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Specific PCR Primers for the Identification of *Salmonella enterica* Serovar Enteritidis in Chicken-Related Samples

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ABSTRACT

In this study, a designed pair of PCR primers, SefB127L-SefB661R, based on the *sefb* gene (accession number L11009) sequences was used in polymerase chain reaction (PCR) for rapid evaluation of *Salmonella enterica* serovar Enteritidis in chicken-related samples. The specificity of this method was checked with 85 *Salmonella* strains and 17 non-*Salmonella* strains. The results showed that only 24 isolates of *S. enterica* serovar Enteritidis exhibited 535 bp PCR product. The detection limit of this PCR method were evaluated using 40 spiked samples under enrichment protocols. The data revealed that microbial extract from as few as 10^1 target cells per gram of the sample culture was required for this assay. Before PCR amplification, pre-culture and the cell lysates, rather than the DNA extracts, were used directly for all tested samples. To verify the usefulness of this PCR process for *S. enterica* serovar Enteritidis examination, 285 endogenous samples including chicken meats, eggs and swabs of chicken-related samples and coop's facilities were tested and compared with that obtained by conventional BAM (Bacteriological Analytical Manual) method. About 1% (three in 285) of the *S. enterica* serovar Enteritidis samples was contaminated, approximately the same as that obtained from BAM method.

Key words: PCR, *Salmonella enterica* serovar Enteritidis, cell lysates

INTRODUCTION

Salmonella is one of the most common pathogens and a major cause of foodborne diseases in human worldwide[1,2,3]. Contaminated poultry products have been identified as the principal sources of *Salmonella* leading to foodborne illness in human[4.5]. The most common serotypes of *Salmonella* isolated from infected human subjects are *Salmonella enterica* serovar Enteritidis[1], which have been increasingly reported in previous studies[6]. Thus a rapid, specific and sensitive detection method for *Salmonella* is important for animal and human health and for the diagnostic industry[7].

The process of isolation and identification of *Salmonella* with traditional biochemical standard methods is laborious and time consuming. It may take up to 5 to 7 days[8] and show poor sensitivity for samples with low level of contamination[7]. Recently, several PCR assays have been developed by targeting various gene sequences, such as a random amplified polymorphic DNA (RAPD) fingerprinting method to differentiate *S. enterica* serovar Enteritidis[9,10], insertion element of *S. enterica* serovar Enteritidis as target DNA[11], 16S-23S rRNA gene designed as PCR primers for detection of *Salmonella*[12], chromosome DNA[13] and restriction analysis of the *S. enterica* serovar Enteritidis virulence plasmid, followed by hybridization with radio-labeled *S. enterica* serovar Typhimurium and *S. dublin* plasmids[14]. A multiplex PCR base assay (m-PCR) was developed for the detection of all serotypes of *Salmonella* within randomly cloned sequence and for the identification of *S. enterica* serovar Enteritidis and *S. enterica* serovar Typhimurium within the *flic* gene and *sefa,* respectively[2,3]. Design and construction of efficient PCR primers for the specific pathogenic bacteria is very important for the detection of pathogens[15].

Despite of the number of validation studies reported in the literature related to the adoption of PCR for *Salmonella* detection, only few studies have reported sensitivity and specificity of the method in detecting and elucidating the ecological development of *S. enterica* serovar Enteritidis in the poultry industry in Taiwan. Therefore, the objective of this study was to develop a PCR-based

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method for the rapid survey of *S. enterica* serovar Enteritidis in chicken-related samples, and to apply for the evaluation of its specificity in analyzing *S. enterica* serovar Enteritidis in naturally contaminated samples.

MATERIALS AND METHODS

I. *Bacterial Strains*

S. enterica serovar Enteritidis and other bacterial strains used in this study are shown in Table 1. These bacterial strains were obtained from the American Type Culture Collection (ATCC), the Centers for Disease Control (CDC), Georgia, U.S.A., the United States Department of Agriculture (USDA), Washington, DC., U. S. A., World Health Organization (WHO), Washington, U. S. A., Department of Health (US), New York, U. S. A., Bureau of Food and Drug Analysis (BFDA), Department of Health, Executive Yuan, Taiwan, R.O.C., Bioresource Collection and Research Center (BCRC), Hsinchu, Taiwan. Bacteria cells were cultivated in Luria broth (tryptone 10 g, yeast extract 5 g, NaCl 5 g in 1000 mL distilled water) overnight at 37°C with rotary shaking. Stock cultures were kept at -80°C in 20% glycerol.

II. *Primers and PCR Conditions*

In this study, the PCR primers designed from the *sefb* gene of *S. enterica* serovar Eenteritidis (accession number L11009) were Sef.B127L (5'-AGATTGGGCACTACACGTGT-3') and SefB661R (5'-TGTACTCCACCAGGTAATTG-3') which produced a DNA fragment of 535 bp. For the PCR assay, the modified method of Wang and Yeh[11] was followed for cell-lysate preparation. The reaction mixture contained 10 μL of diluted heat-lyzed cell culture, 2.5 units of *Taq* polymerase (Promega, Madision, WI, USA), 2 μL each of 10 mM dATP, dTTP, dCTP and dGTP, 5 μL of 10 X reaction buffer (10 mM Tris-HCl (pH 8.3 at 25°C) containing 50 mM KCl, 0.01% Triton X-100, 0.01% gelatin, 6.0 mM $MgCl_2$), and 50 ρmol of each primer in a final volume of 50 μL. The DNA was denatured at 94°C for 2 min and amplified for 35 cycles at 94°C for 40 sec, 60°C for 50 sec and 72°C for 50 sec. A final extension incubation of 2 min at 72°C was included. Amplification reactions were performed on a thermal cycler (Perkin-Elmer GeneAmp PCR System 2400, Foster city, CA, USA). The amplifed products were loaded onto a 1.8% agarose gel. After electrophoresis in 1 X TBE (Tris-Borate-EDTA) buffer at 50 volts, the gel was stained with ethidium bromide before being photographed by ultraviolet illumination.

III. *Detection of S. enterica Serovar Enteritidis in Artificially Spiked Samples*

Salads, chicken meats, and eggs obtained from local markets and feces from healthy chicken collected from farms in southern Taiwan were used for this study. Initially, 25 g of each minced sample were mixed with 225 mL of 0.1% peptone water and homogenized. To evaluate sensitivity for this method, various concentrations (0, 10^1-10^3 cells/g) of *S. enterica* serovar Enteritidis were added to the homogenate and analyzed by preparing DNA for the PCR as described above. A conventional method for the detection of *S. enterica* serovar Enteritidis based on the Bacteriological Analytical Manual (BAM) was used[16]. Additional serotyping testes were used for the identification of *S. enterica* serovar Enteritidis.

V. *Detection of S. enterica Serovar Enteritidis in Endogenous Samples*

A total of 285 chicken and related samples were obtained from 10 local markets and 10 chicken farms in southern part of Taiwan. Conventional method for the identification of *Salmonella* as described in the BAM[16] was used. Twenty-five grams of each subsample was mixed with 225 mL of buffered peptone water (BPW) in a shaking incubator at 37°C for 8 hours. Then 1 mL of this culture was transferred to 9 mL of selenite cystine broth (SCB, Difco, Detroit, MI, USA) at 37°C and shaking for 8-12 hours. All the samples were analyzed by preparing DNA for the PCR as described above.

RESULTS AND DISCUSSION

I. *Specificity of the New Designed PCR Primers*

Two fragments of the DNA sequence of a fimbrial gene (*sefb*)[17] of *S. enterica* serovar Enteritidis (accession number L11009) were selected and designed as primers for the detection of *S. enterica* serovar Enteritidis. These oligonucleotide primers, termed SefB127L-SefB661R, were shown to be different from those analogous primers reported previously by Cohen *et al.*[15] and Soumet *et al.*[2,3].

Under the PCR conditions, all 24 *S. enterica* serovar Enteritidis strains generated PCR products with molecular weight of 535 bp using SefB127L-SefB661R as a primer pair (Table 1 and Figure 1A). *Salmonella* isolates other than *S. enterica* serovar Enteritidis or non-*Salmonella* bacterial strains, including the family of Enterobacteriaceae such as *E. coli, Shigella* and *Citrobacter*, did not generate false-positive results (Table 1). The results showed that SefB127L-SefB661R primer pair was specific for identifying the *S. enterica* serovar Enteritidis strains used in this study. Cohen *et al.*[13] identified *S. enterica* serovar Enteritidis from artificial inoculation of the feces collected from hens using the PCR primers specific for all members of the genus *Salmonella*. In other words, these PCR primers were not performed in detection of *S. enterica* serovar Enteritidis in naturally contaminated samples.

Journal of Food and Drug Analysis, Vol. 17, No. 3, 2009

Table 1. Bacterial strains and results tested with polymerase chain reaction

Species*	No. of isolates	Positive results SefB127L-SefB661R	Species*	No. of isolates	Positive results SefB127L-SefB661R
Non-*Salmonella*			Allandale (US)	1	0
Acinetobacter calcoaceticus (ATCC 19606)	1	0	Arkansas	1	0
Alcaligenes faecalis (ATCC 8750)	1	0	Azteca (PT607)	1	0
Bacillus subtilis (ATCC 21778)	1	0	Bareilly (USDA)	1	0
Brevibacterium linens (ATCC 19391)	1	0	Bousso (PT643)	1	0
Citrobacter freundii (ATCC 8090, 10787)	2	0	Bovismorbificans (PT695)	1	0
E. coli(ATCC 25922, 11775, BFDA E01-E07, BFDA E2416-E2422)	16	0	Bredeny (PT)	1	0
E. coli (EIEC) (ATCC 43983, BFDA 11096, 11098)	3	0	Braenderup (PT703)	1	0
E. coli (EHEC) (BCRC 13085, 13087, 13095)	3	0	Cairo (PT738)	1	0
E. coli (LT & ST ETEC) (ATCC 35401, WHO 110)	2	0	California (US)	1	0
E. coli (LT ETEC) (ATCC 37218, 33849, WHO 112, 117)	3	0	Chailey (PT618)	1	0
Enterobacter aerogenes (ATCC 13048, US)	2	0	Chester (USDA)	1	0
Enterobacter cloacae (ATCC 23355)	1	0	Choleraesusis (PT)	1	0
Hafinia alvei (ATCC 9890)	1	0	Coleypark (US)	1	0
Proteus vulgaris (ATCC 8427)	2	0	Crossness (US)	1	0
Serratia marcescens (ATCC 13880)	1	0	Djakarta (US)	1	0
Shigella flexneri (ATCC 12022, 29903)	2	0	Derby (CDC RF62)	1	0
Shigella sonnei (ATCC 11060)	1	0	Enteritidis (ATCC 13076, US, VSE1-22)	24	24
***S. enterica* serovar**			Eppendrof (PT633)	1	0
Aberdeen (US)	1	0	Essen (PT661)	1	0
Adelaide (US)	1	0	Goerlitz (PT645)	1	0
Agona (PT624)	1	0	Hadar (PT677, US)	2	0
Aalbany (USDA)	1	0	Halmstad (USDA)	1	0
Aamager (US)	1	0	Hartford (USDA)	1	0
Anatum (US) (USDA 807EI, US)	1	0	Havana (US)	1	0

Table 1. (Continued)

Species*	No. of isolates	Positive results SefB127L-SefB661R	Species*	No. of isolates	Positive results SefB127L-SefB661R
Heidelberg (CDC)	1	0	Ohio (PT1007)	1	0
Hvitingfoss (USDA, US)	2	0	Oranienburg (US)	1	0
Indinana (US)	1	0	Panama (PT158, US)	2	0
Infantis (USDA, US)	2	0	Paratyphi B (PT633)	1	0
Johannesburg (USDA)	2	0	Portsmouth (PT748)	1	0
Kentucky (US)	1	0	Richmond (USDA, US)	2	0
Kinshasa (US)	1	0	Rubislaw (USDA, US)	2	0
Kuru (PT793)	1	0	Sandiego (USDA)	1	0
Lagos (PT772)	1	0	Schwarzenground (PT646)	1	0
Lanka (PT660)	1	0	Senftenberg (PT169)	3	0
Limete (PT669)	1	0	Seremban (PT1087)	1	0
Litchfield (PT152)	1	0	Sinstorf (PT606)	1	0
London (PT1004)	1	0	Stanley (PT639)	1	0
Manhattan (PT617)	1	0	Tananarive (PT702)	1	0
Miami (USDA)	1	0	Tennessee (PT721)	1	0
Minnesota (US)	1	0	Thomasville (USDA)	1	0
S. montevideo (US)	1	0	Thompson (US)	1	0
S. muenchen (PT625)	1	0	Trachau (PT919)	1	0
S. muenster (PT1014)	1	0	Typhi (ATCC 10747)	1	0
Nchanga (PT620)	1	0	Typhimurium (PT782, 10240, ATCC 14028, 23566, 13311)	5	0
Newbrunswick (US)	1	0	Vejle (PT1102)	1	0
Newington (USDA)	2	0	Victoria (PT763)	1	0
Newport (PT, US)	2	0	Wassenaar (US)	1	0
Ngor (PT6951)	1	0	Weltevreden (PT658)	1	0
Nigeria (PT696)	1	0	Worthington (PT705)	1	0

*Sources of bacteria used in this study

II. *Detection of S. enterica Serovar Enteritidis in Artificially Spiked Samples*

To assure the positive PCR detection of *Salmonellae* in various samples, especially when target cells are present in very limited numbers, enrichment leading to a predominance of bacteria was carried out[2,3,11,15]. The results showed that after inoculation with 10^1-10^3 cells/g of *S. enterica* serovar Enteritidis into samples and BPW and SCB incubation, *S. enterica* serovar Enteritidis was detected with both conventional culture method and the PCR method (Table 2 and Figure 1B).

The PCR test for detection of *Salmonella* in food samples may be limited by the presence of substances that inhibit the assay[4]. In this study, experiments with artificially challenged samples without pre-enrichment failed to

Journal of Food and Drug Analysis, Vol. 17, No. 3, 2009

Table 2. Detection of artificially contaminated *Salmonella enterica* servoar Enteritidis in food samples and chicken feces

Source	Samples tested*	Samples with positive result							
		PCR				BAM			
		0	10^1	10^2	10^3	0	10^1	10^2	10^3
Salads	10	0	10	10	10	0	10	10	10
Chicken meats	10	0	10	10	10	0	10	10	10
Chicken eggs	10	0	10	10	10	0	10	10	10
Chicken feces	10	0	10	10	10	0	10	10	10
Total	40	0	40	40	40	0	40	40	40

*Samples were spiked with 0-10^3 CFU/g of *Salmonella enterica* servoar Enteritidis.

detect *Salmonella* by PCR. Such result was also reported by Jofré *et al.*[18]. Our results indicated that as few as 10^1 cells/g of target cells in the sample could constitute sufficient cellular material to generate a positive PCR results following enrichment when SefB127L-SefB661R was used as primer. Thus, the combination of pre-enrichment and PCR has the advantage of enhancing the sensitivity of *Salmonella*[2,3,11,15]. Despite the requirement of enrichments, the method described herein was convenient and time effective because of the simple DNA preparation step.

III. *Detection of S. enterica* Serovar Enteritidis *in Endogenous Samples*

Total of 285 chicken-related samples either obtained directly from markets or collected as swabs from chicken farms were detected for *S. enterica* serovar Enteritidis using both the conventional culture method (BAM) and the PCR procedure developed in this study. Table 3 shows that a single 535-bp PCR product after PCR amplification could be detected after pre-enrichment from some chicken eggs purchased from market and chicken feces. Herein we found the same results as those obtained from BAM. The endogenously contaminating microflora in these samples constituted between 6×10^4 to 5×10^6 CFU per gram of the various samples investigated.

These results showed that PCR is useful and specific for the rapid detection of *S. enterica* serovar Enteritidis in tested samples. Detection of *S. enterica* serovar Enteritidis in poultry products or feces contaminated with high micro flora may lower sensitivity, precision and accuracy[2]. Soumet *et al.*[2] analyzed *S. enterica* serovar Enteritidis of 35 poultry houses by Modified Semi-solid Rappaport Vassiliadis medium (MSRV) PCR method after pre-enrichment in phosphate-buffered peptone water for 18-20 hours and MSRV for 18-20 hours. The MSRV-PCR assay and the bacteriological method had an agreement rate of 95.6%. To assure the positive results of detection of *S. enterica* serovar Enteritidis in samples containing high numbers of microflora, a pre-culturing step could improve

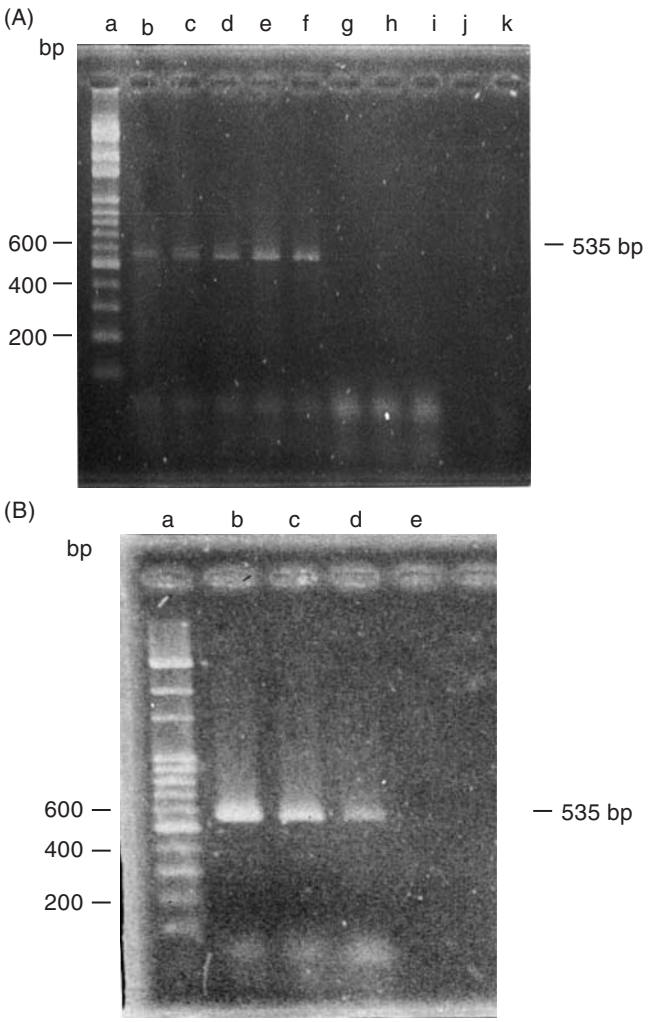

Figure 1. Specificity (A) and sensitivity (B) of polymerase chain reaction for detection of *Salmonella enterica* servoar Enteritidis strain using SefB127L-SefB661R as primers. (A) Lane a: DNA ladder markers, lanes b-f: PCR products amplified from *S. enterica* servoar Enteritidis strains, lane g: *S. anatum*, lane h: *S. raenderup*, lane i: *E. coli*, lane j: *E. coli* (EIEC), lane k: *Bacillus subtilis*. (B) Lane a: DNA ladder markers, lanes b-d: PCR results amplified from 10^3-10^1 CFU target cells/g, Lane e: polymerase chain reaction (PCR) result for blank without inoculation of the target cells.

the detection sensitivity[2,3,11,15], especially using heat lysis to prepare DNA.

In this study, we examined the *S. enterica* serovar Enteritidis from chicken samples and environments of chicken farms. Direct cell lysis after enrichment may be an alternative and rapid method to obtain template DNA for PCR amplification. Moreover, detection of *S. enterica* serovar Enteritidis by the PCR method developed in this study can be completed within thirty hours as compared to the five to seven days required for bacterial culture and a conventional serological method.

Table 3. Detection of endogenous *Salmonella enterica* servoar Enteritidis in chicken and chicken-related samples

Source	Samples tested*	Samples with positive result	
		PCR	BAM
Chicken eggs from markets	20	1	1
Chicken eggs from farms	20	0	0
Facilities of chicken roosts	110	0	0
Chickens bodies from farms	40	0	0
Chicken meats from markets	35	0	0
Chicken feces from farms	60	2	2
Total	285	3	3

*Samples not artificially spiked with *Salmonella enterica* servoar Enteritidis.

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