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Detection of *Salmonella* Serovars from Clinical Samples by Enrichment Broth Cultivation-PCR Procedure†

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To overcome problems associated with application of PCR to clinical samples, we have combined a short cultivation procedure with a *Salmonella*-specific PCR-hybridization assay to specifically identify *Salmonella* serovars from clinical samples of various animal species. The technique was investigated by using fecal samples seeded with known numbers of *Salmonella* organisms and cultivated for different lengths of time in assorted selective and nonselective enrichment media. The ability of PCR to amplify a *Salmonella*-specific DNA product (457-bp sequence covering the *Salmonella invE* and *invA* genes) was examined in Southern hybridizations with an internal oligonucleotide probe. Forty-seven *Salmonella* isolates representing 32 serovars were evaluated, and all *Salmonella* isolates resulted in a 457-bp product that hybridized with the oligonucleotide probe, whereas no hybridizations were evident with 53 non-*Salmonella* organisms. The assay detected as few as 9 CFU of *Salmonella* organisms in pure culture and as little as 300 fg of purified chromosomal DNA. Rappaport-Vassiliadis and tetrathionate broths were inhibitory to PCR, whereas brain heart infusion and selenite-cystine broths were not. The PCR-hybridization assay coupled with a brain heart infusion enrichment culture incubated for 2 h detected as few as 80 CFU of *Salmonella* organisms in seeded feces. We have successfully identified *Salmonella* serovars in clinical samples from swine, horses, and cattle more rapidly than with conventional culture techniques. The sensitivity and specificity of this assay were both 100% compared with culture results. These results indicate that a combined cultivation–PCR-hybridization assay could be applicable and advantageous in the rapid identification of *Salmonella* serovars in routine diagnostic situations.

Salmonellosis is one of the most common infectious diseases in the world in both humans and animals. *Salmonella* infections can be manifested in three forms: gastroenteritis, involving an abrupt onset of nausea, fever, vomiting, and diarrhea; enteric fever (typhoid fever), usually caused by *Salmonella typhi*; and septicemia, characterized by fever, chills, anorexia, anemia, and focal lesions on visceral organs (7). Chronic asymptomatic carriers often arise from a population infected with *Salmonella* serovars, and the difficulty in detecting carriers by culture techniques makes them a potential source of environmental contamination (36).

In recent years, diagnostic laboratories have been concerned with reducing the time required for diagnosis of *Salmonella* infections. The current standard laboratory procedure to culture and identify *Salmonella* serovars takes approximately 4 to 7 days (7). In addition, *Salmonella* serovars are not detectable in certain clinical samples that contain small numbers of organisms (13). Therefore, a more rapid and sensitive method for identification of *Salmonella* serovars from clinical specimens is needed. Several techniques for improving the detection of *Salmonella* serovars, such as selective culture methods (2, 31), DNA hybridization assays (9, 11, 17), and use of immunoglobulins (1, 22, 26), have been developed. However, problems with the sensitivity and specificity of these methods have hampered their routine application (15).

Using PCR-based probes and recombinant DNA hybridizations to detect pathogenic organisms has many advantages over classical culture techniques (15). Amplification of DNA sequences unique to an organism by PCR improves the speed and sensitivity at which organisms can be detected (5, 25). PCR has been used to identify several bacterial species including *Salmonella* serovars from food and clinical samples (3, 6, 8, 12, 19, 23–25, 27, 29, 33, 34, 37). The major obstacle to using PCR for the detection and identification of pathogenic organisms from clinical samples is the presence of substances that are inhibitory to PCR (28, 35). This obstacle can be minimized by direct chemical extraction of nucleic acids (12, 18) or immunomagnetic separation of bacteria with antibodies (33, 34), but these procedures are laborious and expensive.

We have devised a procedure that combines a short cultivation period with PCR and hybridization by using a recombinant DNA probe for the identification of *Salmonella* serovars in culture and clinical specimens. The purpose of this study was to compare the sensitivity and specificity of this procedure with traditional isolation and characterization methods currently used in diagnostic laboratories.

MATERIALS AND METHODS

Bacteria. Bacterial strains (Tables 1 and 2) were obtained from the American Type Culture Collection (Rockville, Md.) and the Kansas State University Department of Veterinary Diagnostic Investigation. All bacterial strains were identified biochemically and serologically (7).

Preparation of DNA. To assess the minimal amount of DNA detectable by PCR and hybridization, chromosomal DNA was extracted from *Salmonella typhimurium* ATCC 29946 as previously described (10), except that 100 μg of lysozyme (Sigma Chemical Co., St. Louis, Mo.) per ml was substituted for

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TABLE 1. *Salmonella* serovars examined in this study

Salmonella serovar[a]	Serogroup	Source or strain	No. of strains
S. agona	B	Laboratory strain[b]	1
S. brandenberg	B	Laboratory strain	1
S. bredeney	B	Laboratory strain	2
S. derby	B	Laboratory strain	2
S. kiambu	B	Laboratory strain	1
S. heidelberg	B	Laboratory strain	1
S. reading	B	Laboratory strain	1
S. schwarzengrund	B	Laboratory strain	1
S. typhimurium	B	ATCC 29946[c]	1
S. typhimurium	B	Laboratory strain	5
S. typhimurium (*copenhagen*)	B	Laboratory strain	3
S. braenderup	C1	Laboratory strain	1
S. choleraesuis	C1	Laboratory strain	2
S. infantis	C1	Laboratory strain	1
S. mbandaka	C1	Laboratory strain	1
S. norwich	C1	Laboratory strain	1
S. hadar	C2	Laboratory strain	1
S. muenchen	C2	Laboratory strain	1
S. newport	C2	Laboratory strain	2
S. albany	C3	Laboratory strain	1
S. bardo	C3	Laboratory strain	1
S. anatum	E1	Laboratory strain	2
S. give	E1	Laboratory strain	1
S. muenster	E1	Laboratory strain	2
S. newbrunswick	E2	Laboratory strain	1
S. thomasville	E3	Laboratory strain	1
S. senftenberg	E4	Laboratory strain	1
S. dublin	D1	Laboratory strain	1
S. enteritidis	D1	Laboratory strain	3
S. cubana	G2	Laboratory strain	1
S. havana	G2	Laboratory strain	1
S. worthington	G2	Laboratory strain	1
S. arizona		Laboratory strain	1

[a] Confirmed by National Veterinary Services Laboratory, Ames, Iowa.
[b] M. M. Chengappa, Department of Veterinary Diagnostic Investigation, Kansas State University, Manhattan.
[c] American Type Culture Collection.

TABLE 2. Non-*Salmonella* strains examined in this study

Microorganism	Source or strain	No. of strains
Actinomyces pyogenes	Laboratory strain[a]	1
Alcaligenes faecalis	Laboratory strain	1
Bacteroides vulgatus	Laboratory strain	1
Citrobacter freundii	Laboratory strain	1
Clostridium bifermentans	Laboratory strain	1
Clostridium perfringens type A	Laboratory strain	1
Clostridium ramosum	Laboratory strain	1
Edwardsiella tarda	ATCC 15947[b]	1
Enterobacter aerogenes	Laboratory strain	1
Enterococcus faecalis	ATCC 29212	1
Escherichia coli	ATCC 25922	1
Escherichia coli	Laboratory strain	6
Escherichia coli	ATCC 14763	1
Escherichia coli	ATCC 43888	1
Escherichia coli	ATCC 43889	1
Escherichia coli	ATCC 43890	1
Escherichia coli	ATCC 43894	1
Escherichia coli	ATCC 43895	1
Fusobacterium necrophorum	ATCC 55329	1
Fusobacterium symbiosum	Laboratory strain	1
Klebsiella pneumoniae	Laboratory strain	1
Leptospira canicola	Laboratory strain	1
Leptospira icterohaemorrhagiae	Laboratory strain	1
Leptospira hardjo	Laboratory strain	1
Leptospira pomona	Laboratory strain	1
Leptospira grippotyphosa	Laboratory strain	1
Morganella morganii	Laboratory strain	1
Pasteurella haemolytica	Laboratory strain	1
Pasteurella multocida	ATCC 1062	1
Peptostreptococcus micros	Laboratory strain	1
Prevotella melaninogenicus	ATCC 25845	1
Proteus mirabilis	Laboratory strain	1
Proteus vulgaris	Laboratory strain	1
Providencia rettgeri	Laboratory strain	1
Pseudomonas aeruginosa	ATCC 27653	1
Serpulina hyodysenteriae	Laboratory strain	4
Serratia marcescens	Laboratory strain	1
Shigella dysenteriae	ATCC 11456A	1
Shigella flexneri	Laboratory strain	1
Staphylococcus aureus	ATCC 29213	1
Streptococcus equisimilis	Laboratory strain	1
Streptococcus suis type 1/2	Laboratory strain	1
Yersinia enterocolitica	Laboratory strain	1
Yersinia pseudotuberculosis	ATCC 29910	1
Candida albicans	Laboratory strain	1

[a] M. M. Chengappa, Department of Veterinary Diagnostic Investigation, Kansas State University.
[b] American Type Culture Collection.

lysostaphin. PCR was conducted on 5-μl amounts of serial 10-fold dilutions of a stock concentration of DNA. For specificity and sensitivity determination, DNA was extracted from all strains of bacteria by a rapid boiled-lysate technique (32). Briefly, bacteria were grown on sheep blood agar plates for 16 h at 37°C. Two colonies were placed in 50 μl of sterile water, boiled for 5 min at 100°C, and centrifuged at 16,000 × *g* for 20 s. A 5-μl aliquot of the supernatant was used in DNA amplification.

Oligonucleotides. Oligonucleotide primers and the internal oligonucleotide probe were selected from the published sequences of the *invA* and *invE* genes of *S. typhimurium* (14, 16) and synthesized in a DNA synthesizer (model 391; Applied Biosystems, Foster City, Calif.). The sequences were 5′-TGC CTACAAGCATGAAATGG-3′ and 5′-AAACTGGACCAC GGTGACAA-3′, corresponding to nucleotides 1219 to 1238 of the *invE* gene and nucleotides 278 to 259 of the *invA* gene, respectively. An additional 20-mer oligonucleotide (5′-CTG-GTTGATTTCCTGATCGC-3′) corresponding to nucleotides 106 to 125 of the *invA* gene was synthesized and used as an internal probe for detection of the amplified products by hybridization.

DNA amplification. The PCR amplification mixture (100 μl) consisted of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (wt/vol) gelatin, 100 μM each of the four deoxynucleoside triphosphates, 1.0 μM (each) primer, and 2.5 U of *Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.). Amplification was conducted on a DNA thermocycler (model 9600; Perkin-Elmer Cetus). PCR was performed for 35 cycles, (1 cycle consisting of 15 s at 94°C, 15 s at 52°C, and 15 s at 72°C), followed by a 2.5-min extension period at 72°C. Amplified products were visualized electrophoretically in ethidium bromide-stained, 1.5% agarose gels. A 123-bp DNA ladder (Gibco-BRL, Gaithersburg, Md.) was used as a molecular size marker. Southern hybridization of PCR products transferred to nylon membranes (MSI, Westboro, Mass.) was conducted as previously described (30). Briefly, membrane filters were prehybridized for 1 h at 48°C in a solution of 10% dextran sulfate, 1 M NaCl, 1% sodium dodecyl sulfate (SDS), and 100 μg of denatured, sonicated salmon sperm DNA (Sigma) per ml. Filters were hybridized for 18 h at 48°C in a solution of 10% dextran sulfate, 1 M NaCl, 1% SDS, and

approximately 100 μg of probe DNA. The internal oligonucleotide probe was labeled with [γ-^{32}P]ATP (Dupont, Boston, Mass.) by using T4 polynucleotide kinase (Promega, Madison, Wis.). After hybridization, membranes were washed twice for 5 min each with 2× SSC–0.1% SDS (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 25°C and once for 30 min with 0.5× SSC–0.1% SDS at 48°C. Membranes were exposed to X-ray film at −70°C.

Sequencing of PCR product. The amplified product from *S. typhimurium* ATCC 29946 was ligated into pT7Blue vector and transformed into NovaBlue cells as recommended by the manufacturer (Novagen, Madison, Wis.). Competent colonies were screened for the amplified fragment. Sequencing of the amplified product was conducted by using T7 and U19 primers in an automated DNA sequencer (model 373A; Applied Biosystems).

Preparation of experimental samples. *S. typhimurium* (ATCC 29946) was grown for 16 h at 37°C in 100 ml brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.). Bacteria were centrifuged at 3,000 × *g* for 10 min, washed, and resuspended in 100 ml of phosphate-buffered saline (PBS). Serial 10-fold dilutions of culture suspension were made, and plate counts were conducted in duplicate to determine the number of viable organisms. One milliliter of 10^9 bacteria was added to a 1-g aliquot of normal dog feces. This sample was centrifuged at 1,000 × *g* for 10 min to remove visible solid material. A 5-μl aliquot of supernatant was removed for direct analysis by PCR-hybridization as described below.

Cultivation of *Salmonella* serovars in enrichment media. To assess a suitable enrichment broth for cultivation of *Salmonella* serovars, four commercially available broths were evaluated along with PCR. One-hundred microliters of supernatant from prepared fecal samples was inoculated into 10 ml of BHI broth (Difco) for nonselective enrichment and into Rappaport-Vassiliadis (RV) broth (Oxoid), selenite-cystine (SC) broth (Difco), and tetrathionate (TET) broth (Difco) for selective enrichment. Broths were incubated at 37°C for 24 h. Twenty-five-microliter aliquots of each culture were plated onto Hektoen Enteric agar plates (Difco) at 0, 2, 4, 8, and 24 h and incubated at 37°C for 18 h. Typical *Salmonella* colonies were counted to determine the approximate number of CFU per milliliter. An additional 5-μl aliquot was removed for PCR. A 1-g aliquot of feces, negative for *Salmonella* serovars by culture and by PCR, was emulsified in 1 ml of PBS and used as a negative control.

PCR on enriched samples. To evaluate the efficiency of PCR and hybridization on prepared fecal samples, 15 μl of Genereleaser (Bioventures, Murfreesboro, Tenn.) was added to a PCR tube containing the 5-μl aliquot that was removed for PCR. A nine-temperature, one-cycle DNA extraction thermocycle program was conducted as recommended by the manufacturer. Briefly, the temperature parameters consisted of 65°C for 15 s, 8°C for 15 s, 65°C for 30 s, 97°C for 45 s, 8°C for 15 s, 65°C for 45 s, 97°C for 15 s, 65°C for 15 s, and 70°C for 1 min. Thermocycling was conducted on a DNA thermocycler (model 9600; Perking-Elmer Cetus). Amplification was conducted as described above by adding 80 μl of a master mixture of amplification reagents to the 20-μl DNA preparation.

Evaluation of clinical samples. Sixteen veterinary clinical samples (Table 3) from animals suspected as possibly having salmonellosis and submitted to the Kansas State University Department of Veterinary Diagnostic Investigation were screened by cultivation–PCR-hybridization. Specimens were plated onto sheep blood, MacConkey, and Hektoen Enteric agar plates and grown for 18 h at 37°C. Suspected *Salmonella* colonies were isolated and characterized biochemically and serologically (7). In addition, the tissue samples were processed by directly swabbing the internal portions of each tissue and placing the swab in 10 ml of enrichment broths (BHI and SC) and incubated for 24 h at 37°C. Five-microliter aliquots were removed at 0, 2, 4, 8, and 24 h and subjected to PCR. An additional 25 μl was plated onto Hektoen Enteric agar and incubated for 18 h at 37°C, and suspected *Salmonella* colonies were characterized biochemically and serologically (7).

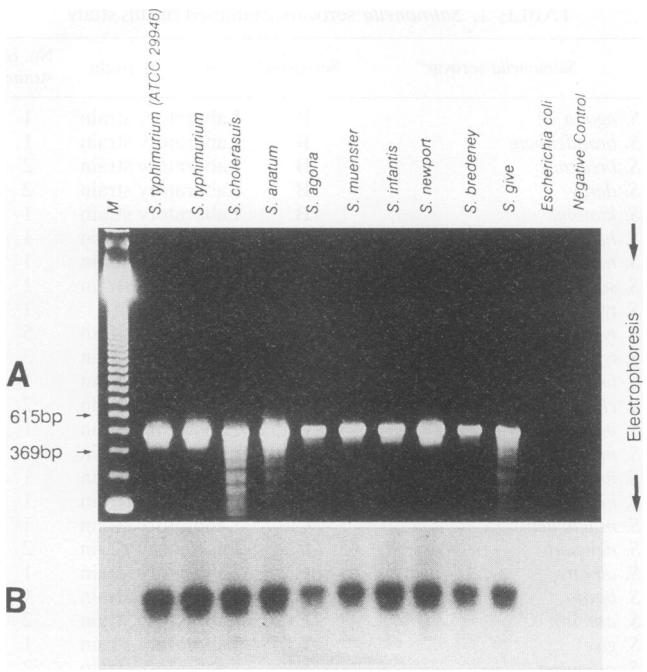

FIG. 1. Specificity of PCR-hybridization assay for amplification of the *invE* and *invA* genes of several different *Salmonella* serovars. (A) Amplified products in 1.5% agarose and visualized by UV transillumination after ethidium bromide staining. Serovars are indicated over the gel. Lane M contains the 123-bp DNA ladder (Gibco-BRL). The Negative Control lane contains no template DNA in the reaction mixture. (B) Southern blot and hybridization with [γ-^{32}P]ATP-labeled internal oligonucleotide probe.

RESULTS

Specificity of PCR. Amplification of DNA from all 47 *Salmonella* strains tested by PCR with primers selected from sequences of the *invE* and *invA* genes resulted in a fragment of the predicted size (Fig. 1A). Southern blots of all amplified products were hybridized with the internal oligonucleotide probe (Fig. 1B). None of the 53 non-*Salmonella* strains assayed resulted in amplified products of 457 bp (Fig. 2). Amplification of *Yersinia pseudotuberculosis* and *Edwardsiella tarda* DNA did produce products that were slightly larger; however, the internal oligonucleotide probe did not hybridize to them. Subcloning of the amplified product from *S. typhimurium* ATCC 29946 into pT7Blue and sequencing of the product indicated that it had the identical sequence as previously described (14, 16).

Sensitivity of PCR-hybridization assay with pure cultures. To determine the minimal detectable concentration of *Salmonella* DNA and the minimal detectable number of *Salmonella* organisms, PCR was conducted on serial dilutions of both purified chromosomal DNA and boiled lysates of known counts (CFU per milliliter) of bacteria. The 457-bp fragment

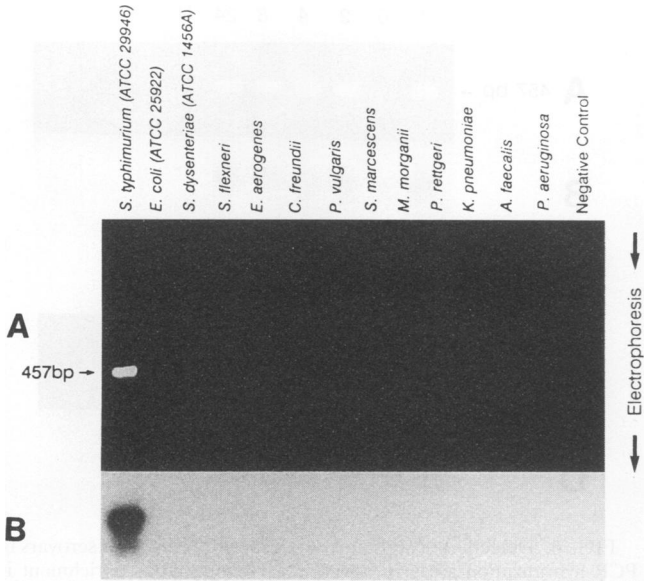

FIG. 2. Specificity of PCR-hybridization assay for cross-reactivity on several different non-*Salmonella* strains of bacteria. (A) Amplified products in 1.5% agarose and visualized by UV transillumination after ethidium bromide staining. The Negative Control lane had no template DNA in the reaction mixture. (B) Southern blot and hybridization with [γ-^{32}P]ATP-labeled internal oligonucleotide probe.

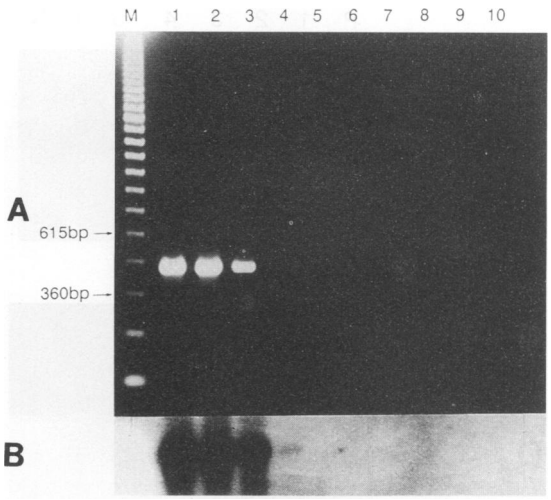

FIG. 4. Sensitivity of PCR-hybridization assay for detection of *invE* and *invA* genes from boiled lysates of *S. typhimurium* and determination of minimal number of CFU. (A) Amplified products in 1.5% agarose and visualized by UV transillumination after ethidium bromide staining. Lane M contains the 123-bp DNA ladder (Gibco-BRL). Lane 1, 9×10^3 CFU; lane 2, 9×10^2 CFU; lane 3, 9×10^1 CFU; lane 4, 9×10^0 CFU; lane 5, 9×10^{-1} CFU; lane 6, 9×10^{-2} CFU; lane 7, 9×10^{-3} CFU; lane 8, 9×10^{-4} CFU; lane 9, 9×10^{-5} CFU; lane 10, no template DNA. (B) Southern blot and hybridization with [γ-^{32}P]ATP-labeled internal oligonucleotide probe.

could be visualized in ethidium bromide-stained gels in reaction mixtures that contained as little as 30 pg of total chromosomal DNA (Fig. 3A). Detection levels were increased 100-fold in Southern blot hybridizations (Fig. 3B). Analysis of PCR products obtained from serial dilutions of bacteria showed that as few as 90 CFU of *S. typhimurium* could be detected in ethidium bromide-stained gels (Fig. 4A) and a 10-fold-greater sensitivity was possible with Southern blot hybridization (Fig. 4B).

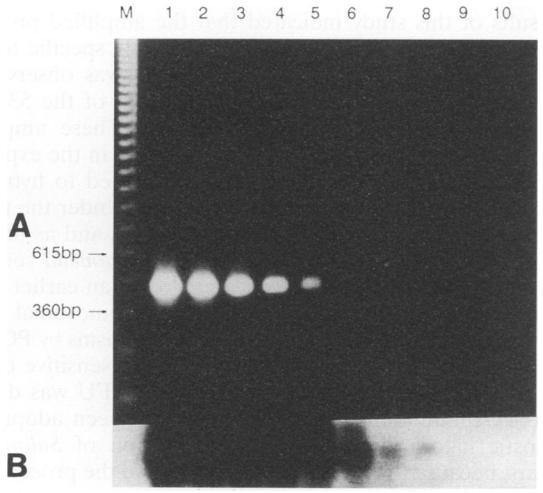

FIG. 3. Sensitivity of PCR-hybridization assay for detection of purified chromosomal *Salmonella* DNA extracted from *S. typhimurium* ATCC 29946. (A) Amplified products in 1.5% agarose and visualized by UV transillumination after ethidium bromide staining. Lane M contains the 123-bp DNA ladder (Gibco-BRL). Lanes 1 to 10 contain different amounts of template DNA as follows: 300 μg (lane 1), 30 μg (lane 2), 3 μg (lane 3), 300 pg (lane 4), 30 pg (lane 5), 3 pg (lane 6), 300 fg (lane 7), 30 fg (lane 8), 3 fg (lane 9), no template DNA (lane 10). (B) Southern blot and hybridization with [γ-^{32}P]ATP-labeled internal oligonucleotide probe.

Sensitivity of PCR-hybridization assay with prepared fecal samples. PCR products were not identified in direct analysis of prepared fecal samples containing *Salmonella* serovars. Therefore, four commercially available enrichment broths were evaluated for culturing *Salmonella* organisms for compatibility with PCR-hybridization analysis. *Salmonella* organisms grew readily in RV, TET, SC, and BHI broths; however, PCR amplifications were consistently successful only when prepared fecal samples were inoculated into BHI or SC broth. Serial 10-fold dilutions of cultures grown in different enrichment broths demonstrated that as few as 80 and 100 CFU of *Salmonella* organisms could be detected in BHI and SC broths after 2 h of incubation, respectively (Fig. 5).

Cultivation–PCR-hybridization on clinical samples. Of the 16 clinical samples collected from animals with clinical signs of salmonellosis (Table 3), seven samples confirmed by culture for *Salmonella* serovars were also identified positively by the PCR-hybridization assay (Fig. 6). *Salmonella* serovars were cultured directly in one of the bovine fecal samples without enrichment. This same sample was positive by the PCR-hybridization assay without incubation in enrichment broths. All samples negative by the PCR-hybridization assay were also negative by culture (Table 3).

DISCUSSION

Diagnosis of *Salmonella* infections at the present time is based primarily on cultivation and identification of the organism from veterinary clinical samples. However, cultivation by traditional methods is a laborious, time-consuming procedure that can take up to 7 days (13). Several factors can interfere with the isolation of *Salmonella* serovars from clinical speci-

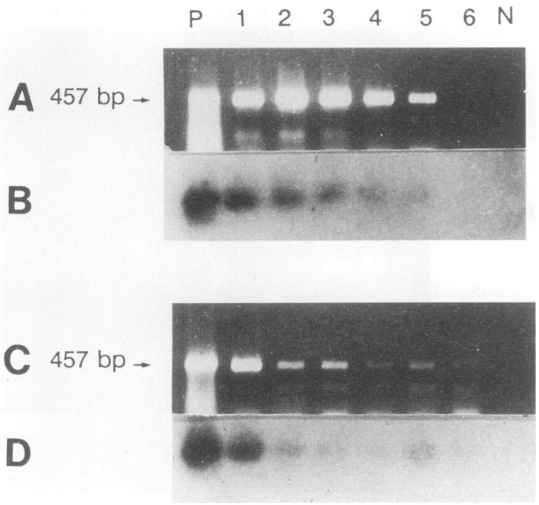

FIG. 5. Sensitivity of PCR-hybridization assay for detection of *invE* and *invA* genes of *Salmonella* serovars in prepared fecal sample after 2-h enrichment in BHI and SC broths. Amplified products from *Salmonella*-seeded feces incubated in BHI broth (A) and SC broth (C) in 1.5% agarose and visualized by UV transillumination after ethidium bromide staining. Lane P, positive control (seeded feces incubated for 24 h in enrichment broth); lane N, negative control (unseeded feces incubated in enrichment broth for 24 h). (A) Lane 1, 8×10^5 CFU; lane 2, 8×10^4 CFU; lane 3, 8×10^3 CFU; lane 4, 8×10^2 CFU; lane 5, 8×10^1 CFU; lane 6, 8×10^0 CFU. (C) Lane 1, 1×10^7 CFU; lane 2, 1×10^6 CFU; lane 3, 1×10^5 CFU; lane 4, 1×10^4 CFU; lane 5, 1×10^3 CFU; lane 6, 1×10^2 CFU. (B and D) Southern blots and hybridization with [γ-^{32}P]ATP-labeled internal oligonucleotide probe.

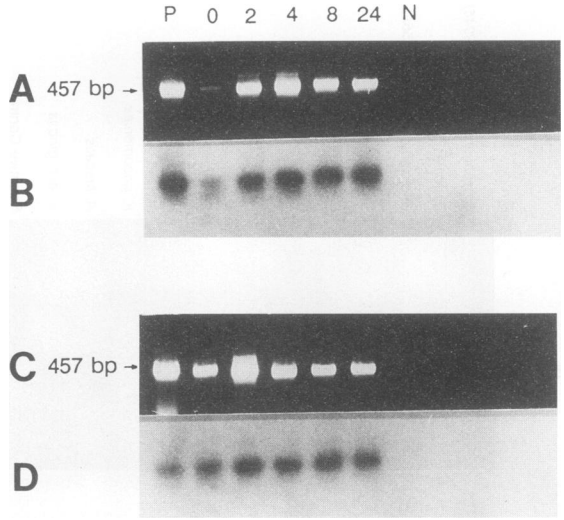

FIG. 6. Detection of *invE* and *invA* genes of *Salmonella* serovars by PCR-hybridization assay in bovine fecal sample after enrichment in BHI and SC broths. Amplified products from fecal sample incubated in BHI (A) and SC (C) broths run through 1.5% agarose and visualized after ethidium bromide staining. Lane P, positive control (seeded feces incubated for 24 h in enrichment broth); lanes 0, 2, 4, 8, and 24, samples incubated for 0, 2, 4, 8, and 24 h, respectively; lane N, negative control (*Salmonella*-free feces incubated for 24 h in enrichment broth). (B and D) Southern blot and hybridization with [γ-^{32}P]ATP-labeled internal oligonucleotide probe.

mens: the condition of the specimen can allow contaminating organisms to inhibit *Salmonella* isolation, antibiotics in infected animals can retard the growth of *Salmonella* organisms, or *Salmonella* organisms may be shed only periodically and in low numbers, particularly in carriers (36).

Use of PCR has increased the rapidity and sensitivity of diagnosing infectious diseases. Earlier studies have described PCR-based probes for detection of pathogenic bacteria, such as verotoxigenic *Escherichia coli* (23), *Yersinia enterocolitica* (37), *Listeria monocytogenes* (3, 27), *Mycobacterium tuberculosis* (19, 20, 29), and *Salmonella* serovars (8, 24, 33, 34). However, most testing procedures describe laborious DNA extraction procedures which are necessary to eliminate substances in clinical samples that can inhibit PCR (12, 18, 33–35). To minimize this problem, the present study utilized PCR technology coupled with an enrichment procedure that not only diluted PCR inhibitors but also resulted in increased numbers of *Salmonella* organisms in reaction mixtures. This combined procedure requires minimal sample manipulation, but is still applicable to most diagnostic laboratories for rapid detection of *Salmonella* serovars.

The primers selected for this study were based on the sequences of the *Salmonella invE* and *invA* genes, genes whose protein products are necessary for invasion of epithelial cells (14, 16). All *Salmonella* strains screened by PCR resulted in visualization of the predicted 457-bp amplified product in ethidium bromide-stained gels. The specificity of all *Salmonella* strains was confirmed by hybridization of a radiolabeled internal oligonucleotide probe. Rahn et al. (24) previously used the *invA* gene sequence for selection of PCR primers, but false-negative reactions were observed. Cano et al. (6) reported using IS*200* as a target for PCR amplification for specific detection of *Salmonella* DNA; however, the potential for false-negative reactions exists, because not all *Salmonella* strains possess IS*200*. In the present study, amplifying a product that overlaps the junction between the *invE* and *invA* genes eliminated the possible occurrence of false-negative reactions.

Results of this study indicated that the amplified products and internal oligonucleotide probe are highly specific to *Salmonella* serovars. Nonspecific amplification was observed in only two (*Y. pseudotuberculosis* and *E. tarda*) of the 53 non-*Salmonella* organisms that were screened. These amplified products were single bands that were larger than the expected size, and the products of both organisms failed to hybridize with the recombinant oligonucleotide probe. Under the tested parameters, as few as 9 CFU in boiled lysates and as little as 300 fg of purified chromosomal DNA of *Salmonella* serovars were detectable. This is an improvement over an earlier study in which 300 CFU and 27 pg of purified chromosomal DNA were needed for detection of *Salmonella* organisms by PCR on the *invA* gene (24). This procedure was more sensitive than a magnetic immuno-PCR assay in which 100 CFU was detectable (34). Immunomagnetic beads have not been adopted in diagnostic laboratories for routine detection of *Salmonella* serovars, because the beads are expensive and the procedure is labor-intensive (15).

The PCR-hybridization assay was as sensitive and specific as traditional culture methods for clinical samples. However, results were obtained more rapidly with the PCR-hybridization assay than with selective culture and subsequent biochemical and serological analyses. A PCR-based procedure would be most useful on clinical samples from animals with suspected *Salmonella* infection but with negative culture results. Low levels of *Salmonella* organisms are common in samples from animals suspected of having a *Salmonella* infection with no

TABLE 3. Results of culture and PCR-hybridization on clinical samples at various incubation times

Origin and specimen no.	Result of test after incubation (h)[a]										Serovar isolated
	Culture[b]					PCR					
	0	2	4	8	24	0	2	4	8	24	
Bovine feces											
93-15382	+	+	+	+	+	+	+	+	+	+	*S. uganda*
93-16530	−	−	−	−	−	−	−	−	−	−	
Equine feces											
93-16417A	−	−	−	−	−	−	−	−	−	−	
93-16417B	−	−	−	−	−	−	−	−	−	−	
93-12873	−	−	−	+	+	−	+	+	+	+	*S. havana*
93-16448A	−	−	−	−	−	−	−	−	−	−	
93-16448B	−	−	−	−	−	−	−	−	−	−	
Bovine intestine 93-15395	−	−	−	−	−	−	−	−	−	−	
Porcine intestine											
93-16436	−	−	−	−	−	−	−	−	−	−	
93-16421	−	−	−	−	−	−	−	−	−	−	
93-16422	−	−	−	−	+	−	−	+	+	+	*S. choleraesuis*
93-15423	−	−	−	+	+	−	−	+	+	+	*S. choleraesuis*
93-15944	−	−	−	−	−	−	−	−	−	−	
Porcine lung											
93-15944A	−	−	−	−	+	−	−	+	+	+	*S. choleraesuis*
93-15944B	−	−	−	−	+	−	−	+	+	+	*S. choleraesuis*
Porcine lymph node 93-15423	−	−	−	−	+	−	−	−	−	+	*S. choleraesuis*

[a] Incubation in enrichment broth at 37°C. Symbols: +, positive for *Salmonella* serovars; −, negative for *Salmonella* serovars.
[b] Identical results were obtained with BHI and SC enrichment procedures.

clinical signs. In such cases, PCR could be applied to samples that were negative by culture to identify the etiological agent. Ramamurthy et al. (25) was able to detect 13 samples that were negative by culture among 94 samples suspected of cholera infection using PCR. More clinical samples need to be investigated to evaluate the usefulness of this technique for identifying carriers of *Salmonella* serovars.

For laboratory diagnosis of *Salmonella* serovars, culturing on selective media after selective enrichment is a routine practice. Several selective enrichment broths are used to increase the detection level of *Salmonella* organisms (2, 7, 31). In the present study, four enrichment broths were tested with the cultivation–PCR-hybridization procedure. *Salmonella* organisms grew readily in all four enrichment media; however, only samples enriched in BHI and SC broths had positive reactions by PCR. Positive reactions were not observed from samples enriched in RV or TET broth, even after 24 h of incubation. Amplification of DNA by PCR can be inhibited by a number of substances (hemoglobin, bile salts, $MgCl_2$, etc.) present in the samples as well as components of the medium used for selective isolation of pathogenic bacteria (21, 28, 35). We used a commercial product (Genereleaser) to help minimize the effect of PCR-inhibitory substances that might be carried over from enrichment broths. This product did not alleviate the problem, because negative results were still observed in RV and TET broths. Bile salts and $MgCl_2$ present in TET and RV broths, respectively, might have been the specific cause of inhibition of PCR with these media. In the present study, use of an enrichment procedure prior to PCR analysis sufficiently diluted PCR-inhibitory substances, while presumably increasing the sensitivity caused by multiplication of organisms. In situations when a medium like TET broth is used, it would be necessary to transfer an aliquot of this medium to another such as BHI broth for a short period of incubation prior to PCR to dilute PCR-inhibitory substances. This transfer would ensure dilution of PCR-inhibitory substances to a point where they no longer interfere with the assay.

Some studies have described PCR procedures for detecting pathogenic organisms directly from clinical samples without prior cultivation of the organism in order to alleviate interference from PCR inhibitors. Extraction of total DNA by a proteinase K and phenol-chloroform treatment (12) or by use of silica (4) or glass beads (5) have been attempted. Immunomagnetic beads attached to specific antibodies to assist in capturing and concentrating organisms prior to DNA extraction have also been used (33, 34). These procedures greatly increase the cost and time required to make diagnostic decisions, and certain chemicals used for extraction of nucleic acids inhibit PCR (28). We believe that the present short enrichment procedure prior to PCR is advantageous, since these media are relatively inexpensive and easy to prepare. Further, these enrichments may still be required in most diagnostic laboratories for antibiotic sensitivity testing and serovar determination of *Salmonella* isolates.

A critical factor in combining cultivation in an enrichment broth with PCR is to select a medium that is not inhibitory to PCR. In this study, 80 and 100 CFU of *Salmonella* organisms in prepared samples were detectable in BHI and SC broths, respectively, in as short as 2 h of incubation. These results indicate that a high level of sensitivity can be obtained with an enrichment process which takes about the same amount of time needed to perform a direct extraction of nucleic acids. In an actual clinical diagnostic setting, however, a longer incubation time may be required (Table 3). The sensitivity of detection of *Salmonella* organisms in prepared feces was approximately 10-fold lower than that in pure cultures. Others have reported similar results of sensitivities with clinical samples (20, 29). Use of enrichment media to cultivate *Salmonella* serovars prior to PCR may not detect nonviable *Salmonella* organisms. Additionally, use of antimicrobial agents in animals

suspected of having salmonellosis may retard the growth of *Salmonella* organisms so that they are not detected (13). Further studies are required to examine procedures that can detect *Salmonella* serovars in these situations.

The best-case scenario for isolation and identification of *Salmonella* serovars by traditional culture methods is approximately 48 h, provided pure isolated colonies were obtained on primary cultivation. This procedure, as described in the present study, would also take approximately 48 h from processing of tissues to enrichment, amplification, and detection of amplified products. However, the time required for this procedure can be shortened considerably by the use of dot blotting of amplified products, rather than Southern transfer of products after electrophoresis in agarose gels. In addition, a nonradioisotopic detection method can be used to decrease the time required for exposure to X-ray film.

Rapid tests for identification of *Salmonella* serovars will complement, not replace, bacterial culture techniques. The need to culture the organism for serotyping, epidemiological purposes, and the development of antibiograms will remain. When combined with a cultivation procedure, a rapid test can significantly increase the number of positive results, while reducing the number of false-negative results. In routine diagnostic use, a large number of samples can be processed in a relatively short amount of time with this procedure. In this study, radiolabeled probes were used to detect amplified products. However, assays using dot blots or liquid hybridization with chemilumnescent or biotinylated probes can be adopted and used routinely in diagnostic laboratories. This would eliminate the need for safety precautions required for radioisotopes and at the same time, decrease the amount of time required for the assay.

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