Detection of Shigella dysenteriae Type 1 and Shigella flexneri in Feces by Immunomagnetic Isolation and Polymerase Chain Reaction

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A combination of immunomagnetic separation (IMS) and a polymerase chain reaction (PCR) procedure was used for direct isolation and identification of Shigella dysenteriae type 1 and Shigella flexneri from feces. Immunomagnetic particles were coated with monoclonal antibody MASFB, which is specific for a common epitope of the O polysaccharides of S. dysenteriae type 1 and S. flexneri. Bacteria bound to the beads were boiled in water, and target DNA was amplified with a primer pair specific for a gene coded on the invasion-associated locus (ial) of the large virulence plasmid of all four Shigella spp. and enteroinvasive strains of Escherichia coli. A 320-bp DNA fragment was generated and detected by an alkaline phosphatase-conjugated probe. Nonviable cells were also captured and detected by this technique. The method is simple and fast (7 h) and has a detection limit of ca. 10 Shigella organisms per g in fecal samples. The combined IMS-PCR assay correctly identified all 57 samples carrying S. dysenteriae type 1 and 68 samples carrying S. flexneri from 238 fecal specimens and also permitted detection of 17 samples carrying Shigella spp. from 113 specimens from diarrheal patients in whom shigellae were not detected by conventional culture.

Shigellosis is a prevalent diarrheal disease in developing countries (10). In Bangladesh, infections caused by Shigella spp. are responsible for the majority of diarrhea-related deaths among children (2, 18).

Shigellae are usually isolated from fecal specimens by in vitro cultivation of the organism followed by identification by biochemical tests and agglutination assays (6, 19, 22). This process usually requires 48 to 72 h. Since shigellae are very fastidious organisms, appropriate collection, rapid transport to the laboratory, and rapid plating of the sample are important for isolation. Such conditions are often difficult to attain, especially in developing countries. As a consequence, the recovery rates of shigellae are usually low.

Virulent species of Shigella are able to invade the epithelial-cell lining of the large intestine, multiply intracellularly, and spread to adjacent cells. The clinical features of dysentery are bloody, urgent, frequent stools and rectal tenesmus. The capacity of penetration and destruction of the colonic epithelium is mediated in part by a large 120- to 140-MDa plasmid (invasiveness plasmid) which codes for several genes of importance for the virulence of shigellae. Loss of the plasmid results in loss of the invasive phenotype and subsequent avirulence (12, 16, 21, 23).

Recently, there have been reports that invasive shigellae and enteroinvasive Escherichia coli (EIEC) species can be detected by using a polymerase chain reaction (PCR) procedure (6, 7). One of the specific DNA fragments of the invasive plasmid, termed invasion-associated locus (ial), contains sequences that are unique for the four different Shigella species and EIEC (7, 25). PCR was performed by amplification of ial-specific DNA fragments, and amplified products were detected with an alkaline phosphatase-labeled oligonucleotide probe (AP probe).

Immunomagnetic particle-based separation (IMS) has been shown to be an efficient tool for separation and isolation of specific cells from heterogeneous cell suspensions (3, 14, 15, 24). We recently developed an IMS technique for the isolation of shigellae directly from fecal samples within 3 to 4 h that had a sensitivity of ca. 10^5 CFU/ml (8). Nonviable shigellae could also be detected.

It is essential for the PCR assay that the sample contain at least one intact DNA strand encompassing the region to be amplified and that impurities be sufficiently diluted so that polymerization is not inhibited (11, 20). Extraction of DNA from feces is somewhat complicated and laborious. Dilution of the extracted DNA is commonplace, and this may reduce the sensitivity of the assay.

This study describes an assay which combines the isolation of shigellae from fecal samples by IMS with identification by PCR. DNAs from the bacteria captured by IMS were released by lysing the cells in boiling water (11, 20). The IMS-PCR assay is sensitive (detects ca. 10 shigellae per g of feces), specific, and rapid for the presence of Shigella dysenteriae type 1 or Shigella flexneri in fecal samples.

MATERIALS AND METHODS

Bacterial strains. Strains used in this study are listed in Table 1.

Bacterial DNA recovery. Whole-cell DNAs were isolated from bacteria by the phenol-chloroform extraction method (22). Cells were lysed with 100 µg of lysozyme (Sigma Chemical Co., St. Louis, Mo.) and then treated with 100 µg of proteinase K (Sigma). DNA was extracted from the cell-lysate mixture with phenol-chloroform-isooamylalcohol, precipitated with absolute ethanol, harvested by centrifugation, and suspended in 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA (TE) (22). Total plasmid DNA was isolated

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TABLE 1. Specificity of ial primers and AP probe for different bacterial strains with and without isolation by MASFB-IMP

<table>
<thead>
<tr>
<th>Strain</th>
<th>No. of strains Tested</th>
<th>Isolated by MASFB-IMP</th>
<th>Not isolated by MASFB-IMP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Probe positive</td>
<td>Probe negative</td>
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<tr>
<td></td>
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<td></td>
</tr>
<tr>
<td>Nonpathogenic E. coli</td>
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<td>10</td>
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<td>EIEC*</td>
<td>8</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>Salmonella spp.</td>
<td>10</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Citrobacter spp.</td>
<td>5</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Proteus mirabilis</td>
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<td>3</td>
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<tr>
<td>Enterobacter agglomerans</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Yersinia enterocolitica</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Morganella morganii</td>
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<td>2</td>
<td>0</td>
</tr>
<tr>
<td>S. dysenteriae type 1*</td>
<td>7</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>S. flexneri*</td>
<td>10</td>
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<tr>
<td>S. flexneri†</td>
<td>4</td>
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</tr>
<tr>
<td>S. boydii*</td>
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<td>4</td>
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<tr>
<td>S. sonnei†</td>
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</tbody>
</table>

* Congo red positive.
† Congo red negative (white colonies on Congo red agar).

according to an alkaline denaturation protocol (22). The isolated DNA was stored in TE buffer.

A direct lysis method was also used for isolation of DNA from bacteria. The cells were lysed, and DNA was released by exposure to deionized water at 95°C for 10 min. Cell debris was removed by centrifugation at 15,000 × g for 5 min.

DNA content was measured at A₂₆₀, and the A₂₆₀/A₂₈₀ ratio was checked. The DNA content was adjusted to 20 μg/ml in TE buffer for PCR standardization.

Fecal samples. A total of 248 fecal samples were analyzed. From each of 190 patients with diarrhea who came to the Diarrhoeal Treatment Center of the International Center for Diarrhoeal Disease Research, Bangladesh, a single fecal specimen was obtained. From the routine Microbiology Laboratory, Huddinge Hospital, Huddinge, Sweden, 58 fecal samples were collected. Fresh fecal samples were plated directly on salmonella-shigella, MacConkey, and xylose-lysine-deoxycholate (Difco Laboratories, Detroit, Mich.) agars. Suspect colonies were further screened on Kligler's iron agar slant and motility indole urea medium (5). Shigella isolates were biochemically identified and serologically grouped by using rabbit polyclonal group- and type-specific antisera (National Bacteriological Laboratory, Stockholm, Sweden). Of the 190 samples collected at the center in Bangladesh, 57 contained S. dysenteriae type 1 and 68 contained S. flexneri.

IMS of Shigella strains from pure culture and feces. Sup-paramagnetic beads (Dynabeads M-450, uncoated; Dynal, Oslo, Norway) (IMP) were coated with the monoclonal antibody MASFB, which binds to the O antigens of S. flexneri serotypes 1 to 5 and S. dysenteriae type 1 (1). The coating with MASFB and the isolation of Shigella spp. by MASFB-IMP was performed essentially as described previously (5). Briefly, 1 ml of pure culture suspension or fecal extract (1 g/5 ml of phosphate-buffered saline [PBS]) was incubated with 400 mg of MASFB-IMP for ~1 h at 22°C on a shaker. Beads with attached bacteria were washed three times in 1 ml of PBS containing 0.04% casein and 0.05% Tween 20, washed a final time in water with the aid of a magnetic separator (MPC-6 Dynal, A/S), and finally suspended in 50 μl of deionized water. For DNA recovery, the beads with attached bacteria were heated at 95°C for 10 min. Cell debris and Dynabeads were separated from the cell lysate by centrifugation at 15,000 × g for 5 min, and 10 to 15 μl of the supernatant was amplified by PCR.

Primers and probe used in PCR. The ial-specific primers and probe (primer 1, 5'-CTG GAT GGT ATG GTG AGG 3'; primer 2, 5'-GGA GCC CAA CAA ATTA TTT CC 3'; probe, 5'-CCA TCT ATG AGA ATA CCT GTG 3') were derived from the nucleotide sequences of a 2.5-kbp HindIII fragment of an EIEC invasive plasmid (7, 25). These are the three unique sequences which are present only in the invasive plasmid of all four Shigella spp. and closely related EIEC strains (Table 2). The primer pair used to generate a 320-bp fragment was prepared by Scandinavian Gene Synthesis AB (Köping, Sweden). The oligonucleotide probe was prepared by Syngene Inc. (San Diego, Calif.) as described previously (9) by using a single modified base containing a 12-carbon linker with a terminal primary amine. The probe was covalently coupled to alkaline phosphatase (AP probe) through the free amino group.

Amplification of target DNA. DNA was amplified by PCR with 5 to 15 μl of purified DNA extract or cell lysate by using 0.2 μg of each of the primers (7). The amplification was performed in 1× PCR buffer containing 200 mM deoxynucleoside triphosphates and 0.5 U of Ampli Taq DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.) in a DNA thermal cycler (Perkin-Elmer Cetus). The following proce-
dure was used: 1 min at 94°C (denaturation), 2 min at 43°C (annealing of primers to a single-stranded DNA), and 3 min at 72°C (DNA polymerase-mediated primer extension) for 25 to 30 cycles and then a 5-min extension at 72°C.

Positive and negative controls were amplified along with the test samples throughout the amplification reaction. Two negative controls were used: one without any template DNA (reagent control) and the other with Salmonella typhimurium DNA. One S. flexneri 2a clinical isolate was used as a positive control. The plasmid profile was checked by agarose gel electrophoresis, and the presence of the invasive plasmid was checked for by growing the strains on Congo red agar (17).

Detection of amplified products. PCR-amplified products (10 to 15 µl) were evaluated initially by electrophoresis with a 1.5% agarose gel and visualization by ethidium bromide staining. As size markers, φX174 replicative-form DNA HaeIII fragments (double-stranded DNA from 72 to 1,353 bp [Gibco BRL, Laboratory Design AB, Lidingö, Sweden]) were included in all gels. Stained gels were photographed under UV light on Polaroid type 57 film. Southern blot analysis by a capillary blotting procedure was performed to verify the amplified product by PCR. In brief, after 1 h of electrophoresis in a 1.5% agarose gel, DNA samples were transferred to a Hybond N+ nylon membrane (Amersham Inc., Buckinghamshire, United Kingdom). The membrane was processed for hybridization (according to the instructions of Amersham). The nylon membrane was prehybridized for 15 min in a solution containing 6× SSC (0.9 M NaCl, 0.09 M sodium citrate [pH 7.0]), 0.5% (wt/vol) sodium dodecyl sulfate (SDS), 5× Denhardt’s solution (0.1% [wt/vol] Ficoll, 0.1% [wt/vol] polyvinylpyrrolidone, 0.1% [wt/vol] bovine serum albumin), and 100 µg of sheared denatured salmon sperm DNA per ml at 42°C. Hybridization with the AP probe took place in the same solution (1 µl of probe per 2 ml of solution) at 42°C for 20 min. The membrane was washed four times at 22°C: in 2× SSC-0.5% SDS for 10 min, in 1× SSC-0.1% SDS for 10 min, in 1× SSC-0.5% Triton X-100 for 5 min, and in 1× SSC for 1 min. After the washes, the membrane was incubated with a mixture of Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate for 1 to 2 h at 22°C. The reaction was stopped by soaking the membrane in distilled water. Positive samples were visualized as purple bands.

Detection of amplified products by dot blot hybridization. The AP probe described above was used for dot blot hybridization. One-tenth of each of the amplified samples (10 µl) was used for dot blot analysis, and the double-stranded amplified DNAs were heat denatured and directly spotted onto Hybond N+ nylon membranes by using a Bio-Rad dot blot manifold at a vacuum pressure of 4 to 5 lb/in². Amplified DNA immobilized on the nylon membranes was then processed for hybridization (according to the instructions of Amersham). The hybridization procedure and solutions used were the same as for the Southern blot hybridization. Positive samples were visualized as purple spots on the membrane.

RESULTS

Sensitivity of PCR. The sensitivity of the PCR assay was investigated with 10-fold serial dilutions in PBS of S. flexneri 2a bacterial cells. Whole-cell DNA (Fig. 1, lane C) and cell lysates of MASFB-IMP-isolated bacterial cells (Fig. 1, lane B) were then subjected to PCR. The lowest number of bacterial cells detected, as determined by viable cell count-
strains (Fig. 3). The specificities of the monoclonal antibody and the primers were verified against a panel of strains (Table 1). The different strains were analyzed by PCR with or without MASF-IMP separation (Table 1). We found, as expected, that the four Shigella spp. and the EIEC strains were identified after amplification with ial primers and detection by the AP probe. Four Congo red-negative and noninvasive S. flexneri strains were not amplified with the ial primers. Only the S. dysenteriae type 1 and S. flexneri strains were detected when IMS was used for isolation prior to PCR amplification and detection with the AP probe (Table 1).

IMS-PCR assay of fecal samples. A total of 238 fecal samples were analyzed. S. dysenteriae type 1 or S. flexneri was isolated with MASF-IMP and cell lysates amplified by PCR and then detected by dot blot hybridization with the AP probe (Fig. 4, Table 2). All samples which were culture positive either with S. dysenteriae type 1 or S. flexneri were identified by the IMS-PCR assay. In addition, the assays identified 17 of the 113 S. dysenteriae type 1 or S. flexneri culture-negative samples as positive. This indicates that the IMS-PCR assay was 15% more sensitive than the conventional culture method.

All the IMS-PCR assays routinely contained negative controls to detect any possible contamination. All controls were negative throughout the study.

DISCUSSION

Because of the fastidious nature of shigellae and the lengthy culture time they require, a rapid and sensitive system for the detection of shigellae is desirable. We here describe an IMS-PCR assay for the isolation of S. dysenteriae type 1 and S. flexneri directly from feces. The assay involves two steps: isolation of bacteria from feces by using specific monoclonal antibody-coated IMP, with the target DNA released by boiling the captured bacteria; and amplification of the recovered DNA by PCR followed by detection with dot blot hybridization. The whole procedure requires 6 to 7 h and has a sensitivity of ca. 10 bacteria per g of feces (Tables 1 and 2). The IMS-PCR assay is about 3 h slower but
about 100 times more sensitive than the IMS technique with immunological detection.

It is essential for the PCR assay that the sample contain at least one intact DNA strand carrying the region to be amplified and that impurities be sufficiently diluted. There are several different protocols for the isolation of DNA: enzymatic digestion, phenol-chloroform extraction, and precipitation with absolute ethanol. One report showed that stool contents may impede the PCR procedure, making it desirable to decrease the contents of nonhomologous DNA, complex polysaccharides, and proteins (4). We bypassed that step by isolating the shigelae from feces by the IMS procedure, which eliminates fecal debris and other contaminating bacterial contents in the subsequent PCR amplification step. The amounts of impurities as nonspecific DNA are negligible in the cell lysates of captured shigelae. The whole-cell lysate can be directly amplified, which increases the sensitivity. The IMS assay exerts positive selection for shigelae, and hence, antibody specificity is critical. The MASFB monoclonal antibody used to coat the immunomagnetic particles recognizes a common epitope present in the repeating unit of the O-antigen polysaccharide chains of both S. dysenteriae type 1 and S. flexneri strains (1). The nal primers do not distinguish shigelae from EIEC strains. The MASFB antibody, however, binds only S. dysenteriae type 1 and S. flexneri strains. The combined IMS-PCR assay is therefore very specific for S. dysenteriae type 1 and S. flexneri types 1a through 5b.

All fecal samples which were either S. dysenteriae type 1 or S. flexneri culture positive were also positive in the IMS-PCR assay, in which detection was by dot blot hybridization (Table 2). When a normal stool specimen had been reconstituted with various concentrations of S. flexneri 2a, the sensitivity of the IMS-PCR assay was such that as few as 10 bacteria could be detected. The reference "gold standard" used for the comparative detection of shigelae in fecal specimens was bacteriological culturing. The explanation for the failure of culture to detect shigelae in 17 fecal samples which were positive by PCR could be either that the fecal samples contained such low concentrations of viable cells that they were below the level of detection by culture or that they contained a large portion of nonviable shigelae. The PCR assay detects the target DNA whether the bacteria are viable or not, provided that gross breakdown of nucleic acid has not occurred. We found our IMS-PCR assay to be 15% more sensitive than the culture method.

A drawback of PCR can be its extreme sensitivity, which can lead to false-positive reactions mainly due to sample contamination by PCR product "carryover" (13). Good laboratory practices are necessary to prevent such carryover of amplified DNA sequences. Controls were included in all assays to detect carryover, but no mishaps were detected.

The lowest limit of detection of amplified product by dot blot hybridization was 3.2 pg of plasmid DNA (Fig. 2). The intensity of the color is proportional to the amount of homologous DNA fixed to the membrane. The IMS-PCR assay can therefore be considered a semiquantitative method and is a potential alternative to traditional culture for diagnosis of shigellosis, since it is both sensitive and rapid.

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