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Original Article

Multiplex Polymerase Chain Reaction Assay for Selective Detection of *Salmonella enterica* Serovar Typhimurium

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SUMMARY: A multiplex polymerase chain reaction (PCR) assay was developed for the identification of *Salmonella enterica* serovar Typhimurium. Three sets of primers were designed for detecting O4, H:i, and H:1,2 antigen genes from the antigen-specific genes *rfbJ*, *fliC*, and *fljB*, respectively. These were evaluated in a multiplex PCR assay by using DNAs from *S. enterica* serovar Typhimurium, 15 other *Salmonella* serovars, and 8 non-*Salmonella* enteric pathogens. Multiplex PCR proved to be capable of identifying *S. enterica* serovar Typhimurium specifically and differentiating it from other *Salmonella* serovars in addition to non-*Salmonella* enteric pathogens. Thus, this multiplex PCR assay can be practically applied to the identificaiton of *S. enterica* serovar Typhimurium.

INTRODUCTION

Salmonella is one of the major pathogens of food-borne disease outbreaks. The genus *Salmonella* is extremely polymorphic. It has been classified into more than 2,000 serovars. Many *Salmonella* serovars cause salmonellosis in humans and animals. *Salmonella enterica* serovar Typhimurium and *S. enterica* serovar Enteritidis are the most frequently isolated serovars from food-borne outbreaks throughout the world (1). *Salmonella* shows different disease syndromes and host specificities according to their antigenic profiles. Therefore, it is necessary and important to discriminate *Salmonella* serovars from each other in order to insure that each pathogen and epidemiology is correctly recognized. The differences within *Salmonella* serovars are based on the surface antigen differences of O and H antigens. The O antigens are derived from the polysaccharide domain of lipopolysaccharide (LPS) in the cell wall, while the H antigens are derived from the flagellin protein in the flagella. O and H antigens are used for the identification of *Salmonella* serovars by slide and tube agglutination tests using O and H antigen-specific anti-sera, and *Salmonella* serovars are determined by the combination of O and H antigen types (phase-1 and -2).

The serological method has been used to identify *Salmonella* serovars. However, when using the serological method, antibodies must be produced for each serovar, and this is extremely complex and time consuming. An easier and simpler method is needed to identify *Salmonella* serovars. Rapid identification methods are based on genomic amplification techniques using distinct target DNA sequences determined by polymerase chain reaction (PCR). Recently, specific identification of *S. enterica* serovars Typhi and Paratyphi A by multiplex PCR, which detects *rfbE*, *rfbS*, *viaB*, and *fliC* genes, has been reported (2), and this method correctly identified *S. enterica* serovars Typhi and Paratyphi A and differentiated these from other *Salmonella* serovars that have similar antigenic structures. We employed a method similar to that described in this paper for designing a method for the specific detection of *S. enterica* serovar Typhimurium. The antigenic structure of *S. enterica* serovar Typhimurium is determined by O antigen (O4), phase-1 (H1) antigen (H:i), and phase-2 (H2) antigen (H:1,2). In this study, these antigenic properties have been used to identify *S. enterica* serovar Typhimurium. In this study, three primer sets were designed from three genes – *rfbJ* (3), *fliC* (4), and *fljB* (5) encoding O:4, H:i, and H:1,2 antigens, respectively, and these were examined for the specific detection of *S. enterica* serovar Typhimurium by applying the multiplex PCR.

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MATERIALS AND METHODS

The DNA from the test strains was prepared by using DNA STAT-60 (TEL-TEST, INC., Friendswood, Tex., USA) and the purified DNA was used as a template for the PCR assay. For the multiplex PCR, three primer pairs were used. All of the primer sequences used in this study are shown in Table 1.The Rfbj primer was selected from the abequose synthase gene (*rfbJ*; GenBank Accession number AE008792), which is necessary for the synthesis of O4 antigen. The Flic primer was chosen from the H1-i antigenic gene (*fliC-i*; *fliC* gene which encodes the H:i antigen gene. GenBank Accession number D13689). The Fljb primer was derived from the H2-1,2 antigenic gene (*fljB*; GenBank Accession number AF045151). PCR was performed in a reaction volume of 25 μl containing PCR reaction buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.3), 50 ng of genomic DNA, 200 μM dNTPs, 1 μM of each primer, and 1 U of Taq DNA polymerase. PCR amplification was performed as follows: the first denaturation cycle at 95°C for 3 min, followed by 30 cycles, each consisting of 1 min of denaturation at 95°C, 1

min of annealing at 65°C, and 30 sec of extension at 72°C, and the final extension cycle of 72°C for 1 min. For each PCR run, a negative control was also included. The reaction was carried out in a DNA thermal cycler (model 9600; Applied Biosystems, Foster City, Calif., USA). The PCR product was electrophoresed on a 2% agarose gel and visualized by UV transillumination after the ethidium bromide staining process.

The strains of *Salmonella* serovars and other bacterial strains used in this study are listed in Table 2. Clinical

Table 1. Nucleotide sequences used as primers in the multiplex PCR

Primer	Target gene	Length (bp)	Sequences	Amplified fragment size (bp)	Source[1)]
Rfbj-s	*rfbJ*	24	5′-CCAGCACCAGTTCCAACTTGATAC	663	AE008792
Rfbj-as		24	5′-GGCTTCCGGCTTTATTGGTAAGCA		
Flic-s	*fliC*	24	5′-ATAGCCATCTTTACCAGTTCCCCC	183	D13689
Flic-as		24	5′-GCTGCAACTGTTACAGGATATGCC		
Fljb-s	*fljB*	24	5′-ACGAATGGTACGGCTTCTGTAACC	526	AF045151
Fljb-as		24	5′-TACCGTCGATAGTAACGACTTCGG		

[1)]: The numbers indicate GenBank-EMBL-DDBL nucleotide sequence database accession numbers.

Table 2. Bacterial strains used in this study and evaluations of the specificity of multiplex PCR

Strains	Strain no.	Antigenic structure			PCR results[1)]		
		O antigen	H-1	H-2[2)]	Rfbj	Flic	Fljb
Salmonella enterica							
Serovar Typhimurium	S-243	4,5,12	i	1,2	+	+	+
Serovar Typhimurium	S-244	4,12	i	1,2	+	+	+
Serovar Typhimurium (another 35 clinical isolates)		4,5,12	i	1,2	+	+	+
Serovar Aberdeen	S-1	11	i	1,2	–	+	+
Serovar Landau	S-125	30	i	1,2	–	+	+
Serovar Saintpaul	S-200	1,4,5,12	e,h	1,2	+	–	+
Serovar Paratyphi B	S-178	4,5,12	b	1,2	+	–	+
Serovar Paratyphi B	S-180	1,4,5,12,27	b	1,2	+	–	+
Serovar Paratyphi B	S-183	1,4,5,12	–	1,–	+	–	–
Serovar Agona	S-9	1,4,12	f,g,s	–	+	–	–
Serovar California	S-37	4,12	g,m,t	–	+	–	–
Serovar Derby	S-59	1,4,12	f,g	–	+	–	–
Serovar Kentucky	S-118	8,20	i	z6	–	+	–
Serovar Newport	S-160	6,8	e,h	1,2	–	–	+
Serovar Onarimon	S-167	1,9,12	b	1,2	–	–	+
Serovar Enteritidis	S-72	1,9,12	g,m	–	–	–	–
Serovar Montevideo	S-146	6,7	g,m,s	–	–	–	–
Serovar Senftenberg	S-210	1,3,19	g,s,t	–	–	–	–
Serovar Typhi	Ty2	9,12,[Vi]	d	–	–	–	–
Serovar Typhi	ATCC19430[3)]	9,12,[Vi]	d	–	–	–	–
Serovar Paratyphi A	RIMD1015[4)]	1,2,12	a	–	–	–	–
Serovar Paratyphi A	NCTC 13[5)]	1,2,12	a	–	–	–	–
Serovar Paratyphi A	NCTC 5702	1,2,12	a	–	–	–	–
Escherichia coli	ATCC11775				–	–	–
Shigella dysenteriae	ATCC13313				–	–	–
Aeromonas hydrophila	ATCC7966				–	–	–
Aeromonas sobria	NIID02-146[6)]				–	–	–
Vibrio cholerae	ATCC14035				–	–	–
Vibrio parahaemolyticus	NIID-98-7				–	–	–
Yersinia enterocolitica	ATCC9610				–	–	–
Citrobacter freundii	ATCC8090				–	–	–

[1)]: +, PCR positives; -, PCR negatives.
[2)]: –, no H-2 phase.
[3)]: American Type Culture Collection, Manassas, Va., USA.
[4)]: Research Institute of Microbial Diseases, Osaka University, Osaka.
[5)]: National Collection of Type Cultures, London, UK.
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Table 3. Epidemiological information of the serovar Typhimurium strains used in this study

	Strain No.	Month/Year of isolation	Place of isolation	Source	Outbreak or sporadic case	Resistance pattern[1)]
1	T00001	Mar-1999	Wakayama	Clinical sample	sporadic	ASTCKN
2	T00002	Apr-1999	Wakayama	Clinical sample	sporadic	ASTCK
3	T00003	Oct-1999	Wakayama	Clinical sample	sporadic	
4	T00004	Dec-1999	Wakayama	Clinical sample	sporadic	
5	T00005	1999	Tokyo	Clinical sample	sporadic	ASTCN
6	T00006	1999	Tokyo	Clinical sample	sporadic	ASTCN
7	T00007	1999	Tokyo	Clinical sample	sporadic	ASTCN
8	T00008	1999	Tokyo	Clinical sample	sporadic	ASTC
9	T00013	Jul-1999	Yamagata	Clinical sample	sporadic	ASTC
10	T00014	Aug-1999	Yamagata	Clinical sample	sporadic	ASTCN
11	T00015	Oct-1999	Yamagata	Clinical sample	sporadic	ASTCK
12	T00017	Aug-1996	Yamagata	Clinical sample	sporadic	ASTCKTmN
13	T00019	Jun-1999	Yamagata	Clinical sample	sporadic	ASTKG
14	T00020	Oct-1999	Yamagata	Clinical sample	sporadic	ASTCKGN
15	T00021	Aug-1999	Yamagata	Clinical sample	sporadic	
16	T00022	Aug-1999	Yamagata	Clinical sample	sporadic	
17	T00023	Nov-1997	Yamagata	Clinical sample	sporadic	ASTCKSxTm
18	T00024	Dec-1996	Yamagata	Clinical sample	sporadic	ASTC
19	T00025	Dec-1996	Yamagata	Clinical sample	sporadic	ASTC
20	T00026	Nov-1997	Yamagata	Clinical sample	sporadic	ASTC
21	T00027	Sep-1997	Yamagata	Clinical sample	sporadic	ASTC
22	T00028	May-1997	Yamagata	Clinical sample	sporadic	ASTC
23	T00052	Jan-2000	Nagano	Stool	sporadic	ASTC
24	T00053	Mar-2000	Okayama	Stool	sporadic	ASTCKGSxTm
25	T00088	Jul-1997	Okayama	Clinical sample	sporadic	ATCKGSiN
26	T00089	Sep-1999	Okayama	Clinical sample	sporadic	ASTSi
27	T00090	Oct-1999	Okayama	Clinical sample	sporadic	ASTCKSiN
28	T00091	Jul-1996	Ehime	Clinical sample	sporadic	ASTCSi
29	T00092	Mar-1995	Ehime	Clinical sample	sporadic	ASTCKSi
30	T00093	Jan-1998	Ehime	Clinical sample	sporadic	ASTCKGSxTmSi
31	T00094	Aug-1998	Ehime	Clinical sample	sporadic	ASTCSi
32	T00095	Oct-1996	Ehime	Clinical sample	sporadic	ASTCKSi
33	T00138	Jul-2000	Hyogo	Stool	outbreak	
34	T00150	Jul-2000	Ibaragi	Stool	outbreak	
35	T00161	Sep-2000	Hyogo	Stool	outbreak	

[1)]: Abbreviations: A; ampicillin, S; streptomycin, T; tetracycline, C; chloramphenicol, K; kanamycin, G; gentamicin, Sx; sulfamethoxazole with trimethoprime, Tm; trimethoprim, Si; sulfisoxazole, N; nalidixic acid.

isolates of serovar Typhimurium were collected from regional public health offices in Japan. The 35 clinical isolates of serovar Typhimurium used for multiplex PCR were isolated from patients who had food-borne diseases and were selected from independent food-borne cases. The epidemiological information regarding clinical isolates is listed in Table 3.

Susceptibility was determined by the disk diffusion method, following National Committee for Clinical Laboratory Standards (NCCLS) recommendations (6). The antibiotic disks (Sensi-disk) were purchased from Becton Dickinson Japan (Tokyo). The antibiotic disks, which contain ampicillin (10 μg/ml), streptomycin (10 μg/ml), tetracycline (30 μg/ml), chloramphenicol (30 μg/ml), kanamycin (30 μg/ml), gentamicin (10 μg/ml), sulfamethoxazole with trimethoprime (S: 23.75/T: 1.25 μg/ml), trimethoprim (5 μg/ml), sulfisoxazole (250 μg/ml), nalidixic acid (30 μg/ml), ciprofloxacin (5 μg/ml), and fosfomycin (50 μg/ml) were used for susceptibility determination.

RESULTS

To identify *S. enterica* serovar Typhimurium using multiplex PCR, three primer sets were used in the same reaction mixture (Table 1). Seven distinct results were obtained from the multiplex PCR according to *Salmonella* serovars (Fig. 1). Three amplified products (663, 526, and 183 bp) in serovar Typhimurium (4,5,12:i:1,2) and serovar Typhimurium (4,12:i:1,2) were found, all of which were derived from the *rfbJ*, *fljB*, and *fliC* genes (Fig. 1, lanes 3 and 4). Another 35 clinical isolates of serovar Typhimurium, which were isolated from independent cases, were tested for multiplex PCR and were correctly identified as seovar Typhimurium using this method (Table 2). In serovars Aberdeen (11:i:1,2) and Landau (30:i:1,2), two positive bands (526 and 183 bp) were amplified from the *fljB* and *fliC* genes (Fig. 1, lanes 5 and 6); in serovars Saintpaul (1,4,5,12:e,h:1,2), Paratyphi B (4,5,12:b:1,2), and Paratyphi B (1,4,5,12,27:b:1,2), two amplified products (663 and 526 bp) from the *rfbJ* and *fljB* were shown (Fig. 1, lanes 7 to 9); in serovars Paratyphi B (1,4,5,12:-:1,-), Agona (1,4,12:f,g,s:-), California (4,12:g,m,t:-), and Derby (1,4,12:f,g:-), one PCR product (663 bp) was amplified from the *rfbJ* gene (Fig. 1, lanes 10 to 13); in serovar Kentucky (8,20:i:z6), one positive band (183 bp) was detected from the *fliC* gene (Fig. 1, lane 14); in serovars Newport

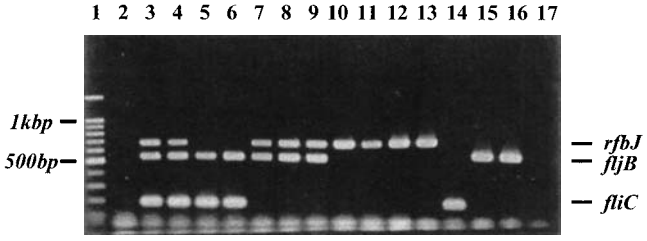

Fig. 1. Multiplex PCR assay using the three primer sets Rfbj, Flic, and Fljb. The lanes contain amplified products from the following sources: 1, DNA size standard (100-bp ladder); 2, negative control; 3, serovar Typhimurium (4,5,12:i:1,2); 4, serovar Typhimurium (4,12:i:1,2); 5, serovar Aberdeen (11:i:1,2); 6, serovar Landau (30:i:1,2); 7, serovar Saintpaul (1,4,5,12:e,h:1,2); 8, serovar Paratyphi B (4,5,12:b:1,2); 9, serovar Paratyphi B (1,4,5,12,27:b:1,2); 10, serovar Paratyphi B (1,4,5,12:-:1,-); 11, serovar Agona (1,4,12:f,g,s:-); 12, serovar California (4,12:g,m,t:-); 13, serovar Derby (1,4,12:f,g:-); 14, serovar Kentucky (8,20:i:z6); 15, serovar Newport (6,8:e,h:1,2); 16, serovar Onarimon (1,9,12:b:1,2); 17, serovar Enteritidis (1,9,12:g,m:-).

(6,8:e,h:1,2) and Onarimon (1,9,12:b:1,2), one amplified product (526 bp) was identified from the *fljB* gene (Fig. 1, lanes 15 and 16); serovar Enteritidis (1,9,12:g,m,:-) possessed none of the antigens O:4, H:i, and H:1,2, and no band appeared (Fig. 1, lane 17). Lane 2 was a negative control of PCR amplification. In addition to the *Salmonella* serovars shown in Figure 1, *S. enterica* serovars Typhi and Paratyphi A and eight non-*Salmonella* enteric pathogens were tested using multiplex PCR employing the three primer sets. None of these pathogens showed any amplified products (Table 2). The sensitivity of this multiplex PCR was 500 ng DNA per tube or 10^5 bacteria per tube. These results clearly show that multiplex PCR is indeed very useful for making a specific detection of *S. enterica* serovar Typhimurium, and three sets of primers specifically detected serovar Typhimurium strains. These results also show that *Salmonella* strains can be identified when the strains possess any antigenic property of O:4, H:i, or H:1,2.

DISCUSSION

Several researchers have already reported about specific detection of *S. enterica* serovar Typhimurium. Ebner and Mathew (7) and Khan et al. (8) have reported specific the detection of *S. enterica* serovar Typhimuriu DT104 by multiplex PCR. They targeted florfenicol (*flo*), integron (*int*), invasion (*invA*), and virulence (*spvC*) genes in the specific detections of serovar Typhimurium DT104. However, Bolton et al. (9) found that 98% of all the serovar Typhimurium tested were positive for *invA* gene and 88% were positive for *spvC* gene. Furthermore, they (9) also reported that *Salmonella* other than serovar Typhimurium had *int* and *spvC* genes. These results indicate that serovar Typhimurium detection by integron (*int*), invasion (*invA*), and virulence (*spvC*) genes does not seem to be specific for serovar Typhimurium.

Soumet et al. have reported that a PCR-based identification system to identify *S. enterica* serovar Typhimurium using the *fliC* gene encoding phase-1 flagellin (H1) (10). That study examined the specificity of serovar Typhimurium and several other *Salmonella* serovars. Unfortunately, this trial cannot be used to distinguish serovar Typhimurium from other *Salmonella* serovars which possess the *fliC-i*. Thus, the *Salmonella* serovars evaluated in that study are not sufficient enough to conclude that the PCR is useful for the specific detection method of *S. enterica* serovar Typhimurium because none of them possess the H:i antigenic property except for serovar Typhimurium.

A PCR-based method has been developed to directly identify *Salmonella* flagellar antigens (11). This method could be applied only to the identification of the most common *Salmonella* second-phase flagellar antigens. None of these methods are useful for making a specific discrimination of *S. enterica* serovar Typhimurium.

In the present study, three sets of primers that targeted the *rfbJ*, *fliC*, and *fljB* genes in a tube were used. This allowed for making a specific identification of O4, H:i, and H:1,2 antigenic properties, because, only *S. enterica* serovar Typhimurium has the antigenic structure combination of O4, H:i, and H:1,2 out of about 2,000 *Salmonella* serovars. The products amplified from *S. enterica* serovar Typhimurium using these primers were sufficiently specific to allow for the detection of the serovar Typhimurium. As a result, the time required to identify *S. enterica* serovar Typhimurium is reduced by the use of multiplex PCR as described here. In conclusion, a multiplex PCR assay to detect *S. enterica* serovar Typhimurium and to discriminate between *S. enterica* serovar Typhimurium and other serovars were developed in this study, and the multiplex PCR described here proved to be specific for detecting of *S. enterica* serovar Typhimurium.

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