Identification of Enteroinvasive Escherichia coli and Shigella Strains in Pediatric Patients by an IpaC-Specific Enzyme-Linked Immunosorbent Assay

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Received 20 November 1996/Returned for modification 4 March 1997/Accepted 27 March 1997

A new method, a monoclonal antibody-based enzyme-linked immunosorbent assay (ELISA) recognizing a secreted, invasion plasmid-coded protein antigen (IpaC), was used to identify enteroinvasive Escherichia coli and Shigella strains among colonies from 859 cultures of fecal samples from children in Kuwait. A total of 33.8% of the samples were diarrheal. By the immunoassay, enteroinvasive E. coli strains were identified from two diarrheal samples but from none of the samples from children without diarrhea. These strains were fully virulent and belonged to serogroup O28ac. In addition, 26 Shigella strains were also recognized by the ELISA, while only 23 were isolated by routine biotyping and serotyping. For two diarrheal patients, Shigella was identified by culture only. The study showed that the IpaC-specific immunoassay is a simple and useful tool for identifying enteroinvasive strains. Furthermore, by reporting the first enteroinvasive E. coli isolates from Kuwait, the study indicates the presence of this group of pathogens as a potential source of diarrhea in the region.

The microbiological diagnosis of bacillary dysentery is based on the selective isolation and biochemical identification of the four Shigella species, S. dysenteriae, S. flexneri, S. boydii, and S. sonnei. The result is then confirmed and refined by determining the serotype of the isolate (3). Recently, in order to improve the sensitivity, specificity, and speed of the diagnosis, new molecular biological methods have been developed. These techniques are based either on nucleic acid hybridization with DNA probes specific for various virulence-related genes (2, 25) or on a PCR technique with primers flanking these sequences (9). Some target genes are located on the invasion plasmid (IP) or, like the ipaH gene, on both IP and the chromosomes of these pathogens (21, 25). These methods were successfully used in case-control studies (5) or investigations aimed at detecting posttreatment Shigella carriage (21).

A definite advantage of these molecular biological techniques is that they also identify the other, often overlooked causative agents of bacillary dysentery, enteroinvasive Escherichia coli (EIEC) strains carrying the same virulence-related genes carried by shigellae (11). The enteroinvasive character had long been recognized in some 10 E. coli serogroups (6); however, not all members of these serogroups express the invasive phenotype. Although invasive isolates are often non-motile, lactose negative, and lysine decarboxylase negative (22), the detection of these markers, similar to that of the O antigens, is not specific or sensitive enough for diagnostic purposes (3). An important reason for our rather limited understanding of the epidemiology of EIEC infections is the difficulty in identifying these strains by simple laboratory techniques.

In order to offer a specific and simple technique for the microbiological diagnosis of enteroinvasive infections, we developed an enzyme-linked immunosorbent assay (ELISA) that can be used to identify EIEC and Shigella strains (17). In the original form of the method, an absorbed rabbit antiserum recognizing IP-coded antigens (tentatively called virulence marker antigen [VMA]) was used (17). Although the procedures for the preparation and standardization of the absorbed sera were cumbersome, the VMA ELISA proved the feasibility of this approach (24). Recently, the assay was further developed and simplified by introducing a monoclonal antibody (MAIC-1) specific for IpaC (8), a component of the tentative VMA (19). While growing in the microcultures of the wells of the ELISA plate, enteroinvasive bacteria secrete IpaC (1), thus sensitizing the plate for antigen detection by the monoclonal antibody (8). So far, however, the specificity and sensitivity of this assay have been assessed with a panel of selected invasive and noninvasive strains only (8), but they have not been evaluated with clinical material. Here we report the results of the first clinical application of the MAIC-1 ELISA to the identification of EIEC and Shigella strains in pediatric patients.

MATERIALS AND METHODS

Patients. A total of 859 fecal samples received from pediatric patients presenting at the Mubarak Al-Kabeer Hospital, Kuwait, were investigated. All fecal samples received were included in the investigation, no preliminary criteria concerning the clinical diagnosis or the symptoms of the patients being used. No special cautions were exercised to exclude from the study samples sent repeatedly from the same patients.

Routine bacteriology, biotyping, and serotyping. The fecal samples were processed in the Microbiology Laboratory of Mubarak Al-Kabeer Hospital according to the guidelines for public health laboratories in Kuwait (13) for common bacterial enteric pathogens, i.e., Shigella, Salmonella, Campylobacter, and enteropathogenic E. coli. The macroscopic appearance of the specimen was recorded as formed, loose, or watery, and the presence of blood or mucus was noted. Loose and watery samples were macroscopically examined for the presence of crythrocytes and leukocytes (RBCs and WBCs, respectively). For the isolation of Shigella, MacConkey (MC) (Oxoid, Unipath Ltd., Basingstoke, United Kingdom) and salmonella-shigella (SS) (Difco Laboratories, Detroit, Mich.) agar plates were inoculated before and after Selenite-F broth (Mast Laboratories Ltd., Hespersons, United Kingdom) enrichment. The plates were investigated by the technical personnel of the Microbiology Laboratory of Mubarak Al-Kabeer Hospital.
Hospital. Colonies suspected of being *Shigella* were isolated on Kliger's iron agar slopes (KIA; Mast Laboratories Ltd.) and urea broth. On the next day, KIA cultures showing reactions typical of *Shigella* were serotyped with a set of *Shigella* typing sera (Murex Diagnostic Ltd., Dartford, England). The species was confirmed in the Vitek automatic system by using the Vitek GNI card (bioMerieux, Marcy l’Etoile, France). Agglutination of eight to nine colonies from the MC agar plate for patients younger than 2 years of age was done with Murex diagnostic pooled sera 2, 3, and 4 for pathogenic agar plate for samples from patients younger than 2 years of age was done with Murex diagnostic pooled sera 2, 3, and 4 for pathogenic *E. coli*. Serotyping of suspect EIEC isolates identified by MAIC-1 ELISA (see below) were carried out with diagnostic sera produced at the Department of Bacteriology, “Bela Johan” National Institute of Public Health, Budapest, Hungary.

**MAIC-1 ELISA.** After isolating colonies for biotyping and serotyping, MC and SS colonies with primary cultures were used to pick colonies for the MAIC-1 ELISA. A person unaware of the culture results selected three colonies, possibly with different morphologies and lactose fermentation patterns, from each plate. The only restriction in choosing various colony types for the immunoassay was that colonies with signs of H2S production on the SS plate were left untouched. Altogether, six colonies from each patient’s sample were individually inoculated into 6 wells of a 96-well sterile tissue culture plates (Nunclon Delta; Nunc, Roskilde, Denmark) (hereafter referred to as ELISA plate) filled with 200 µl of trypic soy broth. Simultaneously, these colonies were also inoculated onto a Congo red (CR) plate (15) as macrocolonies.

No perimeter rows or columns of the 96-well plates were used for the assays. On each plate three wells were inoculated with a virulent (YSH 6500) and a nonvirulent (YSH 6200) *S. flexneri* 2a strain (provided by C. Sasakawa, Tokyo, Japan), serving as positive and negative controls, respectively, in the immunoassay. Inoculated ELISA and CR plates were incubated overnight at 37°C. On the next day the MAIC-1 ELISA was run as described previously (8). Briefly, the microcultures in the wells were discarded and the free binding sites were blocked for 1 h at room temperature with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS; pH 7.2). The plates were washed three times with PBS containing 0.05% Tween 20 (PBS-Tw), and then the IpaC-specific monoclonal antibody was added as a serum-free supernatant of the cell culture medium (RPMI 1640; Gibco BRL, Life Technologies Ltd., Paisley, England) of the hybridoma cell line MAIC-1-3 (8), which was diluted 1:250 in PBS-Tw containing 0.1% BSA (PBS-Tw-BSA). The plates were incubated for 1 h at 37°C, washed, and reacted with anti-mouse immunoglobulin-horseradish peroxidase (P260; Dakopatts, Copenhagen, Denmark). Reactions were developed with 1.2-phe- nylenediamine substrate (Dakopatts), and the optical density (OD) at 490 nm was measured on a Dynatech MR 5000 ELISA reader.

Previously (8), it was shown that a cutoff value for the positive reaction in the ELISA, defined as the mean + 3 standard deviations for nonvirulent isolates, differentiated between enteroinvasive and nonenteroinvasive strains in preliminary experiments with a set of virulent and nonvirulent *Shigella* and EIEC strains from our strain collection, this value was determined for the purpose of the present study to be 0.150. In case of a positive reaction (i.e., OD ≥ 0.150) the corresponding colony taken from the CR plate was retested by the MAIC-1 ELISA. In order to recognize isolates binding to the enzyme-conjugated anti-mouse antibodies (i.e., false-positive results), the assay was also carried out with the anti-mouse-horseradish peroxidase conjugate only, replacing the IpaC-specific antibodies with PBS-Tw-BSA.

Clones that were saved on the CR plate and that showed a positive reaction in the ELISA and all six selected clones from samples from which an enteroinvasive pathogen was isolated in the hospital laboratory were also subjected to biochemical and serological identification, as described above.

**Confirmatory assays for enteroinvasiveness.** All *Shigella* and *E. coli* isolates recognized by the MAIC-1 ELISA were tested for their capacity to invade HEp-2 cells (10). After Giemsa staining of the infected tissue cultures, the invasive potentials of the strains were calculated as the number of infected cells of 200 cells examined and was expressed as the percentage of that for the positive control strain, strain YSH 6000. The *E. coli* isolates exhibiting a positive reaction in the immunoassay were also investigated for the expression of the characteristic pattern of colonization factor encoded proteins detected by sera from convalescent-phase dysenteric patients (16) received from Alf A. Lindberg, Stockholm, Sweden. The detection of bacteria, polyacrylamide gel electrophoresis, and Western blotting (immunoblotting) were carried out as described previously (19). As ultimate proof of the enteroinvasive character, *E. coli* isolates positive by the assays described above were subjected to the Sereny test (i.e., the guinea pig keratoconjunctivitis assay) (20).

**RESULTS**

Altogether, 859 samples were included in the study. On the basis of macroscopic observations, 33.8% of the samples were scored as loose or watery, of which 8.2% contained macroscopic blood. Microscopically, RBCs were detected in 23.4% of the diarrheal samples and WBCs were detected in 27.5% of the diarrheal samples. Sixty-eight nontyphoid *Salmonella* and 12 *Campylobacter jejuni* strains were isolated. One patient excreted both *Salmonella enteritidis* and *C. jejuni*. From the patients younger than 2 years of age, 11 *E. coli* strains belonging to enteropathogenic *E. coli* serogroups were isolated.

The results of the isolation and identification of enteroinvasive bacteria are presented in Table 1. *Shigella* species were identified either by culture or by ELISA in 28 samples, all from different patients. All 28 samples were marked as loose or watery (3.25% of the total and 9.62% of the diarrheal samples). Twenty-six of these samples contained RBCs and WBCs, and 8 of the samples were macroscopically bloody. By biochemical and serological assays, *Shigella* organisms were identified, all from the primary culture plates, in 23 samples, and no further strains were found after enrichment in Selenite-F broth. By MAIC-1 ELISA, members of the genus were detected among colonies from 26 samples. In four of the five samples in which *Shigella* strains were missed by routine culture, the pathogen grew as a few colonies of non-lactose fermenters (NLF) on the MC agar plate only in a heavy lactose fermenter background. In the fifth case a few NLF colonies of *S. dysenteriae* were also missed on the SS plate in a similar background.

The MAIC-1 ELISA did not detect one *S. sonnei* isolate and one *S. flexneri* isolate from diarrheal patients in whom the pathogen was identified by culture (Table 1). By microscopy, RBCs and WBCs were detected in both mucoid samples, while no macroscopic bloody stool was seen. Reinvestigating the colonies tested by ELISA from the *S. sonnei*-infected patient revealed that two of the six colonies (both taken from the MC agar plate) were biochemically *S. sonnei*, indeed, agglutinating in the phase I and II typing sera. However, both clones were CR-negative and lost their invasive capacity, as tested on tissue culture cells, indicating the loss of the IP coding for the target antigen of the immunoassay (data not shown). No phase I, CR-positive, invasive, ELISA-positive colonies were found on reinvestigations of 20 *S. sonnei* colonies from the MC and SS agar plates. In the sample from the other patient excreting *Shigella* but in which *Shigella* was not identified by the immunoassay, two of the six colonies tested proved to be *S. flexneri*. The colonies were taken from MC and SS agar plates, respectively. Similarly to the *S. sonnei* isolate, these colonies did not bind to CR and they were noninvasive (data not shown). However, after reinvestigating the SS and MC agar plates containing the sample from this patient, 5 ELISA-positive, invasive colonies of 20 colonies tested were found.

By the MAIC-1 ELISA we could identify two *E. coli* strains secreting the IpaC antigen. They expressed the O28ac cell wall

**TABLE 1. Identification of enteroinvasive bacteria among colonies isolated from 859 fecal samples**

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of strains identified by ELISA</th>
<th>By biotyping and serotyping</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. dysenteriae</em></td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><em>S. flexneri</em></td>
<td>13</td>
<td>12</td>
<td>14</td>
</tr>
<tr>
<td><em>S. boydii</em></td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>S. sonnei</em></td>
<td>12</td>
<td>11</td>
<td>13</td>
</tr>
<tr>
<td>All <em>Shigella</em></td>
<td>26</td>
<td>23</td>
<td>28</td>
</tr>
<tr>
<td><strong>EIEC</strong></td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>28</td>
<td>23</td>
<td>30</td>
</tr>
</tbody>
</table>

* Total number of strains identified by ELISA and/or biotyping and serotyping.
antigen and fermented lactose, but they were unable to decarboxylate lysine. Both isolates were nonmotile. The isolates formed red colonies on CR plates, and they were invasive in the tissue culture invasion assay, showing 15.2 and 12.3% invasive capacities compared to that of the positive control strain. They expressed the characteristic protein pattern (i.e., VirG and Ipa A, B, C and D) (16) of the virulent strains when tested with reconvalescent-phase human serum by polyacrylamide gel electrophoresis-Western blotting and were positive by the Sereny test (data not shown). The two EIEC strains were isolated from independent patients presenting with diarrhea at the casualty section of the hospital. The stool samples were loose but contained no macroscopic blood. They showed moderate numbers of RBCs and WBCs (i.e., less than 10/high-power field).

The OD values for the Shigella strains identified by ELISA varied between 1.405 and 2.000, while those for the two EIEC isolates were 0.962 to 1.111. Of the total of 5,154 colonies from the 859 samples tested by ELISA, 3 colonies from three independent patients gave false-positive, low OD readings (0.205 to 0.322). All three isolates were shown to bind to the conjugate containing samples from four of five patients in whom shigellae were identified by the immunoassay only but not by culture. It is known that for some Shigella strains, media with selective power better than that of MC agar could be inhibitory (3). If no NLF colonies were seen on the highly selective plates (e.g., agar plates), the culture could mistakenly be declared negative.

The colonies of the two Shigella strains selected for but not identified as IpaC secretors by ELISA had lost their IpaC-producing capacity as well as invasive capacity due to the probable loss of or deletion from the IP. We surmise, however, that encountering noninvasive colonies only from clinical material should be a relatively infrequent phenomenon (one S. sonnei isolate from the 30 patients excreting either Shigella or EIEC in this study) due to the counterselection of the non-virulent clones in the host. In the ELISA the problem of plasmid instability may partially be overcome by testing larger numbers of colonies from individual patients. This was found to be the case for the S. flexneri-infected patient not identified by the immunoassay, for whom, upon reinvestigation, 5 of 20 S. flexneri colonies were still found to secrete IpaC. It should be noted that the loss of or deletion from the IP may result in a negative reaction with the DNA probes or by PCR as well unless the deletion leaves the corresponding sequences intact (18) or genes also present on the chromosome are targeted (25).

The present study did not aim to compare the diagnostic performances of ELISA to that of the molecular biological techniques; therefore, no screening assays other than the ELISA were used to identify EIEC. However, by using the polyclonal antibody- and monoclonal antibody-based versions of this assay, it has previously been found that of the total of ca. 5,500 E. coli strains from various strain collections tested so far, not a single virulent EIEC strain was missed by the method (8, 24). Therefore, we believe that among the total of 5,154 colonies tested in this study, none but those identified as Shigella or EIEC expressed the IpaC antigen, i.e., were enteroinvasive.

Weak false-positive reactions in this assay are known to occur rarely (8). In the present study 3 of 5,154 colonies tested (0.058%) fell in this category, but due to their low frequency and to the fact that they could easily be identified in repeated or parallel assays run without the IpaC-specific antibodies, they hardly indicate a serious diagnostic problem.

The labor requirement of this immunoassay is relatively low, and it could provide a positive identification in 24 h. Since the ELISA technique has been used for various purposes in many laboratories in developing countries, the method could easily fit into the diagnostic armory, even in clinical laboratories with relatively low levels of technical sophistication. One could envision the use of the assay as a screening method, as was done in this study, or it could be applied only to isolates recovered from diarrheal samples in which no other enteric pathogens were identified.

On the basis of these results we believe that the IpaC-specific MAIC-1 ELISA offers a specific and relatively inex-
pensive technique for the identification of enteroinvasive strains. This is particularly true for the members of the EIEC group, for which no other simple alternatives are available.

ACKNOWLEDGMENTS

This work was supported by grants MI O92 from Kuwait University and T 016190 from the National Scientific Research Foundation (OTKA), Hungary.

The skillful technical assistance of Akbar P. Kalandath is highly appreciated.

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