J Microbiol Immunol Infect.
2007;40:222-226

Original Article

A multiplex polymerase chain reaction method for rapid identification of *Citrobacter freundii* and *Salmonella* species, including *Salmonella* Typhi

Chia-Ling Lin[1,2], Cheng-Hsun Chiu[1], Chishih Chu[3], Yhu-Chering Huang[1], Tzou-Yien Lin[1], Jonathan T. Ou[4]

[1]Department of Pediatrics, Chang Gung Children's Hospital, Taoyuan; [2]Department of Pediatrics, Chiayi Christian Hospital, Chiayi; [3]Department of Microbiology and Immunology, National Chiayi University, Chiayi; and [4]Department of Microbiology and Immunology, Chang Gung University College of Medicine, Taoyuan, Taiwan

Received: July 1, 2005 Revised: February 1, 2006 Accepted: May 21, 2006

Background and Purpose: *Salmonella enterica* is one of the most common enteric pathogens worldwide. Conventional methods of isolation of *Salmonella* strains take 4-7 days to complete, are laborious and require substantial manpower. We devised a polymerase chain reaction (PCR) method that simultaneously uses three pairs of specific primers to detect *inv*, *spv*, and *via* genes of *Salmonella*.

Methods: Three primer pairs were designed, including: SPVC-1 and SPVC-2, based on the nucleotide sequences of the *spvC* gene; INVA-1 and INVA-2, based on the *invA* gene; and VIAB-1 and VIAB-2, based on the *viaB* gene. PCR was performed using these three primers to identify 14 clinically important bacterial organisms, including *Citrobacter freundii, S. enterica* serovars Typhi and Paratyphi C, Dublin, and other non-typhoidal *Salmonella* that harbor a virulence plasmid.

Results: The following strains were readily identified using the PCR: (1) *C. freundii*; (2) *S.* Typhi; and *S.* Paratyphi C; (3) *S.* Dublin (virulence antigen-positive); and (4) *Salmonella* serovars that harbor an *spv*-type virulence plasmid. *S. enterica* could also be identified, but required further testing to determine serovar.

Conclusions: This PCR method allows *S.* Typhi to be identified immediately so that appropriate antibiotic treatment can be initiated without delay.

Key words: *Citrobacter freundii*; Polymerase chain reaction; *Salmonella enterica*

Introduction

Salmonella enterica is one of the most common enteric pathogens worldwide. *S. enterica* has more than 2500 serovars, which include various host-specific pathogens. *S. enterica* serotypes Typhi and Paratyphi cause enteric fever in humans and have no other animal reservoirs including primates; *Salmonella* Choleraesuis causes severe salmonellosis in swine; *Salmonella* Gallinarum-Pullorum is specific to fowls; *Salmonella* Dublin causes fever, diarrhea, and severe systemic diseases in cattle. *Salmonella* Enteritidis infects fowls such as chicken without severe illness, but when transmitted to humans, it causes severe diarrhea and, occasionally, bacteremia. *Salmonella* Typhimurium causes systemic disease in mice, the pathogenesis of which closely resembles typhoid fever in humans. It is the most frequent etiologic agent of salmonellosis in humans. In the United States, more than one-half of food poisoning cases were caused by *S. enterica*, and about 70% of salmonellosis was caused by *S.* Typhimurium [1,2]. Non-typhoidal *Salmonella* infections are a major public health issue and are being reported with increasing frequency [1-4].

In addition to salmonellae, numerous other enteric pathogens cause various diarrheal diseases, such as *Escherichia coli, Shigella,* and *Vibrio* [4]. High-risk patients, including human immunodeficiency virus-infected persons, elderly persons, children, and patients

Corresponding author: Dr. Cheng-Hsun Chiu, Department of Pediatrics, Chang Gung Children's Hospital, No. 5-7, Fu-Hsin St, Kweishan, Taoyuan 333, Taiwan.
E-mail: chchiu@adm.cgmh.org.tw

with autoimmune diseases, are prone to bacterial infection and require urgent medical treatment for these infections [5-9]. Therefore, it is important to rapidly detect and identify the etiologic agent when bacterial enteritis is suspected, especially for human-specific typhoid fever, which is a systemic and often fatal disease.

The development of molecular methods for diagnosis of infectious diseases has improved the sensitivity, specificity, quality and availability of diagnosis and treatment. Methods of direct detection of virulence genes of *Salmonella* by polymerase chain reaction (PCR)-based assays, may be useful for pathogen identification [10,11]. All *Salmonella* carry the *invA* gene, which is not carried by any other bacterial species, and is known to enable the bacteria to invade cells [12,13]. The *spvC* virulence gene is located in the virulence plasmid of seven serovars of *Salmonella* that are known to harbor the virulence plasmid, including the most frequent etiologic agents, *S.* Typhimurium and *S.* Enteritidis [12,14,15]. The others are *Salmonella* Abortusovis, *S.* Choleraesuis, *S.* Dublin, *S.* Gallinarum-Pullorum, and *Salmonella* Sendai. Chiu and Ou had developed rapid and definite detection of *Salmonella* strain in feces by PCR assay with these two pairs of oligonucleotide primers [12]. Furthermore, *S.* Typhi and *S.* Paratyphi C have a virulence factor called the Vi (virulence) antigen, that is a required factor for the two strains to be virulent [16]. Two other known bacterial species that have the Vi antigen are *Citrobacter freundii* and *S.* Dublin. According to the genetic characteristics of these pathogens, we devised a multiplex PCR assay that utilizes the sequences of the *invA*, *spvC* and *viaB* genes to rapidly and accurately detect the specific pathogens which cause food-borne diseases.

Methods

Bacterial strains

Fourteen bacterial species and strains isolated from clinical specimens and stocked in our laboratory were used in this study, including 12 *Salmonella* strains representing 7 different serovars, 1 *E. coli* strain and 1 *C. freundii* strain. The serovars of *Salmonella* strains were 2 *S.* Typhimurium, 1 *S.* Choleraesuis, 1 *S.* Dublin Lane, 1 *S.* Gallinarum-Pullorum, 1 *S.* Enteritidis, 5 *S.* Typhi and 1 *S.* Paratyphi C.

PCR primers

The primer pairs used in the PCR were designed according to the nucleotide sequences of the three genes that are already known. The first primer pair, SPVC-1 and SPVC-2, was designed based on the nucleotide sequence from bp 505 to bp 528 for SPVC-1 (ACTCCTTGCACAACCAAATGCGGA) and bp l052 to bp l075 for SPVC-2 (TGTCTTCTGCATTTCGCCAC CATCA), of the *spvC* gene [12,14,15]. This primer pair will amplify and produce a double-stranded fragment of 570 bp, as described previously [12]. The second primer pair, INVA-1 and INVA-2, was synthesized based on the nucleotide sequence of the *invA* gene, from bp 104 to bp 127 for INVA-1 (ACAGTGCTCGTTTAC GACCTGAAT), and bp 324 to bp 347 for INVA-2 (AGACGACTGGTACTGATCGATAAT) [12,13]. This primer pair will amplify and produce a double-stranded fragment of 243 bp as described previously [12]. The third primer pair, VIAB-1 and VIAB-2, was prepared according to the nucleotide sequence of the *viaB* gene (Ou JT, unpublished data), VIAB-1 from bp 5867 to bp 5888 (TGTCGAGCAGATGGATGAGCAT), and VIAB-2, from bp 6362 to bp 6383 (ACGGCTGAAGGT TACGGACCGA). This primer pair will amplify and produce a double-stranded fragment of 516 bp.

PCR assay

About 500 bacteria were added into 50 μL reaction buffer (Promega, Madison, WI, USA) that contained 0.2 μL of *Taq* polymerase (Promega), 3 μL of dNTP (2.5 mM each), 1.5 mM magnesium chloride and 1 μM (each) of primer pairs. The process and conditions of PCR were as follows: incubation at 95°C for 2 min; at 85°C for 5 min, again at 95°C for 1 min to denature the DNA; then at 58°C for 1 min to allow the denatured DNA to anneal, and at 75°C for 1 min for extension reaction. The denaturation, annealing and extension steps were repeated for 5 cycles, then the next denaturation (95°C, 30 sec), annealing (58°C, 1 min) and extension (75°C for 1 min) steps were repeated for 25 cycles. Incubation was completed at 62°C for 2 min and at 72°C for 10 min. The reaction mixture was then electrophoresed in a 3% agarose gel at 40 volts for 3 h. The DNA fragments in the agarose gel were stained with ethidium bromide (0.5 mg/mL) and photographed under ultraviolet light.

Results

The 14 bacterial species and strains used to test this detection method are listed in Table 1. A representative result is shown in Fig. 1. Lane 1 was derived from an *E. coli* strain, which contained none of the genes that the three pairs of primers represented, and therefore, no

Table 1. Laboratory strains used

Strain	Species	Plasmid (kb)	Description
OU3478	*Escherichia coli*	None	Strain DH10B, no plasmid
OU7107	*Citrobacter freundii*	None	Strain WR7004, wild type, Vi$^+$, no plasmid
OU5109	*Salmonella* Typhimurium	None	Strain M206, no plasmid
OU5045	*Salmonella* Typhimurium	90	Strain C5, wild type, harbors virulence plasmid
OU7085	*Salmonella* Choleraesuis	50, 6.7	Harbors two plasmids, one virulence and one non-virulence
OU7178	*Salmonella* Dublin Lane	80	Harbors virulence plasmid
OU7115	*Salmonella* Gallinarum-Pullorum	85	Harbors virulence plasmid
OU7130	*Salmonella* Enteritidis	60	Wild type, harbors virulence plasmid
OU7049	*Salmonella* Typhi	None	Strain FR57, Vi$^-$, no plasmid
OU6050	*Salmonella* Typhi	60, 100	Strain 541 Vi$^-$, two plasmids, but no virulence plasmid
OU6055	*Salmonella* Typhi	None	Strain TY2, Vi$^+$, no plasmid
OU6060	*Salmonella* Typhi	None	Vi$^+$, acquired *viaB* from *Citrobacter freundii,* no plasmid
OU6061	*Salmonella* Typhi	80	Vi$^+$, no virulence plasmid
OU7001	*Salmonella* Paratyphi C	60	Strain ETS32, Vi$^+$, no virulence plasmid

Abbreviations: Vi = virulence antigen; $^-$ = negative; $^+$ = positive

bands were expected and none were observed. Lane 2 was derived from a *C. freundii* strain WR7004, which contained the *viaC* gene but not the other two genes, *invA* and *spvC,* and therefore only a 516-bp fragment appeared. Lane 3 was derived from *S.* Typhimurium strain M206, a virulence plasmid-cured strain. This strain carried only *invA* and no *spvC* nor *viaB,* and thus only produced a 243-bp fragment derived from *invA.* Lanes 4 and 5 were derived from *S.* Typhimurium strain C5 and *S.* Choleraesuis, respectively. They both contained *spvC* and *invA* but no *viaB.* As expected, the lane showed two bands, one of 570 bp (*spvC*) and the other of 243 bp (*invA*). Lane 6 was derived from *S.* Dublin strain, which also carries a virulence plasmid-containing *Salmonella* with Vi$^+$, and therefore was expected to contain the three genes. Accordingly, three bands with corresponding sizes of 570, 516 and 243 bp were observed in lane 6. Lanes 7 and 8 were derived from *S.* Gallinarum-Pullorum and *S.* Enteritidis, respectively, and both were Vi$^-$ and harbored a virulence plasmid. They were therefore expected to carry *spvC* and *invA* but no *viaB.* As expected, these two lanes showed two bands, of 570 and 243 bp. All *S.* Typhi and *S.* Paratyphi did not harbor a *spv*-type virulence plasmid. Lanes 9 and 10 were derived from Vi$^-$ *S.* Typhi strains that were isolated independently. These two strains carry only *invA* and not *spvC* or *viaB.* Both produced only the amplicon of 243 bp in PCR. Lanes 11, 12 and 13 were all derived from independently isolated wild type *S.* Typhi. The PCR showed that they were indeed positive for *invA* and *viaB,* but not *spvC.* Lane 14 was derived from *S.* Paratyphi C strain ETS32, which expressed Vi, and therefore, should contain *invA* and *viaB* but not *spvC,* since it harbored no plasmids. As expected, lane 14 showed two bands, of 516 and 243 bp.

Discussion

All *Salmonella* carry the *invA* gene, which is not carried by any other bacterial species. Therefore, if a 243-bp fragment appeared in PCR with the two primers INVA-1 and INVA-2, it would indicate that the sample bacteria contains an *invA* gene, and is most likely *Salmonella*. Only the following seven serovars have been reported to harbor the virulence plasmid: *S.* Typhimurium, *S.* Enteritidis, *S.* Abortusovis, *S.* Choleraesuis, *S.* Dublin, *S.* Gallinarum-Pullorum, and *S.* Sendai. The plasmids in these serovars differ in sizes but all carry the virulence-related gene *spvC* [14,15]. Therefore, if the sample bacteria had been identified as *Salmonella* by INVA-1 and INVA-2 as mentioned above, this *Salmonella* strain could then be further identified as one of the seven virulence plasmid-containing *Salmonella* serovars. This can be performed by checking for the appearance of a double-stranded 570-bp fragment in the PCR with the primer pair SPVC-1 and SPVC-2.

Human-specific pathogens, *S.* Typhi and *S.* Paratyphi, are the etiologic agents of enteric fever. Therefore, when *Salmonella* bacteria are identified, a further test should be performed to determine whether the isolate is *S.* Typhi or *S.* Paratyphi. *S.* Typhi and *S.* Paratyphi C carry a Vi antigen. The great majority of the other *Salmonella* do not carry the Vi antigen. If PCR could detect this factor with the primer pair VIAB-1 and VIAB-2, the sample bacteria could readily be identified as *S.* Typhi or *S.* Paratyphi C. *Citrobacter*

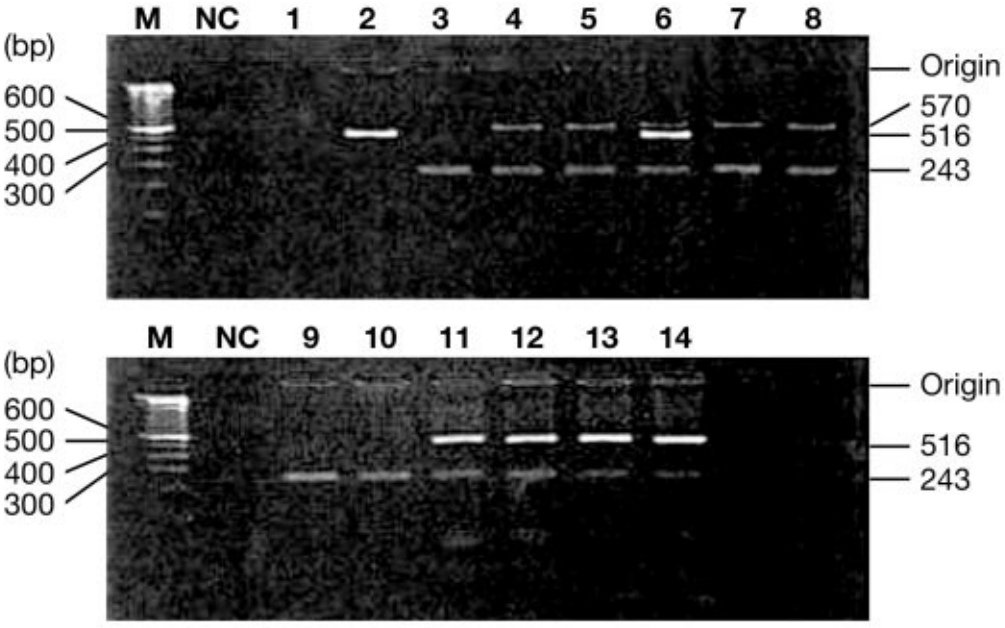

Fig. 1. A result of polymerase chain reaction (PCR) with the simultaneous use of the three primer pairs, INVA-1 and INVA-2, SPVC-1 and SPVC-2, and VIAB-1 and VIAB-2. The numbers on the left indicate the length (in bp) of markers obtained from the molecular size marker in lane M, and the numbers on the right side indicate the position of the fragments produced by PCR. Lane M, 100-bp molecular size (bp) marker; lane NC, none (blank); lane 1, *Escherichia coli*; lane 2, *Citrobacter freundii* WR7004; lane 3, *Salmonella* Typhimurium M206; lane 4, *Salmonella* Typhimurium C5; lane 5, *Salmonella* Choleraesuis; lane 6, *Salmonella* Dublin Lane; lane 7, *Salmonella* Gallinarum-Pullorum; lane 8, *Salmonella* Enteritidis; lane 9, *Salmonella* Typhi FR57 Vi^-; lane 10, *Salmonella* Typhi 541 Vi^+; lane 11, *Salmonella* Typhi TY2 Vi^+; lane 12, *Salmonella* Typhi Vi^+; lane 13, *Salmonella* Typhi Vi^+; lane 14, *Salmonella* Paratyphi C FTS32 Vi^+. Vi = virulence antigen; $^-$ = negative; $^+$ = positive.

freundii and *S.* Dublin are two other known bacterial species that carry the Vi antigen. Among all non-*Salmonella Enterobacteriaceae,* only *C. freundii* carries the Vi antigen; therefore, if only the primer pair for the *viaB* gene, VIAB-1 and VIAB-2, produced a 516-bp fragment, the bacteria would be *C. freundii.* On the other hand, among non-typhoidal *Salmonella,* only *S.* Dublin carries a virulence plasmid as well as the Vi gene. Thus, a 516-bp fragment produced from the multiplex PCR would identify the bacteria as *S.* Dublin.

To verify the value of this multiplex PCR system for the rapid identification of Gram-negative bacteria that cause infections in humans, we randomly collected Gram-negative bacterial isolates from various clinical specimens during a three-month period at Chang Gung Memorial Hospital. Before detailed biochemical characterization was performed, these isolates were subjected to PCR using this rapid identification system. The results of the PCR, clinical diagnosis, and final identification of the isolates are shown in Table 2. PCR methods can be used as an alternative tool for rapid identification of some medically important Gram-negative pathogens, *Salmonella* in particular. Conventional methods of isolation of *Salmonella* strains take 4-7 days to complete, are laborious and require substantial manpower. In addition, very small numbers of viable organisms present in the feces may fail to grow in artificial laboratory media. If problems associated with sample preparation that hamper PCR reaction efficiency can be overcome, this method may be used in the future for direct detection and identification of bacterial pathogens from clinical specimens.

This study has demonstrated that this three-pair primer PCR method can readily identify the following strains: (1) *C. freundii*; (2) *S.* Typhi and *S.* Paratyphi C; (3) *S.* Dublin (Vi^+); (4) *Salmonella* serovars that harbor an *spv*-type virulence plasmid; and (5) *S. enterica* in general. In the last case, further serological testing needs to be carried out to determine specific serovars. In most cases, however, identification to this extent is a helpful indication for medical personnel to initiate therapeutic measures. The advantage of this three-primer method is that it can simultaneously identify more serovars at a time, thus facilitating the search for specific etiologic *Salmonella* organisms. The most clinically important aspect of the method is that it can identify *S.* Typhi immediately, allowing antibiotic treatment to be initiated without further delay.

Table 2. Rapid identification of clinical Gram-negative bacterial isolates by multiplex polymerase chain reaction (PCR)

Clinical diagnosis	No. of isolates	Site of isolation	PCR identification	Final identification
Typhoid fever	2	Blood	2 (*Salmonella* Typhi)	*Salmonella* Typhi (2)
Bacteremia	8	Blood	4 (Non-typhoidal *Salmonella*)	Non-typhoidal *Salmonella* (*Salmonella* Choleraesuis, 2; *Salmonella* serogroup B, 1; and *Salmonella* serogroup D, 1) *Escherichia coli* (2) *Klebsiella pneumoniae* (1) *Acinetobacter* spp. (1)
Gastroenteritis	20	Stools	18 (Non-typhoidal *Salmonella*)	Non-typhoidal *Salmonella* (serogroup B, 8; serogroup D, 6; serogroup C2, 3; and serogroup E, 1) *Campylobacter* spp. (2)
Urinary tract infection	24	Urine	2 (*Citrobacter freundii*)	*Escherichia coli* (17) *Serratia marcescens* (2) *Citrobacter freundii* (2) *Proteus mirabilis* (1) *Klebsiella pneumoniae* (1) *Pseudomonas aeruginosa* (1)

Acknowledgments

This study was supported financially supported by a grant from Crystal Biotechnology Research and Development Company Ltd., Taiwan.

References

1. Centers for Disease Control and Prevention (CDC). *Salmonella* surveillance: annual summary, 2000. Atlanta, GA: US Department of Health and Human Services, CDC; 2001. Available from: http://www.cdc.gov/ncidod/dbmd/phlisdata/salmtab/2000/SalmonellaAnnualSummary2000.PDF
2. Lee LA, Puhr ND, Maloney EK, Bean NH, Tauxe RV. Increase in antimicrobial-resistant *Salmonella* infections in the United States, 1989-1990. J Infect Dis. 1994;170:128-34.
3. Su LH, Chiu CH, Wu TL, Chu C, Chia JH, Kuo AJ, et al. Molecular epidemiology of *Salmonella enterica* serovar Enteritidis isolated in Taiwan. Microbiol Immunol. 2002;46:833-40.
4. Tauxe RV. Emerging foodborne diseases: an evolving public health challenge. Emerg Infect Dis. 1997;3:425-34.
5. Celum CL, Chaisson RE, Rutherford GW, Barnhart JL, Echenberg DF. Incidence of salmonellosis in patients with AIDS. J Infect Dis. 1987;156:998-1002.
6. Levine WC, Smart JF, Archer DL, Bean NH, Tauxe RV. Foodborne disease outbreaks in nursing homes, 1975 through 1987. JAMA. 1991;266:2105-9.
7. Mishu B, Koehler J, Lee LA, Rodrigue D, Brenner FH, Blake P, et al. Outbreaks of *Salmonella enteritidis* infections in the United States, 1985-1991. J Infect Dis. 1994;169:547-52.
8. Saidi SM, Iijima Y, Sang WK, Mwangudza AK, Oundo JO, Taga K, et al. Epidemiological study on infectious diarrheal diseases in children in a coastal rural area of Kenya. Microbiol Immunol. 1997;41:773-8.
9. Lim E, Koh WH, Loh SF, Lam MS, Howe HS. Non-typhoidal salmonellosis in patients with systemic lupus erythematosus. A study of fifty patients and a review of the literature. Lupus. 2001;10:87-92.
10. Malorny B, Hoorfar J, Bunge C, Helmuth R. Multicenter validation of the analytical accuracy of *Salmonella* PCR: towards an international standard. Appl Environ Microbiol. 2003;69:290-6.
11. Pathmanathan SG, Cardona-Castro N, Sanchez-Jimenez MM, Correa-Ochoa MM, Puthucheary SD, Thong KL. Simple and rapid detection of *Salmonella* strains by direct PCR amplification of the hilA gene. J Med Microbiol. 2003;52:773-6.
12. Chiu CH, Ou JT. Rapid identification of *Salmonella* serovars in feces by specific detection of virulence genes, *invA* and *spvC*, by an enrichment broth culture-multiplex PCR combination assay. J Clin Microbiol. 1996;34:2619-22.
13. Rahn K, De Grandis SA, Clarke RC, McEwen SA, Galan JE, Ginocchio C, et al. Amplification of an *invA* gene sequence of *Salmonella typhimurium* by polymerase chain reaction as a specific method of detection of *Salmonella.* Mol Cell Probes. 1992;6:271-9.
14. Gulig PA, Danbara H, Guiney DG, Lax AJ, Norel F, Rhen M. Molecular analysis *of spv* virulence genes of the *Salmonella* virulence plasmids. Mol Microbiol. 1993;7:825-30.
15. Krause M, Roudier C, Fierer J, Harwood J, Guiney D. Molecular analysis of the virulence locus of the *Salmonella dublin* plasmid pSDL2. Mol Microbiol. 1991;5:307-16.
16. Daniels EM, Schneerson R, Egan WM, Szu SC, Robbins JB. Characterization of the *Salmonella paratyphi* C Vi polysaccharide. Infect Immun. 1989;57:3159-64.