Isolation and characterization of enteroaggregative *Escherichia coli* (EAggEC) by genotypic and phenotypic markers, isolated from diarrheal children in Congo

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Objective: To determine the prevalence of enteroaggregative *Escherichia coli* (EAggEC) in African diarrheal children in Lwiro, Congo, to characterize EAggEC isolates by possible genotypic and phenotypic markers, and to evaluate the EAggEC probe pCVD432 in identifying EAggEC.

Methods: The Hep-2 cell adhesion assay and colony-blot hybridization assays were carried out for the identification of EAggEC. O:H serotyping, biotyping, antibiogram and plasmid-profile analysis were done. To detect the *E. coli* LT and ST, ELISA tests were used and, for VT, a vero cell assay was used.

Results: EAggEC strains were isolated from 56 out of 115 diarrheal children (48.7%): the organism was present alone and presumed to cause diarrhea in 22 (19.1%) cases. The rest of the cases were associated with two or more diarrheagenic *E. coli* strains. EAggEC strains were isolated from 25% of total diarrheal children (first day of isolation) and 8.86% of age-matched healthy individuals ($p<0.03$). This isolation rate was significantly higher than the one found for other diarrheagenic *E. coli* strains. In parallel, we evaluated the sensitivity and specificity of EAggEC probe pCVD432, and found that it had 56% sensitivity with 100% specificity compared with the Hep-2 cell test. EAggEC isolates were characterized by serotyping, biotyping, antibiotic resistance pattern, plasmid profiling and toxin production analysis. They did not produce any one of these classical toxins and nor did they relate to any particular serotypes. Plasmid analysis of the 79 EAggEC isolates ($n=315$) showed seven different profiles. Ten resistance patterns were identified and 34 strains were sensitive to all drugs. There was no association between plasmid profiles and antibiotic resistance patterns. All 16 classical *E. coli* biotypes were found in this small EAggEC population.

Conclusions: EAggEC has been emerging as a cause of childhood diarrhea in African children in Congo. From the accumulated data it was found that there is a great heterogeneity in EAggEC populations.

Key words: Africa, children, diarrhea, enteroaggregative *Escherichia coli* (EAggEC), genotype, phenotype, prevalence, Congo

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INTRODUCTION

Escherichia coli is a versatile microorganism associated with a variety of diseases. At least five categories of *E. coli* are well described as causing diarrheal illnesses worldwide: they are enterotoxigenic *E. coli* (ETEC), causing childhood and travelers' diarrhea, entero-hemorrhagic *E. coli* (EHEC), causing bloody diarrhea

and hemolytic uremic syndrome (HUS), enteropathogenic *E. coli* (EPEC), associated with infantile diarrhea, enteroinvasive *E. coli* (EIEC), causing bacillary dysentery which is very similar to shigella infection, and the more recently described enteroaggregative *E. coli* (EAggEC) [1–4]. The role of diffusely adhering *E. coli* (DAEC), has not been clearly established.

On the basis of the patterns of adherence to Hep-2 or HeLa cells, *E. coli* can be classified into three distinct pathotypes [5–7]. They are: localized adherent (LA) *E. coli* strains (mostly EPEC strains), which adhere to the cell surface forming microcolonies on the surface of the cells; diffusely adherent (DA) *E. coli* strains, which adhere to the cell, covering the whole cell surface; and aggregative adherent (AA) *E. coli* strains, which adhere to the cell surface and to glass slides uniformly, giving a characteristic stacked-brick like appearance.

EAggEC strains have been found to be epidemiologically incriminated as a cause of persistent diarrhea [8–10]. The pathogenic mechanisms of EAggEC strains are poorly understood, but recent studies revealed that they are capable of producing a heat-stable enterotoxin called EAST-I, closely related to classical *E. coli* ST toxin, and express bundle-forming fimbriae known as aggregative adherence fimbriae [11,12]. Two antigenically distinct types of aggregative fimbriae have been described. The present study was originally conducted to investigate the efficacy of different types of ORS (oral rehydration saline) in treating diarrheal children at Lwiro children's hospital in Congo in the year 1993, but we also took the opportunity to determine the prevalence of different diarrheagenic *E. coli* strains, particularly EAggEC strains, in that population. To understand the role of EAggEC as a bacterial pathogen it is necessary to characterize the strains completely. So far, to our knowledge, no report has been published on EAggEC strains from African regions. The purpose of this study was to isolate and characterize the EAggEC strains from African diarrheal children at Lwiro children's hospital in Congolese (former Zaire) South Kivu province by using genotypic and phenotypic markers.

MATERIALS AND METHODS

Subjects and strains

All the subjects included in the study were children between 0 and 5 years of age with a history of diarrhea admitted in the large children's hospital at Lwiro in the Congolese South Kivu province. All episodes of diarrhea among the children were included in this study. Diarrhea was defined as the passage of at least three unformed stools within 24 h and an episode was considered persistent if it continued for ≥14 days. An age-matched healthy control group (not case control) was selected without any diarrheal complaint attending the same hospital for either vaccination or any other consultation. A single stool specimen was obtained on each day for five consecutive days of stool sampling from each patient, and a single stool specimen was also collected from each control subject. All the specimens were submitted to the clinical microbiology laboratory and cultured by standard microbiological techniques for *E. coli*, *Salmonella* spp., *Shigella* spp., *Campylobacter* spp., *Yersinia* spp., *Aeromonas* spp., *Vibrio* spp. and *Plesiomonas* spp. [13]. Initially, five different *E. coli* colonies were picked up from each stool specimen, and thus 25 presumptive *E. coli* colonies were collected for each patient. Five *E. coli* colonies were picked up from a single stool specimen for each control subject. After necessary screening procedures, 1964 *E. coli* strains from 115 diarrheal patients were analyzed to determine the different diarrheagenic *E. coli* strains: 137 *E. coli* strains from 34 healthy individuals were analyzed to detect EAggEC only. The isolation rate of EAggEC was compared between the patients and the control groups from the first day of isolation only. For comparison, some prototype strains were also included in this study. The strains were 17-2 (for EAggEC), F-1845 (for DAEC), E-2348/69 (for LA *E. coli*), 933 (for EHEC), M90T (for EIEC) and P307 (for ETEC). *E. coli* HB101 (*pro*A2 *rec*A13 *ara*14 *lac*Y1 *gal*K2 *xyl*5 *mtl*1 *rps*L20 *sup*E44 *hsd*S20) was used as a negative control.

Hep-2 cell adhesion assay

The cell adhesion assay was done by the method of Cravioto et al [5]. Briefly, cells were grown overnight at 37°C in 5% CO_2 to produce a semi-confluent monolayer and inoculated with 20 μL overnight cultures of *E. coli* grown in Luria broth (LB) and incubated for 3 h at 37°C in 5% CO_2 in the presence of 0.5% α-D-mannoside. After incubation, cells were washed, and stained with 10% Giemsa stain, and glass coverslips were examined under oil immersion by light microscopy.

Colony-blot hybridization assay

We performed the hybridization assay on a selected group of *E. coli* strains with EAggEC probe pCVD432 [14]. *E. coli* isolates were grown overnight at 37°C on LB agar media and transferred to Whatman 541 filter papers (Whatman, Inc., Clifton, NJ) by the colony lift technique previously described [15].

Serotyping

All *E. coli* isolates were examined with EPEC antisera by a slide agglutination method [16]. The EPEC sero-

groups were O26, O55, O86, O111, O114, O119, O124, O125, O126, O127, O128, and O142.

O:H serotyping was done on 52 EAggEC isolates at the Central Public Health Laboratory in London using standard methods [16,17]. One hundred and seventy-three somatic (O) and 56 flagellar (H) antisera were used.

Biotyping of EAggEC isolates

EAggEC isolates were biotyped according to the method of Crichton et al [18]. Briefly, biotyping was done on the basis of the fermentation of raffinose, dulcitol and sorbose and decarboxylation of ornithine. *E. coli* can be classified into 16 classical biotypes.

Antibiogram

All EAggEC isolates were tested by the Kirby–Bauer disk diffusion method [19] by following the National Committee for Clinical Laboratory Standards recommendations [20] to determine the susceptibility pattern against a panel of antibiotics. The antibiotics were tetracycline, ampicillin, chloramphenicol, gentamicin, kanamycin, streptomycin, nalidixic acid, trimethoprim–sulfamethoxazole (SXT), trimethoprim, sulfonamides, tobramycin, nitrofurantoin, erythromycin, cefazolin, cefuroxime, ceftriaxone, ciprofloxacin, amoxicillin–clavulanic acid and imipenem. The antibiotic disks were purchased from Rosco Diagnostica, Denmark.

Plasmid analysis

For plasmid analysis, EAggEC isolates were selected by taking the strains from each antibiotic resistance profile, each patient and the positivity and the negativity on colony-blot hybridization. Plasmid DNA was prepared by the method described by Portnoy et al [21] and electrophoresis was done in 0.6% agarose gel prepared and run in 1 × TBE buffer. The sizes of the plasmids were approximated by comparing with the known molecular weights of the different plasmids in control strains.

The control strains were: *E. coli* 39R861 (contains five plasmids of 98.0, 42.0, 23.0, 9.0 and 4.6 MDa); *E. coli* APVR1 (contains three plasmids of 95.0, 41.0 and 32.0 MDa); and *E. coli* V517 (contains eight plasmids of 32.0, 5.2, 3.5, 3.0, 2.2, 1.7, 1.5 and 1.2 MDa).

Detection of toxins

An ELISA method (using GM1 gangliosides) was used to identify the *E. coli* LT toxin [22]. ST toxin was determined by a competitive ELISA method described

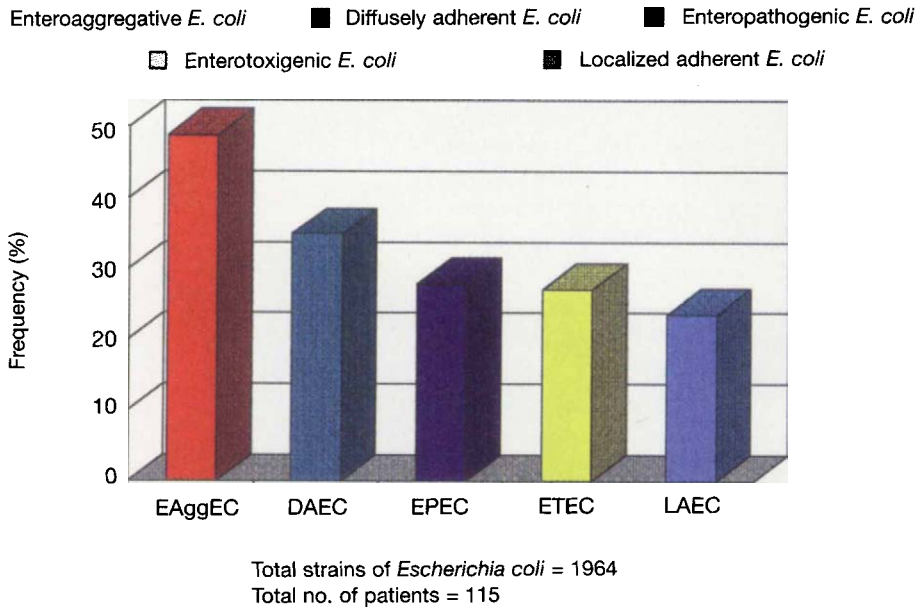

Figure 1 Distribution of different diarrheagenic *Escherichia coli* strains among diarrheal children (n=115) in Lwiro, Congo.

by DeMol et al [23] using a monoclonal antibody against ST-h. *E. coli* cytotoxic activity was determined by cytotoxicity for Vero cells [24,25].

RESULTS

Prevalence of enteroaggregative *Escherichia coli* (EAggEC)

Among 115 diarrheal patients, EAggEC was found in 56 diarrheal children, EPEC was found in 32 children, ETEC (LT/ST or both) was found in 31 children, DAEC was found in 40 children, and LAEC (mostly EPEC) was found in 27 children (Figure 1).

It was found that EAggEC was the predominant group of diarrheagenic *E. coli* associated with 56 diarrheal cases, where this organism was present alone in 22 (19.1%) cases without any association with other diarrheagenic *E. coli*.

The isolation rate of EAggEC from the first day of isolation was compared with the age-matched control group. It was found that 29 (25% of 115 diarrheal cases) patients were infected with EAggEC, which was significantly higher than the figure found in the control group. One hundred and thirty-seven *E. coli* strains from 34 healthy subjects were analyzed, and it was found that 11 strains were EAggEC from three (8.86%) individuals ($p<0.03$, Fisher's exact one tail).

Comparison between the two assay methods, the Hep-2 cell adhesion assay and the DNA probe hybridization assay

Among 315 EAggEC strains, 285 strains were analyzed by colony-blot hybridization assay with EAggEC probe pCVD432. One hundred and sixty-one strains (56%) were hybridized with the EAggEC probe, whereas the rest of the strains were negative in the hybridization assay. To evaluate the specificity of the probe, a colony-blot hybridization assay was also carried out in another filter containing 20 EPEC strains, 20 ETEC strains and 20 randomly selected *E. coli* strains without any known pathogenicity. Six control strains were also included. None of the strains hybridized with the EAggEC probe pCVD432, except for the positive control strain 17-2. Thus the specificity of the probe was 100%.

Serotyping results of EAggEC isolates

No EAggEC isolates agglutinated with any of the EPEC antisera. Of 52 EAggEC strains tested for O:H serotyping, 31 strains did not agglutinate with the currently recognized O serogroups 1–173. Eighteen of the EAggEC strains had the flagellar antigen H18. The distribution of EAggEC serotypes is presented in Table 1.

Table 1 O:H serotyping results of the 52 enteroaggregative *Escherichia coli* isolates and their antibiotic resistance patterns

Strains No.	Serotype		Resistance to antomicrobials and corresponding resistance pattern
	O	H	
601	9ab	11	TSSu
621	9ab	11	TSSu
1542	?	18	TAC
1681	?	–	TACSSuTm
1823	?	18	TASSu
1845	7	–	TACSSuTm
2101	91	32	TACSSuTm
2121	?	18	TAC
2143	?	–	TACSSu
23R1	?	–	TACSSuTM
2641	7	–	TACSSuTm
3042	7	–	TACKSSuTm
3622	86	18	TACSSuTm
3721	131	25	TACKSSuTm
3822	?	9	TACSSuTm
38R1	91	21	T
3921	?	18	TACKSSuTm
3925	?	18	TACKSSuTm
3962	125ac	9	TACSSuTm
4242	130	27	TACKSSuTm
4321	6	10	TACSSuTm
44R2	?	–	T
4522	?	18	TACSuTm
4901	?	18	TAC
4921	?	18	TAC
52R1	?	18	TAC
70R1	?	–	TACSSuTm
73R1	?	18	TAC
7623	?	12	TACSSuTm
7642	7	–	ACKSSuTm
7921	?	34	TACSu
8003	7	6	TACSSuTm
8104	117	27	TACSuTm
8302	?	9	TASSuTm
8421	?	16	TAC
8522	?	18	TCSSuSxt
8545	?	18	TCSSuSxt
8562	?	18	TCSSuSxt
8803	?	1	T
88R1	?	18	TCSSuSxt
8901	7	–	TACSSuSxt
94R1	7	–	TACSSuSxt
9702	?	18	S
9721	?	–	TC
9742	7	–	ACKSSuTm
9901	7	–	ACKSSuTm
10001	7	–	TACSSuTm
11121	R	–	TACSSuTm
11181	?	–	TACSSuTm
11401	?	18	S
11441	?	18	S
115R5	?	–	TCSSuSxt

?, not belonging to any one of the recognized 'O' serogroups; –, non-motile; R, rough; T, tetracycline; A, ampicillin; C, chloramphenicol; S, streptomycin; K, kanamycin; Su, sulfamethoxazole; Tm, trimethroprim; Sxt, septrin (cotrimoxazole).

Biotyping of EAggEC isolates

All 16 classical *E. coli* biotypes were present among 287 EAggEC isolates analyzed for biotyping. The predominant biotypes were biotype 1 (16.7%), biotype 3 (14.3%), biotype 9 (10.8%), and biotype 13 (13.9%). It was found that the strains derived from each patient were mostly associated with a single biotype; in a few cases, they shared two or more biotypes. The strains on the same day from a single stool specimen were apparently associated with a single biotype.

Toxin analysis

EAggEC isolates were negative for classical *E. coli* heat-labile (LT) and heat-stable (ST) enterotoxins. They were also negative for Shiga-like toxins (SLTs) when tested with Vero cells.

Antibiogram

Two hundred and eighty-six EAggEC isolates were analyzed to detect the patterns of resistance against a panel of 19 antimicrobial drugs. Ten resistance patterns were identified and 34 strains were sensitive to all drugs. The most common resistance was encountered with tetracycline (81%), ampicillin (64%), chloramphenicol (64%) and trimethoprim–sulfamethoxazole (44%). The resistance profiles are shown in Table 1.

Plasmid profiling of EAggEC isolates

Plasmid analysis of the 79 representative EAggEC isolates showed seven different profiles. From the accumulated data, it was found that all EAggEC isolates contained at least a single plasmid of 50–80 MDa. The profiles are shown in Table 2 and Figure 2.

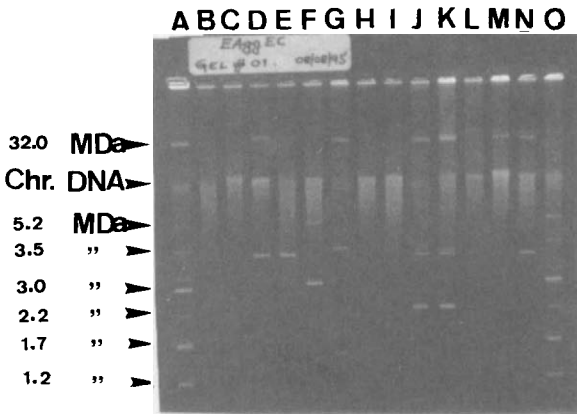

Figure 2 Agarose gel electrophoresis of EAggEC strains representing seven different plasmid profiles. Lanes A and O, reference strain *E. coli* V517 with plasmids of known molecular weights; lanes B and C, strains 2101 and 2102 of profile 1; lanes D and E, strains 73R1 and 3721 of profile 2; lane F, strain 3962 of profile 3; lane G, strain 5261 of profile 4; lane H, strain 4901 of profile 5; lane I, strain 3822 of profile 6; lanes J and K, strains 4321 and 4324 of profile 7; lane L, prototype strain of EAggEC, 17–2; lanes M and N, reference strains *E. coli* APVR1 and *E. coli* 39R861.

DISCUSSION

In this comparative study, EAggEC strains were significantly more often isolated from 115 diarrheal children than from 34 healthy controls. EAggEC strains were associated with 56 (48.7%) diarrheal children where the organism was present alone and presumed to cause diarrhea in 22 (19.1%) cases, suggesting that EAggEC was one of the predominant types of diarrheagenic *E. coli* in that population. We compared the isolation rate of EAggEC between the patients and the control group from the first day of isolation and found that it was significantly higher in the patients than in the control group (25% versus 8.86%, $p<0.03$).

The identification of EAggEC was solely dependent on the cumbersome Hep-2 cell adhesion assay. Sensitive and specific DNA probes are now available but they do not identify all EAggEC strains [14,26]. In our study, we evaluated the sensitivity and the specificity of the EAggEC probe pCVD432 and found that it was able to detect 56% of EAggEC isolates with 100% specificity. Previous studies have also shown that not all EAggEC strains hybridize with the EAggEC

Table 2 Plasmid analysis of the 79 EAggEC isolates showed seven different plasmid profiles

Profile no.	No. of plasmids	Approximate size of the plasmids (MDa)	No. of strains	Percentage (%)
1	01	60–80	32	41
2	02	50–60 and 3.5	08	10
3	03	50–60; 5–6 and 3.0	10	13
4	04	50–60; 5–6; 3–5 and 1.5–1.7	04	05
5	02	60–80 and 30–35	15	19
6	03	60–80; 20–25 and 1.2–1.5	07	09
7	04	50–60; 20–30; 3.0–3.5 and 2.5	03	04

Table 3 Correlation between antibiotic resistance phenotypes and plasmid profiles of the 79 EAggEC isolates

Antibiotic resistance patterns	Profile no. 1	Profile no. 2	Profile no. 3	Profile no. 4	Profile no. 5	Profile no. 6	Profile no. 7	Total no. of isolates	%
1	04	01	02	02	01	02	–	12	15
2	13	01	04	01	04	02	03	28	35
3	01	–	–	–	01	01	–	03	04
4	03	–	03	01	06	–	–	13	16
5	01	–	–	–	–	–	–	01	01
6	01	01	–	–	01	01	–	04	05
7	03	–	–	–	–	–	–	03	04
8	01	02	–	–	01	–	–	04	05
9	02	01	–	–	–	–	–	03	04
10	01	–	–	–	–	–	–	01	01
Sensitive	02	02	01	–	01	01	–	07	09
Total	32	08	10	04	15	07	03	79	
Percentage	41	10	13	05	19	09	04		

probe [27,28]. The reason why not all EAggEC strains hybridize with the probe is not known but it is possible that there is much heterogeneity among the strains and it has been hypothesized that different categories of EAggEC strains may exist [26,29].

Complete O:H serotyping was done on 52 EAggEC isolates (confirmed by DNA hybridization) in the Central Public Health Laboratory in London with currently recognized *E. coli* somatic (O) and flagellar (H) antisera. Out of 52 EAggEC strains, 31 strains did not agglutinate with the currently recognized somatic serogroups 1–173. Further research is needed to resolve this problem. The predominant flagellar serotype in the EAggEC population was H18 (31%), which was in accordance with the findings of others [30].

Biotyping of EAggEC strains cannot discriminate the strains into any particular clusters because all the classical biotypes of *E. coli* were present in that population.

The mechanisms of EAggEC diarrhea are not well understood, though some of the strains can produce a heat-stable enterotoxin (ST-like) known as EAST-I (entero aggregative *E. coli* heat-stable enterotoxin-I). We tried to identify any toxin involvement by analyzing the strains for LT, ST or VT. None of the strains produced any of these toxins.

The present study demonstrated the high incidence of resistance of EAggEC to certain antimicrobial agents. We have seen a great diversity among the EAggEC isolates based on the antibiotic resistance pattern.

The number of plasmids associated with EAggEC strains varied considerably. Some of the strains harbored three to four plasmids, whereas most of the strains (41%) possessed a single plasmid of 60–80 MDa. It was also evident that almost all EAggEC isolates analyzed for plasmid profiling contained at least a plasmid of 50–80 MDa, which suggests that this common character may be associated with large plasmids. We could not find any correlation between plasmid profile and antibiotic resistance pattern (Table 3). A similar drug resistance pattern was not mediated by the same set of plasmids in all strains. Drug resistance patterns did not correlate with an identical plasmid profile. This indicates that the genetic determinants for drug resistance were probably distributed among plasmids of various sizes and on the chromosome.

In the present study, our findings demonstrated that EAggEC was playing a major role in causing diarrhea among the pediatric population in the Democratic Republic of Congo (former Zaire). Future prospective studies are needed to determine the isolation rate of EAggEC among all other diarrheal agents as well as the association of EAggEC with acute and persistent forms of diarrhea. Furthermore, identification of the virulence factors would be helpful in understanding this pathogen more clearly.

We realize that the isolation of EAggEC is still difficult in most of the developing world, since it demands very sophisticated laboratories where the cell-culture and/or hybridization technologies are well set up. Effects should be made to formulate alternative assays that are more suitable and less expensive for screening large numbers of specimens.

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