DEVELOPMENT OF MULTIPLEX PCR FOR RAPID IDENTIFICATION OF FOUR *SALMONELLA* SEROVARS MOST COMMONLY ISOLATED IN JAPAN

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Abstract. More than 2,500 serovars of *Salmonella* species have been reported to date. A multiplex-PCR method was developed and evaluated for discriminating the four *Salmonella enterica* subsp *enterica* serovars, namely, *S*. Enteritidis, *S*. Typhimurium, *S*. Thompson and *S*. Infantis, most commonly isolated in Japan. Twenty-two serovars of 84 *Salmonella* strains and 7 species of non-*Salmonella* strains were evaluated using primer pairs specific for the detection of *Salmonella* spp. Multiplex PCR generated, with 100% specificity, the expected amplicon of 333, 413, 551 and 658 bp of *S*. Enteritidis, *S*. Infantis, *S*. Typhimurium, and *S*. Thompson, respectively, while an additional non-specific amplicon (about 1,000 bp) was observed for *S*. Infantis, but it had no practical impact in the bacterial detection. This multiplex PCR assay can be applied to identify and discriminate clinically significant strains of *Salmonella* serovars rapidly and accurately without the need for serological examination.

Keywords: *Salmonella* spp, discrimination, multiplex-PCR, Japan

INTRODUCTION

The genus *Salmonella* belongs to the family Enterobacteriaceae, and many strains of *Salmonella* are the principal pathogens implicated in human foodborne illnesses (Voetsch *et al*, 2004; Majowicz *et al*, 2010; Hendriksen *et al*, 2011). Regarding *Salmonella* classification into species, subspecies and serovars, *Salmonella* is classified into two species, *Salmonella enterica* and *Salmonella bongori* (Reeves *et al*, 1989). Furthermore, *S. enterica* is divided into six subspecies, namely, *S. enterica* subsp *enterica* (subspecies I), *S. enterica* subsp *salamae* (subspecies II), *S. enterica* subsp *arizonae* (subspecies IIIa), *S. enterica* subsp *diarizonae* (subspecies IIIb), *S. enterica* subsp *houtenae* (subspecies IV), and *S. enterica* subsp *indica*

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(subspecies VI) (Dieckmann *et al*, 2008). More than 2,500 serovars of *Salmonella* spp have been reported to date by typing of surface polysaccharide (O) and phase I and II flagella (H) (Popoff, 2001; Guibourdenche *et al*, 2010). As for serovars, they were classified into as follows: (i) subspecies designation (subspecies I through VI), (ii) O (somatic) antigens separated by a comma if needed, followed by a colon, (iii) H antigens (phase I) separated by a colon, and (iv) H antigens (phase II, if present) (*viz*, *Salmonella* serotype II 39:z10:-) (Popoff *et al*, 2004; Agbaje *et al*, 2011). For serotypes of S. *bongori* (previously belonging to subspecies V), V is still used for consistency (Popoff *et al*, 2004; Agbaje *et al*, 2011).

Salmonella serotyping is a necessary first step in any epidemiological investigation of food-borne outbreaks. *Salmonella* serovars are identified by slide and tube agglutination tests using O and H antigen-specific antisera, although these procedures are both labor intensive and time consuming (Popoff, 2001; Tennant *et al*, 2010). This is because at least three antibody-antigen reactions are required to identify *Salmonella* serovars and often require many further tests for correct typing. The formula for scoring of antigens uses the Kaufmann-White scheme, which is annually updated by the World Health Organization (Popoff, 2001). However, molecular biology methods have been developed for rapid typing of *Salmonella* serovars using multiplex PCR (Hong *et al*, 2009; Tennant *et al*, 2010; Akiba *et al*, 2011). These studies targeted *S. enterica* subsp *enterica* serovar Enteritidis (*S.* Enteritidis) and *S.* Typhimurium mainly isolated in human food-borne illnesses.

In Japan, *S.* Enteritidis, *S.* Typhimurium, *S.* Thompson and *S.* Infantis are the representative *Salmonella* serovars (Ahmed *et al*, 2009; Esaki *et al*, 2013). Although rapid typing of *S.* Enteritidis and *S.* Typhimurium has been reported, there have been few reports of rapid typing of *S.* Thompson and *S.* Infantis (Hong *et al*, 2009; Tennant *et al*, 2010; Akiba *et al*, 2011).

Genes used for PCR-based detection of *Salmonella* spp include 23S (Christensen *et al*, 1998) and *sifB* (de Almeida *et al*, 2014). In this study, we employed *invA*, the common virulence gene in *Salmonella*, *sdf (Salmonella* difference fragment) specific for *S.* Enteritidis and *fliC* specific for each H1 flagellar antigen (i, k, and r) in *S.* Typhimurium, *S.* Thompson, and *S.* Infantis to develop and evaluate a multiplex-PCR method for rapid identification of the four serovars of *Salmonella* spp, most commonly isolated in Japan.

MATERIALS AND METHODS

Salmonella strains

Twenty-two serovars of 84 *Salmonella* strains and 7 non-*Salmonella* strains containing gram-positive bacteria and Enterobacteriaceae from the reference and isolate strains of Kobe University, Kobe Institute of Health, and Hyogo Clinical Laboratory Corporation, Japan between 2009 and 2011 were used to evaluate the multiplex-PCR assay (Table 1). *Salmonella*-positive isolates were further serotyped using the slide agglutination test together with O- and H-antigen antisera (Denka Seiken, Tokyo, Japan).

Multiplex-PCR

The primer sets were designed as follows: sdfF and sdfR to amplified *sdf* unique to *S.* Enteritidis (GenBank accession no. AF370707); 878f and 1275r for *fli*C-r of *S.* Infantis (GenBank accession no. AY353282); H:for and H:i for *fli*C-i (H antigen gene) of *S.* Typhimurium (GenBank accession no. AY649698, AY429608); H:k-F

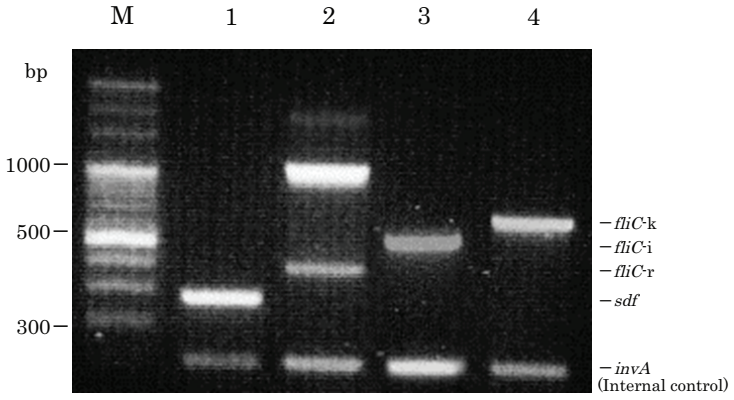

Fig 1–Agarose gel-electrophoresis of amplicons from multiplex PCR of four *Salmonella* serovars. Experimental protocols are described in Materials and Methods. Lane M: 100 bp DNA markers; lane 1: *S. enterica* subsp *enterica* serovar Enteritidis; lane 2: *S.* Infantis; lane 3: *S.* Typhimurium; lane 4: *S.* Thompson.

and H:k-R for *fli*C-k of *S.* Thompson (GenBank accession no. AY649722), and *invA*-F and *invA*-R for *Salmonella* target gene *invA* (GenBank accession no. M90846) as an internal positive control (Table 2) (Agron *et al*, 2001; Kardos *et al*, 2007; Tennant *et al*, 2010). We chose H:k to target *filC-k* because the difference between *S.* Infantis and *S.* Thompson lies with H1 flagellar antigen "r" and "k", but not with the O antigen group 7 and H2 flagellar antigen (1,5) as shown in Table 1.

After an overnight culture at 37°C, DNA extraction was conducted using Illustra™ Bacteria Genomic Prep Mini Spin Kit (GE Healthcare Japan, Tokyo, Japan). PCR mixtures (25 µl) contained 20 ng of DNA, 1X Taq Buffer (TaKaRa, Shiga, Japan), 0.2 mM dNTP Mixture (TaKaRa), and 0.625 U *Taq* DNA polymerase (TaKaRa). The primer sets were added at a concentration of 0.2 µM each, except for H:for and H:i that were used at 0.8 µM each. Multiplex-PCR amplification consisted of an initial heating at 95°C for 6 minutes, followed by 30 cycles of 95°C for 1 minute, 63°C for 12 seconds, and 72°C for 1 minute, and a final step of 72°C for 4 minutes. PCR amplicons were separated on 2% agarose gel-electrophoresis, stained with ethidium bromide and visualized using a UV trans-illuminator. Each multiplex-PCR was conducted in three independent experiments.

RESULTS

The multiplex-PCR was performed using a mixture of all five pairs of primers. Initially, PCR with each primer pair was optimized to ensure that each amplicon was of the correct size (data not shown). Agarose gel-electrophoresis of the multiplex-PCR products revealed the expected amplicon of 333, 413, 551 and 658 bp for *S.* Enteritidis, *S.* Infantis, *S.* Typhimurium and *S.* Thompson, respectively (Fig 1). However, an additional non-specific band (about 1,000 bp) was observed for *S.* Infantis. This additional non-specific band was observed even though several different primers were used but they did not affect the detection of *S.* Infantis. The gene domain of the internal positive control was successfully amplified in each *Salmonella* sample. These results were in agreement with those obtained by the conventional PCR assay using each primer pair separately (data not shown). In order to determine the accuracy of the multiplex-PCR assay, a total of 91 samples containing 22 serovars of 84 *Salmonella* strains and 7 non-*Salmonella* strains were tested, and the expected

Table 1
Bacterial strains for the evaluation of specificity of PCR primers and the results of multiplex PCR.

Strains	O antigens	Phase I H flagellar antigen(s)	Phase II H flagellar antigen(s)	*invA*	*sdf*	*fliC*-r	*fliC*-i	*fliC*-k
Salmonella strains								
S. Typhimurium ATCC14028	O-4	i	1,2	+	-	-	+	-
S. Enteritidis isolates (11 strains)	O-9	g,m	-	+	+	-	-	-
S. Infantis isolates (11 strains)	O-7	r	1,5	+	-	+	-	-
S. Typhimurium isolates (4 strains)	O-4	i	1,2	+	-	-	+	-
S. Thompson isolates (3 strains)	O-7	k	1,5	+	-	-	-	+
S. Saintpaul isolates (3 strains)	O-4	e,h	1,2	+	-	-	-	-
S. Schwarzengrund isolates (3 strains)	O-4	d	1,7	+	-	-	-	-
S. Agona isolates (3 strains)	O-4	f,g,s	(1,2)	+	-	-	-	-
S. Derby isolates (3 strains)	O-4	f,g	(1,2)	+	-	-	-	-
S. Sofia isolates (3 strains)	O-4	b	e,n,x	+	-	-	-	-
S. Bareilly isolates (3 strains)	O-7	y	1,5	+	-	-	-	-
S. Montevideo isolates (3 strains)	O-7	g,m,(p),s	(1,2,7)	+	-	-	-	-
S. Braenderup isolates (3 strains)	O-7	e,h	e,n,z_{15}	+	-	-	-	-
S. Hadar isolates (3 strains)	O-8	z_{10}	e,n,x	+	-	-	-	-
S. Newport isolates (3 strains)	O-8	e,h	1,2	+	-	-	-	-
S. Muenchen isolates (3 strains)	O-8	d	1,2	+	-	-	-	-
S. Havana isolates (3 strains)	O-13	f,g,(s)	-	+	-	-	-	-
S. Worthington isolates (3 strains)	O-13	z	l,w	+	-	-	-	-
S. Cerro isolates (3 strains)	O-18	z_4,z_{23}	(1,5)	+	-	-	-	-
S. London isolates (3 strains)	O-3,10	l,v	1,6	+	-	-	-	-
S. Give isolates (3 strains)	O-3,10	1,v	1,7	+	-	-	-	-
S. Anatum isolates (3 strains)	O-3,10	e,h	1,6	+	-	-	-	-
S. Liverpool isolates (3 strains)	O-1,3,19	d	e,n,z_{15}	+	-	-	-	-
Other bacterial strains								
Escherichia coli ATCC25922				+	-	-	-	-
Klebsiella pneumoniae ATCC13883				+	-	-	-	-
Enterobacter cloacae ATCC23355				+	-	-	-	-
Serratia marcescens ATCC8100				+	-	-	-	-
Pseudomonas aeruginosa ATCC27853				+	-	-	-	-
Staphylococcus aureus ATCC25923				+	-	-	-	-
Enterococcus faecalis ATCC33186				+	-	-	-	-

amplicons were obtained consistently (Table 1).

The multiplex-PCR results were in complete agreement with the traditional serotyping data for the 4 common *Salmonella* serovars, while the amplicons were not generated with 18 other *Salmonella* serovars and non-*Salmonella* strains. The reproducibility of these results was shown by three independent assays.

Table 2
Target genomic regions and primer sequences of the multiplex PCR.

Primer	Sequence	Product size (bp)	Target gene	Serovar	Reference
invA-F	GCCATGGTATGGATTTGTCC	118	*invA*	*Salmonella* specific	This study
invA-R	GTCACGATAAAACCGGCACT				
sdfF	TGTGTTTTATCTGATGCAAGAGG	333	*sdf*	*S*. Enteritidis	Agron *et al*, 2001
sdfR	CGTTCTTCTGGTACTTACGATGAC				
878f	AACAACGACAGCTTATGCCG	413	*fliC-r*	*S*. Infantis	Kardos *et al*, 2007
1275r	CCACCTGCGCCAACGCT				
H:for	ACTCAGGCTTCCCGTAACGC	551	*fliC-i*	*S*. Typhimurium	Tennant *et al*, 2010
H:i	ATAGCCATTTACCAGTTCC				
H:k-F	AACGACGGTATCTCCATTGC	658	*fliC-k*	*S*. Thompson	This study
H:k-R	CAGCCGAACTCGGTGTATTT				

DISCUSSION

Salmonella spp have several kinds of serovars (Popoff, 2001; Guibourdenche *et al*, 2010) and among them, there are many variations depending on habitat environment, with the potential of causing diarrhea in humans (Voetsch *et at*, 2004; Majowicz *et al*, 2010; Hendriksen *et al*, 2011). Furthermore, it is necessary to discriminate rapidly and accurately the serovars of *Salmonella* spp in order for the initiation of appropriate therapeutic managements and prevention of unnecessary antibiotic therapy, which could lead to emergence of antibiotic resistance (Shahada *et al*, 2010; Campioni *et al*, 2012; Kameyama *et al*, 2012).

In the present study, we developed a fast, accurate and technically simple multiplex-PCR method to accurately discriminate the serovars among the most common clinical *Salmonella* isolates in Japan; namely, *S*. Enteritidis, *S*. Typhimurium, *S*. Thompson and *S*. Infantis. *S*. Enteritidis and *S*. Typhimurium are detected frequently worldwide in the clinical setting and many primers for PCR-based detection of these serovars have been reported (Hong *et al*, 2009; Tennant *et al*, 2010; Akiba *et al*, 2011). Recently, multiple drug resistance of *S*. Enteritidis and *S*. Infantis have emerged as a growing problem, and may help spread *Salmonella* as a causative agent of infectious diseases (Taguchi *et al*, 2012; Yamasaki *et al*, 2013). Thus, it is necessary to closely monitor the trend of antibiotic susceptibilities of these serovars. On the other hand, *S*. Thompson and *S*. Infantis are not detected frequently in the clinical setting in many countries compared to Japan, and consequently there are few reports of primer sets for PCR-based detection of *S*. Infantis, and especially not for *S*. Thompson (Kardos *et al*, 2007; Ross and Heuzenroeder, 2008; Akiba *et al*, 2011).

Our multiplex-PCR method for rapid detection may be highly effective for epidemiological and clinical use. In particular clinical application of this method together with antibiotic susceptibility tests could provide rapid detection of antibiotic-resistant strains and might help

track the spread of such antibiotic-resistant *Salmonella* serovars. The intervention and control measured thereby help prevent hospital infections, or antibiotic resistant strains or serovars of *Salmonella* in clinical situations, resulting in prevent ineffective antibiotic use. In this study, all of the serovars tested could be discriminated by the multiplex-PCR assay rapidly with high accuracy and reproducibility, suggesting that even more strains could be possibly added to this screening tool in future studies. A technical point that needs to be overcome was the presence of a non-specific amplicon in the identification of *S*. Infantis, but as this phenomenon was not encountered with other *Salmonella* serovars and bacteria, it was not considered to disturb the diagnosis of *Salmonella* spp.

Our method addressing the four commonest *Salmonella* strains in Japan showed high discriminating power and was easy, rapid, and inexpensive compared with methods based on sequence characterization, including multilocus sequence typing (Laing *et al*, 2011; Achtman *et al*, 2012; Seong *et al*, 2012). This technique would be accessible to any institution or laboratory because it does not require special devices or high costs. Moreover, previous studies used many sets of primers and complicated methods for each O-antigen and H-antigen (Hong *et al*, 2009; Tennant *et al*, 2010; Akiba *et al*, 2011). Compared with these previous studies, our method is easier in that only one kind of primer set for each serotype.

We would like to emphasize the limitations of this study. First, as mentioned above, the number of strains tested is small. Second, this study did not address all of the comparative conventional methodologies. Third, the antibiotic susceptibilities of the tested *Salmonella* spp were not addressed. Fourth, we could not remove the additional non-specific amplicon generated in the detection of *S*. Infantis by the application of several different primers. These limitations will be addressed and hopefully overcome in future studies.

In conclusion, our multiplex-PCR method for discriminating *Salmonella* spp serovars is applicable for rapid identification of highly similar serovars which are previously thought to be very distinct on the basis of their antigenic profiles. Multiplex PCR was able to selectively amplify DNA from individual *Salmonella* strains employing an easy, accessible, low-cost and highly-reproducible protocol. Further investigations with more serovars and kinds of *Salmonella* spp will be necessary in order to draw definitive conclusions for possible clinical application.

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