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Characterization of *Escherichia coli* Strains from Cases of Childhood Diarrhea in Provincial Southwestern Nigeria

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In a study carried out in small-town and rural primary health care centers in southwestern Nigeria, 330 *Escherichia coli* strains isolated from 187 children with diarrhea and 144 apparently healthy controls were examined for virulence traits. Based on the results of colony blot hybridization, strains were categorized as enteropathogenic *E. coli* (1.8%), enterotoxigenic *E. coli* (2.4%), enteroinvasive *E. coli* (1.2%), enterohemorrhagic *E. coli* (0.6%), enteroaggregative *E. coli* (10.3%), diffusely adherent *E. coli* (7.9%), cell-detaching *E. coli* (6.9%), and cytolethal distending toxin-producing *E. coli* (0.9%). *E. coli* strains that hybridized with a Shiga toxin gene probe but lacked other characteristics usually present in enterohemorrhagic *E. coli* constituted 8.4% of the isolates. Ninety-seven *E. coli* isolates adhered to HEp-2 cells in an aggregative fashion but did not hybridize with any of the probes employed in the study. Overall the pathotypes, apart from cytolethal distending toxin-producing *E. coli*, were recovered both from children with diarrhea and from children without diarrhea, though to a lower extent from the healthy children. All diarrheagenic *E. coli* strains were associated with diarrhea ($P < 0.02$). Heat-stable-enterotoxin-producing enterotoxigenic *E. coli* showed significant association with diarrhea ($P < 0.02$), as did strains that demonstrated aggregative adherence to HEp-2 cells ($P < 0.04$), but not those that hybridized with the CVD432 enteroaggregative probe.

Diarrhea is a leading cause of morbidity and mortality among children in developing countries (21). The bacterial pathogen most commonly associated with endemic forms of childhood diarrhea is *Escherichia coli* (24). At least six categories of diarrheagenic *E. coli* have been described: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC) (33). Two additional categories, cell-detaching *E. coli* (CDEC) (23) and cytolethal distending toxin-producing *E. coli* (CLDTEC) (25), have been proposed. Classification is based on the presence of different chromosomal or plasmid-encoded virulence genes in *E. coli* enteropathogens that are absent in most commensal strains, as well as their pattern of interaction with epithelial cells and tissue culture monolayers (33).

The epidemiological significance of each *E. coli* category in childhood diarrhea varies with geographical area. Very few studies have investigated the microbiology of acute childhood diarrhea in Nigeria in particular and in West Africa in general. Many of the studies that have been performed were set predominantly in urban centers, sought only one or two diarrheagenic *E. coli* categories, or were not designed to detect potentially pathogenic *E. coli* (1, 2, 5, 36). This study examined the roles of the different categories of diarrheagenic *E. coli* in diarrhea in children from rural and semiurban locations in southwestern Nigeria.

MATERIALS AND METHODS

Subjects. Ill subjects were outpatients, aged 5 years or younger, attending one of four primary health care centers in Osun State, which is situated in southwestern Nigeria. They were diagnosed by a physician or community health officer as having acute diarrhea, on the basis of frequent watery stools (usually more than three daily), lasting for less than 2 weeks. A large proportion of the patients also had fever and/or respiratory symptoms. Control subjects were apparently healthy children of the same age range and were drawn from the immunization clinics of the same health centers. Neither patients nor controls had been treated with antibiotics in the week preceding sampling. Informed consent was obtained from each child's parent or guardian. Most of the subjects were from low-income families and had no access to pipe-borne water.

Specimen collection and processing. Swabs of stool were inoculated onto the surface of MacConkey and eosin-methylene blue agars (Oxoid, Basingstoke, England) and streaked for isolated colonies. Specimens collected at one center which was some distance away from the laboratory were inoculated into transport media (Bionor, Skein, Norway) and plated out as soon as possible, generally within 24 h. After incubation for 24 and 48 h at 37°C, three or four colonies with typical *E. coli* morphology and one of another morphological type were streaked on fresh plates. Each colony was independently subjected to biochemical tests for identification (8) and antibiotic susceptibility testing. Colonies recovered from the same host and showing the same colony morphology, biochemical profile, and antibiotic susceptibility pattern were considered to be the same isolate for the purpose of the study. Stocks of each isolate were maintained by cryopreservation.

DNA hybridization. All *E. coli* isolates were screened by colony hybridization with the fragment probes shown in Table 1. Colony blots were prepared with Whatman 541 filter papers (Whatman, Maidstone, England). The fragment probes were prepared by extracting plasmids by the method of Birnboim and Doly (12), digesting them with appropriate restriction endonucleases, and purifying fragments by gel extraction. The fragments were then labeled by random priming with [α-^{32}P]dCTP employing a commercially available labeling kit (Amersham Pharmacia Biotech, Piscataway, N.J.) and removing unincorporated nucleotides by passage through Sephadex G-50 microcolumns (Amersham Pharmacia Biotech). Hybridization was carried out under high-stringency conditions by standard techniques (42) and employing a hybridization buffer of the following composition: 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.5% sodium dodecyl sulfate, 10 mM EDTA, 1× Denhardt's solution, and 100 μg of sonicated salmon sperm DNA per ml. Colony blots were hybridized at 65°C overnight, washed with 0.1× SSC–0.1% sodium dodecyl sulfate at 65°C, and exposed to X-ray film at −80°C overnight. Strains that had been characterized in previous studies were used as controls (Table 1).

Hemolysin testing. All the isolates were screened for hemolytic activity on nutrient agar plates containing 7% washed human erythrocytes.

Hemagglutination test. Hemagglutination testing was carried out on all *E. coli* isolates by the method of Evans et al. (18). Bacteria were grown overnight on colonization factor agar. Washed erythrocytes were recovered from freshly collected human type A or bovine blood and resuspended to a final concentration

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TABLE 1. Fragment probes and control strains used for DNA hybridization experiments

Pathotype	Target gene(s)	Fragment probes used (restriction endonuclease[s])	Control strain(s)[a]	Reference(s)
EPEC	LEE A	pCVD453 (*Bgl*II/*Mlu*I)	E2348/69 (O127:H6) (LEE$^+$ *bfpA*$^+$)	16, 31
	LEE B	pCVD461 (*Eco*RI/*Sal*I)		
	LEE C (*eae*)	pCVD443 (*Hin*dIII)		
	LEE D	pCVD460 (*Bgl*III)		
	bfpA	pMSD207 (*Eco*RI)		
EHEC	LEE A to D	As above	EDL933 (O157:H7) (LEE$^+$ *hly*$^+$ *stx1*$^+$ *stx2*$^+$); EDL933cu (*hly* mutant)	28, 31, 34
	hly	pCVD419 (*Hin*dIII)		
	stx1	pNN110-18 (*Sma*I/*Pst*I)		
	stx2	pJN37-19 (*Bam*HI)		
STEC	*stx1* and *stx2*	pJN37-19 (*Bam*HI)	EDL933 (O157:H7)	34
		pNN110-18 (*Sma*I/*Pst*I)		
ETEC	*elt*	pCVD403 (*Bam*HI)	H10407 (LT$^+$ STh$^+$ STp$^+$)	17
	estp	pCVD426 (*Pst*I)		
	esth	pCVD427 (*Eco*RI)		
EIEC	Invasion plasmid	pSF55 (*Hin*dIII)	EI.37	20
EAEC	Aggregative adherence plasmid	pCVD432 (*Eco*RI/*Pst*I)	042 (O44:H18)	9
DAEC	*daaC*	pSLM852 (*Pst*I)	C1845	11
CDEC	*hlyAB*, *pap*, *cnf*	pANN215 (*Hin*dIII)	536, A70.1 (*hly*$^+$ *pap*$^+$ *cnf*$^+$); 536-21 (*hly*$^-$ *pap*$^-$ *cnf*$^-$ mutant)	19
		pRHU845 (*Hin*dIII)		
		pSE266 (*Sau*3AI)		
CLDTEC	*cdtAB*	pCVD448 (*Eco*RV)	E6468/62 (O86:H34)	39, 43
	cdtAB	pCP2100 (*Ava*I/*Sal*I)		

[a] Negative controls: HS (27) and *E. coli* K-12 strains DH5α and C600 (42).

of 3% in phosphate-buffered saline (PBS) or PBS containing 2.5% mannose or *N*-acetylneuramic acid. Four colonies were picked with a sterile toothpick and mixed with a drop of erythrocyte suspension on a sterile slide. Slides were rocked gently and observed for agglutination after 1 min at room temperature or after incubation over ice.

HEp-2 adherence test. All *E. coli* isolates were subjected to HEp-2 adherence tests by the method originally described by Cravioto et al. (14), with slight modifications (48). HEp-2 cells were grown overnight to 50% confluence in Dulbecco's modified Eagle medium (Gibco BRL, Gaithersburg, Md.) containing penicillin, streptomycin, and 10% fetal bovine serum on eight-well chamber slides (Labtek, Scotts Valley, Calif.). Bacteria were grown for 16 h in Luria broth without shaking. The HEp-2 cells were washed three times with PBS, and then the medium was replaced with Dulbecco's modified Eagle medium containing 1% mannose. A volume of 10 μl of bacterial suspension was added per well, and the slides were incubated at 37°C in 5% CO_2 for 3 h. The monolayers were washed three times with PBS, fixed with 70% methanol, and Giemsa stained. Each strain was tested in duplicate, and appropriate controls were included in the test. Strains that adhered to the monolayers were recorded as adhering in localized, diffuse, or aggregative patterns.

FAS. Fluorescent actin staining (FAS) of HEp-2 monolayers infected with each of the *E. coli* isolates was carried out as described by Knutton et al. (26). The HEp-2 monolayers were prepared on chamber slides and inoculated as in the adherence assay. After a 3-h incubation period, the cells were washed three times with PBS, fixed with formalin, permeabilized with 0.1% Triton X-100 in PBS, and stained with fluorescein isothiocyanate-labeled phalloidin (Sigma, St. Louis, Mo.). The HEp-2 cells were observed for actin accumulation at the site of bacterial attachment under incident fluorescent light with a Zeiss Axioskop routine microscope. Each strain was tested in duplicate. EPEC strain E2348/69 and normal flora strain HS were used as positive and negative controls, respectively (27).

Serotyping. Serological typing of the EPEC, EHEC, ETEC, EIEC, and CLDTEC isolates was carried out at the Robert Koch Institute, Berlin, Germany, by tube agglutination with rabbit anti-*E. coli* immune sera produced against a panel of antigenic test strains of *E. coli* containing *E. coli* O groups 1 to 173 and *E. coli* H groups 1 to 56. The methods were performed as described by Ørskov and Ørskov (37).

Statistical analysis. Data derived from children with diarrhea and that from control subjects were compared by a two-tailed chi-square test and Fisher's exact test (6).

RESULTS

Specimens were obtained from 187 children with diarrhea and 144 healthy controls who resided in 23 towns and villages in Osun State, Nigeria. The population in these towns is predominantly agricultural. Children are breast fed, though not exclusively, from birth until at least 6 months. Pipe-borne water is available in only a few of the small towns, and in those cases, not continuously. Coliform counts from water used for drinking purposes in the towns and villages ranged from 4×10^3 to 2.5×10^5 CFU/ml.

A total of 581 fecal bacterial isolates were recovered, of which 330 (56.8%) were identified as *E. coli*. The other bacterial pathogens recovered were *Salmonella*, *Shigella*, and *Aeromonas* spp. Enteric pathogens (*Salmonella*, *Shigella*, and *Aeromonas* spp. and diarrheagenic *E. coli*) were isolated from 77 (41.2%) patients with diarrhea and 38 (26.4%) controls ($P < 0.001$) (Table 2). Other organisms that were identified are listed in Table 2. As indicated in that table, *Acinetobacter baumannii* was recovered from five children with diarrhea and none of the control subjects ($P < 0.02$). *E. coli* strains with potentially diarrheagenic virulence characteristics were isolated from 70 (37.1%) of the 187 diarrhea cases and from 37 (25.7%) of the 144 healthy controls ($P < 0.02$) (Table 3).

E. coli isolates were categorized into different pathotypes

TABLE 2. Organisms isolated from stool specimens

Organism	No. (%) of isolates		
	Recovered from 187 ill subjects	Recovered from 144 healthy subjects	Total (isolated from 330 subjects)
Escherichia coli	196 (60.3)	134 (52.3)	330 (56.8)
Klebsiella	99 (30.5)	98 (38.3)	197 (33.9)
Enterobacter	9 (2.8)	8 (3.1)	17 (2.9)
Citrobacter	4 (1.2)	9 (3.5)	13 (2.2)
Acinetobacter baumannii	5 (1.5)	0	5 (0.9)
Salmonella	3 (0.9)	0	3 (0.5)
Shigella	4 (1.2)	1 (0.4)	5 (0.9)
Aeromonas sobria	1 (0.3)	0	1 (0.2)
Hafnia alvei	1 (0.3)	0	1 (0.2)
Proteus vulgaris	0	1 (0.4)	1 (0.2)
Leclercia adecarboxylata	0	1 (0.4)	1 (0.2)
Pseudomonas	0	3 (1.8)	3 (0.5)
Other nonfermentative gram-negative rods	3 (0.9)	1 (0.4)	4 (0.7)
Total	325	256	581

based on the results of DNA hybridization (Table 3). Tests for hemolysis, HEp-2 adherence pattern, hemagglutination, and actin accumulation were conducted to further characterize strains. The various *E. coli* categories showed characteristic adhesion patterns: localized for EPEC, aggregative for EAEC, diffuse for DAEC and ETEC, and detaching for CDEC. The EHEC isolates showed weak localized adhesion, and the EIEC isolates showed weak diffuse adherence. Strains that hybridized with the *pap* (for P, or pyelonephritis-associated, pili) probe showed diffuse or very weak aggregative adherence. A number of strains that did not hybridize with the tested probes also showed aggregative or diffuse adherence. In particular, 97 *E. coli* isolates showed classic aggregative adherence but failed to hybridize with any of the DNA probes employed in the

TABLE 3. *E. coli* pathotypes identified[a]

Category of diarrheagenic *E. coli*[b]	Ill subjects		Healthy subjects		Total no. (%) of Subjects yielding organisms
	No. (%) yielding organism	Serotype(s)	No. (%) yielding organism	Serotype(s)	
EPEC	4 (2.1)	O113:H19, O119:H6, O130:H26, O142:H6	2 (1.4)	O33:H34, O103:H7	6 (1.8)
ST-producing ETEC*	5 (2.7)	O6:H16, O23:H14, O89:H17, O153:H45, Ont:H21	0 (0)		5 (1.5)
LT-producing ETEC	1 (0.5)	Ont:H18	2 (1.4)	Ont:H−, O21:H−	3 (0.9)
ETEC	6 (3.2)		2 (1.4)		8 (2.4)
EIEC	3 (1.6)	O76:H+nt, O86?:H30, Ont:H−	1 (0.7)	O151:H11	4 (1.2)
EHEC	1 (0.5)	O33:H34	1 (0.7)	O121:H−	2 (0.6)
STEC	17 (9.1)	ND	11 (7.6)	ND	28 (8.4)
EAEC (CVD432-positive)	20 (10.7)	ND	14 (9.7)	ND	34 (10.3)
EAEC (aggregative adherent)**	73 (39.0)	ND	40 (27.7)	ND	113 (34.3)
CLDTEC	3 (1.6)	O8:H9, O157:H−, O157:H−	0 (0)		3 (0.9)
DAEC	18 (9.6)	ND	8 (5.6)	ND	26 (7.9)
CDEC	15 (8.0)	ND	8 (5.6)	ND	23 (6.9)
Total*	71 (38.0)		37 (25.7)		108 (32.7)

[a] ND, not determined; nt, nontypeable; ?, O titer lower than that of control strain.
[b] * and **, differences between ill and healthy subjects were significant at P values of <0.02 and <0.04, respectively.

study. EPEC and EHEC isolates yielded positive FAS tests. P pilus-possessing strains (including CDEC) gave instantaneous mannose-resistant hemagglutination with human (type A) but not bovine erythrocytes. Mannose-resistant agglutination of human and/or bovine erythrocytes was also seen with ETEC and EAEC strains as well as with some probe-negative but HEp-2 cell-adherent strains. Clear zones of hemolysis formed rapidly (after less than 6 h of incubation) on blood agar plates around the CDEC isolates (which also hybridized with the alpha-hemolysin probe). The EHEC isolates (which hybridized with the enterohemolysin probe) yielded only weak hemolysis. There was considerable cross reaction between the two hemolysin probes as well as between the *pap* and DAEC probes.

The serotypes of the EPEC, EHEC, ETEC, EIEC, and CLDTEC isolates are indicated in Table 3. Three strains were O nontypeable. Only two of the EPEC strains isolated in this study belonged to classic EPEC O serogroups (O119 and O142). One EPEC strain belonged to the O103 serogroup, which has occasionally been documented to include EPEC and EHEC strains (10, 29, 47, 49). Two of the ETEC strains (O6 and O153) and none of the EHEC and EIEC strains belonged to serogroups associated with these pathotypes. Two of the CLDTEC strains belonged to the EHEC-associated serotype O157:H−. However, none of the three CLDTEC strains possessed the locus for enterocyte effacement (LEE) or enterohemolysin genes, although both O157 strains hybridized with *stx* probes.

DISCUSSION

In this study of childhood diarrhea in southwestern Nigeria, diarrheagenic *E. coli* strains were recovered more often from children with diarrhea than from healthy controls ($P < 0.02$). The observed differences in recovery from patients and controls for most of the categories of diarrheagenic *E. coli* sought showed the same trend but did not show statistical significance ($P > 0.05$). The notable exception was heat-stable enterotoxin (ST)-producing (but not heat-labile enterotoxin [LT]-producing, or total) ETEC strains, which were associated with diarrhea ($P < 0.02$). The results seen with ETEC strains concur with the results of other studies, in which ST-producing strains showed a greater association with diarrhea than did LT-producing *E. coli* or were more frequently recovered from cases of diarrhea (22, 40). The lack of association with diarrhea observed with the other pathotypes reflects the high incidence of carriage seen with all the pathogens in this study and other studies from developing countries (40, 44). The high rate of carriage can also be attributed to the fact that some of the pathogens encountered most often here, such as DAEC and CDEC, have not been associated with acute diarrhea in several studies (7, 30, 33).

Epidemiological evidence and human challenge studies have demonstrated unequivocally that EPEC, ETEC, EIEC, and EHEC are important causes of diarrhea worldwide (33). Strains from all four of these categories were recovered during this study, but their frequency of isolation was low and they were not significantly associated with diarrhea. The low rate of recovery of EHEC and EIEC in this study and other studies suggests that they may play less important roles in childhood diarrhea in developing countries (4, 40). Although only limited data is available, previous West African studies have reported higher recovery rates for EPEC and ETEC and have usually found them to be significantly associated with childhood diarrhea. The discrepancies seen in this regard between this study and previous studies could be related to differences in study population and methodology. The subjects enrolled in the present study were residents of small towns and villages, whereas those in the previous studies resided in urban cities. Furthermore, the previous studies almost universally employed serotyping for the identification of EPEC and occasionally the less specific latex agglutination tests for the identification of LT-producing ETEC (2, 3, 5, 36, 38). The majority of the ETEC and EPEC isolates in this study did not belong to classic serotypes and so would have been missed had serotyping been the only test used to distinguish the diarrheagenic *E. coli* categories. Other workers have shown that EPEC strains belonging to classic serotypes do not always possess genes encoding virulence factors (45). The diversity of serotypes seen in this study and the apparent lack of correlation of serotype with virulence category suggests that several clones of diarrhea-causing *E. coli* may be responsible for childhood diarrhea in these provincial localities.

It has been demonstrated that enteroaggregative and diffusely adherent strains differ in their ability to cause diarrhea and in their reactivity to the standard probes (32, 33, 46). Furthermore, as previously reported by Qadri et al. (41), we noted varied hemagglutination patterns among the enteroaggregative probe-positive strains. The heterogeneity in these two categories of organisms may account for their frequent recovery from healthy subjects and suggests that more work is needed to identify specific virulence genes in both EAEC and DAEC that may show a better correlation with disease. As many as 97 CVD432 probe-negative strains were able to adhere to HEp-2 monolayers in a classic aggregative manner. Together with probe-positive EAEC, strains demonstrating the aggregative adherence phenotype were isolated from 73 (39%) of the children with diarrhea and 40 (27.7%) of the controls ($P < 0.04$). Further work to evaluate these strains is in progress. The apparent low sensitivity of the CVD432 probe (26.0%) suggests that the HEp-2 adherence test may be of greater value for detecting EAEC in the study area.

CDEC was first described by Gunzburg et al. (23), who found that it was associated with diarrhea in aboriginal children in Australia. It has also been found in Brazil, though without significant association with diarrhea (30). It is noteworthy that most (87.0%) of the CDEC isolates recovered in this study were non-lactose fermenting. In this and other properties, they resembled a cluster of non-lactose-fermenting hemolytic strains isolated previously from stool specimens in Somalia (35). Lactose-negative CDEC strains may be important pathogens on the African continent. Unfortunately, they are likely to be missed (or mistaken for *Shigella* or other non-lactose fermenters) in many studies because of their inability to ferment lactose promptly, as is the case with most *E. coli* strains.

Thirty strains that hybridized with Shiga toxin probes but not with probes for the LEE or enterohemolysin genes were recovered and are here referred to as Shiga toxin-producing *E. coli* (STEC). Such strains have been detected in food specimens and animal feces, but their role as diarrheagenic pathogens remains obscure (33). A distinctive feature of the STEC isolates from this study is that they adhered to HEp-2 cells, usually in an aggregative fashion, although only one of the isolates hybridized with the CVD432 enteroaggregative probe (15). Two of the STEC strains belonged to the EHEC-associated serotype O157:H− but lacked the LEE and enterohemolysin genes. These strains also carried genes encoding the cytolethal distending toxin and were categorized as CLDTEC for the purpose of this study.

Among the non-*E. coli* isolates, the most significant finding was the recovery of *A. baumannii* from cases of diarrhea. This organism was significantly associated with diarrhea ($P < 0.02$)

even though it is rarely isolated from stools. *A. baumannii* can colonize the digestive tracts of hospitalized patients (13), and the lack of reports of recovery from stool specimens by other workers may be related to the difficulty in identifying the organism or to the opinion that it is too unimportant to report. In all cases, *A. baumannii* was isolated from patients infected with an *E. coli* pathogen (STEC [two patients], EPEC [one patient], and EHEC [one patient]), and aside from very strong HEp-2 adherence, no virulence properties could be detected. The frequent occurrence of this organism in cases of illness could suggest a tendency to colonize during enteric infection.

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