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Enteropathogenic *Escherichia coli* Serotype O111:HNT Isolated from Preterm Neonates in Nairobi, Kenya

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This investigation was initiated as a consequence of several cases of diarrhea in a nursery ward for preterm babies in Nairobi, Kenya. Ten lactose-positive colonies were isolated from the stools of each of 30 neonates, regardless of whether they had diarrhea; 229 strains were identified as *Escherichia coli* and 65 strains were identified as *Klebsiella pneumoniae*. Six strains were lost during laboratory handling. No other bacterial, viral, or parasitic enteropathogens were identified. Using synthetic alkaline phosphatase-labeled probes, the bacterial isolates were found to be negative for the presence of genes coding for heat-stable and heat-labile enterotoxins. Seventy-eight *E. coli* strains isolated from a total of 13 neonates possessed the *E. coli* enteropathogenic adhesion factor (EAF) gene, as demonstrated by the use of a cloned radiolabeled DNA fragment probe. These strains possessed similar plasmid profiles constituting a core plasmid profile, and while all adhered to HeLa cells, none produced Vero cell cytotoxins. The EAF gene was located on a 65-megadalton plasmid. Serotyping showed the strains to be of serogroup O111 and serotype H nontypable, a well known enteropathogenic type. Five neonates died during the outbreak, and the fatality rate was 30.7% (4 of 13) for neonates infected with EAF-positive *E. coli* strains compared with 7.7% (1 of 13) for neonates from whom only EAF-negative *E. coli* strains were isolated. *K. pneumoniae* only was isolated from five neonates.

Enteropathogenic *Escherichia coli* (EPEC) was the first *E. coli* isolate to be implicated in diarrheal disease. The causative agents have traditionally been shown to belong to a relatively small group of distinct serotypes (11, 13, 30; T. A. Gomes, M. A. M. Vieira, I. K. Wachsmuth, P. A. Blake, and L. R. Trabulsi, J. Infect. Dis., in press). The classification of a strain as EPEC is complicated by the fact that, within a serogroup, only certain serotypes have been associated with diarrhea. Another more recently recognized limitation is the mounting evidence that there is significant genetic diversity even within isolates of the same serotype (5). Although serogrouping and serotyping have remained the standard reference methods for the identification of EPEC, several laboratories have encountered serious difficulties in the correct serotyping of these *E. coli* strains (6, 10, 24).

EPEC strains adhere to human small intestinal epithelial cells, causing destruction of microvilli without evidence of further invasion (32, 34, 39, 40). In vitro, EPEC strains adhere to HEp-2 cells or HeLa cells in two distinct patterns, localized adherence or diffuse adherence (28, 35). Localized adherence is encoded by genes located on plasmids with molecular sizes ranging from 55 to 65 megadaltons (MDa) (7, 27). The term enteropathogenic adherence factor (EAF) is used for this plasmid-encoded adherence.

Nataro et al. (26) have described the use of a highly specific and sensitive EAF DNA probe to identify classic serotype EPEC strains isolated from patients with infantile enteritis. This probe has been successfully used to identify EPEC strains isolated from diarrheic infants (6, 10; C. A. Bopp, L. K. Giljahn, V. Hundley, E. G. Sowers, T. J. Barret, J. G. Wells, and I. K. Wachsmuth, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, C113, p. 350).

Nearly all episodes of EPEC diarrhea occur in children who are under 2 years of age. Illness is uncommon in older children and adults, even though they carry the organism (9). However, a few outbreaks have been described in adults. Recent studies from several countries in South America, Asia, and Africa have shown EPEC to be either the first or the second most important bacterial cause of diarrhea in infants (2, 8, 10, 15, 38). The frequency with which EPEC occurs in industrialized countries is generally not known (23, 24). EPEC has been shown to cause sporadic outbreaks of diarrhea in children at day care centers in the United States (31, 34; Bopp et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1988).

The aim of the present study was to identify and characterize the causative agent inducing the above-mentioned outbreak of diarrhea and deaths among preterm neonates in a nursery ward in Nairobi, Kenya.

MATERIALS AND METHODS

Patients and sampling. In November and December 1985, several cases of diarrhea occurred in a nursery ward for preterm babies (ages, 4 to 64 days at the time of sampling) at a hospital in Nairobi, Kenya. A stool sample was taken from each of the 30 neonates in the ward, regardless of whether they had diarrhea.

Microbiological examination. Stool samples were inoculated on a MacConkey agar (Oxoid Ltd., London, United Kingdom) plate within 6 h of collection. Ten lactose-positive colonies from each patient were selected at random and were subcultured onto deep tryptic soy agar (Difco Laboratories,

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Detroit, Mich.) slants in screw-cap test tubes and identified to the species level by the criteria listed by Brenner (4). The stool samples were also examined for other enteropathogens, namely, *Shigella* spp., *Salmonella* spp., *Campylobacter* spp., *Klebsiella pneumoniae*, rotavirus, and intestinal parasites, by standard, routine techniques.

Isolation and separation of plasmid DNA. Plasmid DNA was isolated as described by Kado and Liu (18) and separated by electrophoresis at 8°C in a 1% standard low-endosmotic agarose (Bio-Rad Laboratories, Richmond, Calif.) in Tris phosphate-EDTA buffer (0.8 M Tris, 0.008 M sodium EDTA [pH 8.0]) for 3 h at 120 V and 60 mA. Agarose gels were stained for 10 min in distilled water containing 0.5 μg of ethidium bromide (Sigma, Chemical Co., St. Louis, Mo.) per ml and destained for 20 min in distilled water. The gel was then placed on a UV transilluminator (UVP, Inc., San Gabriel, Calif.), and pictures were taken with a land camera (MP-3; Polaroid, Cambridge, Mass.).

Plasmid profile groups. The different plasmid profiles were grouped by the molecular weights of the plasmids. Strains lacking plasmids were classified as a single plasmid profile group.

Preparation and labeling of the EAF DNA probe. An *E. coli* K-12 strain carrying the plasmid pMAR22 containing the 1-kilobase *Bam*HI-*Sal*I fragment constituting the EAF probe was provided by Steve Mosley (Washington State University, Pullman). Plasmid pMAR22 was isolated by the method of Kado and Liu (18) and was deproteinized and concentrated by using a Gene Clean kit (BIO 101, Inc., La Jolla, Calif.) following the recommendations of the manufacturer. The EAF probe was isolated by cutting 17 ng of pMAR22 with 10 U of *Bam*HI and 10 U of *Sal*I (Toyobo Biochemicals, Osaka, Japan) in medium-level salt buffer. The mixture was incubated at 37°C for 2 h, followed by the addition of 1 μl each of *Bam*HI and *Sal*I and further incubation for 2 h at 37°C. The restriction fragments were separated by electrophoresis by using 1% low-melting-point agarose (Bio-Rad) at 5°C and 50 V (giving 35 mA) for 2 h on a horizontal minigel. Linear DNA fragments of known weights (Bethesda Research Laboratories, Middlesex, England) were included in the minigel and were used to estimate the sizes of the pMAR22 *Bam*HI-*Sal*I fragments. The 1-kilobase pMAR22 *Bam*HI-*Sal*I fragment band was cut out of the gel with a scalpel blade and separated from the agarose by using the Gene Clean kit. The probe was radiolabeled by using a random labeling kit (Amersham International, Amersham, England) based on the method of Feinberg and Vogelstein (14).

Hybridization with the EAF probe. Colony blots were made basically as described by Gruinstein and Hogness (16) by inoculating strains onto nylon membranes (Biotechnology Systems, Boston, Mass.) that were placed onto MacConkey agar (Difco) plates, and the blots were cultivated overnight at 37°C. The membranes were then placed (colony side up) on chromatographic papers moistened with 10% sodium dodecyl sulfate for 3 min and then with 0.5 M NaOH, 1.5 M NaCl, and 0.5 M Tris hydrochloride (pH 8) twice for 5 min each time (16). The membranes were dried, and the DNA was then fixed by exposure to UV light for 5 min. The plasmid bands in the agarose gels were transferred to nylon membranes by the procedure described by Southern (37), and the DNA was fixed to the surface by UV activation for 5 min. The colony and Southern blots were hybridized under stringent conditions with the radiolabeled EAF DNA probe, as described by Amersham International (1). The signals were developed by exposure to X-ray diagnostic film (Eastman Kodak Co., Rochester, N.Y.) with Cronex Hi-Plus (E. I. duPont de Nemours & Co., Inc., Wilmington, Del.) intensifying screen (Molecular Biosystems, San Diego, Calif.) at −70°C for 24 h.

TABLE 1. EAF probe hybridization, serotypes, HeLa cell adherence, and Vero cell cytotoxin properties of *E. coli* strains representing the most commonly observed plasmid profiles of strains isolated from neonates[a]

No. of neonates[b]	No. of strains[c]	Serotype	EAF probe	HeLa adherence
13	78	O111:HNT	+	+
4	25	O34:H12	−	−
3	21	ORough:H12	−	−
2	18	ONT[d]:HNT	−	−
1	1	ORough:H18	−	−
1	10	ORough:H4	−	−
1	7	O127:H12	−	−

[a] All *E. coli* strains were negative for Vero cell cytotoxin.
[b] Number of neonates from which strains with this particular plasmid profile were isolated.
[c] Number of strains isolated from the neonates possessing this specific plasmid profile group.
[d] ONT, O not groupable.

Colony hybridization with LTI and STI synthetic enzyme-labeled DNA probes. The colony blots were prepared as described above for the EAF experiments. The heat-labile enterotoxin I and heat-stable enterotoxin I (LTI and STI) alkaline phosphatase-labeled synthetic DNA probes and the hybridization reagents were obtained from Molecular Biosystems. The hybridization was performed as described by Wasteson et al. (42). Known positive and negative *E. coli* strains (LT^+, ST^+, LT^-, and ST^-) were used as controls.

Serotyping, adhesiveness, and Vero cell cytotoxin. Thirteen representative strains of *E. coli* were serotyped by the standard methods described by Edwards and Ewing (12). These strains were also assayed for adherence to HeLa cells (35). Vero cell cytotoxin testing was carried out as described by Konowalchuk et al. (22).

RESULTS

Microbiological findings. Altogether, 300 lactose-positive enterobacterial strains were isolated from 30 neonates. However, six of these strains were subsequently lost during laboratory handling. Of the remaining strains, 229 strains (77.9%) were found to be *E. coli* and 65 strains (22.1%) were found to be *K. pneumoniae*. No other bacterial, viral, or parasitic enteropathogens were found.

Enterotoxins. All 294 strains examined were found by using enzyme-labeled oligonucleotide probes to be negative for both LTI and STI genes.

Plasmid profile groups. A total of 28 different plasmid profile groups of *E. coli* could be defined, of which 7 major plasmid profile groups representing a total of 160 strains from 12 different patients are presented in Table 1.

Serotypes, HeLa cell adherence, and Vero cell cytotoxin. Serotypes, HeLa cell adherence, and Vero cell cytotoxin production of 13 *E. coli* strains representing seven major plasmid profile groups are given in Table 1. All the EAF probe-positive strains belonged to one plasmid profile group and to serogroup O111 with H nontypable (HNT) antigen; these strains adhered to HeLa cells, but did not produce Vero cell cytotoxin.

Localization of the EAF gene. All of the 294 lactose-positive strains were screened for the presence of the EAF

TABLE 2. Variation in the core plasmid profile of the EAF-positive *E. coli* strains

No. of neonates[a]	No. of strains[b]	Mol size (MDa) of plasmids								
12	67		65[c]	42			3.8	1.9	1.7	1.4
3	5		65	42				1.9	1.7	1.4
2	3		65	42		7.6	3.8	1.9	1.7	1.4
1	1		65	44			3.8	1.9	1.7	1.4
1	1	100	65	42		7.6	3.8	1.9	1.7	1.4
1	1		65	42	12			1.9	1.7	1.4

[a] Number of neonates from whom *E. coli* strains with this particular plasmid profile was isolated.
[b] Number of strains which possessed this particular plasmid profile.
[c] The 65-MDa plasmid carrying the EAF gene.

gene by using the probe, and 78 strains were found to be positive. Table 2 shows the variation in plasmids in the EAF-positive strains constituting the largest profile group. The combination of 65; 1.9; 1.7; and 1.4-MDa plasmids was present in all 78 strains which made up this plasmid profile group. Hybridization, on a Southern blot, of plasmids in strains of this profile group (EAF probe positive) showed that the EAF genes were located on the 65-MDa plasmid (Fig. 1).

Colonization patterns of EAF-positive strains and mortality rate of the neonates. In 20 neonates, all the colonies tested proved to be *E. coli*; in five neonates, all were *K. pneumoniae*. In the remaining 5 neonates, a mixture of both species was found. EAF-positive strains were isolated from 13 of the 30 neonates who were investigated. The case fatality rate for neonates who harbored EAF probe-positive *E. coli* was 30.8% (4 of 13) compared with 7.7% (1 of 13) for those harboring EAF-negative *E. coli* strains only. No deaths occurred in any of the five neonates from whom *K. pneumoniae* strains only were isolated from stool samples.

DISCUSSION

Of the 294 lactose-positive enterobacterial strains found in the present study, colony hybridization with an EAF probe

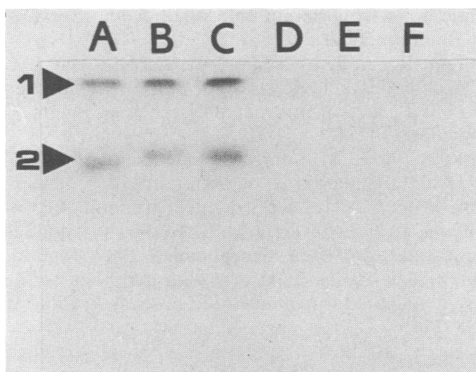

FIG. 1. Hybridization of plasmid DNA transferred to a nylon membrane from an agarose gel by a Southern blot procedure by using the radiolabeled EAF probe. Lanes A, B, and C, Plasmids from serotype O111:HNT strains; lanes D, E, and F, plasmids from O34:H12, ORough:H12, and ONT:HNT strains, respectively (see Table 1). The number 1 indicates the location of the well of the gel, and the 2 indicates the location of the 65-MDa plasmid.

revealed 78 that were positive for *E. coli* strains isolated from a total of 13 neonates. All the EAF probe-positive strains possessed a characteristic core plasmid profile, although some minor divergences in the profiles were observed. Seven of these strains were tested further, and all were found to be serotype O111:HNT. They all adhered locally to HeLa cells, and none produced Vero cell cytotoxins. These strains were typical enteropathogenic types according to previously published information (24), and the homogeneity of the strains indicated that they might have originated from one bacterial clone.

Karch et al. (19) have demonstrated that only 24% of a collection of EPEC strains produced Vero cell cytotoxins; among these several belonged to O group 111. This indicates that EPEC could be capable of causing diarrhea without producing cytotoxin(s). Other workers have also reported that serotype O111:HNT, EAF-positive EPEC strains adhere locally to HeLa cells (6, 26–28; Bopp et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1988).

Serogrouping, serotyping, genetic probing, and investigation of the ability of cell adherence and cytotoxin production are the methods used to identify EPEC. Chatkaemorakot et al. (6) compared the effectiveness of serotyping, mannose-resistant adherence to HeLa cells, and colony hybridization by using the EAF probe in the identification of class I EPEC strains. The use of the EAF probe was found to be more specific and sensitive than either serotyping or the cell adherence test.

Virulence-specific probes have been shown to be useful in investigations of other bacteria that cause diarrhea in humans, such as enterotoxigenic *E. coli* (36, 42), enterohemorrhagic *E. coli* (25), enteroinvasive *E. coli*, *Shigella* bacteria (41), and *Yersinia enterocolitica* (17).

Southern blot hybridization of the plasmids of EAF probe-positive strains showed that the EAF genes were located on a 65-MDa plasmid. This is in agreement with the observations of other workers, who showed that EPEC-localized adhesion to HEp-2 cells is encoded by plasmids with molecular sizes between 55 and 70 MDa (3, 7, 26). The EAF plasmid has been shown to be transmissible (26, 28). In this study, the EAF-positive strains belonged to the classical EPEC serotype O111:HNT. It has not yet been established why the EAF virulence plasmids preferentially reside in strains with a limited number of serotypes. Riley et al. (33) have described a transmissible EAF plasmid that encoded localized adherence, and also a lipopolysaccharide which reacted with serogroup O111-specific antiserum. If the genes encoding a group of lipopolysaccharide O antigens were always cotransferred with the unique virulence genes of *E. coli*, an *E. coli* strain that exhibits a characteristic pathogenic activity would thus belong to a specific serogroup.

The 229 *E. coli* isolates in the present study had several different plasmid profiles. EAF-negative strains representing the most commonly observed plasmid profile groups did not belong to any of the known EPEC serogroups (Gomes, et al., in press) and were negative for HeLa cell adherence and production of cytotoxins.

In the present study, the fatality rate of neonates harboring EAF-positive strains was 30.8% compared with 7.7% for the neonates with EAF-negative *E. coli* and 0% for those infected with *K. pneumoniae*. Outbreaks of EPEC diarrhea in the 1940s and 1950s were reported to be associated with case fatality rates as high as 70%. Although the case fatality rate has shown a decline, the disease is still much more severe than conditions caused by *Shigella* spp., *Salmonella* spp., enterotoxigenic *E. coli*, and nonbacterial pathogens

(24). *E. coli* serotype O111:HNT is one of the classical EPEC serotypes (23) and has been incriminated as the cause of infantile diarrhea in several studies in both developed and developing countries (7, 19–21, 29, 31).

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