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Clinical and Microbiologic Features of *Shigella* and Enteroinvasive *Escherichia coli* Infections Detected by DNA Hybridization

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To determine the clinical and microbiologic features of *Shigella* and enteroinvasive *Escherichia coli* (EIEC) infections, we investigated 410 children with diarrhea and 410 control children without diarrhea who were seen at Children's Hospital, Bangkok, Thailand, from January to June 1985. *Shigella* spp. were isolated from 96 (23%) and EIEC were isolated from 17 (4%) of 410 children with diarrhea and from 12 (3%) and 6 (1%) of 410 control children, respectively. The isolation rates of both pathogens increased with age and peaked in children 3 to 5 years old from whom *Shigella* spp. were isolated from 38% and EIEC were isolated from 9%. *Shigella* spp. were isolated from 52% and EIEC were isolated from 7% of 91 children with bloody diarrhea and from 15 and 3% of 319 children with nonbloody diarrhea. Fifteen (65%) of 23 EIEC were lactose positive, and all belonged to recognized EIEC serotypes. Among children with diarrhea, the stool blots of 76% of 17 children infected with EIEC, 45% of 96 children infected with *Shigella* spp., and 1% of 297 culture-negative children hybridized with the 17-kilobase *Eco*RI digestion fragment of pRM17, a recombinant plasmid containing DNA derived from the 140-megadalton *Shigella flexneri* plasmid. Although EIEC colonies can be reliably detected by DNA hybridization, detection by stool blot is less sensitive. *Shigella* spp. and EIEC are important causes of endemic diarrhea among children >1 year old in Thailand.

Shigella species are important causes of diarrheal disease throughout the world and are probably the most important cause of bacterial dysentery (15, 23). *Shigella* species have distinctive biochemical and serological features that allow them to be easily identified by standard microbiologic techniques (8). Certain serotypes of *Escherichia coli*, known as enteroinvasive *E. coli* (EIEC), have the ability to invade the colon similar to *Shigella* spp. (4, 9). EIEC pose a diagnostic problem because they may be confused with nonpathogenic *E. coli* which comprise the majority of the stool flora. EIEC also have biochemical and serological features common to both *Shigella* spp. and *E. coli* (8, 25). EIEC may be lactose positive or lactose negative, and while they usually are lysine decarboxylase negative and nonmotile, no biochemical test is entirely specific in identifying these organisms. EIEC belong to a number of *E. coli* O serogroups: O28, O29, O124, O136, O143, O144, O147, O152, O164, and 167 (8, 25); some, such as *E. coli* O124, agglutinate in *Shigella dysenteriae* serotype 3 antisera (8). Antisera to identify known EIEC serogroups are not generally available, and the use of such antisera precludes identifying EIEC that are not among the recognized serogroups. These difficulties in identifying EIEC have limited our understanding of this organism as a cause of diarrheal disease.

Virulence of *Shigella* spp. and EIEC is dependent on the ability of these organisms to invade and multiply in intestinal epithelial cells (4, 9). The definitive biological test for enteroinvasion is the Sereny test (21). Enteroinvasion of *Shigella* spp. and EIEC is mediated by a high-molecular-weight (120,000 to 140,000) plasmid (11, 13). DNA sequences encoded by these plasmids are necessary for virulence in both *Shigella* spp. and EIEC. A 17-kilobase *Eco*RI digestion fragment of the 140-megadalton plasmid of *Shigella flexneri* 5 has been cloned (1) and has been used as a probe to identify *Shigella* spp. and EIEC (24, 26).

In this study we describe the clinical and epidemiological features of *Shigella* and EIEC infections in children with all types of diarrheal disease and in children without diarrhea. We also studied the microbiologic characteristics of EIEC identified by DNA hybridization and evaluated the DNA probe to identify *Shigella* and EIEC infections by direct examination of unselected bacterial growth from stool specimens.

MATERIALS AND METHODS

Patient selection. From January to June 1985, we obtained stool specimens from children who were seen in the outpatient clinic of Children's Hospital, Bangkok, Thailand. Stool specimens were obtained from children with a history of diarrhea of <72-h duration. Diarrhea was defined as three or more loose stools in 24 h. Dysentery was defined as diarrhea associated with blood or leukocytes in the stools. The symptoms and clinical course of each child was recorded. For each child with diarrhea (case), one control child was chosen from the same outpatient clinic. Control children were seen for a variety of other minor nongastrointestinal illnesses. The control child was matched within 1 year of age with the case patient, did not have diarrhea in the previous week, and did not have household contacts with diarrhea within the last week. Information and fecal samples were collected from a maximum of five case and control children each day. Stools were examined microscopically for erythrocytes and leukocytes using methylene blue stain (12). A

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two-tailed chi-squared test with Yates correction or Fisher's exact test was used for statistical analysis.

Microbiology. Stools were cultured on MacConkey, Desoxycholate, Hektoen, and thiosulfate-citrate-bile salts-sucrose media (Difco Laboratories, Detroit, Mich.) before and after enrichment in Selenite-F Enrichment (BBL Microbiology Systems, Cockeysville, Md.) and alkaline peptone-water for the isolation of enteric bacterial pathogens as described previously (6, 24). From each child 10 lactose-positive and all lactose-negative *E. coli* isolated on MacConkey media were saved on nutrient agar slants. Ten lactose-positive *E. coli* were tested for enterotoxin production (3, 19). Rotavirus was detected by a monoclonal antibody enzyme-linked immunosorbent assay (ELISA) (14). Intestinal parasites, including *Cryptosporidium* sp., were detected microscopically by standard techniques (2, 16).

From each patient 10 lactose-positive and all lactose-negative colonies were picked from the nutrient agar slants and spotted onto another MacConkey plate. After overnight incubation, Whatman no. 541 filters were pressed evenly over the bacterial growth. Bacteria on the filters were lysed, and the DNA was denatured in 0.5 N NaOH and exposed to 1 M Tris–2 M NaCl for 4 min and then air dried. Stool blots were prepared by inoculation of fecal material onto MacConkey agar. After overnight incubation, the resultant growth (approximate area, 1 by 1 cm) was transferred to Whatman no. 541 filters and processed under stringent conditions as for colony hybridization (22).

The DNA probe used to detect *Shigella* spp. and EIEC was a 17-kilobase *Eco*RI digestion fragment of the 140-megadalton plasmid of *S. flexneri* 5 cloned into pBR325 (1). For DNA hybridization, the 17-kilobase fragment was labeled by nick translation with [α-^{32}P]dATP (New England Nuclear Corp., Boston, Mass.) and hybridized with specimen DNA fixed onto Whatman no. 541 filters as described previously (24). Colonies that hybridized with the probe were confirmed in the Sereny test (21) and the virulence marker antigen (VMA) ELISA (17) and serotyped at the Central Public Health Laboratory Service, Colindale, England (18).

E. coli were differentiated from *Shigella* spp. by biochemical tests such as lactose, sodium acetate, lysine decarboxylase, mucate, ornithine decarboxylase, and gas from glucose (8). Organisms that hybridized with the 17-kilobase probe and were biochemically similar to *Shigella* spp., but failed to agglutinate in *Shigella* group-specific antisera, were also serotyped with *E. coli* antisera.

RESULTS

Isolation rates. In this study we examined 410 children with diarrhea and 410 age-matched children without diarrhea. *Shigella* spp. were the most commonly encountered pathogen, isolated in 23% of children with diarrhea (Table 1). Rotavirus, *Salmonella* sp., and enterotoxigenic *E. coli* were each isolated from approximately 10% of children. EIEC was isolated from 4.2% of the 410 children with diarrhea. There were differences in isolation rates between case children and controls in only half of the pathogens. The greatest difference in isolation rates was found for *Shigella* spp. (Table 1). EIEC was also isolated more frequently from children with diarrhea than from those without diarrhea, $P < 0.05$. EIEC was not isolated from children under 1 year old with diarrhea and was isolated from 6 (8.6%) of 70 children with diarrhea and from 3 (4.3%) children without diarrhea among 3- to 5 year olds. The trend in age-specific isolation rates was similar for *Shigella* serotypes and EIEC (Fig. 1). The isolation rates also increased with age in the control group.

TABLE 1. Enteropathogens isolated from 410 children with diarrhea and 410 children without diarrhea seen in an outpatient clinic, Children's Hospital, Bangkok, Thailand, January to June 1985

Enteropathogen	Isolation rates from children: With diarrhea		Without diarrhea		*P* value[a]
	No.	%	No.	%	
Shigella spp.	96	23.4	12	2.9	<0.001
Rotavirus	41	10.0	1	0.2	<0.001
Salmonella spp.	40	9.8	39	9.5	NS
Enterotoxigenic *E. coli*					
ST or ST/LT[b]	23	5.6	3	0.7	<0.01
LT only	14	3.4	12	2.9	NS
Plesiomonas sp.	23	5.6	22	5.4	NS
Campylobacter sp.	21	5.1	12	2.9	NS
Vibrio spp.	21	5.1	3	0.7	<0.01
Giardia sp.	18	4.4	9	2.2	NS
EIEC	17	4.2	6	1.4	<0.05
Aeromonas sp.	16	3.9	15	3.7	NS
Cryptosporidium sp.	13	3.2	1	0.2	<0.01
No pathogen isolated	155	37.8	299	72.9	<0.01

[a] Chi-squared test with Yates correction. NS, Nonsignificant, $P > 0.05$.
[b] ST, Heat-stable enterotoxin; LT, heat-labile enterotoxin.

Clinical features. *Shigella* spp. were isolated as the only pathogen in 59 (61%) of 96 cases and EIEC was isolated as the only pathogen in 10 (59%) of 17 cases. There was no combination of pathogens that was more frequent than expected by the overall isolation rate, except that *Shigella* spp., the most frequently isolated pathogen, was never isolated with EIEC.

Ninety-one (22%) of 410 patients had bloody diarrhea (dysentery). *Shigella* spp. were isolated from 47 (52%) children with bloody diarrhea and 49 (15%) children with nonbloody diarrhea, $P < 0.001$. None of the other enteropathogens, including EIEC, which was isolated from 7% of 91 children with bloody diarrhea and 3% of children with nonbloody diarrhea, were isolated significantly more often from children with bloody diarrhea.

The clinical characteristics were determined for patients infected with only one pathogen (Table 2). Patients infected with *Shigella* spp. were more likely to come to the clinic less than 24 h after the onset of diarrhea than patients infected with EIEC, $P = 0.04$ by Fisher's exact test. This parameter may indicate a more severe illness among children infected with *Shigella* spp. In other respects EIEC infections were similar to *Shigella* infections. *Shigella* infections were more associated with fever and less with vomiting than rotavirus infections, $P = 0.002$ and $P = 0.001$, respectively. Children infected with *Shigella* spp. were more likely to have fecal leukocytes and erythrocytes than patients infected with rotavirus, $P = 0.002$. Persons with *Shigella* spp. were also more likely to be treated with antibiotics ($P = 0.001$) and less likely to receive intravenous fluids ($P = 0.02$) than patients infected with rotavirus.

Microbiologic features. All *E. coli* that hybridized with the DNA probe were also positive in the VMA ELISA and Sereny test. Lactose-positive EIEC were isolated from 12 (3%) 410 patients with lactose-positive *E. coli*. Lactose-negative EIEC were isolated from 5 (1%) of the 410 patients; however, lactose-negative *E. coli* were only isolated from 50

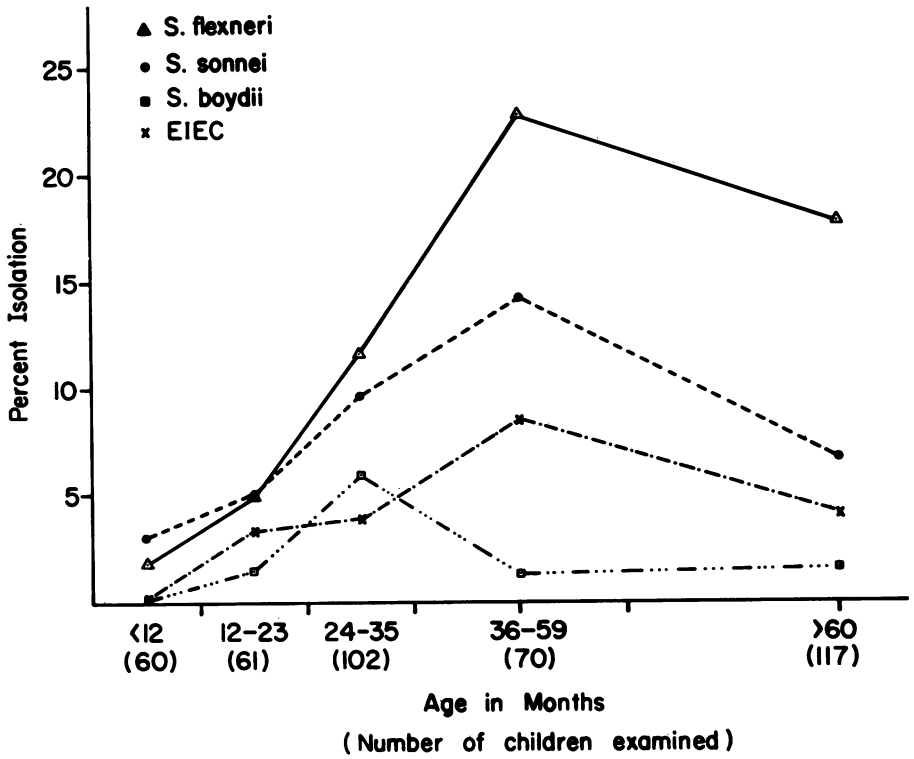

FIG. 1. Age-specific isolation rates of *Shigella* species and EIEC from children with diarrhea, Thailand, January to June 1985.

patients. Thus, EIEC, isolated from 5 (10%) of 50 patients with lactose-negative *E. coli*, were found in higher proportion among lactose-negative *E. coli* than among lactose-positive *E. coli*, $P = 0.035$. Among 12 children with diarrhea infected with lactose-positive EIEC, a mean of 7.3 ± 0.9 (standard error of the mean) of 10 lactose-positive *E. coli* colonies tested were enteroinvasive compared with 1.3 ± 0.6 of 10 lactose-positive *E. coli* colonies from children without diarrhea, $P < 0.02$ by t test. Among five children with diarrhea infected with lactose-negative EIEC, all of 22 lactose-negative colonies were enteroinvasive, and among three children without diarrhea, all of five lactose-negative colonies were enteroinvasive.

All EIEC belonged to recognized EIEC serotypes (Table 3). Of 23 EIEC, 15 (65%) were lactose positive and 8 (35%) were lactose negative. Most (91%) EIEC were lysine decarboxylase negative; *E. coli* O144:H25 isolated from two patients were lactose negative and lysine decarboxylase positive.

Among children with diarrhea, the stool blot technique detected 13 (76%) of 17 children with EIEC, 43 (45%) of 96 children with *Shigella* spp., and 3 (1%) of 297 children with neither EIEC nor *Shigella* spp. Among children without diarrhea, the stool blot was positive in 2 (33%) of 6 children with EIEC, 2 (17%) of 12 children with *Shigella* spp., and 2 (0.5%) of 392 children with neither EIEC nor *Shigella* spp.

Among 17 children with EIEC infections and diarrhea, the stool blots were positive if five or more colonies hybridized with the colony probe (10 of 10) than if less than five colonies hybridized (4 of 7). Blots were also more often positive in children infected with lactose-positive (11 of 12) than lactose-negative (3 of 5) EIEC, and the blot was more often positive if EIEC was isolated as the only pathogen (9 of 10) than if more than one pathogen (4 of 7) was isolated. There were no differences in detecting the various *Shigella* serotypes in the stool blot technique. Stool blots were as often positive with *Shigella* spp. as with lactose-negative EIEC.

TABLE 2. Clinical features and stool examination of children with diarrhea infected with only one pathogen, Bangkok, Thailand, January to June 1985[a]

Pathogen (no.)	Symptoms				Treatment received		Stool examination	
	Diarrhea <24 h[b]	Bloody diarrhea	Fever	Vomiting	i.v. rehydration	Antibiotics	Leukocytes present	Erythrocytes present
Shigella spp. (59)	58	47	98	47	10	61	66	49
EIEC (10)	20	30	90	40	10	70	89	44
Enterotoxigenic *E. coli* (18)	39	17	67	67	66	39	31	13
Rotavirus (31)	32	6	97	97	32	23	22	7

[a] Results are expressed as percentages. i.v., Intravenous.
[b] Duration of diarrhea before coming to the clinic.

TABLE 3. Microbiologic characteristics of EIEC isolated from children at Children's Hospital, Bangkok, Thailand, 1985

E. coli serotype	No. of patients	Characteristic[a]							
		Lactose	LDC	Motility	ODC	Acetate	Mucate	Indole	Glucose (gas)
O28ac:H−	13	+	−	−	−	−	−	+	−
O143:H−	1	+	−	−	−	+	−	+	+
O136:H−	1	+	−	−	+	+	−	+	+
O164:H−	6	−	−	−	−	+	−	d	−
O144:H25	2	−	+	+	+	+	−	+	+

[a] LDC, Lysine decarboxylase; ODC, ornithine decarboxylase; d, variable.

DISCUSSION

In this study of endemic diarrheal disease in Thailand, *Shigella* spp. were the most frequently isolated enteric pathogen and were particularly important in children over 1 year old. However, *Shigella* spp. were not the only cause of invasive diarrheal disease. EIEC may also be a cause of endemic diarrheal disease in the tropics. EIEC was isolated from 4% of all cases of diarrhea and was commonly associated with bloody diarrhea (7%) and among children with diarrhea who were 3 to 5 years old (9%). EIEC accounted for 15% of the bacteria that possessed the invasive plasmid and caused disease by shigellalike enteroinvasion. Thus, EIEC is an important component of the etiology of endemic diarrheal disease.

In a previous study, *Shigella* spp. were isolated from 44% and EIEC was isolated from 5% of 200 children with bloody diarrhea (24). With similar methods in this study, EIEC were detected from 4% of children with diarrhea and significantly less often (0.9%) from children without diarrhea. The number of EIEC colonies per specimen was also significantly less in children without diarrhea, suggesting that asymptomatic children shed fewer organisms. The age-related isolation rates and clinical features of EIEC infections were similar to shigellosis, although the illness appeared to be less severe.

All EIEC isolated in previous study (24) were lactose positive and lysine decarboxylase negative; EIEC from 7 of 10 patients were recognized EIEC serotypes (O28ac:H-, O29:H-), and 3 of 10 belonged to a new serotype (F. Orskov, personal communication). In this study 65% of EIEC were lactose positive, and all belonged to recognized EIEC serotypes. *E. coli* O144:H25, isolated from two patients, were enteroinvasive but lysine decarboxylase positive. These strains were biochemically distinct from those described by Sakazaki et al. (20). EIEC O144:H25 that were lysine decarboxylase negative were recently identified with DNA probes (10). In other studies EIEC isolates were predominantly lactose negative (25); in this study we found that a greater proportion of lactose-negative *E. coli* were enteroinvasive compared with lactose-positive *E. coli*, but lactose-negative *E. coli* were only isolated from 12% of children. DNA hybridization methods are particularly useful in screening large numbers of lactose-positive *E. coli* (5). In a previous study we found that the DNA probe applied to colonies was as sensitive and specific as the Sereny test and VMA ELISA in detecting EIEC (24). Based on these previous results, the same probe was used to screen *E. coli* colonies in the present study, and it was found that all colonies that hybridized with the DNA probe could be confirmed as EIEC by Sereny test, VMA ELISA, and serotyping.

Compared with colony hybridization, the stool blot had a sensitivity of 76% in detecting EIEC cases; compared with culture results, the stool blot had a sensitivity of 45% for *Shigella* cases. The stool blot was considerably less sensitive than colony hybridization for EIEC or culture for *Shigella* detection. Lactose-positive EIEC were identified more often by stool blot than lactose-negative EIEC or *Shigella* species. The growth of lactose-negative organisms on MacConkey agar may have been inhibited by other fecal bacteria. It is possible that examining stool grown on a nonselective medium, such as blood agar, or another enteric medium, such as Hektoen or eosin-methylene blue, might increase the detection of lactose-negative EIEC and *Shigella* spp. by this technique. However, examining stool blots with DNA probes to detect enterotoxigenic *E. coli* has also been less sensitive than examining colonies (7). On the other hand, this probe is remarkably specific in screening DNA from unselected bacterial growth. Hybridization of stool blots is an easier and more rapid method of identifying EIEC than colony blots. Although it is not as sensitive as colony hybridization, the high specificity suggests that stool blot hybridization may be a promising method to detect enteropathogens.

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