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Evaluation of Alkaline Phosphatase-Labelled *ipaH* Probe for Diagnosis of *Shigella* Infections

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The presence of many enteropathogens which are not easily detectable by routine stool culture has led to the development of alternative diagnostic methods. One of these techniques, nucleic acid probe hybridization, has been used to identify *Shigella* spp. and enteroinvasive *Escherichia coli* (EIEC) in stool specimens through the detection of genetic material encoded by a specific large ≈200-kbp virulence-related plasmid. In the present study, an alkaline phosphatase-labelled oligonucleotide probe developed to detect the gene for *ipaH*, a repetitive genetic sequence thought to be present on both the virulence-related plasmid and the chromosomes of all strains of *Shigella* and EIEC, was tested in a developing-country setting through a prospective clinical trial. In a group of 219 Peruvian adults and children with acute gastroenteritis, the *ipaH* probe detected 85% of cases of shigellosis and demonstrated a specificity of 95% when compared with simultaneous detection by several stool culture techniques. Additionally, three cases of EIEC infection which could not be diagnosed by culture methods alone were detected with the *ipaH* probe and were confirmed by plasmid analysis and Sereny testing. These preliminary results suggest that, with further research, the *ipaH* probe should prove to be a useful and rapid adjunct in the diagnosis of acute gastroenteritis in developing countries.

Diagnostic tools for determining the etiology of acute dysentery in developing countries provide useful information for epidemiologists and public health officials interested in the local prevalence of pathogens as well as for clinicians managing individual cases. The standard procedure for identification of bacterial enteropathogens has been the stool culture, a procedure with several practical limitations. Selection of pathogenic bacterial colonies from normal colonic flora on selective culture media can be difficult if the pathogenic colonies are few or if the observer is unskilled, and the protocols for enteropathogen identification usually require 48 to 72 h or longer for their completion. In addition, *Escherichia coli* strains which cause diarrhea by the production of enterotoxins (enterotoxigenic *E. coli* [ETEC]) or by epithelial cell invasion in a manner similar to that by which *Shigella* spp. cause diarrhea (enteroinvasive *E. coli* [EIEC]) cannot be selectively identified by routine microbiologic methods, and special assays are required for the detection of virulence factors by these strains. The difficulty inherent in the identification of EIEC strains has limited their detection in epidemiologic surveillance and individual patient evaluations (3).

DNA probes have been used as an alternative diagnostic method because of their potential for rapid, specific diagnosis. They can also be used to detect the presence of genetic material encoding virulence factors, allowing for the identification of diarrhea-producing *E. coli* or other enteropathogens not readily detectable on stool culture. Recent interest has centered on the use of DNA probes to diagnose *Shigella* spp. and EIEC in stool samples from patients with diarrhea on the basis of the presence of a large ≈200-kbp plasmid that is present only in virulent *Shigella* spp. and EIEC (4) and that encodes many virulence-related proteins common to all *Shigella* spp. and EIEC strains (10, 11). The probes generated from plasmid gene sequences include a 17-kb *Eco*RI restriction fragment (1, 6, 7) and an oligonucleotide probe generated from a 2.5-kb *Hin*dIII restriction fragment (2). Highly specific virulence plasmid probes which detect portions of the *ipaB*, *ipaC*, and *ipaD* genes, which are single-copy genes encoding the production of invasion-related proteins, have also been used to identify *Shigella* and EIEC colonies by in situ hybridization in a laboratory setting (11). Because these probes hybridize exclusively with genes encoded by the large virulence-related plasmid, they do not detect strains which have lost this plasmid. The large plasmid containing genetic sequences detected by these probes can be very unstable in vitro, especially upon subculturing.

Recently, an oligonucleotide probe has been developed to detect the *ipaH* gene, which encodes a 60-kDa antigen and which is found in multiple copies on both the invasion-related plasmid and the chromosomes of *Shigella* spp. and EIEC strains (5). Thus, this genetic marker is not lost after serial passage of *Shigella* organisms in the laboratory. Although the function of the *ipaH* gene is unknown, the gene has been found in all of more than 50 *Shigella* and EIEC strains of many serotypes but in none of over 200 enteric non-*Shigella* bacterial strains tested in our laboratory and in other laboratories (8). The number of copies of the *ipaH* gene varied from 4 to 10 in different strains (9). The multicopy nature of the *ipaH* gene, in conjunction with its demonstrated specificity for *Shigella* and EIEC strains,

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makes the *ipaH* probe a very attractive diagnostic tool for clinical and epidemiologic purposes.

Preliminary work in our laboratory has already shown that the *ipaH* probe that we studied was highly sensitive and specific in the identification of *Shigella* and EIEC bacterial isolates, with positive identification of 55 of 55 stool blots from patients with shigellosis (8). Those studies confirmed that the *ipaH* gene is a more sensitive marker than the *ipaC* gene for detection of *Shigella* and EIEC strains. In the present study, we extended these observations in a preliminary field study to examine the utility of an alkaline phosphatase-labelled *ipaH* probe versus that of a combination of stool culture methods for the diagnosis of *Shigella* and EIEC infections in an area where these organisms are endemic. The purposes of the present investigation were to assess the applicability of the *ipaH* probe for the diagnosis of *Shigella* infections in a field situation by (i) using a more abbreviated growth amplification period, thereby decreasing the time required for a result, (ii) using a nonradioactive enzymatic marker, which is more applicable for diagnostic laboratories in developing countries, and (iii) studying a large number of stool specimens from high-risk patients in order to provide useful data about the sensitivity of the enzyme-labelled probe compared with that of direct bacterial isolation.

MATERIALS AND METHODS

***ipaH* probe.** The DNA probe used in the present study is a 19-mer oligonucleotide with the genetic sequence 5′-d CtG GAG GAC ATT GCC CGG G, where t indicated a modification with suberyl-alkaline phosphatase (Molecular Biosystems, San Diego, Calif.) and d indicates that it is a DNA (versus RNA) probe. The same probe with a radioactive label has been described previously (8).

Patient population and specimen collection. Patients presenting to the Hospital General Cayetano Heredia, Lima, Peru, with diarrhea were enrolled for the project described here between September 1989 and October 1990. The criteria for enrollment were the presence of three or more unformed stools per day with clinical dysentery, which was defined as the presence of gross blood and mucus in the stool or mucus only in conjunction with fecal leukocytes on microscopic examination. The antibiotics taken within the last 72 h were recorded. Stool samples were collected from all patients meeting the entry criteria during the study period, and both whole stool samples and Cary-Blair transport media inoculated at the time of specimen collection were sent to the laboratory. Stool specimens were kept at 4°C both during transport and in the laboratory prior to processing.

The study population included 179 infants and children (mostly under 24 months of age) and 40 adults. Most infants and children were temporary inpatients in a special diarrhea ward located in the emergency room, while all adult patients were outpatients seen in an acute-care clinic. The protocol was approved by the Committee on Human Volunteers of the Johns Hopkins School of Hygiene and Public Health.

Stool culture. In order to optimize the recovery of *Shigella* spp., a three-phase stool culture procedure was implemented. This stool culture procedure consisted of direct plating of the stool specimen onto MacConkey agar by nursing personnel at the time of specimen collection (method A), inoculation of stool samples into Cary-Blair transport medium at the time of specimen collection for culture on numerous media within 4 h as defined below (method B), and at the time of sample processing for probe analysis and within 4 h of specimen collection, direct plating of whole stool sample onto MacConkey and Hektoen agar (method C). Stool specimens inoculated into Cary-Blair transport medium (method B) were used for complete stool culture processing on MacConkey, Salmonella-Shigella, Hektoen, MacConkey-sorbitol, and thiosulfate citrate-bile salt agars and brucella agar with Skirrow's antibiotic supplement; the plate containing brucella agar with Skirrow's antibiotic supplement was incubated under microaerophilic conditions at 42°C for the recovery of *Campylobacter* spp. Bacterial colonies suspicious for *Shigella* spp. were confirmed (i) by the appropriate reactions in tube biochemical tests, including triple sugar iron, lysine-iron agar, urea, and motility, (ii) by agglutination of the suspicious colony by group-specific antisera, and (iii) by API strip biochemicals for final confirmation.

Five colonies of *E. coli*, including slow and rapid lactose fermenters, were isolated on the MacConkey agar plate from culture method B and were saved to test for the presence of EIEC by the Sereny test (i.e., guinea pig keratoconjunctivitis assay). Confirmation of the presence of EIEC by the Sereny test and plasmid analysis (6) was performed on strains from a subset of patient stool samples as described in Results and Discussion.

DNA probe assays. Processing of stool samples for probe detection was done by inoculating a loopful of whole stool onto MacConkey agar plates in 1-cm-diameter circles for growth amplification. A positive control strain (*Shigella flexneri* M90TW; Walter Reed Army Institute of Research) and a negative control strain (*E. coli* HB101; Walter Reed Army Institute of Research) were plated in 1-cm-diameter circles simultaneously along with the stool samples to be assayed. After growth amplification at 37°C for 6 h, a 100-mm-diameter nylon filter (ICN Biochemicals, Irvine, Calif.) was placed on top of the bacterial growth for 10 min at room temperature. Lysis and fixation of bacterial DNA to the filter was performed by placing the membrane serially for 5-min periods on top of Whatman 3MM filters saturated with each of the following solutions: (i) 1% sodium dodecyl sulfate (SDS), (ii) 0.5 N NaOH and 1.5 M NaCl, (iii) 1 M Tris-HCl and 1.5 M NaCl (pH 8.0), and (iv) 5× SSPE (5× SSPE is 0.9 M NaCl, 50 mM $NaPO_4$ [pH 7.7], and 5 mM EDTA). Filter papers were dried for 1 to 2 min between incubations, and after the final incubation, the membrane was dried at 70°C for 1 h. Prehybridization was carried out in 5× SSPE with 1% SDS–0.5% polyvinylpyrrolidone (Sigma) containing 100 μg of yeast tRNA (GIBCO) per ml for 15 min; this was followed by hybridization of each 100-mm-diameter filter paper in 3 ml of the same buffer for 30 min by using 5 μl of the *ipaH* probe (250 nM/ml). The filter was washed twice at 52°C in 1× SSPE–1% SDS for 15 min each time and was given a final rinse at room temperature in 1× SSPE for 15 min. Detection of bound probe was accomplished by incubation of filter papers with 0.33 mg of nitroblue tetrazolium per ml and 0.17 mg of 5-bromo-4-chloro-3-indolyl phosphate per ml in 5 ml of 0.1 M Tris–0.1 M NaCl–50 mM $MgCl_2$ (pH 9.5) as the alkaline phosphatase substrate. Membranes were incubated with substrate for 2 h at room temperature with protection from light, and substrate development was terminated by washing the membrane in distilled water. Probe results from stool samples were read as positive or negative by comparison with control strains on the same filter paper. Indeterminate results, defined as samples showing a slight coloration compared with the negative control, were read as negative.

In a subset of 27 stool specimens, both undiluted stool and a 1:5 dilution (vol/vol) of whole stool in phosphate-buffered

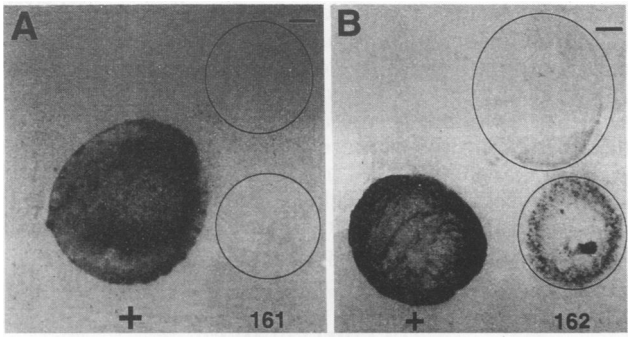

FIG. 1. (A) *ipaH* probe hybridization result for a stool specimen which was culture negative for *Shigella* spp. and EIEC (161) after incubation with substrate to allow detection. Probe hybridization results for the positive control bacterial strain (*S. flexneri* M90TW [+]) and the negative control bacterial strain (*E. coli* HB101 [−]) are shown in panels A and B. Both control strains were from the collection of the Walter Reed Army Institute of Research. (B) Probe hybridization result for a stool specimen which was culture positive for *S. flexneri* (162). The dark coloration of the filter paper circle inoculated with stool specimen 162 indicates a positive *ipaH* probe result, correlating with the presence of *Shigella* spp. in the same specimen.

saline (PBS) were inoculated in parallel on culture media. Diluted stool specimens were processed for hybridization by the same procedure described above for undiluted stool.

RESULTS AND DISCUSSION

Two hundred nineteen patients with dysentery were evaluated by stool culture, and 59 were observed to be culture positive for *Shigella* spp. Of these 59 stools samples, the organism was isolated by method A (immediate plating on MacConkey agar) in only 33 cases (56%), while better recovery was obtained by method B (multiple assays from Cary-Blair transport medium; 56 of 59 stool samples [95%]) and method C (culture from stool sample saved for *ipaH* probe; 50 of 59 stool samples [85%]). The pathogens identified from patients with diarrhea included *S. flexneri* (47 cases), *Shigella sonnei* (4 cases), *Shigella boydii* (7 cases), *Shigella dysenteriae* (1 case), EIEC (3 cases), *Salmonella* spp. (9 cases), *Campylobacter jejuni* (25 cases), *Aeromonas* spp. (19 cases), *Vibrio cholerae* (1 case), and *Vibrio fluvialis* (1 case). For eight patients, two pathogens were isolated from the same stool specimen.

Results of bacteriologic cultures were as follows: *Shigella* spp., $n = 59$ stool samples; EIEC, $n = 3$; other enteropathogens, $n = 50$ (includes 3 stool samples from which both a *Shigella* sp. and another pathogen were isolated); no pathogen isolated, $n = 110$. *ipaH* probe results were as follows. For stool samples from which *Shigella* spp. were isolated, 50 were *ipaH* probe positive and 9 were *ipaH* probe negative. For stool samples from which *Shigella* spp. were not isolated, 7 were *ipaH* probe positive (three cases of confirmed EIEC infection were included in this analysis) and 153 were *ipaH* probe negative. Examples of probe-positive and probe-negative stool samples are shown in Fig. 1. Three of the 50 *ipaH* probe-positive and culture-confirmed *Shigella* cases were detected only by virtue of the direct plating of the stool sample at the bedside (method A) or in the laboratory (method C) and were not detected by routine culture of stool inoculated into Cary-Blair transport medium (method B). These three specimens would likely have been missed by the culture technique used in most epidemiologic surveillance studies but were detected by the *ipaH* probe.

The reasons why 9 of the 59 culture-confirmed cases of shigellosis were not detected by the *ipaH* probe are not clear. For three of these cases probe results were indeterminate, partially explaining this anomaly. Two additional patients were receiving antibiotics at the time of stool sampling, which may have decreased the inoculum of *Shigella* spp. to a level below the threshold of probe detection while allowing a few surviving colonies to be detected by culture. It is possible that low numbers of *Shigella* spp. in stool samples cannot be detected by the *ipaH* probe because of their growth inhibition by other enteric flora.

Of the 160 stool samples which were culture negative for *Shigella* spp., 153 were observed to be probe negative but 7 were probe positive. *E. coli*-like isolates (five colonies for each specimen) were available from four of the seven probe-positive stool specimens that were *Shigella* negative by culture. These *E. coli* isolates were evaluated for the presence of EIEC by the Sereny test, assessing Congo red dye binding ability, and plasmid analysis to detect the presence of a large ≈200 kbp invasion-related plasmid. Three of the four stool specimens analyzed contained EIEC that met the criteria mentioned above. In the fourth probe-positive, *Shigella* culture-negative stool specimen which did not have EIEC by these criteria, the patient had received both ampicillin and dicloxacillin within the previous 3 days. The antibiotic therapy may have eradicated either *Shigella* spp. or EIEC from this specimen. Thus, the *ipaH* probe detected EIEC which could not be detected by stool culture alone.

In an attempt to see whether stool dilution would enhance probe detection of *Shigella* spp. in stool samples by reducing the effect of crowding by other stool flora, a limited number of separate stool specimens were also probed after dilution in PBS. No attempt was made to predict the ideal stool dilution ratio for probe detection in the present study. Twenty-seven culture-confirmed *Shigella*-containing stool specimens were evaluated by probe assays by using both undiluted stool and a 1:5-diluted stool for growth amplification prior to probe processing. Of these 27 specimens, 24 were probe positive by assay of undiluted stool (89%), whereas two additional specimens were found to be probe positive by using diluted stool only (96%). The remaining specimen gave indeterminate results by the *ipaH* probe assay with diluted stool, suggesting that the number of *Shigella* spp. was low. These data confirm the difficulty of probe detection of *Shigella* spp. in culture-proven, undiluted stool samples. Additionally, these preliminary data imply that stool dilution before growth amplification enhances probe detection of *Shigella* spp. in dysenteric stool samples.

By our methodology, the sensitivity of the *ipaH* probe for detection of *Shigella* spp. compared with those of multiple stool culture methods was 84.7% (95% confidence interval [CI], 75.9 to 93.6%) and the specificity was 97% (95% CI, 94.4 to 99.6%). This technique also provided a positive predictive value for the diagnosis of *Shigella* spp. of 93% (95% CI, 86.4 to 99.6%) and a negative predictive value for the diagnosis of *Shigella* spp. of 94% (95% CI, 90.4 to 97.6%). No systematic attempt to detect EIEC by serotyping or HeLa cell invasion assays was possible, so sensitivity and specificity for EIEC detection cannot be inferred from these results. The *ipaH* probe has demonstrated higher sensitivity in a laboratory setting (8), and these preliminary figures probably do not reflect the optimal efficacy of the *ipaH* probe in a field setting. Further investigations of probe analysis

with diluted stool specimens and the use of the polymerase chain reaction should increase the utility of the *ipaH* probe in the future. Because the present study was conducted in a highly selected population with a high prevalence of *Shigella* infections, further studies are also needed in a more typical situation with a lower prevalence of *Shigella* infection.

Several factors may have affected the reported probe sensitivity and specificity. Although the *ipaH* probe detected most cases of bacteriologically confirmed shigellosis accurately, some cases in patients whose stool samples grew few *Shigella* colonies that were seen on the culture plate were missed by the probe. The failure to detect *Shigella* spp. by probe analysis in these patients may have been due to the presence of substances in the stool samples that inhibit hybridization and/or substances (e.g., antibiotics) that inhibit the growth of *Shigella* spp. from undiluted stool when few organisms are present. One must also consider the fact that the probe assay data were compared with combined stool culture data obtained from three parallel stool culture procedures, as described in Materials and Methods. In effect, our stool culture protocol was designed to optimize the recovery of *Shigella* spp. to an extent that standard stool culture procedures in most clinical laboratories would not match. Another factor which may influence the sensitivity of the probe is the variable number of *ipaH* gene copies found in different *Shigella* strains, resulting in an increased ease of detection of strains with a greater number of gene copies. Indeterminate test results, defined as the development of slightly increased coloration compared with that of the negative control, accounted for approximately 5% of negative probe assay results in the 162 probe-negative specimens. In fact, several of these indeterminate assays corresponded to stools with a known positive culture for *Shigella* spp. We cannot exclude the possibility that additional probe-indeterminate but culture-negative specimens contained *Shigella* spp. or EIEC which were not detected bacteriologically.

DNA probe assays required approximately 12 h of actual processing time, including incubations. On a practical level, the assay could be conducted in a reasonable fashion by laboratory technicians working standard 8-h days in 1.5 days (36 h).

One interesting, but not unexpected, finding was the detection via *ipaH* probe analysis of three EIEC strains whose identities were confirmed in retrospect. Resource constraints prevented us from screening all *E. coli* isolates and may have precluded the detection of more EIEC strains. As a practical matter, EIEC strains have been overlooked by many clinical laboratories because of the difficulty in diagnosing them. The inability of the *ipaH* probe to distinguish between *Shigella* spp. and EIEC strains does not present a problem in clinical management, since the clinical manifestations and therapy for patients with dysentery caused by both pathogens are similar. As an epidemiologic tool, the *ipaH* probe shows great promise for screening several stool specimens on a single 15-cm-diameter filter paper, obviating the extra cost and time required for stool cultures and EIEC colony testing. However, further studies are needed to define the efficacy of the *ipaH* probe in the diagnosis of EIEC.

Our preliminary data have demonstrated that the *ipaH* probe hybridization procedure with a nonradiolabelled probe is a useful diagnostic technique for *Shigella* spp. and EIEC in a developing-country laboratory. Because *ipaH* is a multicopy gene, the *ipaH* probe is a very promising tool for epidemiologic field applications. The specificity of the *ipaH* sequence for *Shigella* spp. and EIEC, in conjunction with the added sensitivity afforded by the polymerase chain reaction, has the potential to allow the *ipaH* probe hybridization procedure to be developed into a powerful diagnostic tool. Further assay modifications are essential to develop the full potential of this very promising *ipaH* gene detection technique.

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