JOURNAL OF CLINICAL MICROBIOLOGY, May 1995, p. 1292–1295 Vol. 33, No. 5
0095-1137/95/$04.00+0

Detection of *Salmonella typhimurium* from Rectal Swabs of Experimentally Infected Beagles by Short Cultivation and PCR-Hybridization†

GREGORY G. STONE,[1,2] RICHARD D. OBERST,[2] MICHAEL P. HAYS,[2] SCOTT MCVEY,[2] JOHN C. GALLAND,[3] ROY CURTISS III,[4] SANDRA M. KELLY,[4] AND M. M. CHENGAPPA[2]*

Department of Veterinary Diagnostic Investigation,[1] Department of Pathology & Microbiology,[2] and Department of Clinical Sciences,[3] College of Veterinary Medicine, Kansas State University, Manhattan, Kansas 66506, and Department of Biology, Washington University, St. Louis, Missouri 63130[4]

Received 26 September 1994/Returned for modification 5 December 1994/Accepted 25 January 1995

A rapid and sensitive cultivation and PCR-hybridization procedure for the detection and identification of *Salmonella typhimurium* was evaluated over a 42-day period with eight experimentally infected beagles. Rectal swabs were taken at several times postinfection, inoculated into selenite-cystine broth, and plated onto Hektoen Enteric agar immediately and after incubation for 4 and 24 h. PCRs and hybridizations were also conducted with each sample, and the results were compared with those of standard culture techniques to evaluate the efficiency of the PCR-hybridization procedure. The PCR-hybridization procedure was more sensitive than standard culture techniques at each enrichment incubation ($P < 0.05$). In addition, the PCR-hybridization procedure was significantly better than culture up through 3 days postinfection ($P < 0.05$). A nonspecific amplified product, relatively close in size to the 457-bp specifically amplified product, did not hybridize to an internal oligonucleotide probe or to a random-primed labeled probe. Subsequent sequence information revealed that the product had very little similarity to the 457-bp product but had significant similarity to an *Escherichia coli* aldehyde dehydrogenase gene. This study indicated that a cultivation and PCR-hybridization procedure is significantly better than culture for the identification of *S. typhimurium*. Additionally, the results confirm the importance of determining specificities of PCR products beyond the gel electrophoresis level by hybridization with a specific probe.

The isolation and identification of salmonellae from clinical samples by traditional cultural techniques are time-consuming and laborious procedures. Efforts have been made in diagnostic laboratories to reduce the time required for the identification of *Salmonella* serovars from clinical samples. Rather than identification by biochemical and serological methods, attempts have been aimed at detecting *Salmonella* serovars directly in clinical samples from suspected salmonellosis cases by techniques such as DNA hybridization assays (5, 12) and immunoassays (2, 18). Other studies have used the PCR for the detection of *Salmonella* serovars directly from clinical samples (23, 24); however, these procedures involve time-consuming and expensive DNA extraction methods.

We have recently developed a PCR-based procedure coupled with incubation in an enrichment broth for the detection of *Salmonella* serovars in clinical samples (22). Including an enrichment step in a PCR protocol has several advantages over direct DNA extraction methods. Incubation in an enrichment broth is inexpensive, requires little manipulation, and dilutes substances in the sample that can inhibit PCR. Additionally, a short incubation in an enrichment broth increases the number of viable organisms in the sample, thus increasing the sensitivity of the assay. A set of oligonucleotide primers was constructed from the previously reported nucleotide sequences of the *invE* and *invA* genes of *Salmonella typhimurium* for amplification of a 457-bp fragment covering both genes. These genes, which code for surface proteins that are involved in attachment to and invasion of epithelial cells, are unique and conserved in a number of *Salmonella* serotypes (10, 11). In our previous study, the cultivation and PCR-hybridization procedure successfully detected *Salmonella* serovars more rapidly than conventional culture techniques (22). The present study compares the efficiency of the cultivation and PCR-hybridization procedure with that of standard cultural techniques for the detection of *S. typhimurium* in a group of experimentally infected beagles over a 42-day period.

MATERIALS AND METHODS

Construction of *S. typhimurium* strains. The wild-type, virulent *S. typhimurium* strains χ3761 (6) and SR-11 (20), designated χ3181 after animal passage (13), were genetically modified by using classic genetic methods similar to those previously described by Galán and Curtiss (9) and Lockman and Curtiss (16). χ4127 contains a *phoP13* deletion mutation and is derived from the wild-type strain χ3761. It was constructed by standard transductional methods by using an insertion of Tn*10* (encoding tetracycline resistance) in the gene *phoP* (encoding a transcriptional regulator; *phoP* is part of a two-component regulatory system controlling virulence). Tetracycline-resistant transductants were then plated on fusaric acid medium (17) to select for tetracycline-sensitive derivatives caused by an imprecise excision of the transposon. Several fusaric acid-resistant tetracycline-sensitive derivatives were checked for absence of nonspecific acid phosphatase activity (15), one (χ4127) was selected, and complete synthesis of lipopolysaccharide was confirmed. χ3683 contains an *aroA554* deletion mutation and is derived from χ3181. It was constructed by standard transductional methods by using an insertion of Tn*10* that was closely linked to Δ*aroA* (deficiency in aromatic amino acids biosynthesis) (16). χ3683 also contains a *gyrA* mutation which confers nalidixic acid resistance. The virulence of the *S. typhimurium* mutants χ4127 and χ3683 was assessed by inoculating 6- to 8-week-old female BALB/c mice intraperitoneally or orally with different doses of the mutants. Both χ4127 and χ3683 displayed complete avirulence at greater than 10^4 times the 50% lethal dose of the respective wild-type parent (16).

* Corresponding author. Mailing address: Department of Pathology & Microbiology, College of Veterinary Medicine, Kansas State University, Manhattan, KS 66506. Phone: (913) 532-4605. Fax: (913) 532-4309. Electronic mail address: Chengapa@vet.ksu.edu.

† Kansas Agricultural Experiment Station contribution no. 95-113-J.

Culture conditions. *S. typhimurium* χ4127 and χ3683 were grown in Luria-Bertani broth containing 0.35 M NaCl for 18 h at 37°C. Bacteria were pelleted and resuspended to a concentration of 5×10^8 CFU/ml in phosphate-buffered saline (pH 7.4) containing 0.1% (wt/vol) gelatin.

Animals. Twelve 12- to 16-week-old female beagles were obtained from Ridglan Farms, Inc. (Mount Horeb, Wis.). The beagles were separated into two groups with six dogs per group. Four dogs in one group were challenged orally with 1 ml of one strain of bacteria, four dogs in the second group were challenged orally with the other strain of bacteria, and two dogs in each group served as controls. Dogs were caged individually but were allowed to intermingle twice a day.

Cultivation of *S. typhimurium*. Rectal swabs from all 12 dogs were taken before challenge and at 1, 2, 3, 7, 14, 21, 28, and 42 days postinfection. Swabs were plated directly onto Hektoen Enteric (HE) agar (Difco, Detroit, Mich.) and incubated for 18 h at 37°C. Suspected *Salmonella* colonies were characterized biochemically and serologically for confirmation (4). In addition, swabs were placed onto 10 ml of selenite-cystine broth (Difco) and incubated for 4 and 24 h at 37°C. At each time (0, 4, and 24 h), 25 μl of broth was plated onto HE agar and incubated for 18 h at 37°C. Suspected *Salmonella* colonies were characterized biochemically and serologically. Another 5 μl aliquot of broth was removed for use in the PCR and stored at −20°C until assayed.

DNA amplification. Isolation of nucleic acids was conducted by adding 15 μl of Genereleaser (Bioventures, Murfreesboro, Tenn.) to a thermal cycle tube containing the 5-μl aliquot of enrichment broth removed for PCR. A short DNA extraction procedure was done with the sample, and the DNA was amplified (22) by thermocycling the 20-μl DNA preparation with 30 μl of a master mix of amplification reagents (9600 DNA thermal cycler; Perkin-Elmer, Norwalk, Conn.).

Labeling of DNA probes. The internal oligonucleotide (22) was labeled on the 3′ end with digoxigenin by using terminal transferase (Boehringer-Mannheim, Indianapolis, Ind.) according to the manufacturer's procedure. For the double-stranded DNA probe, the 457-bp amplified product was random primed and labeled with digoxigenin by using Klenow fragment as described by the manufacturer (Boehringer-Mannheim).

Detection of amplified products. Amplified products (20 μl) were electrophoresed in 1.5% ethidium bromide-stained agarose gels for visualization. Amplified products were transferred to nylon membranes (MSI, Westboro, Mass.) and hybridized at 48°C with the internal oligonucleotide (22) and at 68°C with the double-stranded DNA probe. Hybridized products were detected (21) by using alkaline phosphatase-conjugated antidigoxigenin antibody (Boehringer-Mannheim) and AMPPD (Tropix, Bedford, Mass.) chemiluminescent substrate after exposure to X-ray film.

Sequencing of PCR products. Amplified products from nonspecific amplification were ligated into the pT7Blue T-cloning vector and transformed into Nova-Blue cells as recommended by the manufacturer (Novagen, Madison, Wis.). Competent colonies were screened for the amplified product. Sequencing of the product was conducted with T7 and U19 primers in an automated DNA sequencer (model 373A; Applied Biosystems, Foster City, Calif.).

Statistical analysis. The χ^2 test was used to test the association between the proportion of samples positive for *S. typhimurium* and the identification procedure, enrichment, and incubation time by using the Epi Info version 5.01 statistical analysis program (7).

RESULTS

A total of 64 rectal swabs from experimentally infected beagles were analyzed by cultivation and PCR-hybridization in parallel with a standard culture method for the detection and identification of *S. typhimurium*. Each swab was analyzed immediately after the swabbing procedure and after 4 and 24 h of incubation in selenite-cystine enrichment broth, for a total of 192 samples. Another 32 swabs obtained from four uninfected beagles were used as controls, for a total of 96 samples. Twelve swabs obtained prior to infection showed that all samples were negative for *Salmonella* bacteria by both PCR-hybridization and culture. PCR-hybridization detected *S. typhimurium* more often than culture (Fig. 1) for all enrichment broth periods (0, 4, and 24 h). Without enrichment incubation (0 h), the isolation of *S. typhimurium* on HE agar plates resulted in only 1 of 64 samples being positive for *S. typhimurium* over the 42-day period. In contrast, 11 samples were positive by PCR-hybridization over the same period. This is a 16% increase in detection by PCR-hybridization over that by standard culture techniques ($P < 0.05$). The greatest increase in detection by PCR-hybridization (33%; $P < 0.05$) was observed after 4 h of incubation, when 4 samples were positive by culture on HE agar plates and 25 samples were positive by PCR-hybridization. After 24 h of enrichment, 25 samples were positive by culture on HE agar plates and 36 samples were positive by PCR-hybridization, an increase of 17% ($P < 0.05$). Two of the control dogs showed an intermittent positive result by PCR-hybridization but were negative by culture over the 42-day period. The other two control dogs were negative by both PCR-hybridization and culture throughout the study.

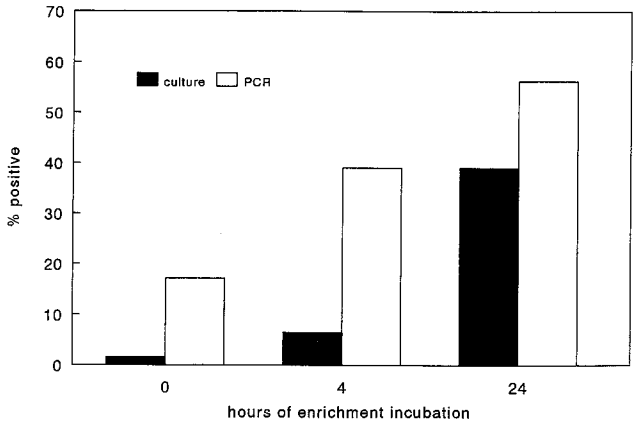

FIG. 1. Comparison of PCR-hybridization and culture results for rectal swabs of eight beagles experimentally infected with *S. typhimurium*. Swabs were taken 24 h after inoculation, placed into selenite-cystine broth, and plated immediately (0 h) or incubated for 4 and 24 h. PCR-hybridization results were significantly better than culture results by χ^2 analysis at all three incubation times ($P < 0.05$).

The detection of *S. typhimurium* by PCR-hybridization up to 3 days after exposure to the organism resulted in a significant difference compared with detection by standard culture techniques (Fig. 2). PCR-hybridization results from swabs taken 1 day postinfection showed a 33% increase in detection over culture ($P < 0.05$). At 2 days postinfection, a 42% increase ($P < 0.05$) in detection was observed, and a 33% increase was observed at 3 days postinfection ($P < 0.05$). The detection of *S. typhimurium* by PCR-hybridization after 3 days was not significantly better ($P > 0.05$) than that by culture. A 12%

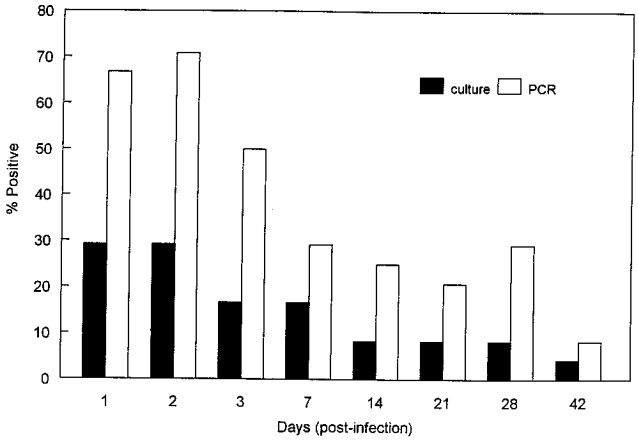

FIG. 2. Comparison of PCR-hybridization and culture results for rectal swabs of eight beagles experimentally infected with *S. typhimurium*. Swabs were inoculated into selenite-cystine broth and incubated for 24 h. Results for swabs taken at 1, 2, and 3 days postinfection were significantly better with PCR-hybridization than with culture by χ^2 analysis ($P < 0.05$).

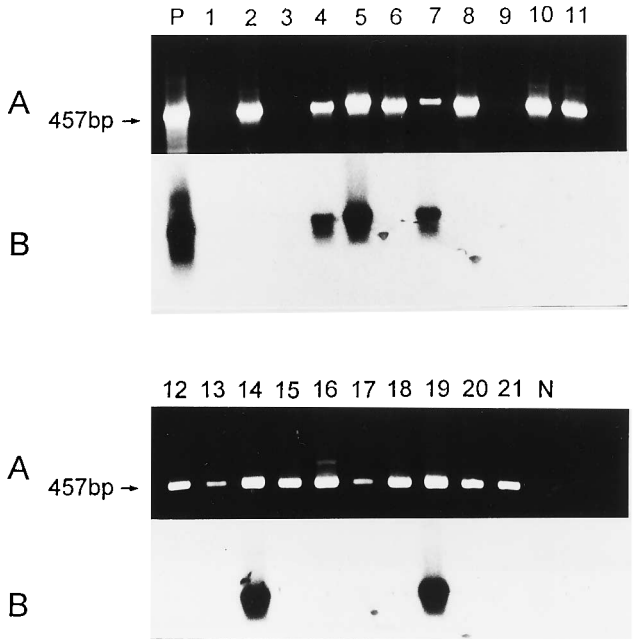

FIG. 3. Detection of *S. typhimurium* by PCR-hybridization for several rectal swabs of experimentally infected beagles after 24 h of incubation in selenite-cystine broth. Amplified products were run through a 1.5% agarose gel (A) and visualized by ethidium bromide staining. Lane P, positive control (seeded rectal swab); lanes 2 to 22, experimental samples; lane N, negative control (no template DNA). (B) Southern blotting and hybridization with the 3′ digoxigenin-labeled internal oligonucleotide probe.

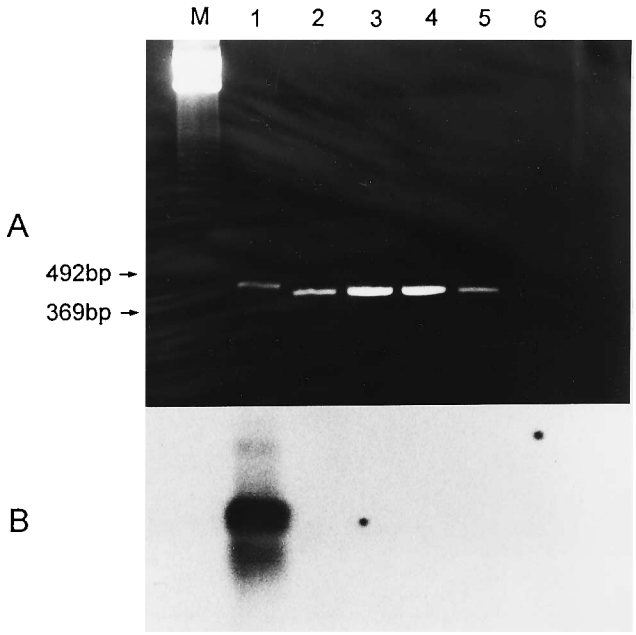

FIG. 4. Gel electrophoresis and hybridization analysis of nonspecifically amplified products. Amplified products were run through a 1.5% agarose gel (A) and visualized by ethidium bromide staining. Lane M, 123-bp DNA ladder (Gibco-BRL, Gaithersburg, Md.); lane 1, 457-bp specifically amplified product from *invE* and *invA* genes of *S. typhimurium* ATCC 29946; lanes 2 to 5, nonspecifically amplified products; lane 6, negative control (no template DNA). (B) Southern blotting and hybridization with the 457-bp random primed, digoxigenin-labeled probe.

increase was observed at 7 days, 17% at 14 days, 13% at 21 days, 21% at 28 days, and 4% at 42 days.

An amplified product relatively close in size to the 457-bp specifically amplified product was observed in samples enriched for 24 h (Fig. 3) but not in samples enriched for less than 24 h. This product did not hybridize with the labeled oligonucleotide that was specific to the 457-bp product. The 457-bp product was random primed, labeled with digoxigenin, and used as a probe to determine homology between the 457-bp product and the nonspecific product. It did not hybridize to the nonspecific product (Fig. 4). To further investigate the homology between the two products, the nonspecific product was isolated, cloned into pT7Blue, and sequenced. The sequence obtained was a 429-bp product that had very little similarity to the 457-bp product from *S. typhimurium* (Fig. 5).

DISCUSSION

Traditionally, the laboratory diagnosis of salmonellae has relied on selective enrichment and subsequent culture on selective media. The introduction of PCR provides a more sensitive and rapid technique for detecting these microorganisms. Accordingly, the results of the present study demonstrated that greater numbers of samples contained *S. typhimurium* when detected by PCR-hybridization than when standard cultural techniques were used. In addition, all samples that were culturally positive for *S. typhimurium* were also positive by PCR-hybridization. Two control dogs showed an intermittent positive result throughout the 42-day period by PCR-hybridization but were negative by culture.

Two major limitations to using PCR as a diagnostic tool are that false-positive reactions can occur from DNA contamination (19) and that false-negative reactions can occur from a number of substances found in clinical samples that inhibit PCR (19). Although DNA contamination can occur in laboratories where PCR is routinely conducted, we feel that the reactions of the two control dogs showing intermittent positive results were true-positive reactions and not caused by laboratory DNA contamination. Because the dogs were allowed to intermingle twice a day, exposure of the control dogs to the bacteria by infected dogs is likely. In this study, the use of an enrichment broth was to dilute PCR-inhibitory substances that might have been present in the samples in order to reduce the possibility of false-negative results (22). The use of an enrichment broth also increases the number of organisms in a sample and increases the likelihood of a positive PCR. Whereas enrichment for as little as 1 h has led to successful PCR detection of *Salmonella* serovars (3, 22), the proportion of positive results by both culture and PCR-hybridization in the current study increased with longer periods of enrichment. However, the overall comparison between PCR-hybridization and culture indicated that the positive results obtained by PCR-hybridization were significantly greater. In addition, longer periods of enrichment incubation are required in order to obtain a positive result from culture.

The bacteria used in this study were attenuated strains of *S. typhimurium* that colonize the gut but do not cause gastroenteric problems. Therefore, infected dogs will shed the organism for a period of time before clearing it completely. The samples taken 1 to 3 days postinfection showed that PCR-hybridization was significantly better than culture for detecting the organism. After 3 days, PCR-hybridization detected 4 to 21% more positive samples than culture; however, these results were not statistically significant.

The importance of confirming the specificity of amplified products by hybridization with labeled probes rather than gel

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          1                                                   50
non459    TGCCTACAAG CATGAAATGG GTATTGACCC AGGTACAACC ATATTGCAGC
sal457    TGCCTACAAG CATGAAATGG CAGAACAGCG TCGTACTATT GAAAAGCTGT
aldH      .......... .......... ...AATTCTT AGCCCTGTTA AAACAGCAGG

          51                                                  100
non459    CGTGCGCT.. ......GACG CGATGCGCCC TGCCCACATC TTTCGTCCAT
sal457    CTTAATTTAA TATTAACAGG ATACCTATAG TGCTGCTTTC TCTACTTAAC
aldH      CGCAAAACTG GCAGCCGGGC CATCCACTTG ATCCCGCAAC CCATGGGCAC

          101                                                 150
non459    ACCGAAGATG CAAGTCCGTA CTGGCT..GT CATTCGCCCA GTTCACCACC
sal457    AGTGCTCGTT TACGACCTGA ATTACT..GA TTCTGGTACT AATGGTGATG
aldH      CTTAATCGAC TGCGCCCACG CCGACTCGGT CCATAGCTTT ATTCGGGAAG

          151                                                 200
non459    TGTTCTTCGT TG.TCGAAGG GCGTAACACT CACTACTGGA CCAAATACCT
sal457    ATCATTTCTA TGTTCGTCAT TCCATTACCT ACCTATCTGG TTGATTTCCT
aldH      GCGAAAGCAA AGGGCAACTG TTGTTGGATG GCCGTAACGC CGGGCTGGCT

          201                                                 250
non459    CTTTTTGCAC GATGGCATCG TCCTGTAATG CGCCAGCCAG CAGCGTCGGC
sal457    GATCGCACTG AATATCGTAC TGGCGATATT GGTGTTTATG GGGTCGTTCT
aldH      GCCGCCATCG GCCCGACCAT CTTTGTGGAT GTGGACCCGA ATGCGTCCTT

          251                                                 300
non459    GCATAGTAAT AGCCATTACC CTTGCGCTTT TCACCGCCAG TGATCACTTT
sal457    ACATTGACAG AATCCTCAGT TTTTCAACGT TTCCTGCGGT ACTGTTAATT
aldH      AAGTCGCGAA GAGATTTTCG GTCCGGTGCT GGTGGTCACG CGTTTCACAT

          301                                                 350
non459    GATGTGCCCT GTCG..... ......... .....CTTTC GCCTCTTCTA
sal457    ACCACGCTCT TTCGTCTGGC ATTATCGATC AGTACCAGTC GTCTTATCTT
aldH      CAGAAGAACA GGCGCTACAG CTTGCCAACG ACAGCCAG.. ........TA

          351                                                 400
non459    CTGCCTTGGT GACGCGTTCG AGATGCGC.C AGCGAGCTTA AAGGTCCAAG
sal457    GATTGAAGCC GATGCCGGTG AAATTATCGC CACGTTCGGG CAATTCGTTA
aldH      CGGCCTTGGC GCGGCGGTAT GGACGCGCGA CCTCTCCCGC GCGCACCGCA

          401                                                 450
non459    CTACCGTAGA CTCGTCATCT GGCGCACCAG ATTTTAACGT CGTCACCGTG
sal457    TTGGCGATAG CCTGGCGGTG GGTTTTGTTG TCTTCTCTAT TGTCACCGTG
aldH      TGAGCCGACG CCTGAAAGCC GGTTCCGTCT TCGTCAATAA CTACAACGAC

          451     460
non459    GTCCAGTTTA
sal457    GTCCAGTTT.
aldH      GGCGATATGA
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FIG. 5. Alignment of the nucleic acid sequences of the 429-bp nonspecifically amplified product (non429), the 457-bp amplified product from the *invE* and *invA* genes of *S. typhimurium* ATCC 29946 (sal457), and the 429-bp portion of the *E. coli* betaine aldehyde dehydrogenase gene (*ald*H) that showed similarity to the nonspecifically amplified product from BLASTN. Sequences were aligned by using the program Pileup from the Genetics Computer Group (8).

electrophoresis detection alone is evident from the results of this study. A nonspecific amplified product was present on ethidium bromide-stained agarose gels at about the same size as the 457-bp specifically amplified product from *S. typhimurium*. Since this product did not hybridize with the internal oligonucleotide or the 457-bp product used as a probe, it was characterized by subcloning into the pT7Blue vector and sequencing. The sequence obtained was a 429-bp product that had very little similarity to the 457-bp fragment. Evaluation of this sequence with GenBank (release 82) by using the BLASTN program (1) showed that 113 bp of a 175-bp fragment (64%) had sequence similarity to the *Escherichia coli* aldehyde dehydrogenase gene (14). Since the product was observed only after incubation of a sample for 24 h in selenite-cystine broth, the possibility exists that it is a portion of an aldehyde dehydrogenase gene from another bacterium and not from a mammalian aldehyde dehydrogenase gene.

This study demonstrates that a rapid diagnostic test using an enrichment broth cultivation and PCR-based procedure is highly sensitive and specific for detecting *Salmonella* serovars from rectal swabs. We should note, however, that rapid tests based on PCR for infectious diseases will not completely replace bacterial isolation, because the infectious agent needs to be cultured for determination of serotype, biotype, and antimicrobial susceptibilities. A PCR-based method, as described in this paper, can be used in association with standard cultural practices to increase the sensitivities of current testing procedures.

ACKNOWLEDGMENTS

This work was supported by a grant from the Kansas Racing Commission and the Kansas Agricultural Experiment Station, Manhattan.

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