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Antibiotic-Resistant Cell-Detaching *Escherichia coli* Strains from Nigerian Children

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The properties of 23 cell-detaching *Escherichia coli* strains that were isolated from stool specimens in Nigeria are described. Common properties of the strains included the presence of genes encoding α-hemolysin (100%), pyelonephritis-associated pili (100%), and cytotoxic necrotizing factor 1 (70%) as well as lactose negativity (70%) and multiple antibiotic resistance (74%). Antibiotic resistance was shown in most cases to be transferable and associated with the presence of class 1 integrons. Phenotypic properties and pulsed-field gel electrophoresis analysis demonstrated that the majority of the strains, particularly multiply resistant, lactose-negative O4:H40 strains, were closely related. Multiply-resistant cell-detaching *E. coli* strains may represent an important reservoir for antibiotic resistance genes.

Five categories of *Escherichia coli* have been consistently associated with diarrhea in epidemiologic studies or produced the signs and symptoms of diarrhea when administered to healthy volunteers (14). These classes, enteropathogenic, enterotoxigenic, enteroinvasive, enterohemorrhagic, and enteroaggregative *E. coli,* possess specific virulence factors that are responsible for their diarrheagenicity (14). Two other categories, diffusely adherent *E. coli* and cytolethal distending toxin *E. coli,* are considered possible diarrheagenic agents because they possess putative virulence genes, but neither has been consistently associated with diarrhea in epidemiologic surveys or volunteer challenge studies (14). These categories are identified by the presence of putative virulence genes and by specific phenotypes, particularly epithelial cell adherence patterns and toxin production (14). Cell-detaching *E. coli* strains (CDEC) are another putative class of diarrheagenic *E. coli*, originally proposed by Gunzburg et al., who found this class to be significantly associated with diarrhea in a cohort of Aborigine children (7).

CDEC were originally defined by their capacity to detach tissue culture cells from solid supports in adherence assays or in a cell-detaching assay (7). In a study of the pathogenesis of CDEC, Elliott et al. reported that such strains possessed pyelonephritis-associated pili (P-pili) and produced α-hemolysin as well as cytotoxic necrotizing factor 1 (CNF1) (4). Furthermore, animal studies demonstrated that CDEC were capable of eliciting diarrhea in the reversible intestinal tie adult rabbit diarrhea model (4). These authors suggested that the term diarrhea-associated hemolytic *E. coli* (DHEC) be used to describe this class of *E. coli*. Not many other epidemiological surveys have sought this relatively recently defined category of potentially diarrheagenic *E. coli*. Of those that have, most have found them not to be associated with diarrhea, and their significance remains unknown (9, 12, 21).

In a recent Nigerian case-control study, although they were not significantly associated with diarrhea, CDEC were the most frequently recovered pathotype after enteroaggregative *E. coli* (21). In that study, only heat-stable toxin-producing *E. coli* and enteroaggregative *E. coli* were significantly associated with diarrhea (21). Considerable variation was noted among strains belonging to other categories of diarrheagenic *E. coli* with respect to virulence factors, biochemical properties, and serotypes (21). In contrast, the CDEC were less diverse in their characteristics: They almost universally hybridized to α-*hly*, *pap,* and *cnf1* probes (for hemolysin, P-pilus. and CNF1 genes, respectively) (21). In addition, the majority of the CDEC isolates (65.2% versus 5.2% of *E. coli* belonging to other groups) were lactose negative. We speculated that they might represent a clone or group of clones which may or may not be pathogenic but are efficiently disseminated within the study environment.

In this paper, we characterize these strains in detail and report O4 and other antibiotic-resistant serogroups. The terminology CDEC (versus DHEC) is retained because these strains were not significantly associated with diarrhea.

The CDEC isolates described in this paper were defined by their ability to detach cells in the cell-detaching assay described by Gunzburg and colleagues (7, 21). DNA probes for CDEC-associated virulence factors (α-*hly*, *pap,* and *cnf1*) as well as probes for virulence factors associated with other diarrheagenic *E. coli* strains were employed in hybridization experiments as described previously (21). Serotyping was performed as described by Ørskov and Ørskov (22) with antisera against *E. coli* O antigens 1 to 173 and H antigens 1 to 56. Phage typing was done according to the protocol of Milch (13), expanded by

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TABLE 1. Probes hybridizing to colony blots of CDEC isolates

Target gene	Properties of target gene	Description of probe (reference)	Positive controls	No. (%) of CDEC strains carrying target gene		
				From children with diarrhea	From healthy controls	Total
hlyA	Hemolysin	2.2-kb *Hin*dIII fragment from pANN215 (6)	Uropathogenic *E. coli* strain 536, CDEC strain A70.1	15 (100)	8 (100)	23 (100)
papA	Adhesin	3-kb *Hin*dIII fragment from pRHU845	536, A70.1	15 (100)	8 (100)	23 (100)
cnfI	Cytotoxic necrotizing toxin 1	950-bp *Bam*HI fragment from pSE266 (4)	536, A70.1	11 (73.3)	5 (50)	16 (69.6)
stx_1 and stx_2	Shiga toxins 1 and 2	pNN110-18 (*Sma*I/*Pst*I), pJN37-19 (*Bam*HI) (17)	Enterohemorrhagic *E. coli* strain EDL933	1 (6.5)	1 (12.5)	2 (8.7)
CVD432, *astA, pet*	Enteroaggregative *E. coli* plasmid (pAA) probe; enteroaggregative heat-stable enterotoxin; and enteroaggregative plasmid-encoded enterotoxin	0.9-kb *Eco*RI-*Pst*I fragment (1); 250-bp *Nru*I-*Cla*I fragment from pJPN61 (15); 2.5-kb *Mlu*I-*Pst*I fragment from pJPN205 (5)	Enteroaggregative *E. coli* strain 042	2 (13.0)	0 (0)	2 (8.7)
intI1	Integrase associated with class 1 integrons	1,175-bp *Rsa*I-*Bam*HI fragment from Tn*21* (24)	K-12 strain C600 carrying plasmid R100	10 (66.7)	4 (50.0)	14 (60.9)
intI2	Integrase associated with class 2 integrons	1,560 bp *Sph*I-*Hpa*I fragment from Tn*7* were used as *intI1* and *intI2* probes (26).	K-12 strain C600 carrying plasmid R483	1 (6.7)	0 (0)	1 (4.3)

specific phages for K1 and K5 and employing a total of 46 phages.

All the CDEC examined hybridized with the probes for *pap* and α-hemolysin. As shown in Table 1, most of the strains hybridized with the *cnf1* probe, but this factor was not found significantly more commonly in strains from children with diarrhea than in those from healthy controls ($P > 0.05$). The presence of aggregative adherence plasmids in two strains and *stx* genes in two strains could account for the diarrheagenicity of three of these four strains irrespective of any cell-detaching properties that they may also possess.

Antibiotic susceptibility testing was done according to the standard disk diffusion method approved by the National Committee for Clinical Laboratory Standards (16). The agents tested were ampicillin (10 μg), chloramphenicol (30 μg), spectinomycin (25 μg), gentamicin (30 μg), nalidixic acid (30 μg), ofloxacin (5 μg), trimethoprim (5 μg) (Oxoid), ciprofloxacin (10 μg), sulfisomidine (250 μg), trimethoprim/sulfamethoxazole (23.8/1.2 μg), tetracycline (30 μg), mezlocillin (30 μg), piperacillin (30 μg), cefotaxime (30 μg), and amikacin (30 μg) (AB Biodisk).

Resistant strains were mated with *E. coli* K-12 strain C600 (*supE44 hsdR thi-1 thr-1 leuB6 lacY1 tonA21* Nal^r Fu^r) to determine if resistance markers were conjugatable (27). Transconjugants were selected on plates containing nalidixic acid (40 mg/liter) (Sigma Chemical Company, St. Louis, Mo.) with either trimethoprim (10 mg/liter) as the lactate (Wellcome), sulfathiazole (40 mg/liter) as the sodium salt (Sigma), tetracycline (40 mg/liter) as the hydrochloride (Pfizer Pharmaceuticals, Lagos, Nigeria), or chloramphenicol (40 mg/liter) (Sigma).

The susceptibility of transconjugants to ampicillin, chloramphenicol, spectinomycin, gentamicin, trimethoprim, sulfisomidine, trimethoprim/sulfamethoxazole, and tetracycline was determined by the NCCLS disk diffusion method. In these in vitro transconjugation experiments, 12 of the 13 isolates showing resistance to more than six antibiotics were capable of transferring most of these resistances horizontally (Table 2). The presence of a class 1 integron was demonstrated in 13 of 15 strains that were resistant to three or more agents by hybridization with a probe specific for the *intI1* gene encoding class 1 integrons (Table 2). A class 2 integron was detected in one strain with the *intI2* probe.

Insert sizes in class 1 integrons were determined by amplification with primers that annealed to the 5′ and 3′ conserved ends (5′-GGC ATC CAA GCA GCA AG-3′ and 5′-AAG CAG ACT TGA CCT GA-3′, respectively) as described by Levesque et al. (11), using *E. coli* C600 carrying plasmid R100 as a positive control. Nine of the isolates yielded a 1.6-kb product with one identical internal *Hin*cII site, and two gave a 750-bp product. Three strains that hybridized with the *intI1* probe and all those that did not failed to yield any product. Colony hybridization of other *E. coli* isolates recovered during the same study demonstrated that CDEC had the highest prevalence of *intI1* and that strains isolated from the same host as a multiply-resistant CDEC invariably carried class 1 integrons (Table 3).

Serotyping revealed that most of the CDEC belonged to serotypes associated with extraintestinal disease. Three strains were O-untypeable. The remaining 20 isolates belonged to 10 different O-groups (12 serotypes), and four O-groups, O1, O4, O16, and O21, accounted for 16 (69.6%) of the isolates. Eight (34.8%) of the CDEC strains belonged to the O4 serogroup, with the serotype O4:H40 (six strains) being the most predominant. Fourteen strains produced a capsule or exopolysaccharide visible by light microscopy. K1 antigens were detected in five and K5 antigens in one of these strains.

We noted that seven of the 15 CDEC isolates from cases and none from controls were lysogenized by phages 4a and 4b ($P = 0.02$, Fisher's exact test), but we did not find any serotype or other subtype to be significantly associated with diarrhea. The significance of the phage type association is not clear, and we can therefore provide no more evidence that they may be enteric pathogens, although the small numbers of isolates in each group weaken the statistical analysis.

Interestingly, serogroups of the strains and the virulence

TABLE 2. Properties of CDEC isolates

Strain	Source	Serotype	Phage type[a]	Biotype	Lactose fermentation	Colicin production	Virulence genes[b]	Antibiotic resistance profile[c]	Conjugative transfer of antibiotic resistance	*intI* class	Class 1 integron insertion amplified by PCR (kb)
C06b	Diarrhea, urban	O1:K1:H7	3, 4	4a/716	−	+	*cnf1*	Cm, Te, Tp, TS, Su, St		1	1.6
D03b	Diarrhea, urban	O1:K1:H7	4a, 4b, 15, 33	4a/717	+	+					No product
G167b	Control, rural	O2:K5:H4	34	23a/752	−	+	*cnf1*, *stx*$_1$, *stx*$_2$	Te			No product
G131a	Control, rural	O4:H1	16	4b/717	−	−	*cnf1*	AC, Ap, Cm, Te, Tp, TS, Sh, St, Su	Ap, Te, Tp, TS, Sh, Su, St	1	No product
E15	Diarrhea, urban	O4:H5	20	4b/717	+	−	*cnf1*	AC, Ap, Mz, Pi, Te, Tp, TS, Sh, St, Su	Ap, Te, Tp, TS, Sh, Su, St	1	1.6
E01b	Diarrhea, urban	O4:H40	16	4b/717	−	−	*cnf1*	Ap, Mz, Pi, Te, Tp, TS, Sh, St, Su	Ap, Te, Tp, TS, Sh, Su, St	1	1.6
E26	Diarrhea, urban	O4:H40	16	4b/717	−	−	*cnf1*	AC, Ap, Mz, Pi, Te, Tp, TS, St, Su	Ap, Tp, TS, Su, St	1	1.6
G30a	Diarrhea, rural	O4:H40		4b/717	−	−	*cnf1*	AC, Ap, Mz, Pi, Te, Tp, TS, Sh, St, Su	Ap, Te, Tp, TS, Sh, Su, St	1	1.6
C62a	Control, urban	O4:H40		4b/717	−	−		AC, Ap, Mz, Pi, Te, Tp, TS, Sh, St, Su	Ap, Tp, TS, Su	1	1.6
C81	Control, urban	O4:H40		4b/717	−	−	*cnf1*	AC, Ap, Mz, Pi, Te, Tp, TS, Sh, St, Su	Ap, Tp, TS, Su	1	1.6
E58	Control, urban	O4:H40		4b/717	−	−	*cnf1*	AC, Ap, Mz, Pi, Te, Tp, TS, Sh, St, Su	Ap, Te, Tp, TS, Sh, Su, St	1	1.6
G76	Diarrhea, rural	O5:H4	22, 24, 33	9b/615	+	−		Ap, Te, Tp, TS, Sh, St, Su	Ap, Te, Tp	1	1.6
G75	Diarrhea, urban	O15:H18	15	10a/676	+	−	*cnf1*	Te, Tp, TS, Sh, St, Su			No product
C51	Control, urban	O16:K1:H-	22, 24, 33	1b/713	+	+		Te, Su			No product
C03	Diarrhea, urban	O16:K1:H6	(4a), 4b, 24, 33	1b/713	+	+	*cnf1*	Te, Sh, Su, St		2	No product
C11a	Diarrhea, urban	O21:H5	4a, 4b	25a/776	−	−	*cnf1*	AC, Ap, Cm, Mz, Pi, Te, Tp, TS, Sh, St, Su	Ap, Tp	1	0.75
G40a	Diarrhea, urban	O21:H5	4a, 4b	9a/714	−	−	*cnf1*	Ap, Cm, Mz, Pi, Te, Tp, TS, Sh, St, Su		1	0.75
G104a	Control, rural	O51:H10		2a/210	−	−	*cnf1*				No product
E05a	Diarrhea, urban	O95:H10	3,6,15, 29,30	2b/611 HCA	−	+	*cnf1*	Cm, Te			No product
E08a	Diarrhea, urban	O158:K1:H14	4a, 4b, 33	9a/634	+	+	*cnf1*, *stx*$_1$, *stx*$_2$	Cm, Te, St, Su			No product
E29a	Diarrhea, urban	Ont:H4	4a, 4b, 6	16a/736	−	−	*pet*, *astA*, *aafII*	AC, Ap, Cm, Mz, Pi, Te, Tp, TS, Sh, Su, St	Ap, Tp, TS, Su, St	1	No product
G02a	Diarrhea, rural	Ont:H4	4a, 4b, (6)	16a/736	−	−	*pet*, *astA*, *aafII*	AC, Ap, Cm, Mz, Pi, Te, Tp, TS, Su, St	Ap, Tp, TS, Su, St	1	No product
G106b	Control, rural	Ont:H10	24	9b/215	−	+					No product

[a] Parentheses indicate a weakly lytic reaction (10 to 50 plaques).
[b] Other than *hlyA* and *pap*.
[c] AC, amoxicillin-clavulanic acid; Ap, ampicillin; Mz, mezlocillin; Pi, piperacillin; Te, tetracycline; Tp, trimethoprim; TS, trimethoprim-sulfamethoxazole; Sh, spectinomycin; St, streptomycin; Su, sulfonamide.

factors they possess—α-hemolysin, P-pili, and CNF1—are associated with *E. coli* isolates from extraintestinal infections. Fecal *E. coli* strains such as these, which display characteristics typical of uropathogenic strains, have been described in several studies. In some studies, they are considered a pool of potential uropathogens (29) and therefore undesirable, even if they do not cause gastrointestinal disease.

We did not find that CDEC were significantly associated with diarrhea in our studies, but this class of *E. coli* was isolated more frequently from cases of diarrhea and showed a stronger correlation with gastrointestinal disease than did other acknowledged diarrheal pathogens such as enteropathogenic and enteroinvasive *E. coli* strains (21). Four of the CDEC isolates belonged to other pathotypes. They include two Ont:H4 enteroaggregative strains from children with diarrhea that possessed genes encoding enteroaggregative heat-stable toxin (*astA*), plasmid-encoded toxin (*pet*), and aggregative adherence fimbriae II (*aaf*) (20). Two other strains, one of which was isolated from a child with diarrhea, carried the *stx*$_1$ and *stx*$_2$ genes and could therefore be classified as Shiga toxin-producing *Escherichia coli*.

Marques et al. (12) reported that in addition to finding no association of this class of organisms with diarrhea in Brazil, CDEC were usually isolated from hosts with diarrhea along

TABLE 3. Class 1 and 2 integrons in other categories of *E. coli*

E. coli isolates	No. of isolates screened	No. (%) of isolates hybridizing to probes for:		
		intI1 only	*intI2* only	*intI1* and *intI2*
CDEC	21	11 (52.4)	1 (4.8)	0 (0)
Enteroaggregative–cell-detaching *E. coli*[a]	2	2 (100)	0 (0)	0 (0)
Enteroaggregative *E. coli*	129	36 (27.9)	4 (3.1)	5 (3.9)
Other diarrheagenic *E. coli*	49	11 (22.4)	0 (0)	0 (0)
Other *E. coli* (normal flora)	130	22 (16.9)	5 (3.8)	3 (2.3)
Total	331	82 (24.8)	10 (3.0)	8 (2.5)

[a] CDEC Ont:H4 isolates E29a and G02a, which bear aggregative adherence plasmids.

with another pathogen. This suggests that the organisms may not be diarrheagenic but may possess the ability to persist in a host with diarrhea. This hypothesis is consistent with the observations of Wold et al. (29, 30) that P-piliated strains adhere to colonic epithelial cells and are persistent gastrointestinal colonizers of persons with asymptomatic bacteriuria. They may therefore represent a significant proportion of the resident flora in children who suffer repeated diarrhea episodes. This could be one explanation for the association of CDEC with diarrhea observed by Nicoletti et al. (18) and Gunzburg et al. (7).

As we have shown here that they often carry multiple antibiotic resistance genes on mobile elements, it is possible that they serve as efficient reservoirs for resistance that could be transferred to other commensals or pathogens. If these strains do have the ability to persist in the gut, the chances that such transfers will occur is high. We probed other *E. coli* isolates recovered during the same study with the *IntI1* and *IntI2* probes. As shown in Table 3, class 1 integrons were more prevalent among pathogenic *E. coli* than normal flora, and the highest prevalence was seen among CDEC. We also found that all five *E. coli* isolates recovered from one subject with a multiply-resistant *IntI1*-positive strain hybridized with the *IntI1* probe.

Strains of serotypes O4:H40, O21:H5, and Ont:H4 were in all cases lactose negative, colicin negative, and resistant to 10 or more antimicrobials. The resistance was associated with the presence of class 1 integrons and transferable in mating experiments. All class 1 integrons identified in all O4:H40 isolates and one O1:K1:H7 isolate had 1.6-kb insertions indistinguishable by *Hin*cII restriction analysis. O21:H5 class 1 integrons had 750-bp insertions, while the insertions in the O4:H1 and Ont:H4 strains could not be amplified under the conditions used.

Class 1 integrons, the prototype being that borne on Tn*21*, have been demonstrated to be important in the evolution and dissemination of antibiotic resistance genes in *E. coli* from many parts of the world (8). Pulsed-field gel electrophoresis (PFGE) of *Sfi*I-digested agarose-embedded DNA was carried out as described by Rios et al. (23), and four of the O4:H40 strains (G30a, C62a, C81, and E58) showed profiles that were indistinguishable (Fig. 1), suggesting that they are clonal (28). The other two O4 strains, E01b and E26, showed a pattern that differed only in the loss of one restriction site and could therefore be considered closely related (28).

O4 isolates with properties similar to those of these strains have been reported from diarrhea cases elsewhere. Nicoletti et al. (18) recovered 23 lactose-negative, α-hemolytic, multiply-resistant O4 *E. coli* from children with diarrhea in Somalia. As in this study, they were unable to present any evidence that the strains were themselves pathogens, but they appeared to be closely related or clonal (3). An O4:H10 isolate for which no conventional virulence factors were found was also reported by Sullivan et al. (25) to be recovered from a case of persistent diarrhea in the Gambia, and a multiply-resistant, hemolytic O4 atypical enteroaggregative isolate capable of mannose-resistant hemagglutination caused an outbreak in a Serbian neonatal ward (2).

We also found indistinguishable electrophoretic patterns for both O21:H5 isolates and both O1:K1:H7 isolates (data not shown). All the isolates that showed a PFGE pattern that was seen more than once were resistant to multiple antibiotics. These data suggest that they may represent successful, multiply-resistant clones distributed within the study area. Interestingly, 11 of these 12 isolates (compared to 5 of the other 11; $P < 0.02$) were isolated from children residing in urban areas, where the population density is considerably greater than in rural locations. Although these strains were not associated with diarrhea, the dissemination of strains such as these is one possible factor that could account for the high prevalence of antibiotic-resistant enteric bacteria within the study area and other urban locations (10, 19). The strains also appear to strongly resemble uropathogenic *E. coli,* and the possibility that they have the potential to cause disease in other niches is worth examining.

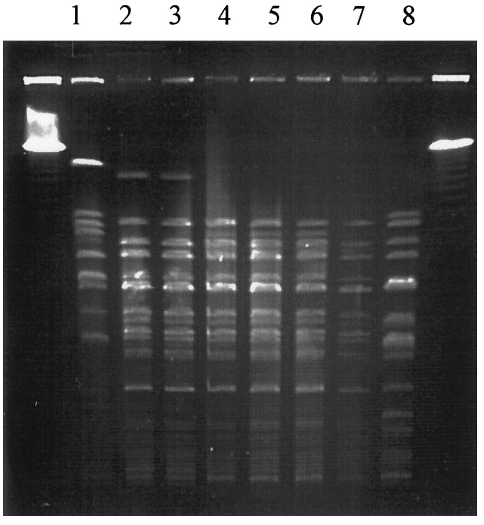

FIG. 1. PFGE of *Sfi*I-digested genomic DNA from CDEC isolates. Lanes: 1, O4:H5 strain E15; 2 to 7, O4:H40 strains E01b, E26, G30a, C62a, C81, and E58, respectively; 8, O4:H1 strain G131a. Size markers are the λ pulsed-field ladder (Bio-Rad).

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