Short communication

Comparison of Direct Culture, Immunomagnetic Separation/culture, and Multiplex PCR Methods for Detection of *Salmonella* in Food

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In this study, an optimized method was developed to detect *Salmonella* by using immunomagnetic separation coupled with culture to selective agar (IMS/culture). To test the effectiveness of the methods and develop a rapid and sensitive detection procedure, direct culture, IMS/culture, and multiplex PCR (mPCR) were compared for the detection of *Salmonella* in 700 food samples. After selective enrichment, all samples were (I) subjected to direct culture, plated on xylose-lysine-tergitol 4 agar, and identified as *Salmonella* via biochemical and serological methods; (II) subjected to IMS then identified as (I); and (III) subjected to DNA extraction and mPCR analysis. A total of 83, 95, and 104 samples were found positive for *Salmonella* by direct culture, IMS/culture, and mPCR, respectively. Results suggested higher sensitivity in mPCR than in direct culture and IMS/culture methods. IMS/culture increased the detection rate of *Salmonella* and compared well with mPCR. This study demonstrated that the use of mPCR in pre-screening of samples and further identification by IMS/culture should enhance the positive identification and increase the number of isolates of *Salmonella*.

Keywords: *Salmonella*, direct culture, immunomagnetic separation/culture, multiplex PCR, food

Introduction

Outbreaks of foodborne salmonellosis in recent years have raised serious concern regarding the rapid diagnosis of pathogens with high specificity and sensitivity. Conventional culture methods for the detection of *Salmonella* are most widely used and considered “gold standard” (Alocilja *et al.*, 2003). However, such methods are laborious, time-consuming, and less sensitive. To overcome these disadvantages, several methods for the detection of *Salmonella* in food have been developed to increase the sensitivity and decrease the detection time. These methods include immunoassays and nucleic acid-based techniques. Among these strategies, immunomagnetic separation (IMS) and polymerase chain reaction (PCR) have been selected as potential approaches.

IMS, which uses anti-*Salmonella* polystyrene beads, can

specifically capture and concentrate target bacteria from food samples, thereby improving the specificity and sensitivity of detection. Moreover, isolated cells can be identified via culture, ELISA, or molecular methods (Cudjoe *et al.*, 1995; Hagren *et al.*, 2008; Jordan *et al.*, 2004; Lynch *et al.*, 2004).

PCR techniques allow highly sensitive detection in few hours and have been described as rapid alternatives to culture for detecting *Salmonella* in food (Glynn *et al.*, 2006; Moganedi *et al.*, 2007). PCR targeting *Salmonella* virulence genes, such as *fimA* (Moreira *et al.*, 2008), *invA* (Rahn *et al.*, 1992; Upadhyay *et al.*, 2010), and *hlyA* (Guo *et al.*, 2000), has been developed. Recently, multiplex PCR (mPCR) targeting two or more genes has been widely used in *Salmonella* detection to increase the specificity (Fach *et al.*, 2009; Kim *et al.*, 2006; Woods *et al.*, 2008).

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Each method presents individual characteristics and suitability for application. To date, few attempts have been focused on the comparison of various methods for the detection of *Salmonella* in different types of food. Although the above methods have achieved some success, a simple, rapid, and robust procedure is still needed to increase the sensitivity and reduce the work of detection, especially when numerous food samples will be examined. Therefore, this study aimed to compare and evaluate the three methods, conventional direct culture, IMS followed by culture to selective agars (IMS/culture), and mPCR analysis, for the detection of *Salmonella* in food samples.

Materials and Methods

Samples A total of 700 food samples purchased from retail markets in China were examined, including chicken (104), pork (117), duck (41), beef (17), mutton (15), fish (109), shrimp (29), milk (36), vegetables (84), mushroom (70), and ready-to-eat food (78). All samples were transported to the laboratory in a chilled container and stored at 4°C prior to examination within 24 h of purchase.

Enrichment and direct culture Samples were prepared and enriched in accordance with the National Food Safety Standards of China (document GB 4789.4-2010). A 25 g sample was randomly collected from each sample and pre-enriched in 225 mL of buffered peptone broth (Huankai, Guangzhou, China). About 1 mL cultures were incubated in 10 mL of selenite cystine broth (SC) (Huankai) at 37°C and 10 mL of tetrathionate brilliant green broth (TTB) at 42°C for 24 h. Loopfuls of SC and TTB cultures were streaked onto xylose-lysine-tergitol 4 (XLT4) selective agar plates (Difco, Detroit, MI, USA) then incubated at 37°C for 24 h.

IMS/culture The immunomagnetic beads used were prepared by coating goat anti-*Salmonella* antibody (CSA-1) (KPL, Gaithersburg, MD) to Dynabeads M-280 Tosylactivated (Invitrogen Dynal AS, Oslo, Norway) following the manufacturer's instructions.

After selective enrichment, IMS was performed simultaneously as follows: 1 mL aliquots of SC broths and 1 mL aliquots of TTB broths were transferred to an Eppendorf tube containing 5 µL of the prepared immunomagnetic beads (10 mg/mL) and incubated at room temperature for 10 min with slight agitation. About 50 µL of PBS containing 1% Tween-20 was added to the tube, mixed, and then separated with Dynal MPC-S. After washing twice with PBS containing 0.05% Tween-20 (PBST), the beads were resuspended in 100 µL of PBST and transferred onto XLT4 plates for incubation at 37°C for 24 h.

Identification and serotyping After incubation, up to five suspect colonies appeared as entirely black or pink to red colonies with black centers (H₂S-positive *Salmonella* strains) or pinkish-yellow colonies (H₂S-negative *Salmonella* strains) were picked up from each plate and then identified using API 20E system (bioMérieux, Marcy-l'Etoile, France). A complete serological

analysis of the *Salmonella* isolates was also performed using antisera (S&A Reagents Lab Ltd., Thailand) with slide agglutination test based on somatic O, phase 1 and phase 2 flagellar antigens in accordance with the Kauffmann–White scheme.

Multiplex PCR Simultaneously, bacterial DNA was extracted from 1 mL suspension of SC and TTB broths using a Column Bacterial Genomic DNA Isolation Kit (Sangon, Shanghai, China) following the manufacturer's protocol. PCR amplification of *invA* and *hlyA* genes was performed to identify *Salmonella* at genus level (Rahn *et al.*, 1992; Guo *et al.*, 2000; Xu *et al.*, 2008). Two sets of primers were synthesized (BGI, Shenzhen, China) to amplify 284 bp (*invA*1: GTGAAATTATCGCCACGTTCCGGGCAA; *invA*2: TCATCGCACCGTCAAAGGAACC) and 497 bp (*hlyA*1: C T G T C G C C T T A A T C G C A T G T; *hlyA*2: CTGCCGAGTGTTAAGGATA) fragments of the target genes. The 25 µL PCR amplification reaction mixtures contained 12.5 µL of 2×GoldStar Taq MasterMix kit (Cwbio, Beijing, China); 0.5 µL each of 10 µM *invA*1, *invA*2, *hlyA*1, and *hlyA*2 primers; 4 µL of extracted DNA; and sterile deionized water. PCR was performed using a T-Professional thermocycler (Biometra, Goettingen, Germany) under the following conditions: initial denaturation at 95°C for 5 min; followed by 35 cycles of 95°C for 45 s, 58°C for 45 s, and 72°C for 60 s; and a final extension step at 72°C for 10 min. A 5 µL aliquot of each amplicon was resolved on a 1.5% agarose gel (Invitrogen, Paisley, UK) with GoldView™ Nucleic Acid Stain (SBS Genetech Co., Ltd., Beijing, China). The gels were examined using the ImageQuant 350 system (GE Healthcare, Chalfont St. Giles, UK).

Results and Discussion

The results for all food samples are summarized in Table 1. Among the 700 food samples tested, 110 were detected positive for *Salmonella*. Direct culture, IMS/culture, and mPCR detected 83, 95, and 104 positive samples, respectively. A total of 122 strains of *Salmonella* were isolated from 103 culture-positive samples, 115 were from IMS/culture and 104 were from direct culture. The distribution of isolated serotypes is presented in Table 2. The isolated *Salmonella* strains represented 9 different serogroups and 32 different serotypes, which were all detected by IMS/culture.

The positive results obtained by the two culture methods

Table 1. Comparison of the number of *Salmonella* positive samples obtained by the three methods in 700 food samples

Direct culture	IMS/culture	mPCR	Number of sample
+	+	+	75
-	+	+	18
+	-	+	4
-	+	-	2
+	-	-	4
-	-	+	7
-	-	-	590

displayed that IMS/culture detected 12 samples more than direct culture. This finding suggested that IMS/culture was more sensitive than direct culture for the isolation of *Salmonella*. This result can be ascribed to the enhanced sensitivity of the IMS method used. The good performance of the IMS/culture method is consistent with previous results (Coleman *et al.*, 1995; Cudjoe and Krona, 1997; Lynch *et al.*, 2004).

Among 103 culture-positive samples, 20 were tested positive by IMS/culture but not by direct culture, and 18 of which were also detected positive by mPCR. The number of *Salmonella* strains in two mPCR-negative samples was low (only one presumptive positive *Salmonella* colony was found on the XLT4 agar plates), which may explain that they were detected by IMS/culture but not by direct culture. IMS/culture captured *Salmonella* from

enrichment broths and was more effective than direct culture in detecting a small number of pathogens in food. This result provides important diagnostic implications in detection cases with very few *Salmonella* organisms in the samples, which can be missed by direct culture.

However, eight samples were tested positive by direct culture but not by IMS/culture, and five of which were also detected positive by mPCR. Six strains of *S. Enteritidis*, one strain of *S. Typhimurium*, and one strain of *S. Heidelberg* were isolated from the eight samples. These three serotypes were detected by IMS/culture among other positive samples (Table 2). Five out of the eight samples with mPCR-positive result simultaneously belonged to frozen chicken. The positive mPCR results suggested that *Salmonella* was successfully cultured in selective enrichment

Table 2. Serotype distribution of 122 *Salmonella* isolates from food samples by IMS/culture and direct culture method

Serogroup	Serotype	Number of <i>Salmonella</i> isolates obtained by	
		IMS/culture	Direct culture
B	<i>S. Typhimurium</i>	18	16
	<i>S. Derby</i>	13	11
	<i>S. Indiana</i>	7	6
	<i>S. Heidelberg</i>	3	4
	<i>S. 4,12:i:-</i>	2	2
	<i>S. Agona</i>	1	1
	<i>S. Shubra</i>	1	1
	<i>S. Remo</i>	1	1
	<i>S. Stanley</i>	1	1
C1	<i>S. Thompson</i>	3	3
	<i>S. Infantis</i>	2	1
	<i>S. Bareilly</i>	2	1
	<i>S. Potsdam</i>	2	1
	<i>S. Ness-ziona</i>	2	2
	<i>S. Isangi</i>	2	2
	<i>S. Virchow</i>	1	0
	<i>S. Montevideo</i>	1	0
C2	<i>S. Tshiongwe</i>	4	2
	<i>S. Kottbus</i>	2	2
C3	<i>S. Albany</i>	3	3
	<i>S. Corvallis</i>	2	2
D	<i>S. Enteritidis</i>	30	29
E1	<i>S. Weltevreden</i>	3	3
	<i>S. Okefoko</i>	1	1
	<i>S. Meleagridis</i>	1	1
	<i>S. London</i>	1	1
	<i>S. Anatum</i>	1	1
	<i>S. Nchanga</i>	1	1
	<i>S. 10:1,v:-</i>	1	1
E4	<i>S. Senftenberg</i>	1	1
F	<i>S. Aberdeen</i>	1	1
Q	<i>S. Wandsworth</i>	1	1

broths, whereas the negative IMS/culture findings showed that the frozen injured cells did not successfully adhere to the beads. Thus, the IMS/culture method for the detection of *Salmonella* may yield false-negative results because of inadequate resuscitation of stressed and injured bacterial cells. By contrast, conventional culture methods were better able to detect severely stressed bacteria (Uyttendaele *et al.*, 2003).

The three other samples with mPCR-negative results were all detected positive by SC broth only and not by TTB broth through direct culture; their positive isolates were all serotyped as *S. Enteritidis*. The SC broth was less selective than the TTB broth and is always suitable for special samples, in which the number of *Salmonella* was low. The negative mPCR results can also prove the presence of low-level *Salmonella* in broths from another standpoint. This finding indicated that the IMS/culture method for the detection of *S. Enteritidis* at low level may yield false-negative results.

Other reasons exist for the eight false-negative results of IMS/culture: (a) matrix effects can influence the IMS, and immunomagnetic beads may have been lost because of extremely high fat content of meat during IMS (Skjerve and Olsvik, 1991); and (b) the culture plates from the eight samples consisted of a heavy growth of other Enterobacteriaceae organisms, which may interfere with the growth of isolated *Salmonella* colonies. This non-specific adherence to the beads of organisms other than *Salmonella* occurred with particular samples that have been previously reported. Cudjoe and Krona (1997) found that the growth of mucoid colonies of *Proteus* spp. and coliforms, such as *Escherichia coli*, *Klebsiella aerogenes*, and *Enterobacter* spp., presents difficulty in the isolation of *Salmonella* in selective agar after IMS.

Furthermore, mPCR detected 97 of 103 culture-positive samples. From the six culture-positive samples that were not detected by mPCR, four isolates were identified as *S. Enteritidis*, one was *S. Heidelberg*, and one was *S. Typhimurium*. The pure culture of these isolates gave a *Salmonella*-positive result after mPCR analysis. Seven other *Salmonella*-positive results were generated by mPCR only (Table 1). The carriage of the target gene of mPCR was confirmed by DNA sequencing. These results were all attributed to the higher sensitivity of molecular detection than that of culture methods. Compared with the culture method, which provides a result after 5 d to 7 d, the mPCR assay detects *Salmonella* after 12 h to 13 h. Hence, the rapid mPCR method allows early intervention and makes preventive consumer protection possible. However, the mPCR assay is prone to yield positive results that cannot be confirmed by culture. Thus, this method may be valuable in primarily screening or evaluating the significance of organisms containing virulence genes.

Conclusions

This study demonstrated that mPCR is more rapid and sensitive

than direct culture and IMS/culture methods. IMS/culture has been shown to be more sensitive than direct culture for the isolation of *Salmonella*. Unlike mPCR, IMS/culture leads to the isolation of a viable organism, thereby contributing to future epidemiological studies on *Salmonella* infection. Thus, the combination of mPCR for primary screening and IMS/culture for confirmation of positive samples as an easy, rapid, and efficient workflow can be recommended for use in large amounts of food samples.

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