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Specific detection of *Salmonella enterica* serotype Enteritidis using the polymerase chain reaction

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SUMMARY

An assay was developed for the specific detection of *Salmonella enterica* serotype Enteritidis, using a novel application of the polymerase chain reaction (PCR). This PCR assay is based on the mismatch amplification mutation assay, an allele-specific reaction, and can discriminate Enteritidis from all other salmonella. PCR primers were selected to amplify a 351-base pair (bp) DNA fragment from the salmonella plasmid virulence A (*spvA*) gene of Enteritidis. A single base difference at position 272 is present between the nucleotide sequence of the *spvA* gene of Enteritidis and other salmonellae. The downstream PCR primer, that encompasses position 272 of the Enteritidis *spvA* gene, was designed to contain a single base mismatch at the penultimate position, resulting in a 1-base mismatch with Enteritidis and a 2-base mismatch with other salmonellae that harbour the virulence plasmid. The upstream primer was completely homologous with the region immediately 5′ to the *spvA* gene. When these primers were used and the annealing and extension reactions were performed at the same temperature, the PCR assay was specific for Enteritidis; no PCR product was detected for 40 other serotypes and 28 different genera examined. In pure culture, 120 colony forming units (c.f.u.) could be detected; a PCR product was observed from template derived from a 5 h enrichment broth culture of chicken seeded with 1 c.f.u. per gram of Enteritidis. This PCR assay is specific, reproducible, and less time consuming than the standard bacteriological methods used to detect Enteritidis.

INTRODUCTION

Salmonella enterica serotype Enteritidis has emerged as the major aetiological agent of salmonellosis in the United States, with the number of cases estimated at 2–4 million yearly [1]. In 1990, 67 Enteritidis outbreaks were reported to the Centers for Disease Control and Prevention (CDC) [2] and 56 outbreaks were recorded in both 1991 and 1992 [3]. The emergence of Enteritidis as a leading source of human disease caused by a bacterial pathogen has also been observed in the international community. The U.K., Italy, Finland, Sweden, Canada and Norway have experienced significant rises in Enteritidis outbreaks [4–6].

Intact eggs have been implicated as a vehicle of transmission of Enteritidis infection to humans. Infected flocks which contaminate their eggs by transovarian infection are suspected reservoirs for Enteritidis. In chickens, serotypes Heidelberg, Enteritidis, Gallinarum, Pullorum and Typhimurium have been found in the ovaries. Currently, flocks are tested for Enteritidis and infected poultry are removed in order to restrict the number of outbreaks. A specific, sensitive and rapid method for the detection of Enteritidis, therefore, would help in the control of human infections.

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Table 2. *Salmonella isolates tested using MAMA-PCR*

Organism	No. tested	Reaction*	Source†	Isolate
Salmonella enterica				
ser‡ Agona	1	–	NVSL	Chicken
ser Anatum	1	–	FDA	Food
ser Berta	4	–	CDC/NVSL	Food
ser Braenderup	2	–	NVSL	Chicken
ser Cerro	1	–	NVSL	Chicken
ser Chester	1	–	FDA	Food
ser Derby	1	–	FDA	Food
ser Dublin	4	–	FDA	Food
bioser§ Gallinarum	6	–	FDA/NVSL/USUHS	Chicken
ser Give	1	–	NVSL	Turkey
ser Hadar	2	–	NVSL	Chicken
ser Havana	1	–	FDA	Food
ser Heidelberg	2	–	NVSL	Chicken
ser Infantis	2	–	FDA	Food
ser Istanbul	1	–	NVSL	Chicken
ser Java	1	–	FDA	Food
ser Johannesburg	1	–	NVSL	Chicken
ser Kentucky	1	–	NVSL	Chicken
ser Lindenburg	1	–	FDA	Food
ser Mbandaka	1	–	NVSL	Chicken
ser Mjimwema	1	–	FDA	Food
ser Montevideo	2	–	NVSL	Chicken
ser Newington	1	–	FDA	Food
ser Newport	1	–	NVSL	Food
ser Ohio	1	–	NVSL	Chicken
ser Oranienburg	1	–	FDA	Food
ser Poona	2	–	FDA	Food
bioser Pullorum	7	–	FDA	Chicken
ser Reading	1	–	FDA	Food
ser Rubislaw	1	–	FDA	Food
ser Saintpaul	1	–	NVSL	Turkey
ser Schwarzengrund	1	–	NVSL	Chicken
ser Senftenberg	2	–	FDA/NVSL	Chicken
ser Sundsvall	1	–	FDA	Food
ser Tennessee	1	–	NVSL	Chicken
ser Thompson	1	–	NVSL	Chicken
ser Typhimurium	3	–	NVSL/Norway**	Food/Clinical
ser Virchow	1	–	NVSL	Chicken
ser Worthington	1	–	NVSL	Chicken
S. typhi	1	–	USUHS	Clinical

* Results of MAMA-PCR; positive (+) and negative indicate presence or absence of a 351-bp product on an agarose gel.

† CDC, Centers for Disease Control and Prevention, Atlanta, GA; FDA, Food and Drug Administration, Washington, DC; NVSL, National Veterinary Service Laboratories, Ames, IA; USUHS, Uniformed Services University of the Health Sciences, Bethesda, MD.

‡ Serotype.

§ Bioserotype.

** Kapperud and colleagues [43].

plates. A large colony was added to 25 μl 0·5 M NaOH, completely suspended by vortexing and maintained at room temperature. After 30 min, 25 μl of 1 M Tris buffer, pH 7·4 and 450 μl of deionized water were added. The lysates were frozen at −20 °C until needed. The PCR assay contained 1 × PCR buffer (Perkin–Elmer Corp., Norwalk, CT), 200 μM of each deoxynucleotide triphosphate (USB), 1 μM of each primer, 1–5 μl of bacterial lysate as template, and 3·0 units of AmpliTaq (Perkin–Elmer) in a final volume of 50 μl. Reactions were overlaid with 1–2 drops of mineral oil (Sigma Chemical Co., St Louis,

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Table 3. *Bacterial genera tested by MAMA-PCR*

Organism	No. tested	Reaction*	Source†
Acinetobacter sp.	1	–	FDA
Aeromonas sobria	1	–	FDA
A. hydrophila	1	–	UCCML
Bacillus cereus	1	–	FDA
Campylobacter coli	2	–	FDA
C. freundii	2	–	FDA
C. jejuni	1	–	FDA
Escherichia coli (EHEC)	1	–	FDA
E. coli (EIEC)	1	–	FDA
E. coli (EPEC)	1	–	FDA
E. coli (ETEC)	1	–	FDA
Enterobacter cloacae	2	–	FDA
Klebsiella pneumoniae	1	–	FDA
Listeria ivanovi	1	–	FDA
L. monocytogenes	1	–	FDA
Pasteurella multocida	1	–	FDA
Pseudomonas aeruginosa	1	–	FDA
P. putida	1	–	FDA
P. vulgaris	1	–	FDA
Shigella boydii	1	–	FDA
S. dysentariae	1	–	FDA
S. flexneri	1	–	FDA
S. sonnei	1	–	FDA
Staphylococcus aureus	1	–	ATCC
Serratia marcescens	1	–	ATCC
Vibrio cholerae	1	–	FDA
Yersinia enterocolitica	1	–	FDA
Y. pseudotuberculosis	1	–	FDA

* Results of MAMA-PCR; positive (+) and negative (−) indicate presence or absence of 351-bp product on agarose gel.

† ATCC, American Type Culture Collection, Rockville, MD; FDA, Food and Drug Administration, Washington, DC; UCCML, University of Chicago Clinical Microbiology Laboratories, Chicago, IL.

S. enteritidis	5′-**TCAGGTTCGTGCCATTGTC**A- 3′
PCR primer 598	5′-**TCAGGTTCGTGCCATTGTCAA-** 3′
S. typhimurium	5′-**TCAGGTTCGTGCCATTGTC**GG- 3′

Fig. 1. Comparison of PCR primer 598 with *spvA* sequences of Enteritidis and Typhimurium. Raised or lowered open letters represent base difference(s) between gene sequence and PCR primer.

MO). For 3-step amplification, templates were denatured at 94 °C for 1·5 min; primers were annealed at 64 °C for 1·5 min and extended at 72 °C for 1·5 min. For 2-step amplification, primers were annealed and extended at 64 °C for 1·5 min and denatured at 94 °C for 1·5 min. All reactions were performed in a Perkin–Elmer 480 thermal cycler. PCR primers were synthesized with an automated DNA synthesizer (Perkin–Elmer Corp.). Those used in the MAMA protocol were designed to have a one base mismatch for Enteritidis one base from the 3′ end, and two bases mismatched at the 3′ end for all other salmonellae (Fig. 1). PCR products (15 μl) were loaded onto a 1% agarose (BRL) gel in Tris-acetate EDTA buffer, pH 7·8, with 2 μl of ethidium bromide (10 mg/ml) added, and electrophoresed at 100 volts. A 123-bp ladder (BRL) was used as molecular weight marker. PCR products were visualized on a transilluminator (UVP Products, San Gabriel, CA).

Sensitivity of PCR assay

Pure cultures of SE-3 were grown overnight at 37 °C in tryptone soya broth (Oxoid). Broth (1 ml) containing 10^0–10^6 c.f.u. of SE-3 was centrifuged at 10000 ***g*** for 5 min. The supernatants were discarded and the pellets were suspended in 50 μl of phosphate-buffered saline (PBS) and 5 μl of lysis solution (50 mg/ml Proteinase K; 3% Brij 35; 0·1 M EDTA, pH 8·0; 0·2 M Tris-HCl, pH 8·0), incubated at 60 °C for 1 h, and boiled at 100 °C for 5 min. For PCR, 5 μl of lysate were used as template. Reaction products (20 μl) were analysed on a 1% agarose gel as described above.

Primers used to amplify Enteritidis specific *spvA* products were 597 (5′-GCAGACATTATCAGTCT-TCAGG-3′) and 598 (5′-TCAGGTTCGTGCCATT-GTCAA-3′) whereas primers 382 (5′-CAGACCAC-CAGTCCGGCAC-3′) and 383 (5′-CAGTCAATGC-TCTCTCGCTG-3′) were homologous to the *spvA* gene of all salmonellae.

Detection of Enteritidis in seeded chicken

Enteritidis (final concentration 1 c.f.u./g) was added to 10 g of chicken meat in a 250-ml Erlenmeyer flask containing 90 ml of lactose broth (Difco Laboratories, Detroit, MI). The flask was incubated for 5 h at 37 °C in a shaking water bath. At 5 min and again at 5 h, 1-ml aliquots were removed and transferred to 1·5 ml centrifuge tubes. The cells were pelleted by centrifugation at 85 ***g*** for 30 sec in an Eppendorf 5402 centrifuge (Brinkman Instruments, Inc., Westbury, NY), suspended in PBS and lysed as described above. To eliminate chicken substances that might be inhibitory to PCR, lysed samples were passed through Centrisep columns (Princeton Separations, Princeton,

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NJ) that were prepared and used according to the manufacturer's protocol. For PCR analysis, 5 μl of column eluate were used as template.

RESULTS

Selection for PCR primers

Differences between the nucleotide sequence of the 4·1-kbp *Eco*R I fragment from Enteritidis and the *spv* genes from Typhimurium [11–14] and Dublin [15] are shown in Table 4. Of the 4118 bp that comprise the *spvABC* genes, only three were unique to Enteritidis.

PCR primers were selected to take advantage of any base mismatch between Enteritidis and both Typhimurium and Dublin. The nucleotide difference at position 272 in the *spvA* gene was incorporated into one PCR primer (21 nt); the other PCR primer was homologous with all reported *spvA* nucleotide sequences. An intentional mismatch with the Enteritidis *spvA* sequence at the penultimate position of the PCR primer (nt 20), incorporated into the primer homologous with Enteritidis, resulted in a 2-base mismatch with the *spvA* gene from Typhimurium and Dublin but only a 1-base mismatch with Enteritidis.

Six primers were synthesized with three different bases in the intentional mismatch site and used as either the upstream or downstream primer. In each primer set, the other primer matched perfectly with the published sequence of the *spvA* gene from Enteritidis, Typhimurium and Dublin. Upstream primers with the mismatches were expected to generate a 450-bp PCR product, whereas downstream primers would yield a 351-bp product. PCR primers with the mismatch in the upstream primer gave the expected 450-bp product with SE-3 DNA as template. However, these sets of primers produced anomalous results when Typhimurium and a few non-salmonella templates were tested.

When the downstream primer had the mismatch and Enteritidis DNA was used as template, the size of the PCR product was consistent with that of the expected 351-bp fragment. The one primer set 597 and 598 that did not yield a product with Typhimurium was used to test all salmonella and non-salmonella isolates.

To confirm that the 351-bp fragment generated with primers 597 and 598 was amplified from the intended target region of the *spvA* gene, the nucleotide sequence of the fragment was determined from one end (100 bp). The sequence matched perfectly with the *spvA* gene of Enteritidis (data not shown).

Table 4. *Nucleotide differences between the spv genes of Enteritidis, Dublin and Typhimurium with accompanying amino acid changes*

Gene	Nucleotide change	Position*	Amino acid change
spvA	G → A†	175	Alanine → Threonine
	T → C†	272	Leucine → Proline
	GT → TC‡	311, 312	Glycine → Valine
spvB	AC → CA§	170, 171	Histidine → Proline
	C → T‡	525	Leucine → Leucine
	AT → TA§	814, 815	Histidine → Leucine
	TG → GT§	922, 923	Leucine → Arginine
	G → T§	1372	Glycine → Valine
spvC	T → C†	669	Glycine → Glycine

* Position in the *spv* gene.
† Change present in both Dublin and Typhimurium.
‡ Change present only in Dublin.
§ Change present only in Typhimurium.

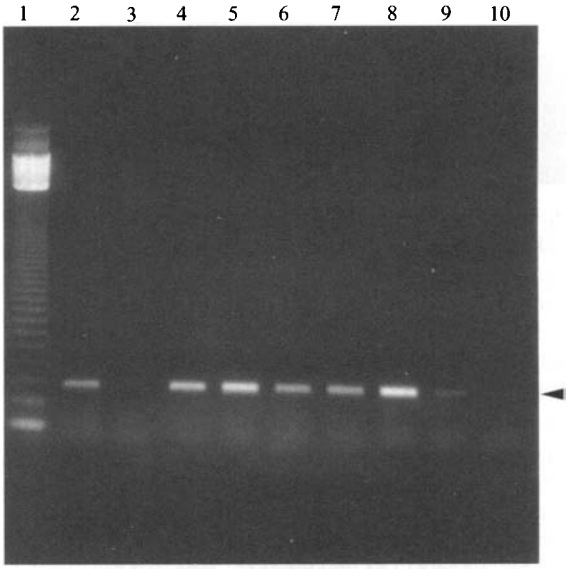

Fig. 2. Agarose gel electrophoresis of PCR products from representative phage-types (PT) of Enteritidis. Isolates of Enteritidis of different phage types were used as templates in the SE-PCR assay. Lane 1, 123-bp standard; lane 2, SE-3; lane 3, PC-1; lane 4, PT-14b; lane 5; PT-13; lane 6, PT-9b; lane 7, PT-4; lane 8, PT-8; lane 9, PT-2; lane 10, PT-not available.

Specificity of PCR assay

The one primer set (597 and 598) which initially differentiated Enteritidis and Typhimurium was tested for Enteritidis specificity by using lysates prepared

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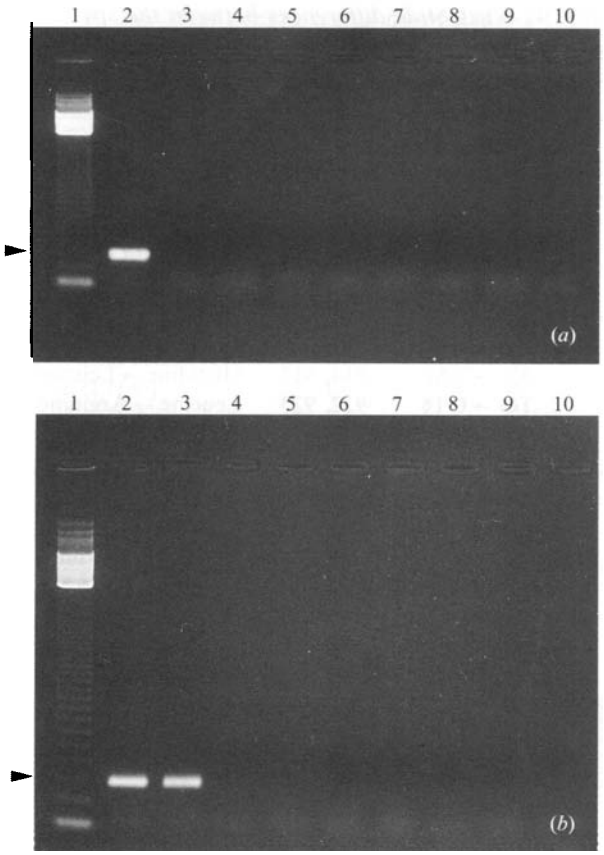

Fig. 3. (*a*) Agarose gel electrophoresis of PCR products from SE-PCR assay using several serotypes of *Salmonella enterica* as template. DNA was isolated from various salmonellae and subjected to SE-PCR. PCR products were run on 1% agarose gel. Lane 1, 123 bp standard; lane 2, SE-3; lane 3, PC-1; lane 4, Typhimurium; lane 5, Gallinarum; lane 6, Pullorum; lane 7, Heidelberg; lane 8, Dublin; lane 9, Hadar; lane 10, Anatum. (*b*) Agarose gel electrophoresis of PCR products from SE-PCR assay using serotypes of *Salmonella enterica* most frequently isolated from chicken as template. DNA was isolated from various samonellae and subjected to SE-PCR. PCR products were run on 1% agarose gel. Lane 1, 123 bp standard; lane 2, SE-3; lane 3, SE-50146 (PT-8); lane 4, Kentucky; lane 5, Berta; lane 6, Braenderup; lane 7, Montevideo; lane 8, Senftenberg; lane 9, Virchow; lane 10, Infantis.

from 34 Enteritidis isolates, 40 *Salmonella* and 28 different genera (Tables 1–3).

Different phage types of Enteritidis were used as templates and, in all cases, PCR products of approximately 350 bp were detected by agarose gel electrophoresis (Fig. 2). A 351-bp product was not amplified from 6 isolates that did not harbour the salmonella virulence plasmid. Figure 3*a*, *b* shows the PCR results of selected salmonella isolates used as templates. Several of these cultures represented the 20 salmonella most often isolated from poultry in the U.S.A.

To demonstrate the presence of the *spvA* gene in some of the salmonella strains tested, PCR primers 382 and 383, homologous with the known sequence of the *spvA* gene from Enteritidis, Typhimurium and Dublin, were used. All Pullorum (7 isolates), Dublin (4 isolates), and Gallinarum (2 of 5 strains) produced the expected size PCR product (data not shown). Plasmid DNA could not be isolated from Gallinarum strains that did not yield the expected amplicon (data not shown). Therefore, in some cases, the lack of a PCR product was due to the absence of the salmonella virulence plasmid.

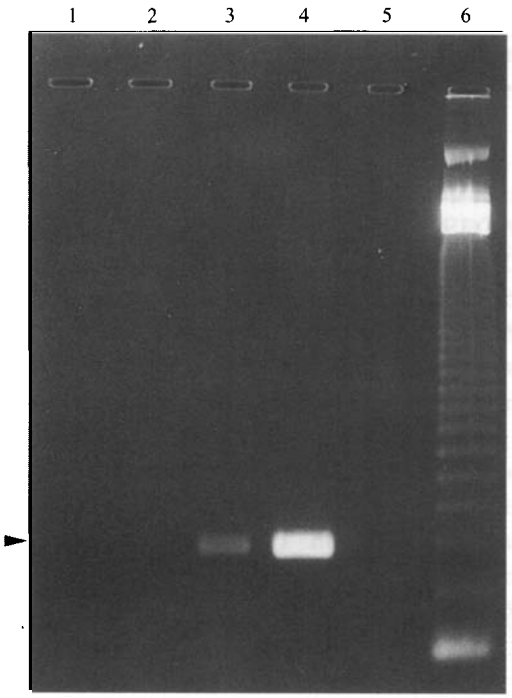

Fig. 4. Agarose gel electrophoresis of PCR products from templates prepared from chicken seeded with SE-3. PCR templates were prepared from chicken meat unseeded and seeded with 1 c.f.u./g of SE-3 and enriched in broth at 37 °C for 5 min and 5 h. Lane 1, unseeded chicken; lane 2, seeded chicken with 5 min enrichment in broth; lane 3, seeded chicken with 5-h enrichment in broth; lane 4, SE-3 plasmid DNA; Lane 5, Typhimurium lysate; lane 6, 123-bp ladder.

Sensitivity of PCR assay

Concentrations of SE-3 (10^0–10^6 c.f.u./ml) were used to determine the lowest limit of detection. In three experiments, the SE-PCR assay routinely detected $1{\cdot}2 \times 10^2$ c.f.u./ml (data not shown).

Detection of Enteritidis in seeded chicken

DNA for PCR was isolated from chicken seeded with 1 c.f.u./g of SE-3 after 5 h of enrichment in broth at 37 °C. DNA fragments of about 350 bp were detected

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by agarose gel electrophoresis from PCR using templates from seeded chicken and SE-3 plasmid DNA as a control (Fig. 4). The expected product was not detected unless the seeded culture was pre-incubated; therefore, pre-enrichment may be necessary for detecting low levels of Enteritidis. The background flora of unseeded chicken did not cause false-positive reactions in the MAMA-PCR assay.

DISCUSSION

Salmonella enterica serotype Enteritidis has emerged as a foodborne pathogen of significant concern in many countries. It has supplanted Typhimurium as the leading cause of salmonellosis in the United States in the past several years and has been the salmonella serotype most frequently isolated from humans during the past 6 years [3]. Gastroenteritis caused by salmonella affects 2–4 million people per year in the United States, second only to the number of cases caused by campylobacter [1, 2]. The disease is characterized by abdominal cramps, diarrhoea, fever, vomiting and headache. In 11% of all cases reported to CDC, hospitalization was required, and of these, 0·3% were fatal [1].

Several recent foodborne outbreaks exemplify the importance of rapid methods for detecting Enteritidis and the need for continued surveillance of poultry flocks and eggs. At an institution for adults and children, several residents became infected by consuming homemade ice cream prepared with contaminated grade A eggs. Recently, contaminated ice cream produced from one manufacturer was responsible for 645 confirmed illnesses with an additional 3213 probable cases [CDC, unpublished data]. Twenty-eight states reported confirmed culture samples to the CDC, and an additional 11 states reported illnesses suspected to be caused by Enteritidis.

Currently available bacteriological methods for detecting and identifying salmonella use selective and differential media, enzyme immunoassays, latex agglutination and DNA-based techniques [21, 22]. Those described in the FDA Bacteriological Analytical Manual [23] and modified by van der Zee [24] require 4 days to complete. Keller and colleagues [25] reported an enzyme-linked immunoabsorbent assay (ELISA) that used 2 monoclonal antibodies (mAbs) specific for Enteritidis. However, these mAbs also reacted with Dublin and *S. enterica* serotype Berta in the ELISA and agglutination assays. A latex particle agglutination test developed specifically for Enteritidis by Thorns and colleagues [26] did not react with all other serotypes; however, it agglutinated Dublin. Other methods based on gene restriction fragment patterns [27, 28] and plasmid profile analyses [29] rely on DNA isolation and restriction digestion, which require several days to complete.

Recently developed DNA-based methods, e.g. probes and PCR, target the genus *Salmonella*. However, a DNA probe specific for Enteritidis has been reported [30]. Alternatively, PCR assays for identifying salmonella have had varying degrees of success. Way and colleagues [31] used a multiplex PCR to detect salmonella and tested this assay with environmental samples. Using the *phoP* primers from this multiplex PCR assay, Pillai and colleagues [32] screened for Typhimurium from chicken caecal matter. The sensitivity of their assay was 100 c.f.u. from pure culture and 700 c.f.u. from caecal matter.

The nucleotide sequence of the *spvABC* genes of Enteritidis (this study) was compared with sequence data generated for other salmonella *spvABC* genes [11–15]. In the 4·1-kbp *Eco*R I fragment that contained these genes, only 13 bp differed between Enteritidis and Typhimurium or Dublin, and only 3 bp were unique to Enteritidis. PCR primers were synthesized to target one of these specific sites within the *spvA* gene. The PCR-assay was designed so that amplification would occur only from the *spvA* gene of Enteritidis, although this gene is present in other salmonellae. Initially, the synthesized PCR primers were homologous with the *spvA* gene of Enteritidis and differed by 1 bp with other *spvA* genes. These primers were tested with several salmonellae templates but did not discriminate between Enteritidis and other salmonella.

To improve the specificity of this reaction, we designed a PCR-assay on the basis of the MAMA-PCR described in which mutant alleles that differed from the wild-type gene by 1 bp were selectively amplified. In the SE-PCR assay, one PCR primer (21 nt) had an intentional mismatch to the *spvA* gene of Enteritidis at the penultimate position (nt 20) to allow MAMA-PCR to discriminate between Enteritidis and all other salmonellae. According to the data provided by Kwok and colleagues [33], mismatches at the 3′-terminus affect the relative amplification efficiencies. Although most mismatches did not affect the amplification of PCR products, 4 base pairings significantly affected amplification: A:G, G:A, and C:C mismatches reduced the efficiency by 100-fold; A:A reduced efficiency by 20-fold. They also reported on mismatches at the penultimate

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position coupled with a mismatch at the 3′-terminus [33]. In most cases, the overall efficiency of amplification was reduced 100-fold. We found that the substitution of A to G in the penultimate position of primer 598 along with the mismatch at the 3′ end of this oligo was the only combination that allowed specific amplification from Enteritidis and no other salmonella.

Test results (Tables 1–2) of different Enteritidis phage types and isolates from various geographical areas that contained the salmonella virulence plasmid were all positive by the MAMA-PCR assay. It is noteworthy then, that this bp change was conserved in the *spvA* gene of all Enteritidis and not present in other salmonella. In most studies where plasmid profiles of Enteritidis isolates were examined, 95–100% of strains carried the salmonella virulence plasmid, as judged by gel electrophoresis [34, 35]. However, in a few isolated instances, 55–73% of strains examined lacked this plasmid [36, 37]. Since the PCR assay targeted the plasmid-borne *spvA* gene, no amplification would occur in these samples and a false negative would result. However, recent loss of the plasmid in a bacterial population does not necessarily preclude a positive result. Even though a variable proportion of the population will not contain the plasmid, a certain number will retain the *spvA* gene and thus be detectable by the SE-PCR assay. A false negative result would occur only if the entire bacterial population of Enteritidis was devoid of the virulence plasmid as occurs when the reservoir or source of an incident or outbreak in plasmid free.

Our study demonstrates that PCR technology can be applied to a rapid detection system, targeting a well conserved gene from a bacterial genus but able to amplify the desired product from one serotype based on the 1 bp difference found in the nucleotide sequence of the targeted gene. The advantages and disadvantages of using PCR for the detection of foodborne pathogens have been discussed elsewhere [38]. Because food matrices differ, an isolation method is needed to prepare a PCR template to be used with foods that may harbour Enteritidis and yet retain the sensitivity required for a correct diagnosis. The number of false-negative reactions is always a major concern, particularly if the suspected food contains low number of, or injured, pathogens. Therefore, it may be advantageous to enrich samples in broth culture before attempting to isolate bacterial DNA templates for PCR. For example, in a recent outbreak caused by Enteritidis, ice cream samples were found to contain less than 1 c.f.u. per g, as determined by the most probable number method [P. Sherrod, FDA, personal communication, 1995]. This finding emphasizes the need for pre-enrichment of food samples in broth to obtain a satisfactory number of cells for detection.

The SE-PCR assay can be applied to available methods for extracting DNA from foods or clinical samples for rapid detection of Enteritidis. PCR-based assays have been used to detect other foodborne pathogens such as *Vibrio vulnificus* [39], *Escherichia coli* [40], *Shigella* spp. [41], and *Campylobacter* spp. [42]. We used the MAMA-PCR technique to detect Enteritidis in seeded chicken, and found as little as 1 c.f.u. per g of chicken meat by PCR and agarose gel electrophoresis. Our study demonstrates that the SE-PCR assay can discriminate between Enteritidis and other bacterial isolates both in pure culture and in a seeded food sample.

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