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NOTES

Selective Amplification of *tyv* (*rfbE*), *prt* (*rfbS*), *viaB*, and *fliC* Genes by Multiplex PCR for Identification of *Salmonella enterica* Serovars Typhi and Paratyphi A

Kenji Hirose,[1]* Ken-Ichiro Itoh,[2] Hiroshi Nakajima,[3] Takayuki Kurazono,[4] Masanori Yamaguchi,[4] Kazuo Moriya,[5] Takayuki Ezaki,[6] Yoshiaki Kawamura,[6] Kazumichi Tamura,[1] and Haruo Watanabe[1]

Department of Bacteriology, National Institute of Infectious Diseases, Tokyo 162-8640,[1] Department of Microbiology, National Institute of Public Health, Tokyo 108-8638,[2] Department of Microbiology, Okayama Prefectural Institute for Environmental Science and Public Health, Okayama 701-0298,[3] Saitama Institute for Public Health, Saitama 338-0842,[4] Saga Prefectural Institute of Public Health, Saga 849-0925,[5] and Department of Microbiology, Gifu University, School of Medicine, Gifu 500-8750,[6] Japan

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The PCR primers for O, H, and Vi antigen genes, *tyv* (rfbE), *prt* (*rfbS*), *fliC-d*, *fliC-a*, and *viaB*, were designed and used for the rapid identification of *Salmonella enterica* serovars Typhi and Paratyphi A with multiplex PCR. The results showed that all the clinical isolates examined of *Salmonella* serovars Typhi and Paratyphi A were accurately identified by this assay.

Enteric fever remains an important public health problem in many countries of the world. Typhoid fever and paratyphoid fever are still serious public health problems in many geographic areas and are endemic in most countries, especially those of Southeast Asia and Africa. Recently, multiple-drug- or fluoroquinolone-resistant strains of *Salmonella enterica* serovars Typhi and Paratyphi A have been emerging on the Indian subcontinent, spreading to, and becoming major problems, throughout the world (1, 4, 6). Typhoid fever and paratyphoid fever are sometimes-fatal infections of adults and children that cause bacteremia and inflammatory destruction of the intestine and other organs and that require urgent treatment by the administration of appropriate antibiotics. The diagnosis of typhoid fever or paratyphoid fever is made by ordinary culture methods and biochemical tests. The classic diagnosis method for typhoid fever or paratyphoid fever requires at least 4 or 5 days for positive results. A rapid, alternative method is needed for the diagnosis of typhoid fever or paratyphoid fever. Some researchers have already reported serovar Typhi detection methods with PCR that use the *fliC-d* gene (7), the Vi capsular antigen gene (3), and the 16S rRNA gene (9). As only one gene was targeted for the identification of serovar Typhi in these methods, strains of *Salmonella* serovars other than serovar Typhi were detected in some cases. In this study, we developed a more specific diagnosis method for both typhoid fever and paratyphoid fever based on a multiplex PCR technique that detected the Vi antigen gene (*viaB*), H antigen genes (*fliC-d* and *fliC-a*), and O antigen synthesis genes (*tyv* and *prt*). This system enabled us to identify and differentiate serovars Typhi and Paratyphi A, which are clinically important human pathogens, by only a single PCR, when we isolated the bacteria from blood or stool cultures from clinical patients.

The bacterial strains used in this study were collected from the regional public health office in Japan, and all isolates were identified by biochemical and serological tests. A suspension of bacteria was heated at 100°C for 10 min. The samples were then used for the PCRs. We designed the primers tyv-s and tyv-as for detection of the tyvelose epimerase gene (*tyv*, previously called *rfbE*) and the primers fliCcom-s and fliCd-as for detection of the *fliC-d* gene (phase-1 flagellin gene for d antigen [H:d]) of *Salmonella* serovar Typhi. The primers parat-s and parat-as were designed for detection of a paratose synthase gene (*prt*, previously called *rfbS*), and the primers fliC-com-s and fliCa-as were designed for detection of a *fliC-a* gene (phase-1 flagellin; H:a). The primer sequences used in this study are listed in Table 1. The gene *prt* encodes CDP-paratose synthase, which converts CDP-4-keto-3,6-dideoxyglucose to CDP-paratose. The gene *prt* is present in both serovars Typhi and Paratyphi A. The gene *tyv* encodes CDP-tyvelose epimerase, which converts CDP-paratose to CDP-tyvelose. The *tyv* gene is present in both serovars Typhi and Paratyphi A, but the *tyv* gene of serovar Paratyphi A does not produce active CDP-tyvelose epimerase due to the 1-bp deletion which causes the frameshift mutation and converts codon 4 of Tyv to a stop codon (8). We used this deleted region for the design of primer tyv-s to specifically detect the *tyv* gene of serovar Typhi but not of serovar Paratyphi A. The primers for the *viaB* gene were previously reported by Hashimoto et al. (3). The researchers reported the two kinds of primers. One specifically detects the *Salmonella* Vi antigen gene, and the other detects both the

* Corresponding author. Mailing address: Department of Bacteriology, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku, Tokyo 162-8640, Japan. Phone: 81-3-5285-1111, ext. 2230. Fax: 81-3-5285-1163. E-mail: hirosek@nih.go.jp.

TABLE 1. Primers for multiplex PCR amplification of *Salmonella enterica* serovars Typhi and Paratyphi A

Gene and primer (oligonucleotide sequence)	Length (bp)	Amplified fragment size (bp)	Source[b]
tyv (rfbE)			
tyv-s (5′-GAG GAA GGG AAA TGA AGC TTT T-3′)	22	615	M29682
tyv-as (5′-TAG CAA ACT GTC TCC CAC CAT AC-3′)	23		M29682
prt (rfbS)			
parat-s (5′-CTT GCT ATG GAA GAC ATA ACG AAC C-3′)	25	258	M29682
parat-as, (5′-CGT CTC CAT CAA AAG CTC CAT AGA-3′)	24		M29682
viaB			
vi-s (5′-GTT ATT TCA GCA TAA GGA G-3′)	19	439	D14156
vi-as (5′-CTT CCA TAC CAC TTT CCG-3′)	18		D14156
fliC			
fliCcom-s (5′-AAT CAA CAA CAA CCT GCA GCG-3′)	21		L21912
fliCd-as (5′-GCA TAG CCA CCA TCA ATA ACC-3′)	21		L21912
fliCa-as (5′-TAG TGC TTA ATG TAG CCG AAG G-3′)	22		X03393
fliCcom–fliCd-as		750 (489)[a]	
fliCcom–fliCa-as		329	

[a] Number in parentheses represents size of PCR product of H:j gene.
[b] Primers were designed using sequences corresponding to indicated GenBank-EMBL-DDBL nucleotide sequence database accession numbers.

Salmonella and the *Citrobacter freundii* Vi antigen genes. We used the primers which detected only the *Salmonella* Vi antigen gene in this assay. The *fliC-d* and *viaB* genes are present in serovar Typhi, and the *fliC-a* gene is present in serovar Paratyphi A.

The PCR was carried out with a 50 μl mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 1 U of *Taq* DNA polymerase (Promega, Madison, Wis.), 0.2 mM deoxynucleoside triphosphate, a 0.1 μM concentration (each) of primers (tyv-s, tyv-as, parat-s, fliCcom-s, fliCd-as, and fliCa-as, a 0.2 μM concentration (each) of primers parat-as, vi-s, and vi-as, and 5 μl of the DNA sample. The PCR was carried out under the following conditions: 25 cycles with heat denaturation at 95°C for 30 s, primer annealing at 55°C for 60 s, and DNA extension at 72°C for 90 by a DNA thermal cycler (model 9600; Applied Biosystems, Foster City, Calif.). The amplified DNA was separated by 2% agarose gel electrophoresis, stained with ethidium bromide, and visualized by UV transillumination.

The multiplex PCR using five sets of primer pairs, which were targeted for the *viaB*, *prt, tyv, fliC-d*, and *fliC-a* genes, correctly identified *Salmonella* serovars Typhi and Paratyphi A and differentiated the two serovars by the combinations of the different-size bands produced: four positive bands, which consist of *viaB, prt, tyv* and *fliC-d* PCR products, in serovar Typhi and two positive bands, which consist of *prt* and *fliC-a* PCR products, in serovar Paratyphi A (Fig. 1). As expected, the *prt* primers in this study reacted with both serovars Typhi and Paratyphi A, yielding PCR products of the same size. The presence in both serovars Typhi and Paratyphi A of the *prt* gene was consistent with the findings of a previous report (8). The primers for *tyv* specifically detected the *tyv* gene of serovar Typhi. The *prt* primers also detected strains belonging to the O2 and O9 groups of *Salmonella*, and the *tyv* primers detected isolates of the *Salmonella* O9 group (Table 2). The primer pairs for *fliC-d* and *fliC-a* specifically detected the *fliC-d* and *fliC-a* genes, respectively, for the *Salmonella* serovars, and were able to distinguish *fliC-d* and *fliC-a* genes from other *Salmonella* serovar *fliC* genes. The primers for *fliC-d* also detected the *fliC-j* gene, which is an alternate phase of serovar Typhi H-1 antigen genes (5). Since *fliC-j* is a 261-bp deletion derivative of the *fliC-d* gene (2), the PCR product was smaller (Fig. 1).

To examine possible cross-reactions of the selected *viaB, prt, tyv*, and *fliC* primers among major enteric pathogens, including the several genera of the family *Enterobacteriaceae*, some strains were tested by the multiplex PCR assay; none showed positive results (Table 2). To further evaluate the primer specificities for *Salmonella* species, we tested several kinds of salmonella serovars. Detection of both *prt* and *fliC-a* correctly identified serovar Paratyphi A. Detection of the combination of *viaB, tyv*, and *fliC-d* correctly identified serovar Typhi. Hashimoto et al. (3) reported that the PCR primers specific for the Vi antigen gene also reacted with the chromosomal DNAs of serovars Paratyphi C and Dublin which possessed Vi antigen genes. However, our system, which targeted not only the Vi antigen gene but also serogroup O9 and H:d genes for identification, discriminated serovar Typhi from serovars Paratyphi C and *S*. Dublin. Furthermore, our system differentiated serovar Typhi from serovar Strasbourg (9,46:d:1,7), and serovar Ndolo (1,9,12:d:1,5) possessed O9 and H:d genes but not the Vi antigen gene (Table 2). Similarly, other O9 and H:d group strains without the Vi antigen, such as serovars Tarshyne (9,12:d:1,6), Eschberg (9,12:d:1,7), Bangui (9,12:d:e,n,z_{15}), Zega (9,12:d:z_6), Jaffna (1,9,12:d:z_{35}), Ontario (9,46:d:1,5), Quentin (9,46:d:1,6), Olten (9,46:d:e,n,z_{15}), and Plymouth (9,46:d:z_6), may be also discriminated from serovar Typhi by this assay. Here we used purified colonies of each strain for our PCR. We plan to examine whether our system is usable for direct detection from clinical samples.

Taken together, the methods described here may make possible the detection and/or identification of clinically important strains of *Salmonella* serovars Typhi and Paratyphi A strains

TABLE 2. Bacterial strains used to evaluate the specificities of multiplex PCRs and the multiplex PCR results

Bacterium	Strain no.	Antigen structure				PCR result[a]				
		Phage type	O antigen	H-1	H-2[b]	*tyv*	*fliC-d*	*viaB*	*fliC-a*	*prt*
Salmonella enterica										
Serovar Typhi	990116	D1	9,12,[Vi]	d	−	+	+	+	−	+
Serovar Typhi	990120	E1	9,12,[Vi]	d	−	+	+	+	−	+
Serovar Typhi	990005	UVS1	9,12,[Vi]	d	−	+	+	+	−	+
Serovar Typhi	990006	A	9,12,[Vi]	d	−	+	+	+	−	+
Serovar Typhi	990007	E1	9,12,[Vi]	d	−	+	+	+	−	+
Serovar Typhi	990008	E1	9,12,[Vi]	d	−	+	+	+	−	+
Serovar Typhi	990009	E1	9,12,[Vi]	d	−	+	+	+	−	+
Serovar Typhi	990012	E1	9,12,[Vi]	d	−	+	+	+	−	+
Serovar Typhi	990014	E1	9,12,[Vi]	d	−	+	+	+	−	+
Serovar Typhi	990037	D1	9,12,[Vi]	d	−	+	+	+	−	+
Serovar Typhi	980096	46	9,12,[Vi]	d	−	+	+	+	−	+
Serovar Typhi	980111	DVS	9,12,[Vi]	d	−	+	+	+	−	+
Serovar Typhi	980077	UVS1	9,12,[Vi]	d	−	+	+	+	−	+
Serovar Typhi	980014	UVS1	9,12,[Vi]	j	−	+	+[c]	+	−	+
Serovar Typhi	GIFU9954		Rough	d	−	+	+	+	−	+
Serovar Paratyphi A	000055	1	1,2,12	a	[1,5]	−	−	−	+	+
Serovar Paratyphi A	000056	1	1,2,12	a	[1,5]	−	−	−	+	+
Serovar Paratyphi A	990110	2	1,2,12	a	[1,5]	−	−	−	+	+
Serovar Paratyphi A	970083	2	1,2,12	a	[1,5]	−	−	−	+	+
Serovar Paratyphi A	960007	3	1,2,12	a	[1,5]	−	−	−	+	+
Serovar Paratyphi A	000001	4	1,2,12	a	[1,5]	−	−	−	+	+
Serovar Paratyphi A	000041	4	1,2,12	a	[1,5]	−	−	−	+	+
Serovar Paratyphi A	990081	5	1,2,12	a	[1,5]	−	−	−	+	+
Serovar Paratyphi A	970032	5	1,2,12	a	[1,5]	−	−	−	+	+
Serovar Paratyphi A	990046	6	1,2,12	a	[1,5]	−	−	−	+	+
Serovar Paratyphi A	990103	6	1,2,12	a	[1,5]	−	−	−	+	+
Serovar Chester	99023		1,4,[5],12	e,h	e,n,x	−	−	−	−	−
Serovar Agona	99076		1,4,[5],12	f,g,s	[1,2]	−	−	−	−	−
Serovar Oranienburg	99026		6,7,14	m,t	[z_{57}]	−	−	−	−	−
Serovar Infantis	99063		6,7,14	r	1,5	−	−	−	−	−
Serovar Litchfield	99087		6,8	l,v	1,2	−	−	−	−	−
Serovar Hadar	99114		6,8	z_{10}	e,n,x	−	−	−	−	−
Serovar Enteritidis	99109		1,9,12	[f],g,m,[p]	[1,7]	+	−	−	−	+
Serovar Javiana	99112		1,9,12	l,z_{28}	1,5	+	−	−	−	+
Serovar Senftenberg	99017		1,3,19	g,[s],t	−	−	−	−	−	−
Serovar Grumpensis	99089		13,23	d	1,7	−	+	−	−	−
Serovar Poona	99108		1,13,22	z	1,6	−	−	−	−	−
Serovar Typhimurium	1363		1,4,[5],12	i	1,2	−	−	−	−	−
Serovar Enteritidis	1364		1,9,12	[f],g,m,[p]	[1,7]	+	−	−	−	+
Serovar Weltereden	1365		3,10[15]	r	z_6	−	−	−	−	−
Serovar Durban	S-222		9,12	a	e,n,Z_{15}	+	−	−	+	+
Serovar Strasbourg	S-214		9,46	d	1,7	+	+	−	−	+
Serovar Ndolo	S-154		1,9,12	d	1,5	+	+	−	−	+
Serovar Paratyphi C	GIFU12823		6,7,[Vi]	c	1,5	−	−	+	−	−
Serovar Dublin	GIFU13011		1,9,12[Vi]	g,p	−	+	−	+	−	+
Citrobacter freundii			Vi+			−	−[d]	−	−	−
Yersinia pseudotuberculosis			1b			−	−	−	−	−
Yersinia pseudotuberculosis			2a			−	−	−	−	−
Yersinia pseudotuberculosis			2b			−	−	−	−	−
Yersinia pseudotuberculosis			4a			−	−	−	−	−
Yersinia pseudotuberculosis			4b			−	−	−	−	−
Yersinia pseudotuberculosis			5b			−	−	−	−	−
Yersinia enterocolitica			O3			−	−	−	−	−
Yersinia enterocolitica			O5			−	−	−	−	−
Yersinia enterocolitica			O8			−	−	−	−	−
Yersinia enterocolitica			O9			−	−	−	−	−
Vibrio cholerae eltor Ogawa			O1			−	−	−	−	−
Vibrio cholerae eltor Inaba			O1			−	−	−	−	−
Vibrio cholerae			O139			−	−	−	−	−
Vibrio cholerae			non-O1, non-O139			−	−	−	−	−

Continued on following page

TABLE 2—*Continued*

Bacterium	Strain no.	Antigen structure				PCR result[a]				
		Phage type	O antigen	H-1	H-2[b]	*tyv*	*fliC-d*	*viaB*	*fliC-a*	*prt*
Vibrio mimicus						–	–	–	–	–
Vibrio parahaemolyticus						–	–	–	–	–
Vibrio fluvialis						–	–	–	–	–
Aeromonas hydrophila						–	–	–	–	–
Aeromonas sobria						–	–	–	–	–
Aeromonas caviae						–	–	–	–	–
Escherichia coli						–	–	–	–	–
Shigella dysenteriae						–	–	–	–	–
Shigella flexneri						–	–	–	–	–
Shigella boidyii						–	–	–	–	–
Shigella sonnei						–	–	–	–	–

[a] +, PCR positives −, PCR negative.
[b] −, no H-2 phase.
[c] H1-j antigen.
[d] Our primers for the *viaB* gene did not react with Vi antigen genes of *C. freundii.*

within a few working days of the arrival of specimens in the diagnostic microbiology laboratory.

In this study, we used the primers for *viaB, tyv, prt, fliC-a*, and *fliC-d* genes only. However, if specific primers are designed for the amplification of other flagellin genes and/or other O-antigen synthase genes, it might be possible to identify other human-pathogenic *Salmonella* serovars by the combination of O- and H-antigen-specific gene-targeted PCR primers with multiplex PCR.

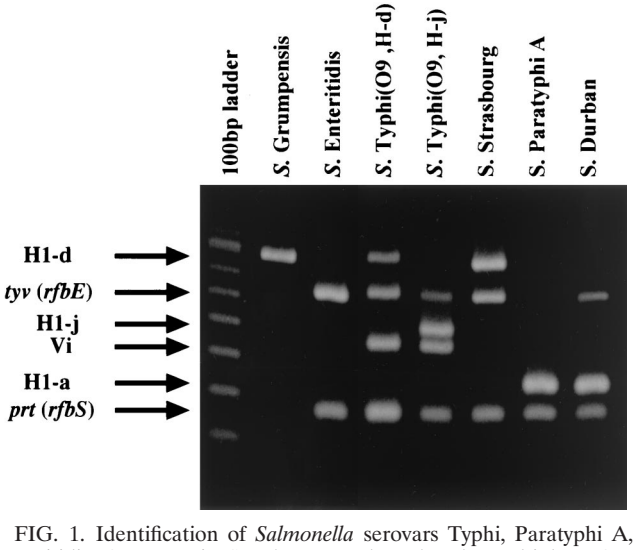

FIG. 1. Identification of *Salmonella* serovars Typhi, Paratyphi A, Enteritidis, Grumpensis, Strasbourg, and Durban by multiplex PCR. After the PCR, the PCR products were separated by 2% agarose gel electrophoresis.

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