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Specific Detection of *Salmonella* spp. by Multiplex Polymerase Chain Reaction

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Three sets of oligonucleotide primers were used in the polymerase chain reaction (PCR) assay to detect *Salmonella* species. *phoP* primers specific to the *phoP/phoQ* loci of coliform pathogenic bacteria such as *Salmonella*, *Shigella*, *Escherichia coli*, and *Citrobacter* species served as presumptive indicators of enteric bacteria. In addition to the *phoP* primers, the *Hin* and the *H-1i* primers, which targeted a 236-bp region of *hin*/H2 and a 173-bp region of the *H-1i* flagellin gene, respectively, were used. Both *Hin* and *H-1i* primers are specific to motile *Salmonella* species and are not present in *Shigella*, *E. coli*, or *Citrobacter* species. Thus, by multiplex PCR amplification, *Salmonella* species including *Salmonella typhi*, *Salmonella typhimurium*, *Salmonella paratyphi* A, and *Salmonella enteritidis* can be specifically detected. Optimal reaction conditions have been described to demonstrate this specific, sensitive detection of *Salmonella* species. By using agarose gel electrophoresis for detection of the PCR-amplified products, the sensitivity of detection was 10^2 CFU after 25 cycles of PCR and 1 (10^0) CFU after a 50-cycle double PCR. The efficacy of these primers was demonstrated on environmental isolates which had previously been confirmed as *Salmonella* species by the use of conventional cultural techniques. In addition, positive amplifications resulted from *Salmonella* species in environmental samples including soil and water.

The application of sewage sludges and effluents on agricultural lands throughout the United States has increased the potential for microbial contamination of field soil and groundwater. In particular, enteric bacteria such as *Salmonella* species can be responsible for increased health hazards. *Salmonella typhimurium* is a facultative intracellular pathogen which causes the salmonellosis of humans and animals and is also the causative agent of human typhoid fever, a worldwide problem with over 30 million cases annually (12). Although many different conventional culture media and enrichment regimes have been proposed for isolating *Salmonella* species from food or environmental samples (1, 2, 15, 22), these organisms are still difficult to culture, detect, or enumerate from the complex microbial communities of natural ecosystems (27). The ability of *Salmonella* species to enter a viable but nonculturable state after lengthy exposure to soil or groundwater under ambient conditions of temperature and low nutrient concentration may contribute to this difficulty (18, 19). Because of limitations in the methodologies employed, precise diagnosis of *Salmonella* species in environmental samples on current enrichment media is difficult. In addition, conventional plate counting methods are tedious and time-consuming and usually underestimate the numbers (8, 23, 31).

The development of polymerase chain reaction (PCR) technology has the potential to solve these problems. PCR is a rapid in vitro procedure for enzymatic amplification of specific DNA sequences which increases the number of copies of the target sequence. This allows increased sensitivity of detection of a DNA sequence present in trace amounts in mixed populations (25, 28–30). Recently, gene-specific probes have been used for monitoring *Salmonella* species in food samples (10, 11). Although there are some reports on the detection of enteric pathogens in environmental samples (4, 5), there are no reports of primers which can detect only *Salmonella* species in soil or water environments. In this study, multiplex PCR amplification with three sets of primers, *phoP*, *Hin*, and *H-1i*, permitted specific and sensitive detection of *Salmonella* species. Since *phoP* has the ability to simultaneously detect coliform pathogenic bacteria, which include *Salmonella*, *Shigella*, *Escherichia coli*, and *Citrobacter* species, it can be used as an indicator to monitor environmental samples for enteric bacteria. *phoP* primers selected from the *phoP/phoQ* loci are part of the phosphorylation regulon which regulates the expression of genes involved in virulence and macrophage survival of *Salmonella* species. *Hin* and *H-1i* genes which are involved in the control of phase variation of *Salmonella* species are only present in *Salmonella* species and are not present in *E. coli*. Thus, *phoP* primers used in conjunction with *Hin* and *H-1i* primers can subsequently be used to confirm motile *Salmonella* species, including *Salmonella typhi*, *Salmonella typhimurium*, *Salmonella paratyphi* A, and *Salmonella enteritidis*.

MATERIALS AND METHODS

Bacterial strains. Thirty-six known bacterial strains, including 11 *Salmonella* species, 6 *E. coli* strains, 4 *Shigella* species, 3 *Citrobacter* species, *Bacillus subtilis*, *Enterobacter cloacae*, *Enterobacter aerogenes*, *Erwinia carotovora*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Rhizobium leguminosarum* bv. phaseoli, *Serratia marcescens*, *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Vibrio natriegens*, were analyzed. In addition to this, numerous environmental isolates were also analyzed. The sources of the known bacteria and the phase characteristics for the *Salmonella* species are listed in Table 1. The bacteria were cultivated and maintained in Nutrient Broth (Difco Laboratories, Detroit, Mich.) at 37°C, unless Ameri-

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TABLE 1. Bacterial strains utilized to evaluate the specificity of multiplex PCR, phase characteristics of *Salmonella* species, and multiplex PCR results

Strain	ATCC no.	Phase[a]	PCR result		
			phoP[b]	*Hin*	*H-1i*
Salmonella typhi	6539	1	+	+	+
Salmonella paratyphi A	9150	1 and 2	+	+	+
Salmonella gallinarum	9184	NM	+	−	−
Salmonella enteritidis	13076	1	+	−	+
Salmonella choleraesuis	13312	1 and 2	+	+	+
Salmonella pullorum	19945	NM	+	−	−
Salmonella sp.	35664	2	+	+	−
Salmonella arizonae	13314	2	+	+	+
Salmonella typhimurium	14028	1 and 2	+	+	+
Salmonella typhimurium	19585	1 and 2	+	+	+
Salmonella typhimurium	23564	1 and 2	+	+	+
Escherichia coli	8739		+/−	−	−
Escherichia coli	11303		+/−	−	−
Escherichia coli	13706		+/+	−	−
Escherichia coli	12435		+/−	−	−
Escherichia coli	15224		+/−	−	−
Escherichia coli	25922		+/−	−	−
Citrobacter freundii	8090		+/−	−	−
Citrobacter diversus	27028		+/−	−	−
Citrobacter freundii	43162		+/−	−	−
Shigella boydii type 1	9207		+/−	−	−
Shigella flexneri type 2b	12022		+/−	−	−
Shigella sonnei	11060		+/−	−	−
Shigella sonnei	25931		+/−	−	−
Bacillus subtilis	21332		−	−	−
Enterobacter cloacae	13047		−	−	−
Enterobacter aerogenes[c]			−	−	−
Erwinia carotovora[c]			−	−	−
Klebsiella pneumoniae[c]			−	−	−
Proteus vulgaris[c]			−	−	−
Pseudomonas aeruginosa	9027		−	−	−
Rhizobium leguminosarum bv. phaseoli[c]			−	−	−
Serratia marcescens[c]			−	−	−
Staphylococcus aureus	10832		−	−	−
Streptococcus pyogenes	19615		−	−	−
Vibrio natriegens	14048		−	−	−

[a] Phase in *Salmonella* species applies only to the antigenic properties of the H components of the flagellated bacteria and their corresponding agglutinins. NM, nonmotile (no H antigen).

[b] +/−, There is no amplification with *phoP* primers under stringent reaction conditions: annealing temperature, 75°C; concentration of each primer, 25 ng/50-μl reaction mixture; concentration of deoxynucleoside triphosphates, 200 μM/50-μl reaction mixture; hot start.

[c] Provided by Department of Microbiology and Immunology, University of Arizona.

can Type Culture Collection (ATCC) instructions specified otherwise.

Primer design. Oligonucleotide sequences of *phoP*, *Hin*, and *H-1i* for the multiplex PCR amplification of *Salmonella* species are listed in Table 2. *phoP* defines a 299-bp region on the 2.3-kb *phoP*/*phoQ* gene loci of *Salmonella typhimurium* (21). The second pair of primers are derived from the 1.5-kb *hin*/H2 sequences of *Salmonella typhimurium* and amplifies a 236-bp region (38). The *H-1i* primer pair is specific for a 173-bp region of the *H-1i* flagellin region of *Salmonella typhimurium* (17). Primers were synthesized with a 380A Applied Biosystems DNA Synthesizer (Foster City, Calif.) and purified by polyacrylamide gel electrophoresis.

PCR amplification conditions. PCR amplification of the target sequence was performed with the GeneAmp kit with *Taq* DNA polymerase in a DNA thermal cycler (Perkin-Elmer Cetus Corp., Norwalk, Conn.). The PCR mixture contained the reaction buffer (50 mM KCl, 10 mM Tris-Cl [pH 8.3], 1.5 mM $MgCl_2$, 0.01% [wt/vol] gelatin), 200 μM (each) deoxynucleoside triphosphates, 50 ng of *phoP* primers, 1.25 U of *Taq* polymerase, template DNA, and deionized water for a final volume of 50 μl. For multiplex PCR, 100 ng (each) of *Hin* and *H-1i* primers was used. The reaction mixture was subjected to PCR under the following conditions: heat denaturation at 94°C for 2 min and then an additional 30 cycles with heat denaturation at 94°C for 1.5 min, primer annealing at 62°C for 30 s, and DNA extension at 72°C for 1.5 min. After the last cycle, samples were maintained at 72°C for 7 min to complete synthesis of all strands.

Detection of amplified DNAs. PCR products were analyzed by gel electrophoresis. Fifteen microliters of each sample was loaded onto a 1.6% electrophoresis-grade agarose gel (Sigma Chemical Co., St. Louis, Mo.) and run in 1× TBE buffer at 3.2 V cm^{-1} for 1.5 to 2 h (20). Gels were stained with 1 μg of ethidium bromide solution per ml and visualized with a UV transilluminator (UVP Inc., San Gabriel, Calif.).

Specificity of primers. The specificity of multiplex PCR for *Salmonella* species was evaluated with the 30 ATCC strains and 6 non-ATCC strains listed in Table 1. In addition, the efficacy of the primers was also evaluated on 15 known environmental isolates (Table 3), of which 12 had previously been confirmed as *Salmonella* species by conventional techniques.

Sensitivity of amplification. Sensitivity of the PCR amplification was determined by the use of whole-cell lysates from *Salmonella typhimurium* cells. The methodology to evaluate the sensitivity of primers was adopted from that of Debue et

TABLE 2. Primers for the multiplex PCR amplification of *Salmonella* species

Oligonucleotide sequence	Length (bp)	Position and orientation on DNA sequences[a]	Amplification region (bp)	T_m (°C)	Reference
phoP					
337-L, 5′-ATGCAAAGCCCGACCATGACG-3′	21	1052 → 1072	299	67.4	21
338-R, 5′-GTATCGACCACCACGATGGTT-3′	21	1331 ← 1351		67.4	
Hin					
1750-L, 5′-CTAGTGCAAATTGTGACCGCA-3′	21	169 → 189	236	66.8	38
1751-R, 5′-CCCCATCGCGCTACTGGTATC-3′	21	384 ← 404		66.7	
H-1i					
1788-L, 5′-AGCCTCGGCTACTGGTCTTG-3′	20	624 → 643	173	65.8	17
1789-R, 5′-CCGCAGCAAGAGTCACCTCA-3′	20	777 ← 796		65.8	

[a] Arrows indicate orientation of the oligonucleotides.

TABLE 3. Multiplex PCR identification of environmental isolates

No.	Presumptive organism	Phase[a]	PCR result		
			phoP	*Hin*	*H-1i*
B-6601034	*Salmonella heidelberg*	1 and 2	+	+	+
B-7211645-10	*Salmonella heidelberg*	1 and 2	+	+	±[b]
M-7011529-1	*Salmonella poona*	1 and 2	+	+	−
M-6212478-1	*Salmonella muenchen*	1 and 2	+	+	+
M-7008395-4	*Salmonella pomona*	1 and 2	+	+	+
MP5-5126-4	*Salmonella dublin*	1	+	−	+
O-7210564-2	*Salmonella california*	1	+	±	+
O-711484-3	*Salmonella lomita*	1 and 2	+	+	+
SDPHS 103-1	*Salmonella typhimurium*	1 and 2	+	+	+
SDPHS 107-1	*Salmonella typhimurium*	1 and 2	+	+	+
SDPHS 114-2	*Salmonella typhimurium*	1 and 2	+	+	+
SDPHS 117-2	*Salmonella typhimurium*	1 and 2	+	+	+
MP5-9959-1	*E. coli* O147:K:H7		+	−	−
MP5-6120-2	*E. coli* O5:K:H11		+	−	−
MP4-354-1	*E. coli* O8:K:NM		+	−	−

[a] Phase in *Salmonella* species applies only to the antigenic properties of the H components of the flagellated bacteria and their corresponding agglutinins.
[b] ±, faint amplification band shows in picture.

al. (7). One milliliter from late-log-phase cultures (10^8 cells per ml) was centrifuged for 10 min at 14,000 × *g*, and the pellet was washed in 0.1% peptone water and then resuspended in 100 μl of double-distilled water. Cell suspensions were directly lysed by boiling at 98°C for 10 min. Lysed bacterial cells were serially diluted in double-distilled water. Aliquots were assayed for amplification with *phoP*, *Hin*, and *H-1i* primer sets individually and also in the multiplex system under the same reaction conditions as described previously.

Efficacy of primers for detection of presumptive *Salmonella* isolates from environmental samples. Fifteen environmental isolates from animal (dairy cattle, pigs, and broiler chickens) feed lots and waste sludges (provided by J. G. Songer, University of Arizona) were used. Twelve were positively identified as *Salmonella* species by standard biochemical procedures and serotyping. Three of them were determined not to be *Salmonella* species. All environmental isolates were cultured in nutrient broth at 37°C to late log phase and subjected to multiplex PCR amplification as described previously.

Efficacy of primers for detection of *Salmonella* species in soil. Two soils were used in this study: Brazito sandy loam and Pima silty clay loam. Soil community DNA was extracted from soil after direct lysis within the soil (32). Community DNA was purified by $CsCl_2$ ethidium bromide equilibrium density centrifugation (32). Following removal of ethidium bromide via treatment with isopropanol, the DNA was further purified by ethanol precipitation and qualified by $A_{260/280}$ readings in a U-2000 Double-Beam UV/VIS Spectrophotometer (Hitachi, Tokyo, Japan) (20, 32). Control nonseeded soils as well as soils seeded with *Salmonella typhimurium* ATCC 14028 at a rate of 10^8 cells per g of soil were extracted.

Efficacy of primers for detection of *Salmonella* species in environmental water samples. Four well water samples and one surface pond water sample were collected from the Marana and Avra Valley area of southern Arizona. The surface pond water sample was collected from a lagoon where municipal effluent had been discharged and stored.

For each well water sample, a 1-liter sample was aseptically pumped through a 13-mm 0.22-μm-pore-size polycarbonate membrane with a syringe with an attached Swinnex filter holder at a pressure of 50 cm Hg. The filter membrane and trapped cells were placed in a 1.5-ml polypropylene tube with 600 μl of H_2O. The filter was heated at 65°C for 1 h. The tube was vigorously vortexed for 1 min, and the membrane was recovered. The cell suspension was microcentrifuged at 14,000 × *g* for 10 min, after which the liquid was discarded and the cell pellet was resuspended in 20 μl of H_2O. This cell suspension was transferred into a 0.5-ml PCR microcentrifuge tube containing the PCR reaction mixture minus enzyme, and the cells were lysed by boiling at 98°C for 10 min. After cooling to room temperature, the enzyme was added prior to subsequent PCR analysis. A positive control was performed to confirm that chemicals and particulates from the well water did not interfere with PCR amplification. One liter of each well water was inoculated with *Salmonella typhi* ATCC 6539 late-log-phase culture at approximately 5×10^5 cells and concentrated as described previously. The surface pond water, which contained excessive amounts of colloidal debris, could not be filtered. Instead, presumptive *Salmonella* isolates were obtained by using the pond water to inoculate enrichment media, which were then grown on bismuth sulfite selective media (2). Isolates which grew at 37°C that were deep black and had a metallic sheen were subjected to PCR multiplex amplification.

RESULTS AND DISCUSSION

Salmonella typhimurium causes infections in humans that are manifested by typhoid fever, septicemia, and gastroenteritis. The *phoP/phoQ* loci are part of the regulon which regulates the expression of genes involved in virulence and macrophage survival of *Salmonella* species (21). Strains carrying *phoP* mutations are avirulent, unable to survive in macrophage, and extremely sensitive to peptides having antimicrobial activity such as the host-derived defensins (12). The survival and growth of *Salmonella* species within the macrophage phagolysosome is felt to be essential for typhoid pathogenesis (6, 14). Theoretically, for specific detection of *Salmonella* species by PCR amplification, a 299-bp region of the unique regulatory gene *phoP/phoQ* was an appropriate selection for the target organism. However, since it has been reported that *phoP/phoQ* homologous DNA sequences are present in other organisms including *Shigella*,

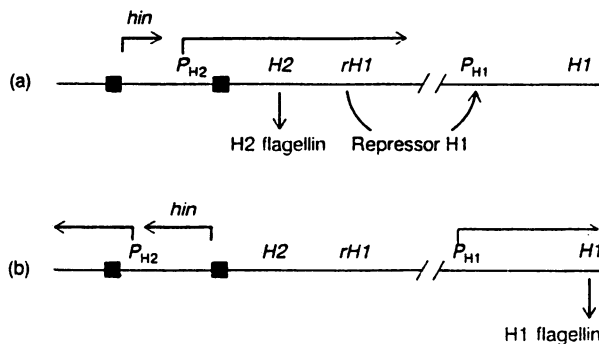

FIG. 1. Molecular basis of variation. (a) With *hin* and the *H2* promoter (P_{H2}) in the rightward orientation, *H2* and *rH1* are expressed. The *H2* product is H2 flagellin. The *rH1* product is a repressor that binds near the *H1* promoter (P_{H1}) and prevents expression of the *H1* gene. (b) With the *hin-H2* promoter segment inverted, *H2* and *rH1* cannot be expressed; thus, no H2 flagellin or H1 repressor is made. With no repressor, the *H1* gene is active, so H1 flagellin is made. The black boxes denote inverted repeats at the borders of the invertible segment.

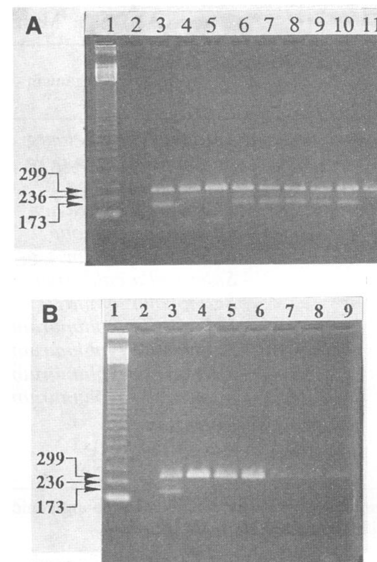

FIG. 2. Multiplex PCR results of the specificity of *phoP*, *Hin*, and *H-1i* primers on various strains. (A) *Salmonella* ATCC strains. Lanes: 1, 123-bp DNA ladder as a size standard; 2, negative control (no DNA); 3, *Salmonella typhi* (ATCC 6539, phase 1); 4, *Salmonella paratyphi* A (ATCC 9150, phase 1 and 2); 5, *Salmonella enteritidis* (ATCC 13076, phase 1); 6, *Salmonella choleraesuis* (ATCC 13312, phase 1 and 2); 7, *Salmonella arizonae* (ATCC 13314, phase 2); 8, *Salmonella typhimurium* (ATCC 14028, phase 1 and 2); 9, *Salmonella typhimurium* (ATCC 19585, phase 1 and 2); 10, *Salmonella typhimurium* (ATCC 23564, phase 1 and 2); 11, *Salmonella* sp. (ATCC 35664, phase 2). (B) *E. coli* ATCC strains. Lanes: 1, 123-bp DNA ladder; 2, negative control (no DNA); 3, positive control (*Salmonella typhimurium* ATCC 14028); 4, *E. coli* (ATCC 8739); 5, *E. coli* (ATCC 11303); 6, *E. coli* (ATCC 12435); 7, *E. coli* (ATCC 13706); 8, *E. coli* (ATCC 15224); 9, *E. coli* (ATCC 25922).

E. coli, and *Citrobacter* species (13), *phoP* primers, under less stringent conditions, can amplify DNA from other coliform bacteria. Therefore, *phoP* primers were used in this study as a presumptive indicator to initially screen for possible *Salmonella* species and other coliform pathogens. To confirm the presence of *Salmonella* species, primers which could specifically detect all *Salmonella* species were necessary. Some of the phenotypic characteristics that distinguish *Salmonella typhimurium* from *E. coli* are encoded by genes that are associated with phase variation in *Salmonella* species (3). The genes of the H2 antigen and the *hin* inversion system, which are responsible for flagellar phase variation, are present in *Salmonella* species and are controlled by a novel kind of regulatory unit in which a site-specific recombinational event regulates gene expression (26, 36, 37). A 995-bp invertible DNA fragment adjacent to the *H2* gene, which specifies one of the flagellar antigens, can exist in either orientation with respect to the *H2* structural gene. This orientation of the inversion region controls expression of the *H2* gene, i.e., in one orientation the adjacent *H2* gene is expressed, and in the opposite orientation the adjacent *H2* gene is not expressed (38). The *hin* (for H inversion) gene is located within the inversion region (between nucleotides 76 and 648) and accounts for approximately two-thirds of the sequence (38). *hin* encodes a protein (*hin* protein) necessary for the inversion of the H2 controlling region (Fig. 1) (33). Thus, the particular phase of a *Salmonella* species can be defined on the basis of the flagella antigenic state (Table 1).

Specificity of primers. The *Hin* primer set was selected from the *Salmonella typhimurium hin*/H2 region, which targets a 236-bp DNA fragment in *Salmonella* phase 2, as well as phase 1-phase 2 inversion species (at times, certain species may exhibit both antigenic types). The primer set *H-1i* which amplifies a 173-bp DNA sequence in *Salmonella* phase 1 species was located in the *H-1i* flagellin gene, also from *Salmonella typhimurium*. Multiplex PCR was performed by adding the above sets of primers in the same reaction to determine the amplification of known *Salmonella* species-specific DNA and discriminate against non-target DNA.

Table 1 shows the multiplex PCR results. All coliform pathogenic bacteria showed a 299-bp *phoP*/*phoQ* amplification product, whereas only motile *Salmonella* species resulted in Hin and/or H-1i amplifications. *Bacillus subtilis*, *Enterobacter cloacae*, *Enterobacter aerogenes*, *Erwinia carotovora*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Rhizobium leguminosarum* bv. phaseoli, *Serratia marcescens*, *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Vibrio natriegens* gave no amplification products with this multiplex PCR. More importantly, with *phoP* primers under stringent reaction conditions (Table 1), there was no amplification for *Shigella* and *Citrobacter* species and all *E. coli* except *E. coli* ATCC 13706. Figure 2 shows the multiplex PCR amplifications of ATCC *Salmonella* motile species (Fig. 2A) and ATCC *E. coli* strains (Fig. 2B) with the three primer sets in a multiplex PCR reaction. Amplification bands due to *phoP*/*phoQ* are seen in all tested *Salmonella* species. In addition, all *E. coli* species also produced amplification bands because of the lower annealing temperature of 62°C that was used in this assay relatively to the high annealing temperature of 75°C that was used on the *E. coli* strains in Table 1. The 236-bp product due to the *hin* target occurred with all tested *Salmonella* strains except the *Salmonella enteritidis* (ATCC 13076) phase 1 strain (Fig. 2A, lane 5). The 173-bp *H-1i* band was detected with all *Salmonella* phase 1 strains and phase 2 strains except *Salmonella* sp. (ATCC 35664) phase 2 strain (Fig. 2A, lane 11). The reason that there is no *hin* amplification by the *Hin* primers with this *Salmonella* strain may be (i) there is no H antigen (for some

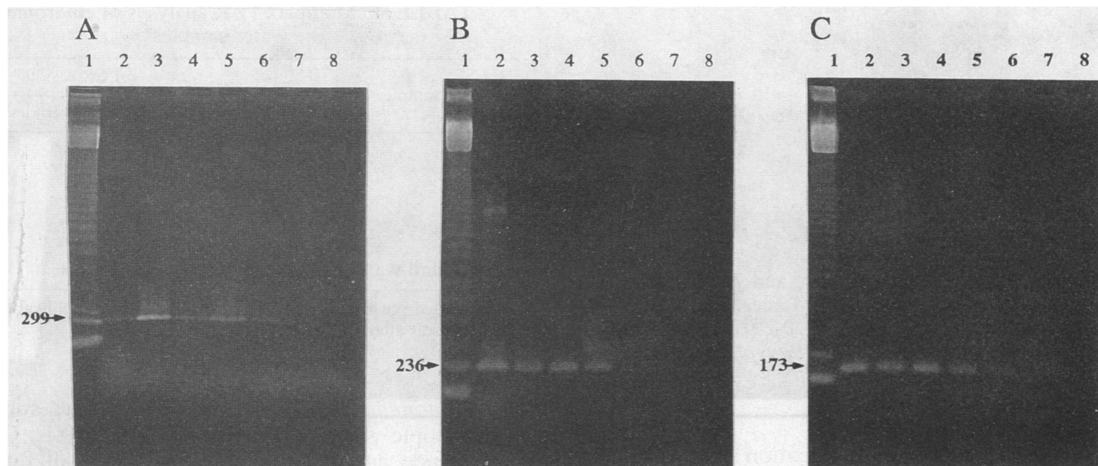

FIG. 3. Sensitivity of *Salmonella typhimurium* detection in terms of whole cells with *phoP* (A), *Hin* (B), and *H-1i* (C) primers. Lanes: 1, 123-bp DNA ladder as a size standard; 2, 6×10^6 CFU; 3, 6×10^5 CFU; 4, 6×10^4 CFU; 5, 6×10^3 CFU; 6, 6×10^2 CFU; 7, 6×10^1 CFU; 8, 6×10^0 CFU.

reason it was lost during evolution) (38), (ii) the number of copies of the H antigen is too low to be amplified, or (iii) poor homology of this H antigen with the primers. Note however, that both strains (ATCC 35664 and 13076) with only two bands would still be identified as *Salmonella* spp. because of the presence of two amplification products. The *H-1i* primer set was selected from *Salmonella typhimurium*. Although the homology of the H1 antigens of other *Salmonella* strains is similar to that of the *Salmonella typhimurium H-1i* gene (17, 34, 35), the size of the H1-i amplification fragment can be slightly different because of slight differences in homology. For example, *Salmonella paratyphi* A (Fig. 2A, lane 4) is slightly different. Some environmental isolates also showed slight differences (see Fig. 4). Some *Salmonella* species such as *Salmonella gallinarum* and *Salmonella pullorum* are nonmotile (no H antigen) and therefore give no amplification bands with *Hin* and *H-1i* primers (Table 1). Use of *lamB* primers (16) differentiated these nonmotile *Salmonella* species from other enteric bacteria (data not shown). Overall, the use of the multiplex PCR analysis allowed specific detection of different species of *Salmonella*.

Sensitivity of amplification. PCR amplification allows very sensitive detection of specific DNA sequences. Sensitivity of amplification was dependent on the number of copies of target DNA and was evaluated by use of whole cells or extracted pure DNA. *Salmonella typhimurium* cells were collected from late-log-phase cultures. After centrifugation for 10 min at $14,000 \times g$, pellets were washed, resuspended, and directly lysed by boiling at 98°C for 10 min. Lysed bacterial cells were serially diluted, and aliquots were assayed for sensitivity with *phoP*, *Hin*, and *H-1i* primer sets individually and also in a multiplex system. Figure 3 shows the PCR amplification of *Salmonella typhimurium phoP/phoQ*, *hin*, and *H-1i*. Target DNA was derived from whole cells, and amplification products were visualized by ethidium bromide staining. The data show that after 25 cycles, 10^3 CFU was easily visualized (Fig. 3, lanes 5). Amplification of 10^2 CFU was only visualized with *phoP* primers (Fig. 3A, lane 6), whereas for *Hin* and *H-1i* primers, 10^2 CFU was just discernible (Fig. 3B and C, lanes 6). Similar results also occurred with the multiplex system (data not shown). Sensitivity can be improved by the use of double PCR or gene probes with dot blot or Southern hybridizations. Double PCR was performed by adding 10 μl of PCR products from the first 25 cycles to a fresh reaction mixture and then running another 25 cycles of PCR. The results showed that for all primers, after 50 cycles, as few as 10^0 CFU could be detected via ethidium bromide staining (data not shown). Sensitivity of primers is highly dependent on (i) the method of analysis, e.g., cells, cell lysates, or extracted pure DNA, and (ii) the age of the culture used. In addition, sensitivity usually decreases when detecting cells in environmental samples.

Efficacy of primers on known environmental samples. To test the efficacy of primers for monitoring environmental samples, 15 known environmental isolates were analyzed. Twelve presumptive isolates previously isolated from animal feed lots and waste sludges and confirmed to be *Salmonella* species by conventional cultural methods, as well as three isolates previously confirmed to be *E. coli*, were tested. Table 3 and Figure 4 show positive 299-bp *phoP/phoQ* bands

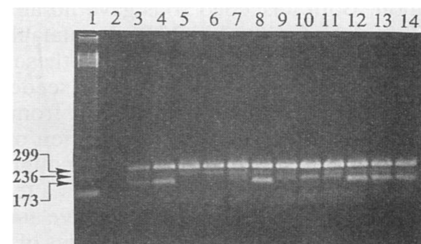

FIG. 4. Multiplex PCR results for the specificity of *phoP*, *Hin*, and *H-1i* primers on presumptive *Salmonella* strains. Lanes: 1, 123-bp DNA ladder as a size standard; 2, negative control (no cells or DNA); 3, *Salmonella typhimurium* ATCC 14028 as a positive control; 4, B-6601034 (*Salmonella heidelberg*); 5, B-7211645-10 (*Salmonella heidelberg*); 6, M-7011529-1 (*Salmonella poona*); 7, M-6212478-1 (*Salmonella muenchen*); 8, M-7008395-4 (*Salmonella pomona*); 9, MP5-5126-4 (*Salmonella dublin*); 10, O-7210564-2 (*Salmonella california*); 11, O-711484-3 (*Salmonella lomita*); 12, SDPHS 103-1 (*Salmonella typhimurium*); 13, SDPHS 107-1 (*Salmonella typhimurium*); 14, SDPHS 114-2 (*Salmonella typhimurium*).

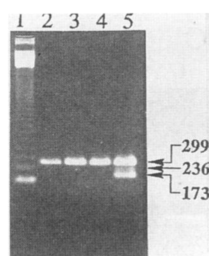

FIG. 5. Multiplex PCR with *phoP*, *Hin*, and *H-1i* primers on presumptive *E. coli* environmental isolates. Lanes: 1, 123-bp DNA ladder; 2, MP5-9959-1 (*E. coli*); 3, MP5-6120-2 (*E. coli*); 4, MP4-354-1 (*E. coli*); 5, positive control (*Salmonella typhimurium* ATCC 14028).

for all environmental isolates (amplification of *Salmonella typhimurium* SDPHS 117-2 was not shown on Fig. 4). Most multiplex PCR results of environmental isolates are in agreement with the phase characteristics, which are based on the antigenic flagellar state of each isolate (9), as listed in Table 3. Two *Salmonella heidelberg* environmental isolates were tested with their antigenic formula, indicating that they were phase 1-phase 2 inversion species. But, whereas one isolate, B-6601034 (Fig. 4, lane 4), showed all three amplification bands, the other isolate, B-7211645-10 (Fig. 4, lane 5), showed only the *phoP/phoQ* and *hin* bands. Again, the presence of two bands does identify the isolate as a *Salmonella* spp. Also, no amplification of three non-*Salmonella* isolates (*E. coli* MP5-9959-1, *E. coli* MP5-6120-2, and *E. coli* MP4-354-1) resulted with the *Hin* and *H-1i* primers (Fig. 5). Thus, the multiplex PCR analysis was effective in identifying *Salmonella* spp. of environmental origin.

Efficacy of primers for detection of *Salmonella* species in soil. Brazito sandy loam and Pima silty clay loam were chosen for this study because they are soil types commonly found in the Sonoran Desert of the southwestern United States. The physical and chemical properties of these two soils are quite different, especially in the silt and clay content; Pima silty clay loam has 50% silt and clay compared with the 20% of the Brazito sandy loam. The extraction methodology used in this study allowed the recovery of microbial DNA from soil samples, by lysis of the cells directly in the presence of the soil and then by cesium chloride-ethidium bromide equilibrium density centrifugation purification. Both unseeded soils gave no amplification after 30 or 50 cycles of multiplex PCR, presumably because the *Salmonella* species are not indigenous to these soils. The average amount of recovered DNA from seeded Brazito sandy loam was 1.6 times greater than that from the Pima silty clay loam most likely because of sorption of DNA on soil colloids in the clay loam (24). Equal amounts of DNA extracted from each seeded soil were used as a template for multiplex PCR. Both seeded soils gave positive amplification products for all three primer sets after 50 cycles of PCR (data not shown). However, amplification bands were less pronounced from the DNA recovered from Pima silty clay loam, perhaps because of increased sorption of DNA on clay colloids.

Efficacy of primers for detection of *Salmonella* species in environmental water samples. None of the well water samples produced positive PCR amplification bands (Table 4). However, all seeded well water samples did give positive amplification, indicating that there were no PCR inhibitory substances in the groundwater samples. Several presumptive *Salmonella* isolates obtained from the surface pond water sample were subjected to PCR analysis. One isolate did produce amplification products from all three primer sets, demonstrating that a *Salmonella* species was originally present in the environmental water sample.

TABLE 4. Multiplex PCR analyses of environmental water samples[a]

Water sample	PCR results[b]		
	phoP	*Hin*	*H-1i*
Well water			
1	–	–	–
2	–	–	–
3	–	–	–
4	–	–	–
Surface pond water	+	+	+

[a] All seeded positive controls resulted in positive amplification bands.
[b] PCR result after 30 cycles.

Conclusions. Overall, these data indicate that the multiplex PCR analysis utilizing *phoP*, *Hin*, and *H-1i* primer sets can distinguish *Salmonella* species from other enteric bacteria, including *E. coli*. This multiplex analysis is sensitive and rapid and allows detection of *Salmonella* species in environmental samples.

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