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Enteroaggregative *Escherichia coli:* a public health hazard in Yaoundé, Cameroon?

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ABSTRACT

The prevalence of various pathotypes of *Escherichia coli* was investigated during a case-control study conducted in children diarrhoea in Yaoundé. Isolates obtained from the stools samples of children aged 6 months to 5 years were selected on phenotypic basis, and identified by virulence genes detection using polymerase chain reactions. The most prevalent pathotype was enteroaggregative *Escherichia coli* (25.8%). Enteropathogenic *Escherichia coli* (3.6%), enterotoxigenic *Escherichia coli* (1%), and enteroinvasive *Escherichia coli* (0.2%) followed. No shiga toxin-producing *Escherichia coli* were identified. Enteroaggregative *Escherichia coli* was not associated with diarrhoea (cases 26.1%, controls 25.5%; P=0,887), unlike enteropathogenic *Escherichia coli* (cases 6.7%, controls 1%; P=0.003). Investigations into documented potentials of enteroaggregative *Escherichia coli* in causing diarrhoea and other related pathologies indicated that it could be a major public health threat in Cameroon despite the fact that it was not found associated with clinical diarrhoeal cases in this study.

Keywords: Cameroon, diarrhoeagenic *Escherichia coli*, public health.

INTRODUCTION

Diarrhoea is a major cause of morbidity and mortality throughout the world, especially among children in countries with limited resources, due to poor hygiene and sanitation (Adachi et al., 2002; Nguyen et al., 2005; Nguendo Yongsi et al., 2008). It is the third most frequent cause of medical attention in Yaoundé, Cameroon (Fotsing-Kwetché, 2002).

Unlike *Salmonella enterica* and *Shigella* that are typical pathogens in humans, *Escherichia coli* is made up of pathogenic and non-pathogenic strains. These two categories cannot be distinguished based on their biochemical properties, not even on their serotypes as is the case with *Salmonella* and *Shigella*. For this reason, it is difficult to assess their role in causing diarrhoea. Based on their virulence and pathophysiology, six pathotypes of *E. coli* are known to be involved in gastroenteritis (Nataro et al., 1998; Nataro and Kaper, 1998).

Diarrhoeagenic bacteria routinely investigated in Cameroon include *Salmonella, Shigella* (by serotyping) and enteropathogenic *E. coli* (by serogrouping). Barely 28% of

diarrhoea episodes are elucidated as caused by these pathogens (Fotsing-Kwetché, 2002).

Recent studies conducted in Nigeria (Okeke et al., 2000a, 2000b) and in Gabon (Presterl et al., 2003) showed that enteroaggregative *E. coli* (EAEC) was frequently isolated in this sub-region of Africa. Due to its heterogeneity, research on EAEC is very expensive (Weintraud, 2007). In developing countries, the high prevalence of EAEC contrasts with the lack of resources for their detection. Efforts to design convenient and efficient protocols in the contexts of resource limitation are, however, being made (Wakimoto et al., 2004; Jenkins et al., 2006). It was reported (Nataro and Kaper, 1998) that identification approaches based on aggregative adherence phenotype (the gold standard) could include both pathogenic and non-pathogenic strains (sharing factors conferring a common phenotype, but lacking the necessary virulence determinant).

In Cameroon, very little public health implications have been identified for diarrhoeagenic *E. coli*. Subsequent to a case-control study on children with and without diarrhoea, conducted in Yaoundé from December 2001 through February 2002, and from April through June 2002, the prevalence of *E. coli* pathotypes known to play a major role in diarrhoea was investigated. To characterize EAEC, a glass assay was adapted from the modified cell culture technique (Okeke et al., 2000b). EAEC (identified by aggregative phenotype) bearing the virulence gene *astA,* were then assumed to be potentially diarrhoeagenic. Beyond pathotypes characterization, whether EAEC could be regarded as public health worries was also discussed in this paper.

MATERIALS AND METHODS

Setting, sampling, and culture methods

This study was conducted in Yaoundé, during the dry season (December 2001 through February 2002), and the rainy season (April through June 2002). Cases and controls were children aged six months through five years. Children who defecated three or more unformed stools in 24 hours or the ones from whom this was observed, but in which resolution took place 72 hours earlier were regarded as cases. Controls included children from whom no such signs were observed within two weeks from the day of sample collection. Cases and controls were children of the same age, sex and from the same neighbourhood. Parents or guardians´ consent to participate in this study was obtained. Once collected, the specimens were kept in a refrigerated container, and immediately transported to the laboratory for pathogen screening. The isolates represented the predominant (more than 70% of the population) colony phenotype observed in culture after twenty-four hours at 37 °C. Once selected, they were cryopreserved at -20 °C in a brain-heart infusion made up of 30% (v/v) glycerol, for subsequent virulence genes screening.

Reference strains used

The reference strains used in this study included strains E2348/69 (Nataro et al., 1985) for enteropathogenic *E. coli* (EPEC), 17.2 (Baudry et al., 1990) for enteroaggregative *E. coli* (EAEC), C600VT2 (O`Brien et al., 1984) for shiga toxin 2 (*stx2*)-producing *E. coli* and H19VT1 for shiga toxin 1 (*stx1*)-producing *E. coli* (STEC), EDL1493 (Pasteur Institute of Dakar, Senegal) for heat stable (ST) and/or heat labile (LT) toxin-producing enterotoxigenic *E. coli* (ETEC). Strains M90T for enteroinvasive *E. coli* (EIEC), and Hb101 that served as negative control were kindly provided by the Pasteur Institute of Bangui, Central African Republic.

Selection of potential pathogenic strains

Selection criteria for potential pathogenic strains of *E. coli* were phenotypic and included serogrouping and mannose-resistant haemagglutination assay, according to Evans et al. (1979), Zamora et al. (1997) and Baraduc et al. (2000).

Serogrouping

Colonies harvested from an overnight culture at 37 °C on Mueller Hinton agar underwent slide agglutination assay, using the classical twelve monovalent antisera (BioRad, France) directed against O-antigens known to occur in EPEC. Tests were carried out according to the manufacturer's instructions.

Mannose-resistant haemagglutination assay (MRHA)

This was carried out, as a slide agglutination

assay in the presence of 2.5% D-mannose according to Okeke et al. (2000b).

Confirmation assays

Selected isolates that showed MRHA- and/or agglutinated the antisera used underwent a glass adherence assay, and were further tested for the presence of *astA*, *eae, stx1, stx2, st, lt and ipaH* genes by PCR.

Glass adherence testing

EAEC is the only pathotype, which adheres on both cell lines and glass with stacked-brick or honeycomb phenotype. Prior to screening, a series of twelve duplicated experimental tests were reproducibly performed (using reference strains) on Hep-2 cells with the modified cell culture technique (Okeke et al., 2000b) and on cover slides. This technique with slides free from cells was carried out using the following procedure:

All bacterial strains were grown overnight in tryptic soy broth without shaking. 250-µl of Dulbecco's modified Eagle medium containing 0.5% D-mannose was transferred to each well of a twenty-four well cell culture cluster (Corning Incorporated, USA) previously equipped with a 8 mm diameter glass slide. Then 10 µl of the bacterial suspension (0.5 Mcfarland) was added per well. The set was incubated for three hours at 37 °C and 5% CO_2. After incubation, the preparation was washed three times with phosphate-buffered saline, fixed with 70% methanol for five minutes, stained with 10% Giemsa for 15 minutes and examined by oil immersion light microscopy (magnification, 100x). Each isolate was tested in duplicate and examined by two bacteriologists. Adherence to the glass with a stacked-brick or honeycomb phenotype indicated a positive test.

PCR assays

- Lysate preparation

A bacterial suspension prepared from sterile distilled water and bacterial colonies harvested from an overnight culture at 37 °C was heated for 10 minutes at 100 °C, and, immediately transferred into ice for 1 minute. After a 10 minutes centrifugation at 10000g, the supernatant containing the bacterial DNA was collected for polymerase chain reaction (PCR), using the primers listed on Table 1. Primer sequences for *astA* were obtained from Yamamoto and Echeverria (1996), and all the others from Gassama et al. (2001). The different tests were conducted according to the following considerations:

- Reaction volume composition

The reaction mixture consisted of a 10x reaction buffer, 25mM $MgCl_2$, 10 mM dNTPs, 100mM of each primer, 5U/µl Taq polymerase, 10µl of the appropriate DNA suspension. This was completed to 50µl with sterile water.

- Amplification parameters

Amplification was achieved in a thermal cycler, GeneAmp® PCR System 9700™, (Applied Biosystem). For all the genes, predenaturation was achieved at 94 °C for five minutes, denaturation at the same temperature for one minute, polymerization at 72°C for one minute, and final extension at 72 °C for seven minutes. The time for annealing was also the same for all the genes (45 seconds), but the required temperature varied (*eae* 65 °C; *stx1, sta, lt, ipaH* 51°C; *stx2* 50 °C, and *astA* 56 °C). Thirty-five cycles were required to complete the process. The amplified product was resolved by a 1.5% agarose gel electrophoresis stained with ethidium bromide, completely soaked in a 1x TBE buffer for 40 minutes at 100 milivolts. The gel was then visualised under ultra violet transillumination. A positive result was determined by the presence of an expected size PCR product, with a 100 bp molecular weight marker.

Confirmation criteria

Confirmation of EAEC was based on the fact that though the *astA* gene can be detected in other pathotypes, it is predominantly associated with attaching and effacing lesions-producing (*eae*-positive) *E. coli,* and EAEC identified by Hep-2 cell culture (Nataro and Kaper, 1998; Presterl et al., 2003).

An exclusion diagnosis was then designed accordingly. Isolates that were positive for *astA*, exhibited stacked-brick (or honeycomb) adherence and negative for other genes were classified as potentially diarrhoeagenic EAEC. Those that were positive for *stx* (*stx1* and/or *stx2*) genes were regarded as STEC. Those that were positive for *eae* and negative for other tests were

Table 1: Primers used in this study.

Designation	Primer sequence	Target gene	Expected amplicon size in base pairs (bp)	Pathotype
Eae1	5'-GGC TCA ATT TGC TGA GAC CAC GGT T-3'	*eae*	494	EPEC
Eae2	5'- GCA AAT TTA GGT GCG GGT CAG CGT T-3'			
East1	5'-CCA TCA ACA CAG TAT ATC CGA-3'	*astA1*	111	EAEC
East2	5'-GGT CGC GAG TGA CGG CTT TGT-3'			
SLTI-1	5'-GAA GAG TCC GTG GGA TTA CG-3'	*stx1*	130	STEC
SLTI-2	5'-AGC GAT GCA GCT ATT AAT AA-3'			
SLTII-1	5'-TTA ACC ACA CCC ACG GCA GT-3'	*stx2*	346	
SLTII-2	5'-GCT CTG GAT GCA TCT CTG GT-3'			
Sta 1	5'-CTG TAT TGT CTT TTT CAC TC-3'	*sta*	182	ETEC
Sta 2	5'-GCA CCC GGT ACA AGC AGG AT-3'			
LT 1	5'-GCG ACA AAT TAT ACC GTG CT-3'	*lt*	707	
LT 2	5'-CCG AAT TCT GTT ATA TAT GT-3'			
IpaH 1	5'-GCT GGA AAA ACT CAG TGC CT-3'	*ipaH*	424	EIEC
IpaH 2	5'-CCA GTC CGT AAA TTC ATT CT-3'			

Eae: enterocyte attachment/effacement; East: enteroaggregative stable toxin; SLTI: shiga-like toxin I; SLTII: shiga-like toxin II; Sta: stable toxin a; LT: labile toxin; IpaH: invasion plasmid H.

identified as EPEC. All *lt* and/or *st*-positive isolates were identified as ETEC, while *ipaH*-positive *E. coli* were classified as EIEC. Isolates were not classified if they harboured both the *eae* and *astA* genes, or exhibited aggregative adherence phenotype, but negative for all the genes targeted in this study.

Statistical analyses

Statistical analyses were conducted using the Chi-square (χ^2) test, adjusted with Yates correction when necessary. The associated likelihoods were accessed at 5% (P value <0.05 indicated significant difference).

RESULTS

Specimens from 384 children were screened for diarrhoeagenic *Escherichia coli*. Samples collected during the dry season included specimens from 57 cases, and 63 controls. During the rainy season, specimens from 123 cases and 141 controls were examined.

Five hundred and nine strains of *E. coli* isolated and cryopreserved underwent selection tests for potential virulence properties. Amongst these isolates, 136 belong to an identifiable O-serogroup [the most prevalent serogroups included O119 (25%), O111 (17%), O126 (13%), and O127 (11%)], and 197 showed a MRHA phenotype. Two hundred and sixty nine isolates (53%) fulfilled at least one of the selection criteria and were subjected to confirmatory PCR tests.

Results obtained after confirmation tests are presented on Table 2.

Overall, EAEC was not associated with diarrhoea (26.1% in cases, and 25.5% in controls; P=0.887). EPEC was significantly associated with diarrhoea (6.7% in cases and 1% in controls; P=0.003). Of the 136 strains that belonged to an identifiable O-serogroup, 57 (41.9%) were ultimately identified as EAEC. Four strains of ETEC were identified, two of which were isolated from cases. One strain of EIEC was isolated from one case. No STEC was detected in this study. Regarding adhesion phenotype, all *astA*-positive *E. coli* were also positive for adhesion phenotype (91% of adhering isolates). Seven isolates presenting aggregative adherence phenotype were negative for all the genes targeted, while three were positive for both *eae* and *astA*.

DISCUSSION

In the present study, EPEC were significantly associated with diarrhoea (6.7% in cases and 1% in controls). It has been assumed (identified by serogrouping) to be the most common pathotype of *E. coli* in Yaoundé. Its prevalence was then estimated at 26% (Fotsing-Kwetché, 2002).

This low infection rate reflects a regional tendency. In the case-control study conducted among children less than five years in Nigeria (Okeke et al., 2000b), a prevalence rate of 1.8% for EPEC was reported among the cases. During a similar study in Gabon (Presterl et al., 2003), no EPEC cases were reported in children of eleven years or younger. The rate of infection due to EPEC in the central African sub-region is probably lower than expected in countries with limited resources (Baraduc et al., 2000).

The ETEC, EIEC and STEC were the least frequent pathotypes observed in this study. Their overall frequencies were 1%, 0.3% and 0%, respectively. They were also less frequent in Nigeria (Okeke et al., 2000b), (2.4%, 1.2% and 0.6 %, respectively). In Gabon (Presterl et al., 2003), 4.6% were positive for ETEC. No STEC or EIEC isolates were identified in that study.

Concerning EAEC, though increasingly acknowledged as a global pathogen, its role in causing diarrhoea remains controversial.

AstA-positive EAEC were not significantly associated with diarrhoea in this study (26.1% in cases, 25.5% in controls). These results, in accordance with those reported from Nigeria (Okeke et al., 2000b), contrasted with those obtained during a similar investigation in Brazil (Zamboni et al., 2004) and in India (Anvikar et al., 2008). The question whether *astA*-positive EAEC could be regarded as a health issue in Cameroon, in spite of its lack of association with diarrhoea is then posed.

Nataro et al. (1998) observed that EAEC was an emerging global pathogen. Nishikawa et al. (1999) also reported an outbreak due to a strain with *astA* as the single virulence determinant, and proposed that diarrhoeal specimens should be examined for the enteroaggregative heat stable toxin 1 (EAST-1, the transcription product of *astA*)-producing *E. coli,* to determine how these

Table 2: Distribution of aetiologies with regard to clinical category.

Pathotype	Dry season				Rainy season				Total					
	cases		controls		cases		controls		cases		controls		χ^2 value	P value
	57		63		123		141		180		204			
EAEC	22	38.6%	21	33.3%	25	20.3%	31	22.0%	47	26.1%	52	25.5%	0.02	0.887
EPEC *	2	3.5%	0	0.0%	10	8.1%	2	1.4%	12	6.7%	2	1.0%	**8.80**	**0.003**
ETEC	1	1.8%	1	1.6%	1	0.8%	1	0.7%	2	1.1%	2	1.0%	0.14	0.708
EIEC	0	0.0%	0	0.0%	1	0.8%	0	0.0%	1	0.6%	0	0.0%	ND	
EHEC	0	0.0%	0	0.0%	0	0	0	0.0%	0	0.0%	0	0.0%	-	
	25		22		37		34		62		56			

* Significant association with diarrhoea
EAEC: Enteroaggregative *E. coli*; EPEC: Enteropathogenic *E. coli*; ETEC: Enterotoxigenic *E. coli*; EIEC: Enteroinvasive *E. coli*; EHEC: Enterohemorrhagic *E. coli.*
ND: not determined

organisms are distributed worldwide. McVeigh et al. (2000) gave evidence of the enterotoxic activity of the EAST-1 protein. The EAST-1 enterotoxin was also found to be associated with intestinal inflammatory response (Nataro et al., 1998; Jiang et al., 2002), most frequently in children (Piva et al., 2003; Zamboni et al., 2004). Several other authors (Okeke et al., 2000b; Piva et al., 2003) used the *astA* determinant to identify EAEC in different settings.

In addition, the present study indicates association between the *astA* gene and the adherence phenotype (91% of adhering isolates are positive for *astA*). Adhesion and toxin production are major mechanisms involved in EAEC diarrhoea. Why strains were not associated with diarrhoea remains a puzzle. Genetic and environmental factors interact to produce a phenotype, including virulence (Martinez and Baquero, 2002).

The absence of association between *astA*-positive EAEC and diarrhoea could be related to a set of parameters among which are: the patient's general health status, the strain involved, the diagnosis techniques used including case definition, and the level of endemicity. In fact, it was observed (Adachi et al., 2002) that in the regions where EAEC is endemic, it is frequent in food. In an earlier study (Ndayo et al., 2000), it was reported that food sold in the streets of Yaoundé and Douala (towns in Cameroon) were strongly contaminated by *E. coli.* The study did not, however, investigate the different pathotypes. The genes involved could also influence the onset of diarrhoea (strains of this pathotype are very heterogeneous as regard virulence determinants). Wakimoto et al., (2004) highlighted the fact that strains possessing the transcriptional activator, *aggR,* exhibited stronger biofilm formation, typical aggregative adherence, and were likely more virulent than those that did not have it. In the case of high rates of contamination, vulnerable subjects including international travellers and immunocompromised persons are the most often exposed to diarrhoea caused by EAEC.

EAEC can cause diarrhoea in hosts other than children, and produce other pathologies than diarrhoea among children. It has been reported in association with diarrhoea in adults (Okeke et al., 2003), travellers (Huang et al., 2003), and HIV-infected subjects (Gassama et al., 2001; Mossoro et al., 2002). Growth retardation and malnutrition due to EAEC were also reported in children (Nataro et al., 1998; Nataro and Kaper, 1998). Accordingly, the pathogenic potentials of EAEC are very likely underestimated when children diarrhoea is regarded as the sole pathology.

In fact, up to 41.9% of seroagglutinating strains were ultimately identified as *astA*-positive EAEC. It is likely that most strains diagnosed by seroagglutination (the routine procedure in the setting) as EPEC belong to this category. The need to better characterize *E. coli* in the routine procedure is an urgent research priority in Yaoundé. Works are underway to identify others potential aetiologies of childhood diarrhoea.

Conclusions

The predominant pathotype identified in this study was EAEC, followed by EPEC that was less frequently isolated than expected. The isolation rates of different pathotypes were similar to those observed in other countries of the sub-region. *AstA*-positive EAEC could be a major public health danger in Yaoundé. These highlight the need to improve the protocol used in this study in order to better characterize EAEC and implement it in routine process.

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