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Multiplex PCR method for differentiating highly pathogenic *Yersinia enterocolitica* and low pathogenic *Yersinia enterocolitica*, and *Yersinia pseudotuberculosis*

Thi Hien BUI[1)], Shunsuke IKEUCHI[1)], Yukiko SASSA-O'BRIEN[1)], Takeshi NIWA[1)], Yukiko HARA-KUDO[2)], Takahide TANIGUCHI[1)] and Hideki HAYASHIDANI[1)]*

[1)]Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu-shi, Tokyo 183-8509, Japan
[2)]National Institute of Health Sciences, 3-25-26 Tonomachi, Kawasaki-ku, Kawasaki-shi, Kanagawa 210-9501, Japan

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ABSTRACT. A multiplex PCR method for rapid and sensitive diagnosis, differentiating three pathogenic *Yersinia* groups such as the highly pathogenic *Y. enterocolitica*, including serotype O8, low pathogenic *Y. enterocolitica*, and *Y. pseudotuberculosis,* was developed. Four primer pairs were chosen to detect the genes *fyuA, ail, inv,* and *virF*, responsible for the virulence in pathogenic *Yersinia* species. Under the multiplex PCR conditions, the unique band patterns for the highly pathogenic *Y. enterocolitica,* low pathogenic *Y. enterocolitica,* and *Y. pseudotuberculosis* were generated from *Yersinia* strains. The detection limit of this method was 10^1–10^3 CFU per reaction tube. This multiplex PCR method could detect highly pathogenic *Y. enterocolitica* O8 from the wild rodent fecal samples that were culture-positive. Therefore, the new multiplex PCR method developed in this study is a useful tool for rapid and sensitive diagnosis, distinguishing three pathogenic *Yersinia* groups.

KEY WORDS: detection, diagnosis, multiplex PCR, *Yersinia enterocolitica*, *Yersinia pseudotuberculosis*

Pathogenic bacteria of the *Yersinia* genus, including *Yersinia enterocolitica* and *Yersinia pseudotuberculosis,* are known to cause yersiniosis [2, 4, 5]. From over 60 *Y. enterocolitica* serotypes, only nine serotypes (O3, O4,32, O5,27, O8, O9, O13, O18, O20, and O21) are pathogenic to humans [2, 5]. Among them, serotypes O3, O5,27, and O9 are called "European strains", and show low pathogenicity to humans. In contrast, the remaining six serotypes, which are called "American strains", are highly pathogenic to humans [3, 4]. Generally, human *Yersinia* infection causes gastroenteritis with clinical symptoms including abdominal pain, diarrhea, and fever, however, highly pathogenic *Y. enterocolitica* serotypes including serotype O8 and *Y. pseudotuberculosis* sometimes cause septicemia [2, 4, 5]. In the highly pathogenic *Y. enterocolitica* serotypes, recently serotype O8 has been increasing in Japan [9, 15, 23] and in some European countries such as Germany and Poland [18, 19]. Therefore, a sensitive and rapid method for detecting these pathogens is required.

The diagnostic methods for pathogenic *Yersinia* are mainly based on conventional isolation and identification procedures [7, 13]; however, these methods are time-consuming and laborious. Recently, some PCR methods have been developed to detect *Y. enterocolitica* and *Y. pseudotuberculosis*, allowing rapid diagnosis [6, 20]. A few multiplex PCR methods have been developed to detect both pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* [16, 21]. However, the multiplex PCR method to detect highly pathogenic *Y. enterocolitica,* including serotype O8, low pathogenic *Y. enterocolitica,* and *Y. pseudotuberculosis,* has not been established.

Therefore, the study aimed to develop a rapid multiplex PCR method for the detection and identification of highly pathogenic *Y. enterocolitica*, low pathogenic *Y. enterocolitica,* and *Y. pseudotuberculosis,* and evaluate the performance of the method in the detection of highly pathogenic *Y. enterocolitica* O8 from clinical samples.

*Correspondence to: Hayashidani, H.: eisei@cc.tuat.ac.jp

MATERIALS AND METHODS

Bacterial strains

A total of 25 strains of pathogenic *Yersinia*, including 6 strains of low pathogenic *Y. enterocolitica* serotypes, 9 strains of highly pathogenic serotypes, and 10 strains of pathogenic *Y. pseudotuberculosis* were used in this study. Moreover, non-pathogenic *Y. enterocolitica* serotype O8,19, *Y. aldovae, Y. intermedia, Y. kristensenii, Y. rohdei*, *Escherichia coli*, and *Salmonella enterica* subsp. *enterica* serovar Enteritidis were used to verify the specificity of the multiplex PCR method (Table 1). These strains were stored in skim milk at −80°C until analysis.

DNA extraction for multiplex PCR

All bacterial strains were plated on trypticase soy agar (TSA, Becton, Dickinson and Co., Franklin Lakes, NJ, USA) and incubated for 24 hr at 25°C. After suspending the bacterial cells of each strain in TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA), genomic DNA was extracted using the alkali-heat DNA extraction method described previously [8]. Briefly, 200 µl of the bacterial suspension was centrifuged at 10,000 × *g* for 10 min. The collected pellet was resuspended in 85 µl of sterilized 50 mM NaOH, followed by heating at 100°C for 10 min. After cooling on ice, the suspension was neutralized with 15 µl of sterilized 1 M Tris-HCl (pH 7.0) and centrifuged at 10,000 × *g* for 10 min. The supernatant containing the DNA template was collected and used for the multiplex PCR.

Primer selection

The target genes were selected based on their ability to identify all pathogenic *Yersinia*, including highly pathogenic *Y. enterocolitica*, low pathogenic *Y. enterocolitica,* and *Y. pseudotuberculosis*. These genes included *fyuA* (ferric yersiniabactin uptake receptor A), present on chromosomal DNA of highly pathogenic *Y. enterocolitica* [17]; *ail* (attachment invasion locus),

Table 1. Bacteria strains and band patterns of each bacteria by the polymerase chain reaction (PCR) method

Species	Biotype	Serotype	Strain	PCR results			
				inv	*fyuA*	*ail*	*virF*
Yersinia enterocolitica	4	O3	S3-3	-	-	+	+
Y. enterocolitica	4	O3	S3-8	-	-	+	+
Y. enterocolitica	1B	O4,32	IP96	-	+	+	+
Y. enterocolitica	2	O5,27	S5-250	-	-	+	+
Y. enterocolitica	2	O5,27	S5-203	-	-	+	+
Y. enterocolitica	1B	O8	Ye16-58	-	+	+	+
Y. enterocolitica	1B	O8	NY9306089	-	+	+	+
Y. enterocolitica	1B	O8	IP843	-	+	+	+
Y. enterocolitica	2	O9	S9-87	-	-	+	+
Y. enterocolitica	2	O9	Pa117	-	-	+	+
Y. enterocolitica	1B	O13a,13b	WA285	-	+	+	+
Y. enterocolitica	1B	O13a,13b	WAT568	-	+	+	+
Y. enterocolitica	1B	O18,13b	IP896	-	+	+	+
Y. enterocolitica	1B	O20	IP1106	-	+	+	+
Y. enterocolitica	1B	O21	IP1110	-	+	+	+
Y. pseudotuberculosis		1b	SP-20	+	-	-	+
Y. pseudotuberculosis		1b	SP-1526	+	-	-	+
Y. pseudotuberculosis		2b	1608	+	-	-	+
Y. pseudotuberculosis		3	SP-148	+	-	-	+
Y. pseudotuberculosis		3	SP-1726	+	-	-	+
Y. pseudotuberculosis		4b	SP-2067	+	-	-	+
Y. pseudotuberculosis		4b	SP-2118	+	-	-	+
Y. pseudotuberculosis		5a	SP-328	+	-	-	+
Y. pseudotuberculosis		5a	SP-334	+	-	-	+
Y. pseudotuberculosis		6	SP-901	+	-	-	+
Y. enterocolitica	1A	O8,19	NY8904001	-	-	-	-
Y. aldovae			JCM 5892	-	-	-	-
Y. intermedia			JCM 7579	-	-	-	-
Y. kristensenii			JCM 7576	-	-	-	-
Y. rohdei			JCM 7376	-	-	-	-
Salmonella enterica subsp. *enterica* serovar Enteritidis			NS9506003	-	-	-	-
Escherichia coli			JCM 5491	-	-	-	-

found uniquely on the chromosome of pathogenic *Y. enterocolitica* strains [14, 20]; *inv* (invasin), present on the chromosome of pathogenic *Y. pseudotuberculosis* [20]; and *virF* (virulence regulon transcriptional activator), which is encoded on a 70 kilobase plasmid (pYV) of pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* [4]. PCR primers targeting the *fyuA* gene were designed for this study. A region of the *fyuA* gene sequence of *Y. enterocolitica* serotype O8 (GenBank accession no. Z35486.1), which lacks homology with the *fyuA* gene sequence of *Y. pseudotuberculosis* was chosen to design *fyuA* gene-specific primers using the Primer-BLAST software [22]. The primer pairs for *inv, ail,* and *virF* were designed by Thoerner *et al.* [20]. The details of primers to each target genes are shown in Table 2.

Multiplex PCR method

Initially, monoplex PCR using each primer pair was performed to observe the distribution of target genes among pathogenic *Yersinia* species. After validation of each pair, these four primer pairs were combined to confirm that each PCR product was the correct size. Subsequently, the multiplex PCR conditions were optimized. Each reaction mixture (15 µl) contained 0.1 µM of each primer, 1X Green buffer of Gotaq Flexi DNA polymerase kit (Promega Corp., Madison, WI, USA), 2.5 mM $MgCl_2$, 200 µM dNTP, 0.05 U Gotaq DNA polymerase (Promega), and 5 µl of template DNA. The reaction was performed in a T100 Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) under the following conditions: initial denaturation step for 2 min at 95°C, followed by 30 cycles of 95°C for 30 sec, 56°C for 30 sec, 72°C for 30 sec, and a final extension at 72°C for 5 min. The PCR products were then subjected to electrophoresis on 1.5% agarose ME (Fujifilm Wako Pure Chemical Corp., Osaka, Japan) gel in 1X Tris-acetic acid EDTA buffer (Fujifilm Wako Pure Chemical Corp.) at 100 V for 30 min and stained with AtlasSight DNA Stain (Bioatlas, Tartu, Estonia).

Sensitivity test of developed multiplex PCR

The sensitivity of the developed multiplex PCR was examined using *Y. enterocolitica* O3 (strain S3-3), *Y. enterocolitica* O8 (strain YE16-58), *Y. pseudotuberculosis* 1b (strain SP-20), and *Y. pseudotuberculosis* 4b (strain SP-2067). The bacterial cells of each strain from colonies on TSA were suspended in TE buffer to achieve a final concentration of 10^9 CFU/ml. To examine the detection limits for the developed multiplex PCR, a serial 10-fold dilution of these strains with TE buffer was performed. Genomic DNA from each dilution was obtained using the alkaline-heat DNA extraction method described above and was used for multiplex PCR amplification. Aliquots of the serial dilutions were plated in duplicates onto TSA and grown at 25°C for 24 hr to determine the number of colony-forming units (CFU).

Detection of pathogenic Yersinia from fecal samples

To evaluate the performance of the multiplex PCR developed in this study to detect pathogenic *Yersinia* in clinical samples, a total of 45 wild rodent feces contaminated with *Y. enterocolitica* O8 were used. The fecal samples (0.5 g) were suspended in 4.5 ml of phosphate-buffered saline (PBS; pH 7.6), and 200 µl of the PBS suspension was subjected to DNA extraction. Genomic DNA was extracted immediately after the fecal samples were homogenized in PBS without enrichment. It was purified using the QIAamp DNA stool mini kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions, with 100 µl elution buffer added for DNA collection and used for multiplex PCR amplification. *Y. enterocolitica* O8 was isolated from wild rodent feces using the cold enrichment culture method and was identified as described previously [10].

RESULTS

The specificity of the developed multiplex PCR

The results of the specificity test for the monoplex and multiplex PCR are shown in Table 1. Detection of the *fyuA*, *ail*, *inv*, and *virF* genes correlated well with the genotypic traits of highly pathogenic *Y. enterocolitica*, low pathogenic *Y. enterocolitica,* and *Y. pseudotuberculosis*. Typical examples of multiplex PCR assays for pathogenic *Yersinia* species are shown in Fig. 1. Among the 25 different pathogenic *Yersinia* strains, only highly pathogenic *Y. enterocolitica* showed an extra PCR product of 253 bp, which corresponded to a part of the *fyuA* gene. The 351 bp fragment of the *ail* gene was observed in all the pathogenic *Y. enterocolitica*

Table 2. Oligonucleotide primers used in this study

Target genes		Sequences (5′-3′)	Product length (bp)	Target pathogens	References
inv	F	CGGTACGGCTCAAGTTAATCTG	183	Pathogenic *Yersinia pseudotuberculosis*	[20]
	R	CCGTTCTCCAATGTACGTATCC			
fyuA	F	GGCCGTAAGCTCTCACTT	253	Highly pathogenic *Y. enterocolitica* (American strains)	This study
	R	ACACCATATCAACGGTACGC			
ail	F	TAATGTGTACGCTGCGAG	351	Pathogenic *Y. enterocolitica*	[20]
	R	GACGTCTTACTTGCACTG			
virF	F	GGCAGAACAGCAGTCAGACATA	561	Pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis*	[20]
	R	GGTGAGCATAGAGAATACGTCG			

F, forward primer; R, reverse primer.

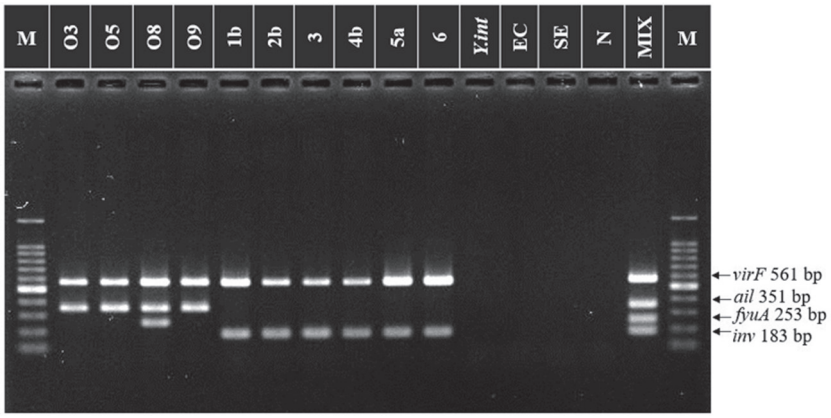

Fig. 1. Agarose gel electrophoresis results for the developed multiplex PCR method with representative isolates of pathogenic *Yersinia* serotypes. Lanes O3, O5, O8, and O9, *Y. enterocolitica* serotype O3, O5,27, O8, and O9, respectively; lanes 1b, 2b, 3, 4b, 5a, and 6, *Y. pseudotuberculosis* serotype 1b, 2b, 3, 4b, 5a and 6, respectively; lane Y.int., *Y. intermedia;* lane EC, *Escherichia coli*; lane SE, *Salmonella enterica* subsp. *enterica* serovar Enteritidis. Lane N, multiplex PCR in the absence of template DNA. Lane MIX, Mix DNA of *Y. enterocolitica* O8 and *Y. pseudotuberculosis* 1b. Lane M, 100 bp DNA ladder (TaKaRa Bio Inc., Kusatsu, Japan).

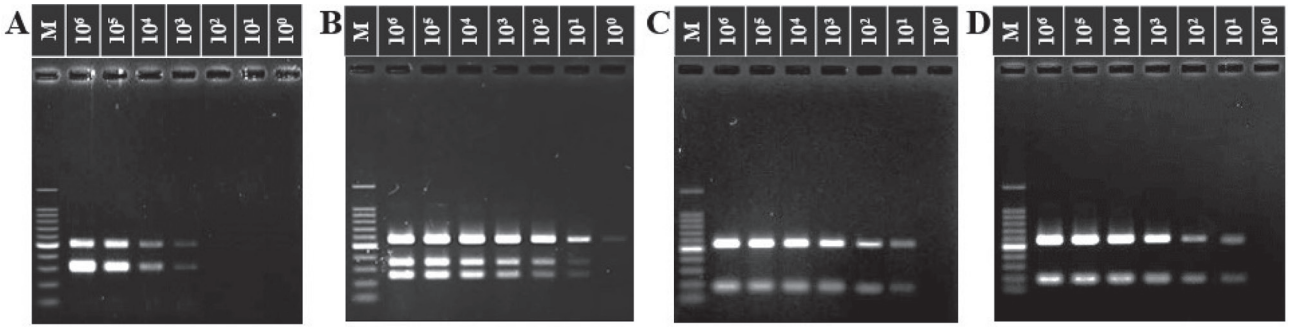

Fig. 2. Detection limits of the multiplex PCR developed in this study. The numbers above each lane represent 10^6, 10^5, 10^4, 10^3, 10^2, 10^1, and 10^0 CFU per reaction tube of template DNA of *Yersinia enterocolitica* O3 (strain S3-3) **(A)**; *Y. enterocolitica* O8 (strain YE16-58) **(B)**; *Y. pseudotuberculosis* 1b (strain SP-20) **(C)**; and *Y. pseudotuberculosis* 4b (strain SP-2067) **(D)**. Lane M, 100 bp DNA ladder (TaKaRa Bio Inc., Kusatsu, Japan).

serotypes, and the 183 bp fragment of the *inv* gene was detected in all the *Y. pseudotuberculosis* serotypes. The amplicon of 561 bp, which corresponded to a part of the *virF* gene, was observed in all the pathogenic *Yersinia* serotypes tested. The pattern with two bands, 253 bp, and 351 bp, indicated the presence of a highly pathogenic *Y. enterocolitica* strains. The single-band, 351 bp corresponded to low pathogenic *Y. enterocolitica* strains, and 183 bp corresponded to *Y. pseudotuberculosis* strains. The 561 bp band indicated the presence of a virulent plasmid of the *Yersinia* strains. Thus, the highly pathogenic *Y. enterocolitica*, pathogenic *Y. enterocolitica*, and *Y. pseudotuberculosis* can be differentiated by three different band patterns. No targeted gene products were amplified from the negative controls (Fig. 1).

The sensitivity of the developed multiplex PCR

The results showed that multiplex PCR was able to detect pathogenic *Yersinia* with 10^1–10^3 CFU per reaction tube. Among the four strains tested, *Y. enterocolitica* O8 (strain YE16-58) and two strains of *Y. pseudotuberculosis* 1b (strain SP-20) and 4b (strain SP-2067) were detectable at 10^1 CFU per reaction tube. However, more than 10^3 CFU per reaction tube was required to detect *Y. enterocolitica* O3 (strain S3-3) (Fig. 2).

Detection of pathogenic Yersinia from fecal samples

The multiplex PCR results were in agreement with those from the culture method (Table 3 and Fig. 3). Among the 45 naturally contaminated wild rodent fecal samples tested, simultaneous amplification of the *virF*, *ail,* and *fyuA* genes was observed in three (6.7%) samples, indicating the presence of highly pathogenic *Y. enterocolitica* O8. These samples were the same as those of the culture-positive samples. No PCR product was observed in the culture-negative samples.

Table 3. Comparison of multiplex PCR method with culture method in detecting pathogenic *Yersinia* from wild rodent fecal samples

Methods	No. of samples	*Y. enterocolitica*	*Y. pseudotuberculosis*
Multiplex PCR	45	3 (6.7%)§	0
Culture		3 (6.7%)§	0

§ *Yersinia enterocolitica* O8.

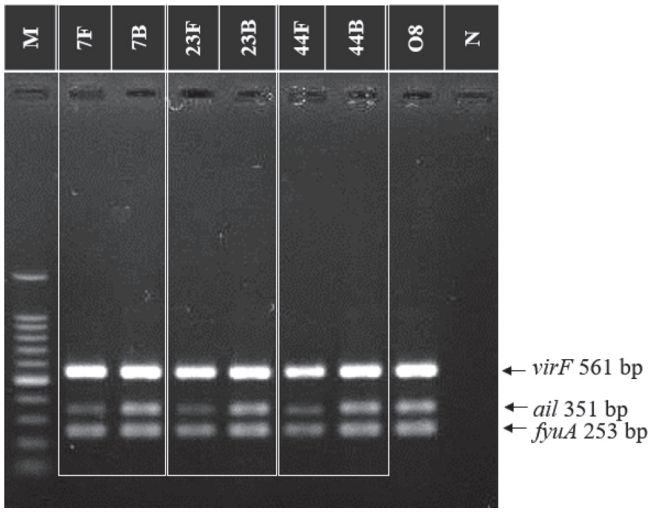

Fig. 3. Band pattern of the developed multiplex PCR for *Yersinia* from fecal samples and *Yersinia* isolates from wild rodents. Lane M, 100 bp DNA ladder (TaKaRa Bio Inc., Kusatsu, Japan). Lanes 7F, 23F, 44F, mice fecal samples number 7, 23, and 44, respectively; 7B, 23B, 44B, *Yersinia* isolates from mice fecal samples number 7, 23, and 44, respectively. Lane O8, positive control *Y. enterocolitica* O8. Lane N, multiplex PCR in the absence of template DNA.

DISCUSSION

A few multiplex PCR methods to detect *Y. enterocolitica* and *Y. pseudotuberculosis* have been reported [16, 21]. However, no reports are available on the multiplex PCR method for simultaneous detection and identification of low and high pathogenic *Y. enterocolitica* at the same time. A rapid, specific, and sensitive multiplex PCR method, which can detect and distinguish the three pathogenic *Yersinia* groups consisting of highly pathogenic *Y. enterocolitica*, including serotype O8, low pathogenic *Y. enterocolitica*, and *Y. pseudotuberculosis,* was developed in this study. A new primer pair targeting *fyuA* was designed to detect highly pathogenic *Y. enterocolitica*. This *fyuA* primer pair was combined with the *ail, inv,* and *virF* primer pairs described previously by Thoerner *et al.* [20] to allow both detection and differentiation among the three pathogenic *Yersinia* groups. The primer pairs, *ail, inv,* and *virF,* were initially designed and used in conventional monoplex PCR assays [20]. Under the multiplex PCR conditions, with a mixture of these four pairs of primers (Fig. 1), three groups of pathogenic *Yersinia,* were distinguished. Moreover, the detection limit of the multiplex PCR method was 10^1–10^3 CFU per reaction tube, which demonstrated a high sensitivity level [11, 16]. A spike experiment using *Yersinia*-free pig fecal samples was performed, and the multiplex PCR method developed in this study could detect pathogenic *Yersinia* at 10^1–10^3 CFU per reaction tube from spiked fecal samples (data not shown). However, few reports [1, 12] have stated that some primer sets of PCR methods for detecting pathogenic *Yersinia* showed high sensitivity in spiked fecal samples but not in naturally contaminated samples. Therefore, the multiplex PCR method was applied to detect pathogenic *Yersinia* from wild rodent feces contaminated with *Y. enterocolitica* O8 to determine the feasibility of this method as a diagnostic tool. The multiplex PCR method developed in this study could detect *Y. enterocolitica* O8 from the same rodent fecal samples that were culture-positive (Table 3, Fig. 3). While the conventional culture method is time-consuming and laborious [7, 13], the multiplex PCR method can be completed within one day. Therefore, the multiplex PCR developed in this study seems to be a useful method for rapid and sensitive diagnosis, distinguishing three pathogenic *Yersinia* groups such as highly pathogenic *Y. enterocolitica*, including *Y. enterocolitica* O8, low pathogenic *Y. enterocolitica*, and *Y. pseudotuberculosis*.

CONFLICT OF INTEREST STATEMENT. The authors declare no conflicts of interest

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