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Comparison of Three Stool-Processing Methods for Detection of *Salmonella* Serogroups B, C2, and D by PCR

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Three different stool sample-processing methods (centrifugation, immunomagnetic separation, and selective enrichment cultivation) for the identification of *Salmonella* serogroups by PCR were studied. The corresponding sensitivities in an ethidium bromide stained-agarose gel were 10^5, 10^3, and 10 bacteria, respectively. The PCR assay with overnight enrichment performed as well as, or even better than, the conventional culture technique. Of 485 clinical stool samples, PCR correctly identified all 230 culture-positive samples as well as mixed *Salmonella* infections in four cases.

PCR technology has revolutionized many facets of molecular medicine and microbiology over the past few years, and the development of rapid and sensitive PCR methods for detection of infectious microbial agents from clinical or food specimens has been of great interest to many microbiologists (3, 11). A handful of PCR assays for direct detection of enteropathogenic *Escherichia coli*, *Shigella* spp., *Vibrio cholerae*, *Clostridium difficile*, and group A rotarvirus in human feces have recently emerged (2, 4, 13, 15).

In our previous report a PCR assay for detection and identification of *Salmonella* serogroups A, B, C2, and D was described (8). The specificity of this assay is based on the designed primer sets targeting variable regions of the DNA sequences of the *rfb* gene clusters that are involved in biosynthesis of the *Salmonella* lipopolysaccharide (LPS) O antigens (12). To evaluate the applicability of the *Salmonella* PCR assay in routine diagnostic microbiology laboratories, we compared the PCR assay with the conventional culture technique for identifying salmonellae from clinical stool specimens. Because fecal specimens often contain a substance(s) which may interfere with the PCR assay, leading to false-positive or false-negative results (11, 15), we investigated three alternative techniques (centrifugation, immunomagnetic separation [IMS], and selective enrichment cultivation) for processing stool samples in attempts to eliminate any potentially interfering materials derived from the host tissues. In this study, we also demonstrated the capability of our PCR assay to identify mixed infections by different *Salmonella* serogroups.

Salmonella typhimurium IS248 (serogroup B), *Salmonella newoport* IS50 (serogroup C2), and a clinical isolate of *Salmonella enteritidis* (serogroup D) were from previous studies (8, 10) and used as controls to spike stool samples. About 0.2 g of normal stool materials (obtained from healthy donors and confirmed by cultures as salmonella negative) in a sterile microcentrifuge tube was mixed with 1-ml aliquots of bacterial suspensions at 10-fold dilutions (<10 and 10^1 to 10^7 CFU). The samples were then subjected to three different processing methods before the PCR assay.

(i) Centrifugation. Particulate fecal substances were removed by low-speed centrifugation (300 × *g*) for 3 min. After high-speed centrifugation (17,000 × *g*, 3 min), soluble substances were discarded with the supernatant and the pelleted bacteria were resuspended in 1 ml of phosphate-buffered saline (PBS). Following two more cycles of the low- and high-speed centrifugation, the final pellet was suspended in distilled water and heated to 95°C for 10 min to extract the bacterial DNA. After a brief high-speed centrifugation, 10 μl of the straight supernatant was subjected to PCR analysis.

(ii) IMS. Magnetic beads (M-280; Dynal AS, Oslo, Norway) coated with purified monoclonal antibody MATy-O9, which is specific for the LPS O9 antigen (10), were used to isolate serogroup D *Salmonella* cells from the stool samples. After a low-speed centrifugation to remove particulate matters, 0.2 mg of the antibody-coated magnetic beads was added to the stool supernatant. The separation procedure was as described before (9), and bacteria bound to the beads were heated for DNA extraction as mentioned above.

(iii) Selective enrichment broth cultivation. Each spiked stool suspension (0.5 ml) was inoculated into 5 ml (each) of the two *Salmonella* enrichment media, Rappaport broth (Oxoid Ltd., Hampshire, United Kingdom) and Selenite broth (Difco Laboratories, Detroit, Mich.). The stool suspensions were then cultured at 37°C with gentle shaking, and 0.5-ml aliquots were taken for PCR analysis at 2 h, at 4 h, and after overnight incubation. Bacterial cells were collected by centrifugation at 17,000 × *g* for 3 min, washed twice with PBS, and then similarly heated in 0.1 ml of distilled water for DNA extraction.

A total of 485 stool samples collected from diarrheal patients were tested for *Salmonella* identification by PCR analysis with the overnight enrichment culture method. Stool swabs were first streaked on selective agar (salmonella-shigella, MacConkey, or xylose-lysine-deoxycholate) (Difco) and inoculated into the Rappaport enrichment broth for overnight incubation at 37°C. Subsequent isolation and identification of salmonellae was based on standard biochemical tests and serological typing using anti-H and anti-O sera (1, 6).

For PCR analysis of clinical samples, a primer cocktail combining three pairs of 18-mer oligonucleotides targeting the *Salmonella rfbJ* (serogroup B) (5′-AGA ATA TGT AAT TGT CAG-3′ and 5′-TAA CCG TTT CAG TAG TTC-3′), *rfbJ* (serogroup C2) (5′-ATG CTT GAT GTG AAT AAG-3′ and 5′-CTA ATC GAG TCA AGA AAG-3′), and *rfbS* (serogroup D) (5′-TCA CGA CTT ACA TCC TAC-3′ and 5′-CTG CTA

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TABLE 1. Detection of salmonellae in 485 clinical stool samples by the culture method and PCR with overnight enrichment culture

Clinical isolate	No. of samples	No. of samples positive by:	
		Culture	PCR
Salmonella serogroup(s) (no. of serotypes identified)			
B (7)	70	70	69 (+1[a])
C1 (6)	30	30	0
C2 (5)	28	28	28
D (2)	132	132	132
E (2)	5	5	0
B + D (2)	2[b]	0[c]	2
C2 + D (2)	2[b]	0[c]	2
Other	216	0	3[d]

[a] One samples was positive by PCR after repeated washings.
[b] Considered as mixed *Salmonella* infections.
[c] Samples were positive in culture but identified as only one serogroup.
[d] Samples were identified as serogroup B. When these samples from the enrichment broth were subcultured on selective agar plates and a large number of colonies were subjected to further PCR analysis, no reproducible results were obtained.

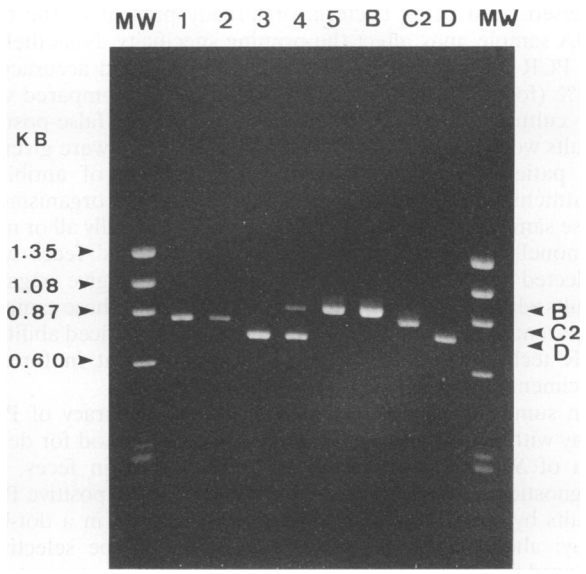

FIG. 1. Detection and identification of *Salmonella* serogroups in clinical stool samples by the enriched PCR assay. *Salmonella* reference strains *S. typhimurium* IS248 (lane B) and *S. newoport* IS50 (lane C2) and a well-defined clinical isolate of *S. enteritidis* (lane D) were controls and gave amplified DNA bands of 882, 820, and 720 bp, respectively. Stool samples were *Salmonella* serogroup C2 (lanes 1 and 2), serogroup D (lane 3), serogroup B (lane 5), and a mixed infection with serogroups B and D (lane 4). Molecular size markers were *Hae*III-digested φX174 DNA (lane MW).

TAT CAG CAC AAC-3′) genes was used (8). The final reaction mixture volume was 100 μl including 0.2 μg of each primer, 10 μl of DNA extracts, and 2 U of *Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.). The reaction was run in a DNA thermal cycler (Perkin-Elmer Cetus) through 30 cycles: 94°C for 1 min (denaturation), 45°C for 1 min (annealing), and 72°C for 2 min (extension). The resulting PCR products yielded distinctive DNA bands at 882 bp (for serogroup B), 820 bp (serogroup C2), and 720 bp (serogroup D) as revealed in 1.5% agarose gel electrophoresis (8).

An obligatory feature of any assay for direct detection of infectious agents in clinical specimens is a satisfactory performance in the presence of host tissues (e.g., blood, stool, or secretions). In this regard, we compared and determined the sensitivities of three different methods (centrifugation, IMS, and enrichment cultivation) for processing stool specimens for analysis by PCR. In order to give a positive PCR signal on gels, our results showed that the centrifugation method required at least 10^5 bacteria whereas the IMS was approximately 1,000-fold more sensitive, requiring 10^2 to 10^3 bacteria. An increase in the amount of the magnetic beads from 0.2 to 0.4 mg did not significantly improve the sensitivity. For the enrichment culture method, the detection limits were about 10^6 and 10^3 bacteria for the specified incubation times (2 and 4 h, respectively). After overnight cultivation, *Salmonella* cells were detectable with an initial inoculum of <10 bacteria in stool samples. Enrichment in either Rappaport or Selenite broth gave similar results.

The cost-effectiveness of each processing method is also of concern. The centrifugation process is simple and cheap. Both the particulate and soluble substances were mostly removed by the low- and high-speed centrifugation. However, some target bacteria might be lost in the processing steps and some interfering substances may remain in the final DNA supernatant, leading to lower PCR sensitivity. The IMS technique promises to be rapid and sensitive, and its major advantage is the selective isolation of pathogenic agents of interest from other microflora and inhibitory substances (9). Nevertheless, the IMS technique continues to be relatively expensive and limited by antibody availability. Inevitably, such critical factors diminish the popularity of IMS in routine diagnostic microbiology laboratories. As a result, the enrichment culture method appears to be more practical and affordable in most clinical laboratory settings, especially in developing countries. An overnight enrichment cultivation was optimal in this study for processing tens of stool samples at one time, and the subsequent PCR analysis for *Salmonella* identification was accomplished the following day. This enriched PCR assay was then compared with the standard culture technique for *Salmonella* detection in 485 stool samples (Table 1).

The enriched PCR assay performed as well as, or even better than, the culture technique. Of the 485 samples, 265 were culture positive for salmonellae. Among them, all of the 70 serogroup B *Salmonella* samples, 28 serogroup C2 samples, and 132 serogroup D samples were correctly identified by PCR. In addition, PCR detected in four cases mixed infections that were identified only as single infections by culture. Two different amplified DNA bands which corresponded to *Salmonella* serogroups B and D and serogroups C2 and D were observed in these samples (Fig. 1). The serotypes were confirmed by subculturing the enrichment broth on selective agar plates and subsequent identification of six to eight colonies by biochemical and serological tests. However, it is obvious that mixed infections of different serotypes in a same serogroup could not be detected.

Of 216 *Salmonella*-negative cultures, 3 were positive for serogroup B by PCR. In order to confirm that these results were due to nonspecific amplification, a large number of colonies from these samples were subcultured on selective agar and again tested by PCR. No reproducible PCR products were detected. The possibility of getting a false-positive result by PCR is not entirely unexpected from such complex fecal specimens, because unknown interfering substances, either

released from other bacteria or already present in the final DNA sample, may affect the priming specificity. Nonetheless, the PCR assay has demonstrated high predicted accuracy of 100% (for positive) and 98.6% (for negative) compared with the culture technique. Interpretations of the false-positive results would be more complicated if antibiotics were given to the patients prior to culture. In the course of antibiotic treatment(s), the number of viable pathogenic organisms in these samples would be less and less, and eventually all or most salmonellae would be dead when the patients' feces were collected for culture. Thus, the culture would give negative results while PCR would identify salmonellae in these samples. Apparently, these findings might reflect the enhanced ability of PCR technology to detect target DNA present in forensic specimens or from dead organisms.

In summary, we have demonstrated the accuracy of PCR assay with the enrichment broth processing method for detection of *Salmonella* serogroups B, C2, and D in feces. For diagnostic purposes, it is essential to confirm the positive PCR results by hybridization with specific *rfb* probes in a dot-blot assay; alternatively, positive specimens could be selectively cultured for the appropriate pathogen(s). Several attempts are being made to accommodate the PCR assay more feasibly in a clinical laboratory setting. (i) To shorten the time delay from overnight enrichment, DNA template sources will be obtained from 4- to 6-h broth cultures instead. (ii) To improve the overall detection sensitivity, a recently introduced PCR-enzyme immunoassay technique that also allows us to simultaneously detect a large number of samples on microplates (7) is under evaluation. (iii) To extend the present PCR analysis to all *Salmonella* major serogroups (A to E), since the sequences of the *rfb* genes of serogroups C1 and E are now available (5, 14), we are designing specific probes for distinguishing these salmonellae from others. In combination with all the *rfb* (A to E) primers, it would be possible to detect and/or identify most clinically important *Salmonella* species. With further improvement and appropriate modifications, PCR will offer us a better alternative for reliable diagnosis of most *Salmonella* infections within 24 h of arrival of specimens.

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