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Use of the polymerase chain reaction for *Salmonella* detection

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J. KWANG, E.T. LITTLEDIKE AND J.E. KEEN. 1996. A primer set of oligonucleotides (S18 and S19) from the *ompC* gene of *Salmonella* has been evaluated for specific detection of Salmonella by polymerase chain reaction (PCR). This primer set successfully amplified 40 Salmonella serovars (60 isolates), but not 24 non-Salmonella bacteria (42 isolates) that have been tested so far. The uniqueness of these primer sequences was also confirmed. The sensitivity of PCR detection in extracted chromosomal DNA for *Salm. typhimurium* was 1 pg. The sensitivity for boiled whole bacteria was 400 cells. The detection of *Salm. typhimurium* in ground beef samples required 4–6 h enrichment with an initial inocula of 100 bacteria.

INTRODUCTION

Salmonellae are Gram-negative, facultative, intracellular parasites that invade the mucous membrane and are spread primarily by faecal oral transmission. There are more than 2200 different *Salmonella* serotypes (Pelzer 1989) and most of these serotypes are human pathogens. Cattle and poultry have been implicated as major sources of *Salmonella*-contaminated food products that cause human salmonellosis. However, *Salmonella* infection in food-producing animals is often clinically not apparent, which may result in non-detection of *Salmonella*-infected animals at pre- and post-harvest inspections (Pelzer 1989).

Several polymerase chain reaction (PCR) methods of detecting *Salmonella* have been published utilizing specific gene sequences for targeting (Widjojoatmodjo *et al.* 1991; Doran *et al.* 1992; Rahn *et al.* 1992; Aabo *et al.* 1993; Cohen *et al.* 1993; Way *et al.* 1993; Stone *et al.* 1994). The outer membrane proteins are major structural proteins of *Salmonella* and other Gram-negative bacteria. It has been shown that the *ompC* nucleotide sequences for *Salmonella* and *Escherichia coli* have 77% homology (Mizuno *et al.* 1983). A pair of unique sequences have been identified from the *ompC* gene of *Salmonella*. This report describes the usefulness of these unique sequences for PCR detection of *Salmonella* organisms.

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MATERIALS AND METHODS

Bacterial strains

In this study, 40 *Salmonella* serovars (60 isolates) and 24 non-*Salmonella* Gram-negative bacteria (42 isolates) were used. These cultures were collected from the American Type Culture Collection (ATCC, Rockville, MD), Centers for Disease Control and Prevention (CDC, Atlanta, GA), National Veterinary Services Laboratories (NVSL, Ames, IA) and National Animal Disease Center (NADC, Ames, IA) (Tables 1 and 2).

Isolation of chromosomal DNA from bacteria

All chromosomal DNA were prepared as previously described (Cohen *et al.* 1993).

Oligoprimers and polymerase chain reaction (PCR)

Three sets of PCR primers (S18–19, S21–22 and S29–30) were degenerated from the *Salmonella ompC* gene (Puente *et al.* 1989). The sequences and function of each primer set and their locations are shown in Table 3 and Fig. 1. PCR amplification was performed in a 100 μl reaction using 100 ng of genomic DNA. Reagents used for PCR were purchased

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Table 1 Non-*Salmonella* bacteria strains tested by PCR

Strains	Source
Actinobacillus suis	ATCC
Aeromonas hydrophila	ATCC
Alcaligenes (patent strain)	ATCC
Alcaligenes faecalis faecalis	ATCC
Cedecea lapagei	ATCC
Citrobacter freundii	ATCC
Edwardsiella tarda	ATCC
Enterobacter cloacae	ATCC
Escherichia coli (15)*	NVSL and ATCC
Haemophilus somnus	ATCC
Hafnia alvei	ATCC
Klebsiella pneumoniae	ATCC
Leminorella grimontii	ATCC
Pasteurella haemolytica	ATCC
Pasteurella multocida Type-A	ATCC
Pasteurella multocida Type-D (5)*	ATCC and NADC
Proteus mirabilis	ATCC
Pr. vulgaris	ATCC
Providencia rettgeri	ATCC
P. stuartii	ATCC
Pseudomonas aeruginosa	ATCC
Serratia marcescens	ATCC
Shigella sonnei	ATCC
Yersinia pseudotuberculosis	ATCC

*Indicates number of strains tested.

Table 2 *Salmonella* spp. tested by PCR

Salmonella serovars	Source
var. 9, 12 :non-motile	NVSL
agona (2)*	NVSL and CDC
anatum	NVSL
arizonae	ATCC
berta	CDC
braenderup	CDC
brandenburg	CDC
broughton	NVSL
cerro	NVSL
cholerasuis	ATCC
cholerasuis var. *kunzendorf* (2)*	NVSL and NADC
derby (2)*	NVSL and CDC
dublin (2)*	NVSL and ATCC
duesseldorf	NVSL
enteritidis (2)*	ATCC and CDC
enteritidis, phage Type-8	NVSL
gallinarum	ATCC
give	NVSL
hadar	NVSL
heidelberg (2)*	NVSL and CDC
infantis	CDC
istanbul	NVSL
javiana	CDC
kentucky	NVSL
johannesburg	NVSL
montevideo	CDC
muenchen	CDC
newport (2)*	ATCC and CDC
ohio	NVSL
oranienburg	CDC
paratyphi-A	ATCC
poona	CDC
pullorum	ATCC
reading	CDC
saintpaul	CDC
thompson	CDC
typhi	CDC
typhimurium (4)*	NADC
typhimurium LT2 (wild type)	ATCC
typhimurium var. *copenhagen* (6)*	NVSL and NADC

*Indicates number of serovars tested.

from Perkin-Elmer (Norwalk, CT) and mixtures were prepared according to the instructions of the manufacturer. The cycling reaction was programmed for 30 cycles and set to heat at 95°C for 30 s, cooled to 56°C for 45 s, and then incubated at 72°C for 60 s.

DNA sequencing

Nucleotide sequencing was done by using a fmol DNA sequencing system according to the manufacturer's specifications (Promega, Madison, WI).

Southern hybridization

Southern transfer was performed by using Zeta-probe blotting membrane according to the supplier's instructions (Bio-Rad, Richmond, CA). Probe labelling and hybridization were performed by using the Dig DNA labelling and detection kit according to their recommended protocol (Boehringer Mannheim, Germany).

Sensitivity of PCR detection

Salmonella typhimurium bacteria and extracted chromosomal DNA were used to evaluate the sensitivity of detection in the PCR assay. A bacterial culture of 4×10^8 cell ml^{-1} was serially diluted 10-fold to 4×10^1 cell ml^{-1}. These cell suspensions were then centrifuged ($10\,000 \times g$) for 1 min. The pellets were then resuspended in 100 μl of water and boiled for 10 min. Ten microlitres of each dilution were added directly

Table 3 Sequence of primers used in PCR

Primer code	Sequences (5′ → 3′)	Position
S18–19	ACC GCT AAC GCT CGC CTG TAT AGA GGT GGA CGG GTT GCT GCC GTT	730–888
S21–22	AAC GGC GAT CGC GCC ACG GTT GGT ACC AAA ACG GGT AGC GTT	754–861
S29–30	CAG TAT CAG GGC AAA AAC GGC TTC AAA GTT CTG CGC TTT GTT	562–921

Primer sequence is cited from Puente *et al.* (1989).

to the PCR mixture. *Salmonella typhimurium* chromosomal DNA, measured by spectrophotometry, was diluted 10-fold in water from 1 μg to 100 fg/100 μl. Ten microlitres of each diluted DNA were used for PCR amplification.

Detection of *Salmonella* in ground beef

Ground beef (5 g) was added to 45 ml of trypticase soy broth and heat-treated at 60°C for $1\frac{1}{2}$ h to kill any possible *Salmonella* bacterial contamination. The mixture was then inoculated with 100 viable bacteria and incubated at 37°C for 2, 4, 6, 8, 10, 12 and 22 h. One millilitre of samples from each incubation time was boiled for 10 min and centrifuged at 10 000 × *g* for 1 min. The 10 μl of supernatant fluid were used for PCR amplification.

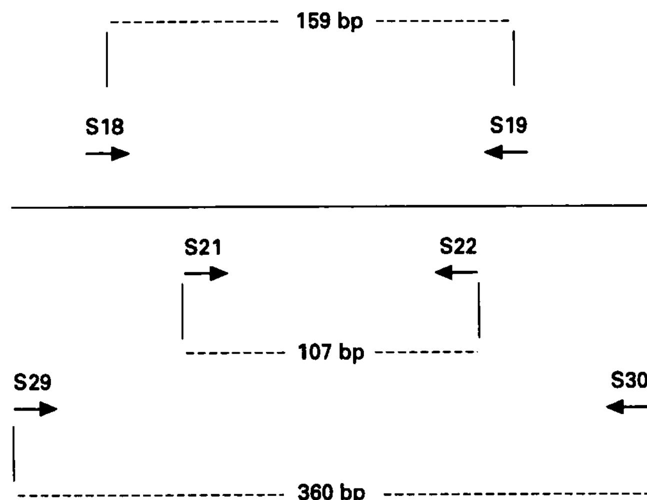

Fig. 1 Schematically illustrated three sets of PCR primers and their related location. On the *ompC* gene, the S21–22 amplified product is located inside of the S18–19. S29–30 amplified product is located outside the S18–19. S18–19 primers used for specific detection of *Salmonella*. S21–22 primers were used to amplify a 107 bp fragment and served as an internal probe for *Salmonella* sequence identification. S29–30 primers amplified products used as template for DNA sequencing analysis to confirm the uniqueness of the S18–19 primer

RESULTS AND DISCUSSION

The PCR amplified product of 159 bp by S18–19 primer was present in samples from all 40 *Salmonella* serovars (60 isolates) tested. However, the primers did not amplify any specific fragment from the 24 non-*Salmonella* bacteria (42 isolates). These data suggest that S18–19 primers could be unique to *Salmonella* sequences, which are not conserved in non-*Salmonella* organisms. Representative agarose gels for analysing these PCR products are shown in Fig. 2a.

To verify the identification of the S18–19 PCR product from *Salmonella*, 7 μl of each of the PCR products were analysed by Southern hybridization with a digoxigenin labelled probe. This DNA probe was derived from S21–22 amplified 107 bp fragments by using *Salm. typhimurium* genomic DNA as a template. As shown in Fig. 2b, the probe unanimously detected all *Salmonella* spp. and confirmed that the PCR products were indeed generated from target *Salmonella ompC* gene.

In order to confirm that the S18–19 primer sequences were unique to *Salmonella* but not to non-*Salmonella* bacterial genomes, nucleotide sequencing of S29–30 amplified products from *Salm. typhimurium*, *Salm. enteritidis*, *E. coli* 23513 and *Enterobacter cloacae* were performed.

Comparison of the S18–19 primer sequences among these organisms (*E. coli* K12 *ompC* gene sequence also included) showed that S18–19 were highly conserved between *Salmonella* isolates. In contrast, non-*Salmonella* bacteria showed either deletion or insertion of residues or were highly variable as compared to *Salmonella* S18–19 primer sequences (Table 4). Thus, these regions were poorly conserved between *Salmonella* and non-*Salmonella* bacteria.

The sensitivity of PCR detection after 30 cycles amplification of the target sequences in extracted chromosomal DNA from *Salm. typhimurium* was 1 pg (Fig. 3a). The sensitivity of PCR detection from the boiled bacterial cells was

Table 4 Comparative analysis of the part of the *ompC* genes of *Salmonella typhimurium*, *Salm. enteritidis*, *Escherichia coli* K12, *E. coli* 23513 and *Enterobacter cloacae*

(A)	
Salm. typhimurium	GATCAGAA( )CAACACCGCTAACGCTCGCCTGTATGGTAACGGCGAT
Salm. enteritidis	--------( )-----------G-------------------------
E. coli K12	-C------( )--C-G-T---T--ATC( )------------C
E. coli 23513	--------( )--G-C-GCTGT--ATC( )-------------
Enterobacter cloacae	-C------AGCTGT--CAG-G--CT--TTC( )----T-------C
(B)	
Salm. typhimurium	ACGCTACCCGTTTTGGTACCTCTAACGGCAGCAACCCGTCCACCTCTTACGGTTTTGCCAAC
Salm. enteritidis	----A-------------------------A---T---------------------------
E. coli K12	----A--T--CG-A---T--CTG( )----GG--G---
E. coli 23513	----A--T--CG-A---T--CTG( )----GG--G---
Enterobacter cloacae	-T-GC-GT--CG-A--TT--GCG( )--C-GG--G-T-

–, Indicates residue identical to *Salm. typhimurium*.
( ), Indicates the absence of residue.
Double underlines in (A) and (B) were S18 and S19 sequences, respectively.
E. coli K12 sequence is from Mizuno *et al.* (1983).

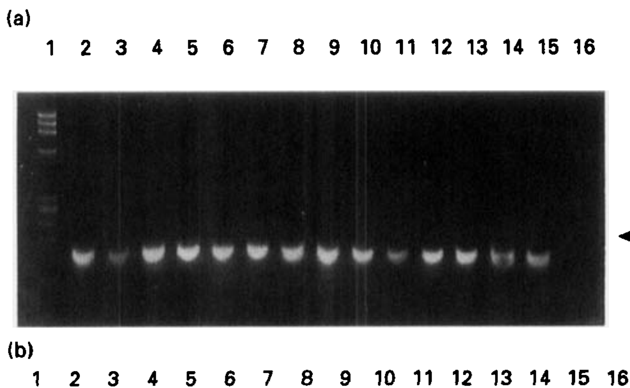

Fig. 2 (a) Agarose gel electrophoresis of the representative PCR products amplified with primers S18–19. Lanes: 1, ϕX174 RF DNA/*Hae*III fragments molecular standard; 2–15, *Salmonella typhimurium*, *Salm. cerro*, *Salm. typhimurium* var. *copenhagen*, *Salm. anatum*, *Salm. dublin*, *Salm. enteritidis*, *Salm. kentucky*, *Salm. montevideo*, *Salm. newport*, *Salm. cholerasuis*, *Salm. cholerasuis* var. *kunzendorf*, *Salm. derby*, *Salm. heidelberg*, *Salm. agona*; 16, *Citrobacter freundii*. The predicted amplification product of 159 bp was seen only in *Salmonella* DNA (arrow). (b) Southern blot of gel in (a)

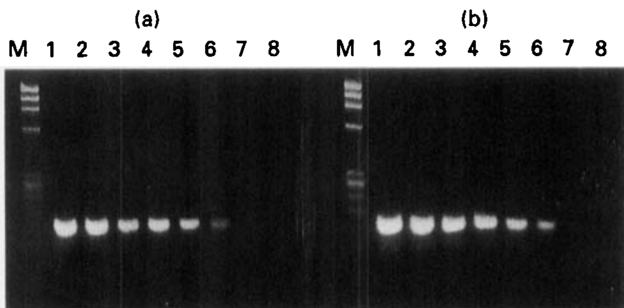

Fig. 3 Sensitivity of the PCR detection after 30 cycles amplification. (a) Extracted *Salmonella typhimurium* chromosomal DNA used as template. Lanes: M, ϕX174 RF DNA/*Hae*III fragments molecular standards; 1–8, PCR product from 100 ng, 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg. (b) Boiled *Salm. typhimurium* bacterial used as template. Lanes: M, ϕX174 RF DNA/*Hae*III fragments molecular standards; 1–8, 4×10^7, 4×10^6, 4×10^5, 4×10^4, 4×10^3, 4×10^2, 4×10^1, 4×10^0

400 cells of *Salm. typhimurium* (Fig. 3b). These detection limits are more sensitive in comparison with other methods such as an enzyme-linked immunosorbent assay (Curiale *et al.* 1990). The detection of *Salm. typhimurium* in ground beef samples required 4–6 h enrichment with an initial inoculum of 100 bacteria.

In this study, unique sequences for *Salmonella* in the *ompC* gene were identified and these sequences were conserved among all the *Salmonella* spp. that have been tested. These 40 *Salmonella* serovars (60 isolates) included those serotypes most frequently associated with disease in cattle, swine, humans, chickens and turkeys. The use of the PCR with a single primer pair, as described in this report, appeared to be highly specific for the *in vitro* detection of *Salmonella* organisms. Further investigations will concentrate on using these primers in an assay format suitable for *Salmonella* detection in biological samples, such as faecal material, carcass samples, lymph nodes and other tissues.

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