Immunomagnetic Separation and DNA Hybridization for Detection of Enterotoxigenic Escherichia coli in a Piglet Model

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Enterotoxigenic Escherichia coli (ETEC) strains were detected by stool blot hybridization assays using different oligonucleotide probes for the colonization fimbrial antigen F4, heat-stable enterotoxin I (ST I), and heat-labile enterotoxin (LT I) genes. Forty-eight fecal samples and seven samples of intestinal content from ETEC-challenged newborn piglets were processed in two ways: (i) by direct inoculation of bacterial suspension onto nylon membranes overlaying blood agar and (ii) by immunomagnetic enrichment of F4+ ETEC using magnetic beads coated with F4 monoclonal antibodies before inoculation onto nylon membranes. In samples obtained from nondiarrheic piglets pre- and postchallenge, E. coli genes for F4, ST I, and LT I could be detected only after immunomagnetic enrichment. No difference between the two methods in detection of these E. coli genes was observed when stool blots from diarrheic piglets were examined. By using magnetic separation, it was easy to decrease background bacterial flora, intestinal cells, and fecal debris and thus produce purer specimens. The method evaluated in this animal model appeared simple and quick and increased the sensitivity of detection of ETEC strains 100-fold compared with the direct stool blot hybridization assays. Prior bacterial isolation and identification were not necessary.

MATERIALS AND METHODS

Experimental design. The animals used were 10 colostrum-fed piglets from a nonvaccinated primiparous sow reared in a conventional herd. The sow was not vaccinated against E. coli diseases. Colostrum and serum collected on the day of birth did not contain antibodies against the F4 antigen (assessed by enzyme-linked immunosorbent assay). Five of the piglets were randomly selected and challenged orally when 1 day old with 3 ml of a broth culture containing 107 cells of the hemolytic, F4-positive, ST I-positive, and LT I-positive E. coli strain NVH 3906 per ml. Their litters were kept in the same pen and thus acted as in-contact control animals. Clinical symptoms were recorded during the observation period of 9 days postchallenge, and no treatment was given to the piglets.

Specimens. Stool specimens from each piglet were obtained before challenge and during the observation period. Diseased piglets were autopsied, and samples of jejunal content were collected. Altogether, 10 stool samples obtained prechallenge, 38 stool samples obtained postchallenge, and 7 samples of jejunal content were examined.

Preparation of specimens. Rectal and jejunal swabs were put into 2 ml of phosphate-buffered saline (pH 7.2). Debris was allowed to settle for 30 min. The samples were then processed in two ways: (i) direct inoculation of 1 μl of the fecal suspension onto nylon membranes (Hybond N; Amersham International, Amersham, United Kingdom) overlaying blood agar or (ii) immunomagnetic separation of F4+ ETEC strains with magnetic beads (Dynabeads M-450; Dynal, Oslo, Norway) coated with F4 monoclonal antibodies and subsequent inoculation onto nylon membranes. Immunomagnetic separation was performed essentially as described by Lund et al. (11). Briefly, 400 μg of monodisperse immunomagnetic beads was added to 1 ml of the fecal suspension, incubated, washed, and reconstituted to 40 μl. Subsequently, 1 μl of beads with bacteria attached to them was inoculated onto nylon membranes. Control strains of E. coli with appropriate F4, ST I, and LT I phenotypes were

Enterotoxigenic Escherichia coli (ETEC) strains belonging to serogroup O149 and possessing the F4 antigen are common causative agents of diarrhea in Norwegian piglets (3, 10). The identification of pathogenic strains on the basis of biochemical reactions and serological tests usually requires isolation of a pure culture from the total bacterial population present in stools. ETEC strains can also be identified by detecting genes for enterotoxins and adhesins by using nucleic acid hybridization (4, 5). This method has been used to detect genes in isolated strains of E. coli as well as directly in bacterial growth from stool specimens (2, 13). Examination of stool blots may be faster and easier to perform than testing individual E. coli colonies. However, stool specimens may prove to be less suitable for use in direct hybridization assays. Echeverria et al. (4) reported that nonhomologous DNA or bacterial cell wall remnants in stool specimens interfered with the access of specific probes to target cell DNA. Several authors have tried to improve methods of processing the specimens, including isolation of crude nucleic acids from stool samples (2, 13, 18). Immunomagnetic beads are superparamagnetic, monosized polymer particles coated with antibodies (19). These beads have been used successfully to enrich both bacteria and mammalian cells of certain specificities from mixed cultures (9, 11, 16).

In this study, colony blot hybridization assays were performed to detect genes encoding F4 fimbriae and heat-stable enterotoxin I (ST I) and heat-labile enterotoxin I (LT I) production. We compared two hybridization procedures, direct hybridization and hybridization preceded by immunomagnetic separation, with regard to the ability of the two methods to detect F4+ ETEC strains in stool specimens and samples of intestinal contents from piglets.

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included on each membrane. All specimens and control strains were inoculated in duplicate.

**Oligonucleotide probes.** The probes for detection of ST I genes consisted of a mixture of two 22-mer probes encoding ST Ia and ST Ib as reported by Hill et al. (8). The probe for detecting the genes coding for LT I was a 20-mer oligonucleotide as described by Furrer et al. (6). The probe for detection of genes encoding the F4 fimbrae corresponded to the nucleotide sequence of the F4 gene from positions 146 to 164 (7, 20). All the oligonucleotide probes were synthesized on an Applied Biosystems synthesizer (Applied Biosystems International, Foster City, Calif.) and labelled with $^{32}$P at the 5' end by using T4 polynucleotide kinase (Bethesda Research Laboratories, Oxbridge, Middlesex, England) according to the method of Sambrook et al. (14). The labelled probes were all purified on a Sephadex G-50 column (5, 14).

**Hybridization assays.** Replica stool blots were prepared as described by Moseley et al. (12). DNA was fixed to the membranes by UV light exposure and hybridized with F4, ST I, and LT I probes. Hybridization experiments were performed under stringent conditions as previously described (1, 14).

**Estimation of the number of hemolytic and nonhemolytic E. coli colonies and control experiments.** The numbers of hemolytic and nonhemolytic colonies of E. coli in the suspensions were assessed by a standard plate count procedure. The method did not allow detection of colonies occurring in numbers of $<10^2$ CFU/ml. Hemolytic colonies on blood agar plates were identified by appropriate biochemical reactions. Colony blot assays for detection of F4, ST I, and LT I genes were performed with the applied oligonucleotide probes. Immunomagnetic beads were inoculated onto nylon membranes in order to examine unspecific binding of the probes used in the study.

**RESULTS**

**Clinical results.** All five challenged piglets and two of the in-contact piglets died within 3 days postchallenge. With the exception of one of the in-contact piglets (piglet F), all of the piglets which died had severe diarrhea before death. Three in-contact piglets remained healthy throughout the observation period.

**Quantitative results.** The results are presented in Tables 1 and 2. Fecal specimens obtained from the piglets prechallenge were dominated by nonhemolytic strains of E. coli occurring at levels of approximately $10^6$ to $10^7$ CFU/ml. Hemolytic colonies of E. coli were not detected. In fecal specimens from challenged piglets obtained 7 h postchallenge, the challenge strain occurred in amounts of approximately $10^4$ to $10^5$ CFU/ml in all samples, three of which did not contain nonhemolytic colonies of E. coli. Only hemolytic

### Table 1. Amount of hemolytic and nonhemolytic E. coli in fecal and jejunal content samples from challenged piglets

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Time obtained postchallenge (h)</th>
<th>Results for piglet:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$^{\text{A}} \log_{10}$ (CFU/ml)$^b$</td>
<td>$^{\text{B}} \log_{10}$ (CFU/ml)</td>
</tr>
<tr>
<td></td>
<td>N H</td>
<td>N H</td>
</tr>
<tr>
<td>Fecal</td>
<td>0</td>
<td>6 &lt; 2</td>
</tr>
<tr>
<td>Fecal</td>
<td>7</td>
<td>6 7+</td>
</tr>
<tr>
<td>Jejunal</td>
<td>20</td>
<td>&lt; 2 7+</td>
</tr>
</tbody>
</table>

$^a$ log$_{10}$(CFU per milliliter) of hemolytic (H; challenge strain) and nonhemolytic (N) E. coli strains in indicated samples.

$^b$ +, positive in all DNA hybridization assays after immunomagnetic enrichment for F4, ST I, and LT I; −, negative in all such assays.
colonies of *E. coli* were detected in the jejunal content of all five challenged piglets, whereas a mixed bacterial flora of nonhemolytic and hemolytic strains of *E. coli* was detected in the jejunal contents of the two diseased in-contact piglets. In specimens from the three healthy in-contact piglets, nonhemolytic strains of *E. coli* dominated the bacterial flora throughout the observation period, being present at levels of approximately 10^7 to 10^9 CFU/ml. During the period from 20 to 32 h postchallenge, the hemolytic challenge strain appeared for the first time in fecal specimens from three of the five in-contact piglets. In the healthy piglets, the hemolytic challenge strain appeared irregularly and in low numbers, ranging from 10^2 to 10^3 CFU/ml, in samples during the observation period. The challenge strain could not be detected in the samples collected from one of the in-contact piglets (J).

**Hybridization results.** The results are presented in Tables 1 through 3. ETEC strains carrying genes encoding F4 fimbriae, ST I, and LT I were found in 2 of the 10 samples obtained before challenge when immunomagnetic separation was used. None were detected by the direct method. F4, ST I, and LT I genes were detected at almost the same rate in samples from diseased piglets (both fecal specimens and jejunal content), irrespective of the method of preparation. None of the samples from one of the diseased in-contact piglets (P) hybridized with the probes. F4-, ST I-, and LT I-encoding genes were present in 6 of 30 samples obtained postchallenge from the three healthy piglets. These were detected only when immunomagnetic separation was used. In the case of piglet G, ST I gene-positive samples were obtained at 26 h and at 2 and 6 days postchallenge. In the case of piglet I, the fecal specimens containing the F4, ST I, and LT I genes were obtained at 32 h and at 2 days postchallenge, while all of the samples collected from piglet J were negative.

**Results of control experiments.** All of the hemolytic colonies examined were identified as the *E. coli* challenge strain. Unspecific binding of the probes to immunomagnetic beads was not detected.

**DISCUSSION**

In the case of nondiarrheic piglets carrying low numbers of F4+ ETEC strains, immunomagnetic separation was a prerequisite for the detection of these strains from fecal specimens both before and after challenge. Two piglets harbored strains of *E. coli* possessing the F4, ST I, and LT I genes prechallenge. The strains were not isolated in pure culture. Upon direct culture or by dilution of the fecal specimens and subsequent culture, hemolytic colonies were not detected. Repeated hybridization assays confirmed previous results. The presence of ETEC strains in healthy piglets is not an unusual finding; a study by Söderling and Möllby demonstrated ETEC strains in 3% of healthy piglets from unvaccinated herds (17). When 1 µl of the fecal suspensions was used as the inoculation volume on nylon membranes, the limit of detection of the challenging strain by the direct hybridization method was 10^2 CFU/ml. When immunomagnetic enrichment was included, the sensitivity was increased 100-fold. However, a negative result was obtained for jejunal content from piglet F, although the hemolytic challenge strain was present at a level of 10^4 CFU/ml (Table 2). This piglet did not show signs of diarrhea before death. In the case of piglet G, a positive result was detected 6 days postchallenge, whereas no hemolytic colonies were observed upon culture (Table 2). According to the literature cited (4, 8, 13, 20), the applied probes are specific and do not hybridize with nonhomologous DNA under stringent hybridization conditions. The number of colonies of the challenge strain in this sample was probably just below the detection limit of the plate count procedure.

Echeverria et al. (4), and Coll et al. (2) found it necessary to decrease the content of nonhomologous DNA, complex polysaccharides, and proteins in stool specimens when performing direct hybridization assays. Immunomagnetic separation offers several advantages for working with clinical specimens, as it avoids time-consuming cultivation and bacteriological diagnostic work. The beads with the attached bacteria are easily separated from suspensions by applying a magnet to the outside of the tube wall. During the washing steps, fecal debris, intestinal epithelial cells, and bacteria not attached to the beads are discarded. The procedure does not require expensive equipment; only a specially designed magnet is needed. Access to specific antibodies recognizing appropriate bacterial surface antigens is, however, a prerequisite. As regards the stool specimens from healthy piglets, F4+ *E. coli* was selected and liberated from background flora by means of immunomagnetic beads, producing a sample containing the specified agent in only a few minutes. Fecal specimens and samples from jejunal content obtained from challenged piglets contained almost a pure monoculture of the *E. coli* challenge strain. Almost identical results were obtained with and without immunomagnetic separation (Table 3), indicating that interfering nonhomologous DNA or other substances were probably of only minor importance when the samples from diarrheic piglets were tested.

Three in-contact piglets did not present symptoms of diarrhea during the observation period. They shed the challenge strain of *E. coli* irregularly and in low numbers or not at all. We assume that the intestines of these piglets may not have been colonized. One explanation might be lack of specific F4 receptor sites on the epithelial cells (15).

Herd health programs require sensitive methods in order to detect carrier animals acting as reservoirs of important pathogens. Immunomagnetic separation in conjunction with the use of DNA probes is a potential alternative to traditional techniques for screening specimens from such animals. In the present study, more *E. coli* infections were identified by the examination of stool blots when this procedure was preceded by immunomagnetic separation than when such an enrichment step was omitted. We therefore propose the use of immunomagnetic beads to enrich bacterial agents occurring in fecal or other specimens.
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REFERENCES