *Salmonella* detection limits of 2.5 cfu/25 g for salmon and minced meat, 5 cfu/25 g of chicken meat, and 5 cfu/25 ml for raw milk, respectively [59]. Malorny et al. reported a duplex qPCR assay to detect *S. enteritidis* in whole chicken carcass rinses and eggs, with a detection limit of 3 cfu/50 ml of chicken carcass rinses and 3 cfu/10 ml of homogenized egg content [60]. Bohaychuk used qPCR for *Salmonella* detection in poultry cecal contents and carcasses with reported sensitivities ranging from 97 to 100% for various matrices [61]. Although qPCR is an effective tool to detect *Salmonella* with high sensitivity and specificity, it does have several limitations, which are listed in **Table 1**.

2.2.2. Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA)-based approaches are the most prevalent antibody-based assay for pathogen detection in foods [62]. This immunological approach has been used to detect *Salmonella* in poultry production (poultry feed, feces, litter, carcass rinsing, and water samples) and has provided a better sensitivity and shorter time frame than that of culture-based methods [46]. Improvements by combination with other advanced technologies have been made to the basic ELISA method for

Salmonella detection. For example, incorporation of monoclonal antibodies can improve the sensitivity of the assay, and it can quantify *Salmonella* among poultry probiotic bacteria such as *Veillonella* [63]. In this study, the detection limit for *S. Typhimurium* was determined to be 5.5×10^4 cells/ml in pure culture. Dill combined monoclonal and polyclonal antibodies and a commercial filtering system to detect *S. Typhimurium* cells in a chicken rinsate, with detection limit of fewer than 100 *S. Typhimurium* cells [64]. As the advantages of ELISA methods for *Salmonella* detection in foods and animal feeds, they are now widely used for detection of *Salmonella* in animal-producing foods [65]. The comparison of ELISA methods with culture-based methods is performed and listed in **Table 1**.

Method	Advantages	Disadvantages
Culture-dependent methods	—Accurate	—Labor and time cost
Single and multiplex PCR	—More rapid than culture-based methods (<24 h vs. 5 ~ 7 days) —High specificity and sensitivity —Multiplex PCR (several pathogens at a time) —Labor saving —Multidetection of several *Salmonella* serotypes (5 ~ 6) in one reaction	—Costs more than culture-based methods and ELISA —Difficulty in distinguishing live and dead cells —Technically can be challenging (optimized PCR condition) —Enrichment to detect viable cells —Requires post-PCR processing of products (electrophoresis) —PCR inhibitors
qPCR	—Not influenced by nonspecific amplification; amplification can be monitored at real time —No post-PCR processing of products (gel electrophoresis) —Rapid cycling (25 min) —Confirmation of specific amplification by melting curve —Specific, sensitive, and reproducible	—Difficulty in multiplex assay —Need skilled person and support —High equipment cost —mRNA lability —Possibility of cross contamination
Antibody-based method	—More rapid than culture-based methods (2 days vs. 5 ~ 7 days) —Can be automated to reduce assay time and manual labor input —Able to handle large numbers of samples —More specific than cultural methods	—Not high sensitivity —Difficult to multidetect —False-negative results —Difficulty to differentiate damaged or stressed cells —Need to pre-enrichment —High cross-reactivity with close antigens in bacteria
Aptamer-based method	—Inexpensive, stable, and can be chemically synthesized than antibody —Time saving (2 h vs. 5 ~ 7 days of culture-based methods) —Automated to reduce manual labor input —Large numbers of sample detection at one time —Higher specificity than cultural methods	—High false-positive results —Difficulty in detecting damaged or stressed cells —Pre-enrichment for production of cell surface antigens —Possibility of cross contamination

Table 1. Advantages and disadvantages of detection methods.

2.2.3. Aptamer-based detection assay

Besides antibodies, other biomolecules have been investigated to selectively capture and enrich *Salmonella* from cultures, among which aptamer is the most prevalent one [66]. Aptamers are single-stranded oligonucleotides, DNA, or RNA that can fold into unique 3D structures based on their primary nucleotide sequence, rendering them capable of binding to specific ligands, like antibody interacting with an antigen [67]. Aptamers offer some advantages over antibodies in that they are relatively inexpensive to synthesize and they provide more batch-to-batch consistency [68]. However, few studies have reported their specific use in detecting *S. Typhimurium* from river water and fecal samples [66, 69]. Bacteriophages have also been explored as a means to capture *Salmonella* cells. Phages may offer some advantages over antibodies given their inherent specificity for host cells, their ease of production in bacteria versus animals or eukaryotic cell culture, and their relative stability in harsh conditions such as pH and temperature extremes [70].

Relative to culture-independent detection, researchers have focused on methods to concentrate whole cells within the sample before the pre-enrichment step. The enriched whole *Salmonella* allows for direct detection from food and environmental samples. The enrichment steps mainly rely on filtering liquids, rinsates, or mechanically disintegrated (i.e., blended or stomached) samples. Therefore, this approach has been widely used in large volumes of water, but the testing of food samples was problematic due to the food particles difficult to go through filter membranes [71]. To overcome this problem, endopeptidases have been added to apply in food samples. These degrade the small, soluble proteins and peptides so that they are unable to clog the filter and pass through with the permeate. The United States has awarded the method with grant prize. The Food and Drug Administration also recommends the method for food safety guard, (http://www.foodsafetychallenge.com), which signified its potential to greatly enhance the detection of *Salmonella* directly from foods.

2.3. Conclusion

In summary, the mentioned methods here have utility advantages for *Salmonella* detection in the food safety sector. It is important to emphasize that none of the methods will be recommended or even suited for every situation in detecting all food varieties for *Salmonella*. Application to specific food samples will be dictated by method performance. As noted previously, the performance of these methods depends on several factors, such as matrix-driven effects, general specificity and sensitivity, and their technical complexity. Meanwhile, other extrinsic factors would affect the performance, including user skill set and technical prowess, cost of the equipment, and cost per sample. Hence, the systematic validation to evaluate the methods should be considered according to its specific utility and application across the food supply.

In order to meet the current requirement of rapid detection, it is clear that several approaches have emerged including PCR-based, antibody-based, aptamer-based, and other approaches encompassing those stemming from the current genomic era. A clear character of method development direction is moving toward greater automation, cost-saving, and time-saving

network integration. It is important to mention that outputs from one approach would serve to strengthen directly or tangentially other approaches. At last, it seems that a suite of tools is emerging for the food safety microbiologist, each with its specific advantages and disadvantages but all with the ability to rapidly and accurately detect *Salmonella* in certain cases and early in its contamination of the human and veterinary food supply.

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