J Microbiol Immunol Infect
2002;35:147-151

Identification of *Salmonella enteritidis* isolates by polymerase chain reaction and multiplex polymerase chain reaction

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Received: January 22, 2002 Revised: February 5, 2002 Accepted: February 25, 2002

The polymerase chain reaction and the multiplex polymerase chain reaction were developed for detection of *Salmonella* and for identification of the serotype *enteritidis*. Three sets of primers were selected from different genomic sequences amplifying a 429 bp fragment specific for the genus *Salmonella* within a randomly cloned sequence, including a 250 bp fragment within the *spv* gene, and a 310 bp fragment within the *sef*A gene specific for *Salmonella enteritidis*. The polymerase chain reaction and the multiplex polymerase chain reaction were used for detecting *S. enteritidis* isolated from stool samples during outbreaks of foodborne gastroenteritis between 1992 and 1998 in Taiwan. The *sef*A gene was detected in all 27 strains of *S. enteritidis* by this polymerase chain reaction method. Multiplex polymerase chain reaction could detect 3 genes in all strains, but could not detect the *spv* gene in 2 strains. The sensitivity of the polymerase chain reaction and the multiplex polymerase chain reaction were 10^4 and 10^5 cells/mL, respectively. In double polymerase chain reaction, the sensitivity increased to 100 cells/mL. These data indicate that the specificity and sensitivity of the polymerase chain reaction and the multiplex polymerase chain reaction make them potentially valuable tools for diagnosis of *S. enteritidis* infection and that they may be used for the identification of *S. enteritidis* responsible for sporadic enteritis cases.

Key words: *Salmonella enteritidis*, polymerase chain reaction, multiplex polymerase chain reaction

Salmonella is the most important pathogen responsible for foodborne illness in many countries [1,2]. This illness leads to many hospitalizations each year and can be fatal. Therefore, *Salmonella* control is necessary at all the key steps of food production to ensure safe products for consumers. In the past 10 years, the incidence of foodborne cases of infection caused by *Salmonella enterica* serotype *enteritidis* (*Salmonella enteritidis*) has increased dramatically in Taiwan [1,2]. *Salmonella* infection develops in humans frequently from the consumption of contaminated poultry, eggs, or meat [3]. Traditional detection methods for *Salmonella* are based on cultures using selective media and characterization of suspicious colonies by biochemical and serological tests. These methods are generally time-consuming. To overcome this drawback, immunological and genetic detection methods have been developed [4]. Recently, polymerase chain reaction (PCR) has been used as a highly sensitive, specific, and rapid test for the presence of pathogenic bacteria in clinical samples.

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In this study, PCR and multiplex PCR were used to detect *S. enteritidis* in isolates from outbreaks of foodborne gastroenteritis in Taiwan. The PCR target gene was the *sef*A gene, which is specific for *S. enteritidis* [5]. The object of this study was to develop a PCR-based assay capable of simultaneously detecting *Salmonella* sp. and rapidly identifying *S. enteritidis*. Thus, we selected 3 multiplex PCR target sequences, which were the *spv* (*Salmonella* plasmid virulence) and *sef*A (*S. enteritidis* fimbrial antigen) genes and a randomly cloned sequence specific for the genus *Salmonella* [6-8], to establish a suitable assay for the analysis of *S. enteritidis*.

Materials and Methods

Bacterial strains and media

Twenty-seven *S. enteritidis* strains isolated from stool samples during outbreaks of foodborne gastroenteritis between 1992 and 1998 in Taiwan were obtained from the Center for Disease Control of the Department of Health, Taiwan. The Center for Disease Control had previously performed biotyping and serotyping of these isolates. Strains were cultivated by inoculation of one loopful on to 5 mL tryptic soy broth (TSB, Difco Laboratories Inc, Detroit, MI, US) and incubated at

37°C for 18 h. A portion of the culture was diluted and placed on tryptic soy broth agar (TSA, Difco) for future transfer.

Polymerase chain reaction

A colony of each bacterial strain grown overnight on TSA was suspended in 1 mL of water and boiled for 10 min. As a template, 10 µL of the cell lysate was added to 15 µL of the amplification mixture, containing 10 mM Tris-HCl (pH 8), 1.5 mM $MgCl_2$, 50 mM KCl, 0.5 µM each primer, 250 µM deoxynucleotide triphosphates (Takara Biotechnology Co., Japan) and 1 U *Taq* polymerase (Takara Biotechnology). SEFA2 and SEFA4 [5] amplified a specific fragment of 310 bp on the DNA region from the fimbrial antigen SEF14 encoded by the *sef*A gene [9] within *S. enteritidis*. The set of primers used are shown in Table 1. The amplifications were carried out using a GeneAmp 2400 Thermocycler (Perkin Elmer Instruments, Norwalk, CT, US) for 40 cycles of 60 sec for denaturation at 94°C, 60 sec for annealing at 61°C, and 90 sec for primer extension at 72°C, followed by a terminal extension at 72°C for 10 min. The amplification product was analyzed by electrophoresis in 1.8% agarose gel. The specificity of this primer set was further determined with other serotypes *Salmonella* and non-*Salmonella* strains. A positive result for the *sef*A gene was indicated by the presence of a 310 bp DNA fragment. A negative result was indicated by the absence of any amplified DNA fragment.

Multiplex polymerase chain reaction

For the multiplex PCR, 3 sets of primers were used: (1) ST11-ST15 shown to be specific for the genus *Salmonella* [8]; (2) S1-S4 selected from a gene associated with virulence and specific for *S. enteritidis* [7]; and (3) according to the results of PCR assay, SEFA2-SEFA4 primers were chosen for specificity within *S. enteritidis*. All of the sequences used are shown in Table 1. The multiplex PCR mixture was made as previously described [5,6]. The amplification was carried out using the same thermocycler for 35 cycles of 30 sec for denaturation at 94°C, 90 sec for annealing at 56°C, and 30 sec for primer extension at 72°C, followed by a terminal extension at 72°C for 10 min. The amplification products were analyzed by electrophoresis. In addition, *Salmonella* non-*enteritidis* (ie *Salmonella typhimurium*, *Salmonella virchow*, *Salmonella welterveden*, *Salmonella chailey*, *Salmonella newport*, *Salmonella schwarzengrund*, and *Salmonella stanley*) and non-*Salmonella* strains (ie, *Escherichia coli*) were used to determine the specificity of these testing primers.

Sensitivity of polymerase chain reaction and multiplex polymerase chain reaction

Pure cultures were grown overnight at 37°C in TSB. Following the procedure described by Liu *et al* [10], broth (1 mL) containing 10^0 to 10^6 cells/mL was centrifuged at 10 000 g for 5 min. The PCR and multiplex PCR were performed using the same program described above. In the double PCR, the first PCR product was used as the DNA template. The PCR also used the same program described above.

Results

Specificity and sensitivity of polymerase chain reaction

The PCR detected the *sef*A gene in all of the 27 isolated strains and in *S. enteritidis* CCRC 10744. The *sef*A gene was not detected in any of the *Salmonella* non-*enteritidis* and non-*Salmonella* strains in this study (Table 2 and Fig. 1). Different concentrations of strain (10^0-10^6 cells/mL) were used to determine the lowest limit of detection. The limit of detection was 10^4 cells/mL in the first PCR and 10^0 cells/mL in the double PCR (Fig. 2).

Table 1. Primers used for identification of *Salmonella enteritidis* by polymerase chain reaction and multiplex polymerase chain reaction

Target sequence	Primer	Sequence	Amplification product (bp)	Reference
Random	ST11	5'-GCCAACCATTGCTAAATTGGCGCA-3'	429	Soumet *et al*,
sequence[a]	ST14	5'-GGTAGAAATTCCCAGCGGGTACTGG-3'		1999 [6]
spv[b]	S1	5'-GCCGTACACGAGCTTATAGA-3'	250	Soumet *et al*,
	S4	5'-ACCTACAGGGGCACAATAAC-3'		1999 [6]
*sef*A[c]	SEFA2	5'-GCAGCGGTTACTATTGCAGC-3'	310	Woodward and
	SEFA4	5'-TGTGACAGGGACATTTAGCG-3'		Kirwan, 1996 [5]

[a]Randomly cloned sequence specific for the genus *Salmonella*.
[b]*Salmonella* plasmid virulent gene.
[c]*S. enteritidis* fimbrial antigen gene.

Table 2. Specificity of multiplex polymerase chain reaction using 3 primer pairs with different bacterial strains

Strain	No. of strains	PCR-positive results by multiplex PCR with amplified products of		
		429 bp	310 bp	250 bp
Salmonella enteritidis	27	27	27	25[a]
Salmonella enteritidis CCRC 10744	1	1	1	1
Salmonella typhimurium	1	1	0	0
Salmonella virchow	2	2	0	0
Salmonella welterveden	1	1	0	0
Salmonella chailey	1	1	0	0
Salmonella newport	1	1	0	0
Salmonella schwarzengrund	1	1	0	0
Salmonella stanley	1	1	0	0
Escherichia coli	3	0	0	0

Abbreviation: PCR = polymerase chain reaction

[a]PCR product could not be detected by using S1-S4 primers in 2 *Salmonella enteritidis* isolates.

Specificity and sensitivity of multiplex polymerase chain reaction

For multiplex PCR, 3 primer pairs were tested. The ST11-ST15 primer pair amplified a 429 bp fragment for all strains of the 8 serotypes of *Salmonella* most frequently found from stool samples of patients with foodborne gastroenteritis. This fragment was not detected for non-*Salmonella* strains. In this study, the specific sequence could be detected in all *Salmonella* strains for the genus *Salmonella* (Table 2). With SEFA2-SEFA4 primers, a 310-bp amplified fragment was noted for all the tested strains of *S. enteritidis* only. However, S1-S4 primers could not be detected in 2 isolated strains (Fig. 3). These results show that the SEFA2-SEFA4 primer set amplified only the strains of *S. enteritidis* producing the expected 310 bp fragment. Multiplex PCR could detect *sef*A gene in all of the 27 isolated strains of *S. enteritidis*. Detection of both *spv* and *sef*A gene did not occur in any of the *Salmonella*–non-*enteritidis* and non-*Salmonella* strains. Under the multiplex PCR conditions used in this study, these results confirmed that the *sef*A gene, from which this primer set was derived, is a good candidate for the specific detection of *S. enteritidis* [6,7]. The limit of detection was 10^5 cells/mL in the multiplex PCR (Fig. 4).

Discussion

In previously reported PCR assay using the S1-S4 primers, which were chosen from a *spv* gene of a virulent plasmid of *S. enteritidis*, to detect these strains

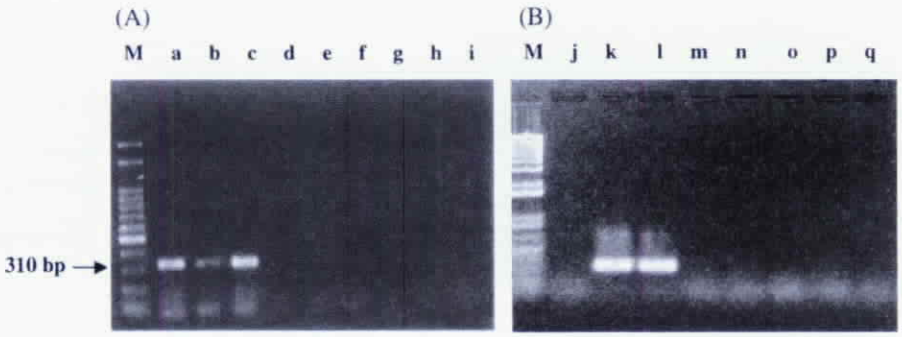

Fig. 1. Specificity of polymerase chain reaction for detection of *Salmonella enteritidis sef*A gene with primer pair SEFA2/SEFA4. **(A)** Lane M: 100 bp ladder. Lane a-i: *S. enteritidis* clinical isolates (a,b); type strain *S. enteritidis* CCRC 10744 (c). Lane d-f: *Salmonella*–non-*enteritidis* strains; *S. typhimurium* (d); *S. virchow* (e); *S. welterveden* (f). Lane g-i: non-*Salmonella* strains; *E. coli* CCRC 14824 (g); *E. coli* CCRC 15372 (h); *E. coli* JM 109 (i). **(B)** Lane M: 100 bp ladder. Lane j-q: blank (j); *S. enteritidis* clinical isolates (k); type strain *S. enteritidis* CCRC 10744 (l); *S. chailey* (m); *S. newport* (n); *S. schwarzengrund* (o). *S. stanley* (p); *S. virchow* (q). The strains tested in m-q were isolated in 2000.

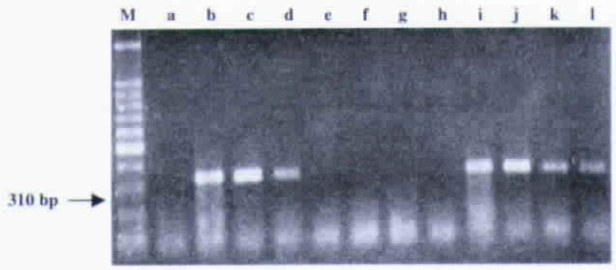

Fig. 2. Sensitivity of polymerase chain reaction for detection of *Salmonella enteritidis sef*A gene with primer pair SEFA2/SEFA4. Lane M: 100 bp ladder; Lane a: negative control; lanes b-h: 10^6 (b), 10^5 (c), 10^4 (d), 10^3 (e), 10^2 (f), 10^1 (g), 10^0 (h) cells/mL of *Salmonella enteritidis* clinical isolate, respectively. Lanes i-l: double polymerase chain reaction products, 10^3 (i), 10^2 (j), 10^1 (k), 10^0 (l) cells/mL.

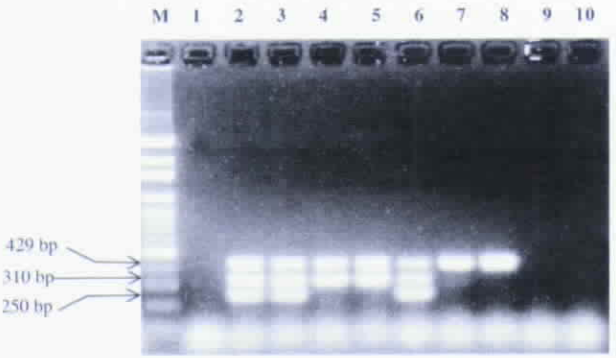

Fig. 3. Detection of *Salmonella enteritidis* by multiplex polymerase chain reaction. Lane M: 100 bp marker. Lane 1-10: free DNA control (1); *S. enteritidis* isolates (2-5); *S. enteritidis* CCRC 10744 (6); *S. typhimurium* (7); *S. stanley* (8); *Escherichia coli* CCRC 14824 (9); *E. coli* CCRC 15372 (10).

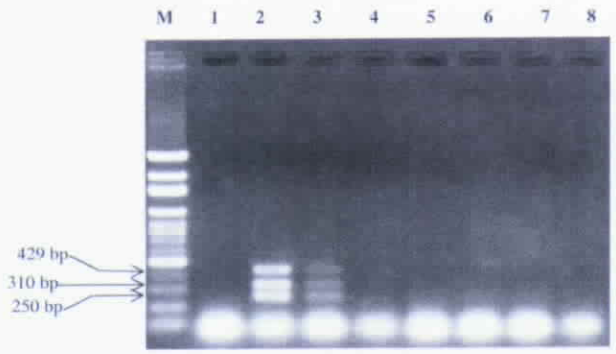

Fig. 4. Sensitivity of 3 primers used in this study used for detection of *Salmonella enteritidis* by multiplex polymerase chain reaction. Lane M: 100 bp ladder; Lane 1: free DNA control. Lane 2 to 8 were the serial dilution of cell numbers of an *S. enteritidis* strain from 10^6 -10^0 cells/mL. The multiplex polymerase chain reaction conditions were 94°C/30 sec, 56°C/90 sec, 7°C/30 sec. Total cycles for multiplex PCR were 35. The detection sensitivity for multiplex PCR showed the minimal cell number required to give a positive reaction was 10^5 cells/mL.

[5]. However, Wood *et al* [7] found that this gene is only present in 30% of *S. enteritidis* strains isolated from poultry. Similar results were obtained in this study. The *spv* gene could not be detected in 2 isolated strains (data not shown). For the presence of strains of *S. enteritidis* to be confirmed, a new pair of primers would need to be selected. *S. enteritidis* elaborates a fimbrial antigen, designated SEF14 [11]. The gene encoding the major subunit protein of this antigen, *sef*A, has been cloned and its deoxyribonucleic acid sequence has been determined [11]. The *sef*A gene could be detected in all of the isolated strains in this study. In addition, both of the primers of these 2 genes could distinguish *S. enteritidis* from *Salmonella* non-*enteritidis* and non-*Salmonella* strains. Multiplex PCR gave a positive result for all *Salmonella* strains yielding a specific fragment of 429 bp and an additional distinct 310 bp amplified product of *sef*A, which allowed identification of *S. enteritidis*.

Study of the sensitivity of detection for PCR and multiplex PCR showed the minimal cell number required to give a positive reaction were 10^4 and 10^5 cells/mL, respectively. Moreover, when a double PCR run with SEFA2-SEFA4 primer pair was performed, the sensitivity of detection increased to 100 cells/mL. These preliminary data indicate that the specificity and sensitivity of the PCR and multiplex PCR make them potentially valuable tools for the diagnosis of *S. enteritidis* infection and that they may also be useful for the identification of *S. enteritidis* responsible for sporadic enteritis cases.

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