

Original Article

Molecular and phenotypic typing of enteropathogenic *Escherichia coli* isolated in childhood acute diarrhea in Abuja, Nigeria

Casmir Ifeanyiichukwu Cajetan Ifeanyi¹, Nkiruka Florence Ikeneche², Bassey Enya Bassey³, Stefano Morabito⁴, Caterina Graziani⁴, Alfredo Caprioli⁴

¹ Department of Veterinary Microbiology, Faculty of Veterinary Medicine, University of Abuja, Abuja, Nigeria

² Department of Medical Microbiology, College of Health Science, University of Abuja, Abuja, Nigeria

³ World Health Organization, Nigeria Country Office, UN House, Plot 617/618 Diplomatic Drive, Central Business District, Garki, Abuja, Nigeria

⁴ EU Reference Laboratory for *E. coli*, Department of Veterinary Public Health and Food Safety, Istituto Superiore di Sanita, Rome, Italy

Abstract

Introduction: Enteropathogenic *Escherichia coli* (EPEC) causes infectious diarrhea among children in developing countries. However, in Nigeria, due to limited laboratory resources, the genetic diversity of its virulence factors, which include intimin subtypes, remains undefined. **Methodology:** EPEC isolates from diarrheic children 60 months of age and younger in Abuja, Nigeria, were analyzed. Polymerase chain reaction (PCR) for EPEC virulence gene, Hep-2 cell adherence, and serotyping were performed. EPEC strains were further subtyped by PCR for the identification of intimin subtype genes α (alpha), β (beta), γ 1 (gamma-1), and ϵ (epsilon). Antibiotic resistance and extended-spectrum beta-lactamase (ESBL) production was determined by Clinical and Laboratory Standards Institute guidelines.

Results: Overall, 18 (4.5%) out of 400 children with acute diarrhea had EPEC infection. Typical EPEC (tEPEC) strains were detected in 14 (3.5%), whereas 4 (1.1%) were atypical EPEC (aEPEC). A total of 15 (83.3%) of the EPEC isolated belonged to β intimin subtype gene, while the remaining 3 EPEC isolates possessed the intimin ϵ subtype. No α and γ intimin subtypes were detected. Traditional EPEC serotypes O114:H14 were detected only in tEPEC strains. Marked resistance to β -lactam agents were observed but no ESBL-producing tEPEC or aEPEC was detected.

Conclusions: This is the first report of intimin subtype genes in Abuja, Nigeria. EPEC isolates of diverse serotypes resistant to β -lactam antimicrobial agents were observed. These data will be useful in facilitating the characterization of intimin variants of EPEC and some Shiga toxin-producing *E. coli* (STEC) in humans and other animal species.

Key words: gastroenteritis, enteropathogenic, intimin gene, bundle-forming pilus.

J Infect Dev Ctries 2017; 11(7):527-535. doi:10.3855/jidc.9338

(Received 23 August 2016 – Accepted 17 december 2016)

Copyright © 2017 Ifeanyi *et al.* This is an open-access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Introduction

Enteropathogenic *Escherichia coli* (EPEC) implicated in gastrointestinal infections represents a major causative agent of infant diarrhea [1]. It has been recognized to exhibit a great pathogen-attributable risk of death in infants 11 months of age and younger [2]. Studies have shown that its morbidity and mortality rates, especially in developing countries, are quite significant [3].

The hallmark of EPEC infection is the formation of the attaching and effacing (A/E) lesions on the gut mucosa, characterized by microvilli destruction [4,5]. This lesion is the direct consequence of an adhesin known as intimin that allows the *E. coli* to adhere to the intestinal epithelium of its host cells [6]. Its adherence to the intestinal mucosa causes a rearrangement of the

actin in the host cell, resulting in significant deformation [7]. However, the exact mechanisms of diarrhea in EPEC infection are not completely understood [8,2].

EPEC have the locus for enterocyte effacement (LEE), a chromosomal pathogenicity island that houses the translocated intimin receptor (*tir*) gene, the outer membrane protein intimin for attaching and effacing (*eae*) gene, but do not carry genes for the phage-borne Shiga toxins (*stx*) of enterohemorrhagic *E. coli* (EHEC) [6,9,10]. The intimin is localized in the central region of LEE where it binds with its Tir receptor. This protein-receptor interaction is what is responsible for the tight association of the EPEC pathogen with the host cell [11,12].

The intimin gene *eae* has been severally cloned from different EPEC and EHEC strains isolated from humans and animals [12]. The diversity of the *eae* gene sequence has been the subject of several investigations. It has also been hypothesized that the heterologous sequences of the *eae* genes of EPEC and EHEC explain the different host cell tropism (small bowel/large bowel) that exist between the two pathogroups [10]. The differentiation of intimin alleles remains an important tool for strain typing in routine diagnostics as well as in epidemiological studies [11].

EPEC are classified into typical and atypical strains based on the presence of a plasmid called the *E. coli* adherence factor (EAF) [2,13,14]. *E. coli* strains that are *eae*⁺*bfpA*⁺*stx*⁻ are classified as typical EPEC (tEPEC) [15,16]. Most of these strains belong to the classic O:H serotypes [15], and produce the localized adherence (LA) phenotype associated with the production of bundle-forming pili (BFP) [15,17]. Conversely, *E. coli* strains that are *eae*⁺*bfpA*⁻*stx*⁻ are classified as atypical EPEC (aEPEC) [15]. These strains display either localized-like (LAL), diffuse (DA), or aggregative adherence (AA) patterns [18]. The LAL pattern in aEPEC is associated with the *E. coli* common pilus and other known adhesins [17,19]. Previous studies have significantly associated aEPEC with both endemic diarrhea in children and diarrhea outbreaks [20,21]. Similarly, epidemiological reports on the prevalence of EPEC subgroups in both developed and developing countries indicate that aEPEC are more prevalent than tEPEC [22,23]. However, the actual geographic distributions of the two EPEC subgroups remain controversial, since data can be highly influenced by laboratory resources [24].

Traditionally, the standard diagnostic test for EPEC until the 1980s was mainly based on O- or O:H-serotyping using pooled sera [9,25]. It is now confirmed that serotyping alone can lead to overestimated identification of EPEC [24]. Although relatively unreliable, serotyping paved the way for the phenotypic means associated with pathogenesis for delineating EPEC and the PCR-based molecular method that have led to better identification of EPEC genotypic features and the discovery of its major virulence factors [9,24]. Unfortunately, molecular methodologies are not readily available in Nigeria, as is the case in most developing countries of the world. We believe that vaccine development for pediatric EPEC should be of high priority not only because it is an important cause of potentially fatal infant diarrhea in developing countries, but also because of the high mortality and morbidity rates associated with it [2].

EPEC was reported to be the most prevalent DEC pathotype followed by enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enterohemorrhagic *E. coli* (EHEC), and enteroinvasive *E. coli* (EIEC) in our previous study that determined the diarrheagenic *E. coli* pathotypes isolated from children with diarrhea in Abuja, Nigeria [26]. This underscores the need to further characterize by phenotypic and molecular methods the EPEC strains isolated from the study. In this study, we aimed to evaluate the serotypes, antibiotic resistance, and the intimin subtypes genes associated with typical and atypical EPEC strains isolated from children with acute diarrhea in Federal Capital Territory Abuja, Nigeria.

Methodology

E. coli strains

A total of 18 EPEC strains isolated from fecal cultures of childhood diarrheal disease attributable to diarrheagenic *E. coli* (DEC) in Abuja, Nigeria, were characterized in this study [26].

Extraction of genomic DNA

The procedure described by Zhang *et al.* [11] was employed in the extraction of genomic DNA using NucleoSpin Tissue columns (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. One milliliter (1 mL) of overnight growth of the *E. coli* strains in tryptone broth (Oxoid, Basingstoke, England) was centrifuged for 5 minutes at 8,000 g.

The supernatant was removed and the pellet re-suspended in 180 µL buffer T1 by pipetting up and down. Thereafter, 25 µL of proteinase K was added; the entire mixture was then vortexed vigorously and incubated at 56°C with occasional vortex until complete lysis was obtained.

The lysed suspension was vortexed, and 200 µL of buffer B3 was added and incubated at 70°C with vigorous vortexing for 10 minutes. To adjust the DNA binding conditions, 210 µL of ethanol (96%–100%) was added to the suspension and vortexed vigorously. To bind the DNA, the suspension was applied to the NucleoSpin Tissue column and was inserted into a collection tube (microcentrifuge tube). The tube was centrifuged for 1 minute at 11,000 g. The flow-through was discarded and the column placed back into the collection tube. The centrifugation was repeated until the sample was completely drawn through the matrix and the flow-through discarded at each instance.

The silica membrane of the matrix was washed first by the addition of 500 µL of buffer BW, followed by centrifugation at 11,000 g for 1 minute. The flow-

through was thereafter discarded. The membrane was washed a second time by the addition of 600 μ L of buffer B5 to the column, followed by centrifugation at 11,000 g for 1 minute. Again, the flow-through was discarded thereafter.

To dry the silica membrane and remove the residual ethanol, the column was again centrifuged at 11,000 g for 1 minute. To elute the highly pure DNA, the NucleoSpin Tissue column was inserted into a 1.5 mL microcentrifuge tube, and 100 μ L of pre-heated buffer BE (70°C) was added. This was incubated at room temperature for 1 minute and subsequently centrifuged at 11,000 g for 1 minute. The highly pure DNA was stored at -20°C until use. The quality and purity of the extracted DNA were checked by agarose gel electrophoresis, which was done using agarose 0.8% in 0.5X TBE (pH 8.0) buffer according to the protocol of Sambrook *et al.* for submarine gel electrophoresis [27]. Ethidium bromide (1 %) was added at 5 μ L/100 mL. The wells were charged with 5 μ L of DNA preparations mixed with 1X BPB dye. Electrophoresis was carried out at voltage 5V/cm for 60 minutes at room temperature. DNA was visualized under a UV transilluminator.

Detection of pathogenic *E. coli*

Detection of EPEC virulence genes and other DEC was performed by polymerase chain reaction (PCR) protocols using specific primers for DNA amplifications of nine virulence genes of distinct DEC categories as previously described [26]. The primers used have been previously described by various studies [8,1,28-33] and assayed for the following genes: *eaeA* gene (a structural gene for intimin in VTEC and EPEC), *bfpA* (a structural gene for the bundle-forming pilus of EPEC), VT (*vtx1* and *vtx2* of VTEC), *eltB* and/or *estA* (enterotoxins LT and ST of ETEC), *astA* (heat-stable

enterotoxin I gene), *ipaH* (invasion plasmid antigen [gene of EIEC]), and the anti-aggregation protein transporter gene (*aat*; previously reported as CVD 432) of EAEC. Strains that tested positive for *eae* gene but negative for genes *vtx1* and *vtx2* were selected for HEP-2 cell-adherence assay and intimin gene subtyping [34]. Reference strains used in this PCR included EHEC EDL933 (O157:H7), EPEC 2348/69 (O127:H6), ETEC H10407, ETEC Jep5683, EIEC 11741, and EAEC 17-2. The non-pathogenic *E. coli* strain HB 101 was used as a negative control.

Intimin (*eae*) gene subtyping

The different types of *eae* genes were identified using SK1 as forward primer in all PCR approaches in combination with SK2, LP2, LP3, LP4, and LP5 according to the protocol described by Schmidt *et al.* [35,36]. Amplifications were performed in a total volume of 20 μ L containing 2.0 Mm MgCl₂ and deoxynucleoside triphosphates (dNTPs) at 200 mM, 50 pmol of each primer, 5 μ L of 10-fold concentrated polymerase synthesis buffer, and 2.5 U of Taq DNA polymerase (Fermentas, Burlington, Canada).

The PCR tubes containing the mixture were tapped gently and quickly spun at 10,000 rpm for 15 seconds. The PCR tubes with all the components were transferred to a thermal cycler. The PCR samples were incubated, the DNA denatured to anneal the primers, and the annealed primers extended at the various temperatures and durations stipulated in Table 1. The amplification products were analyzed by electrophoresis on 1% agarose gel. EPEC O127:H6 strain E2348/69, EHEC O157:H7 strain EDL933, EHEC O26:H11 strain H19, and EHEC O103:H2 strain PMK5 were used as reference strains for α , γ , β , and ϵ *eae* intimin types, respectively [12, 33,37,38].

Table 1. PCR primers and conditions for amplification of intimin subtypes.

Primer	Primer sequence	Target gene	Reference strain(s)	PCR conditions	Reference
SK1 ^c	CCCGAATTCGGCACAAGCATAAGC	Conserved region of <i>eae</i> (863 bp)	EDL933,	94°C, 30 s; 52°C, 60 s; 72°C, 60 s ^{a,b}	[35]
SK2	CCCGGATCCGCTCGCCAGTATTCG		E2348/69		
LP2	CCCGAATTCCTATTTTACACAAGTGGC	<i>eae</i> - α (2,807 bp)	E2348/69	94°C, 30 s; 55°C, 60 s; 72°C, 120 s ^{a,b}	[36]
LP3	CCCGAATTCCTATTCTACACAAACCGC	<i>eae</i> - γ (2,792 bp)	EDL933	94°C, 30 s; 48°C, 60 s; 72°C, 90 s ^{a,d}	[36]
				94°C, 30 s; 55°C, 60 s; 72°C, 90 s ^b	
LP4	CCCGTGATACCAGTACCAATTACGGTC	<i>eae</i> - β (2,287 bp)	RDEC-1	94°C, 30 s; 55°C, 60 s; 72°C, 120 s ^{a,b}	[12]
LP5	AGCTCACTCGTAGATGACGGCAAGCG	<i>eae</i> - ϵ (2,608 bp)	PMK5	94°C, 30 s; 55°C, 60 s; 72°C, 120 s ^{a,b}	[12]

^a Before the first cycle the sample was denatured for 2 min at 94°C; ^b After the last cycle, the sample was extended for 5 min at 72°C; ^c Primer SK1 was used as forward primer in all PCR approaches in combination with SK2, LP2, LP3, LP4, LP5; ^d 3 cycles; ^e 28 cycles.

Serotyping

Serotyping of isolates was performed in the National Microbiology Laboratory (NML), Public Health Agency (PHC) of Canada. The determination of O and H antigens was carried out according to the method described elsewhere, employing all available O (O1–O185) and H (H1–H56) antisera [39].

Antimicrobial susceptibility

The characterized 18 EPEC strains were tested for susceptibility to the following antibiotics: nalidixic acid (NAL; 30 µg), ciprofloxacin (CIP; 5 µg), cephalixin (CEPH; 30 µg), cefuroxime (CEFU; 30 µg), amoxicillin (AMX; 25 µg), ceftriaxone (CFX; 30 µg), and amoxicillin-clavulanic acid (AMX-CLAV; 30 µg) (Oxoid, Basingstoke, UK), using the standard Kirby-Bauer disk diffusion susceptibility testing method. *E. coli* ATCC 25922 was used as a control. Multidrug resistance (MDR) for this study was defined as resistance to ≥ 2 classes of antimicrobials agents; using Clinical and Laboratory Standards Institute (CLSI) guidelines, each organism was classified either resistant or susceptible [40,41].

Phenotypic screening of extended-spectrum β-lactamase (ESBL) production

EPEC isolates were tested for extended-spectrum beta lactamase (ESBL) production by the double disk

diffusion (DDD) method using both CTX (30 µg) combined with CLAV (10 µg), and CAZ (30 µg) combined with CLAV (10 µg) (Oxoid, Basingstoke, UK). The zones of inhibition of each isolate were tested on Mueller-Hinton agar plates (Oxoid, Basingstoke, UK). ESBL production was confirmed by a ≥ 5 mm increase in diameter of the inhibition zone of the cephalosporin-plus-clavulanate disk when compared to the cephalosporin disk alone. *Klebsiella pneumonia* ATCC 700603 was used as an ESBL producing control and *E. coli* ATCC 25922 as a negative control [42].

HEp-2 cell adherence assay

EPEC were tested to detect adherence to HEp-2 cell according to the methods previously described [34,43]. Reference EPEC strain 2348/69 (serotype O127:H6), *E. coli* strains E17-2 (serotype O3:H2), and C1845 (serotype O75:NM) showing LA, AA, and DA, respectively, were included as positive control in adherence assays, and *E. coli* K12 was used as a negative control. EPEC isolates that adhered to the HEp-2 monolayers were recorded as adhering in a localized, diffuse, or aggregative pattern.

Ethics approval

The study was approved by the institutional ethics committee boards of the University of Abuja Teaching

Table 2. Distribution of intimin gene types and phenotypic characteristics of enteropathogenic *E. coli* strains isolated from children with diarrhea in Federal Capital Territory, Abuja, Nigeria.

Isolate No.	Serotype (O:H)	Patient demographic features			<i>eae</i>	Intimin type				<i>bfpA</i>	Adherence pattern
		Patient age (months)	Patient sex	Diarrhea type		α	β	ε	γ		
002	O114:H14	07	M	Mucoid	+	-	+	-	-	+	LA
004	O33:H6	11	M	Watery	+	-	+	-	-	+	LA
025	O128ab:H18	24	M	Watery	+	-	-	+	-	+	LA
065	O69:H32	60	F	Watery	+	-	+	-	-	-	LAL
068	O114:H14	16	F	Watery	+	-	+	-	-	+	LA
081	O132:HNM	14	M	Watery	+	-	-	+	-	+	LA
212	ONT:H10	12	M	Watery	+	-	+	-	-	+	LA
215	ONT:H10	10	F	Watery	+	-	+	-	-	+	LA
219	ONT:H6	11	M	Watery	+	-	+	-	-	+	LA
236	O132:HNM	14	M	Watery	+	-	-	+	-	+	LA
246	ONT:H10	10	F	Watery	+	-	+	-	-	+	LA
275	O69:H32	01	M	Watery	+	-	+	-	-	-	LAL
293	ONT:HNT	60	F	Watery	+	-	+	-	-	+	LA
306	O69:H32	60	F	Watery	+	-	+	-	-	-	LAL
317	ONT:HNT	06	M	Watery	+	-	+	-	-	+	LA
347	ONT:HNT	24	F	Mucoid	+	-	+	-	-	+	LA
348	ONT:HNT	24	F	Mucoid	+	-	+	-	-	+	LA
377	O69:H32	01	F	Watery	+	-	+	-	-	-	LAL

LA: localized adherence; LAL: localized-like adherence; α: alpha; β: beta; ε: epsilon; γ: gamma.

Hospital Gwagwalada Abuja and National Hospital Abuja Nigeria.

Results

In this study, 18 EPEC isolates were characterized. A total of 15 (83.3%) of the EPEC strains were isolated from patients presenting with watery diarrhea. Only 3 of the patients had mucoid diarrhea. The mean age (in months) of patients was 20.3 ± 18.9 SD; 10 (55.6%) of the patients were males and 8 (44.4%) were females. PCR results revealed that 14 isolates carried *eae* and *bfpA* genes (typical EPEC strains), and 4 possessed only the *eae* gene (atypical EPEC strains). A total of 11 (61.1%) typical EPEC were positive for intimin type β and displayed LA on Hep2 cell. The atypical EPEC strains (22.2%) possessed an *eae-β* subtype and displayed LAL on Hep2 cell. No EPEC isolate displayed diffuse adherence (DA). The results of the intimin gene types and phenotypic characteristics of the EPEC are presented in Table 2. EPEC strains belonged to 6 O serogroups, 7 H flagellar antigen types, and 8 O:H serotypes. The 11 EPEC strains were of 2 O serogroups (O69 and ONT) and expressed 4 H flagellar antigens (H6, H10, H32, and HNT); 55.5% belonged to only 3 serotypes: O69:H32 (3 strains), ONT:H10 (3 strains), and ONT:HNT (4 strains). The remaining

strains were O114:H14 (2 strains), O132:HNM (4 strains), O33:H6 (1 strain), and O128ab:H18 (1 strain).

Following the screening of the EPEC isolates, intimin gene type β (15 strains, consisting of 4 each of O69:H32 and ONT:HNT strains, 3 ONT:H10 strains, 2 traditional EPEC serotypes O114:H14, and 1 each of ONT:H6 and O33:H6) were detected from 2 patients. Just 2 O132:HNM and 1 O128ab:H18 strains were positive for the intimin type ε. The intimin α and γ were not detected among the EPEC isolates screened.

Table 3 illustrates antimicrobial resistance profiles of (typical and atypical EPEC) isolates studied: AMX and AUG: 6 (33.3%); CEPH and CEFU, 1 (5.6%); AMX, CEPH, and CEFU: 2 (11.1%); AMX, AUG, and CEPH: 3 (16.7%); AMX, AUG, CEPH, and CEFU: 3 (16.7%); NAL, AMX, AUG, CEPH, and CEFU: 3 (16.7%). Typical EPEC displayed high resistance profile to 5 antimicrobials (NAL, AMX, AUG, CEPH, and CEFU) tested. *E. coli* serotypes O114:H14; ONT:H10 and ONT:HNT (all typical EPEC) were resistant to NAL (quinolone). All the serotypes (typical EPEC and atypical EPEC) studied were resistant to amoxicillin. However, typical EPEC and atypical EPEC isolates were 100% sensitive to CFX and CIP (fluoroquinolones).

The growth inhibitory zone diameters of both the typical EPEC and atypical EPEC strains tested for

Table 3. Antimicrobial phenotypes of enteropathogenic *E. coli* isolated from children younger than 5 years of age in Federal Capital Territory, Abuja, Nigeria.

Isolate No.	Serogroup	<i>bfpA</i> gene	β-lactamase phenotypic profile				Antimicrobial resistance profile				
			CTX (30 µg)	CTX (30 µg)/CLAV (10 µg)	CAZ (30 µg)	CAZ (30 µg)/CLA (10 µg)					
002	O114	+	S	S	S	S	AMX,	AUG			
004	O33	+	S	S	S	S	CEPH,	CEFU			
025	O128ab	+	S	S	S	S	AMX,	CEPH,	CEFU		
065	O69	-	S	S	S	S	AMX,	AUG			
068	O114	+	S	S	S	S	NAL,	AMX,	AUG,	CEPH,	CEFU
081	O132	+	S	S	S	S	AMX,	AUG			
212	ONT	+	S	S	S	S	AMX,	AUG,	CEPH,	CEFU	
215	ONT	+	S	S	S	S	AMX,	AUG			
219	ONT	+	S	S	S	S	AMX,	AUG			
236	O132	+	S	S	S	S	AMX,	AUG,	CEPH,	CEFU	
246	ONT	+	S	S	S	S	NAL,	AMX,	AUG,	CEPH,	CEFU
275	O69	-	S	S	S	S	AMX,	AUG,	CEPH		
293	ONT	+	S	S	S	S	NAL,	AMX,	AUG,	CEPH,	CEFU
306	O69	-	S	S	S	S	AMX,	AUG,	CEPH		
317	ONT	+	S	S	S	S	AMX,	AUG			
347	ONT	+	S	S	S	S	AMX,	AUG,	CEPH,	CEFU	
348	ONT	+	S	S	S	S	AMX,	AUG,	CEPH		
377	O69	-	S	S	S	S	AMX,	CEPH,	CEFU		

ONT: O untypeable; S: susceptible; NAL: nalidixic acid; AMX: amoxicillin; AUG: augmentin; CEPH: cephalexin; CEFU: cefuroxime; CTX (30 µg): cefotaxime (30 µg); CTX (30 µg)/CLAV (10 µg): cefotaxime (30 µg) combined with clavulanic acid (10 µg); CAZ (30 µg): ceftazidime (30 µg); CAZ (30 µg)/CLAV (10 µg): ceftazidime (30 µg) combined with clavulanic acid (10 µg)

ESBL production exceeded the zone inhibition diameter indicator criteria for ESBL production. In this study, both the typical EPEC and atypical EPEC strains did not display the resistance patterns of ESBL producers. There was no substantial dissimilarity between the EPEC virulence category susceptibility patterns to phenotypic ESBL confirmatory antimicrobials.

Discussion

EPEC are a major cause of infantile diarrhea in developing countries, including Nigeria. We previously reported the predominant isolation of EPEC pathotype and the detection of atypical EPEC from diarrheal infected children in Abuja, Nigeria [26]. To elucidate the pathogenic, immunologic, clonal, and epidemiologic importance of these EPEC strains, we subtyped their intimin genes and evaluated their phenotypic characteristics.

In earlier studies, the identification of EPEC was mainly based on serotyping. It is now clear that this approach can lead to the overestimation of EPEC among infected patients. Recent studies have shown, however, that they are better and more accurately identified based on their genotypic features [24]. Generally, EPEC are categorized into two subtypes: typical and atypical. EPEC isolates, which are positive for both *eaeA* and *bfpA* genes, are considered typical EPEC; those that are positive for only *eaeA* gene and lack especially the *bfpA* gene and other DEC genes, are designated as atypical EPEC [16,44].

In this study, we analyzed EPEC isolates and based on *eaeA*⁺*bfpA*⁺*stx*⁻ or *eaeA*⁺*bfpA*⁻*stx*⁻ genes profile and classified them into typical and atypical EPEC, respectively. We observed that 14 typical EPEC strains showed the LA adherence phenotype. This adhesion as observed in this study has been reported to be a marker for EPEC pathogenicity mediated by the presence of the EAF plasmid [45]. This plasmid codes for the fimbria denoted BFP and is responsible for the LA pattern characteristic of typical EPEC [2]. The atypical EPEC strains analyzed in this study were positive only for *eae* gene, and lacked the EAF plasmid and the STEC gene and presented the LAL pattern. These findings are in agreement with those in a previous report which showed that LAL pattern is a characteristic of the strains of most serotypes and is mediated mainly by intimin [46].

We also examined EPEC isolates for intimin subtypes α , γ , β , and ϵ and observed that typical EPEC strains only had two different intimin types (β and ϵ). Among the types, intimin β was the most common

subtype identified in both typical and atypical EPEC strains and was distributed over six serotypes. Contrary to our findings, earlier studies had reported detection of intimin θ and γ allele from children with diarrhea in Uruguay and Denmark, respectively [8,47]. Remarkably, the predominance of intimin β in this study is in consonance with several reports that describe intimin β as the most ubiquitous found among human and animal A/E pathogens [48].

The intimin ϵ expressed by three typical EPEC strains in this study was first found in human and bovine STEC [9]. It has recently emerged and been genotyped as the most predominant intimin type among atypical EPEC strains from children with and without diarrhea [47]. Conversely, our report of intimin ϵ expression by typical EPEC strains reflects the dynamics in the expression of intimin alleles and variants among *E. coli* pathotypes.

The O-non-typeable serotypes that expressed intimin β were observed significantly in this study among the typical EPEC strains, while atypical EPEC strains that had intimin β exclusively belonged to O69:H2. This finding is in agreement with several recent reports from Brazil and Iran of the occurrence of non-typeable EPEC serogroups (tEPEC and aEPEC) [49]. Most of the EPEC isolates did not belong to the classical EPEC O-serogroups, similar to what has been recently described in children from Iran and India [50,51]. The detection of EPEC by serological screening for certain *E. coli* O-serogroups is still the method of choice in most of the clinical diagnostic laboratories worldwide [52]. However, had serotyping alone been used to distinguish the diarrheagenic *E. coli* categories, EPEC isolates other than those of the classic serotype would have been missed.

The patterns of antimicrobial resistance phenotypes were strikingly different among the individual EPEC isolates. The observed levels of resistance by EPEC (typical and atypical) for amoxicillin agrees with the findings in a report from a recent study in India of high rates of more than 90% resistance to amoxicillin by EPEC [53]. In the present study, the finding of high rates of resistance to amoxicillin-clavulanic acid (augmentin) is in consonance with a recent report from Burkina Faso, in which more than 70% of *E. coli* isolates were resistant to amoxicillin/clavulanic acid [54]. The increased resistance by diarrheagenic *E. coli* to this class of antibiotic within our sub-region can contribute to development and residual circulation of MDR-encoding genes, especially those mediated by chromosomally encoded enzymes such as *bla*_{CTX-M}, *bla*_{TEM}, and *bla*_{SHV} determinants.

There is similarity in the antimicrobial resistance phenotyping of nalidixic acid (quinolone) with the recent report of EPEC strain resistance to nalidixic acid [41]. This can be a probable indication of the emergence of low-level EPEC resistance to nalidixic acid in our country. Typical EPEC exhibited more resistance to the antimicrobials tested compared to the atypical EPEC. This is similar to reports from earlier studies [49,52]. Classification of MDR, based on published criteria, stipulates that a strain of EPEC is considered to be multidrug resistant if it is non-susceptible to at least three families of antibiotics [55]. Curiously, the MDR phenotypes described in the present study included classes of antimicrobials commonly used in the two most fatal pediatric infections (diarrheal and respiratory). These findings highlight the need for continuous surveillance of the antimicrobial resistance patterns in enteric bacterial pathogens.

EPEC strains encoding for ESBLs isolated from children have widely been reported in various parts of the world [56-59]. Nevertheless, no ESBL-producing EPEC strain was detected in this study. This result is in agreement with an earlier study in Brazil, where none of the EPEC were characterized as ESBL producers [49].

Though the intimin-subtyping scheme applied in this study may not have been comprehensive and substantial, we believe that intimin subtypes and variants may exist undetected in the country. Therefore, we hope that these data will be useful in facilitating the characterization of *eae* among *E. coli* isolates recovered from humans and other animal species.

Conclusions

Our results demonstrate the diversity in the serotypes of EPEC strains. This is the first report of intimin subtype genes in Abuja, Nigeria. It reinforces the need for a comprehensive intimin subtyping to elucidate the immunological and epidemiological significance of EPEC infection, particularly atypical EPEC, in childhood acute gastroenteritis in Abuja, Nigeria.

Acknowledgements

The authors thank all those who helped and collaborated for the success of this work. We thank the staff of the Enteric Diseases Program (WHO Collaborating Centre) at the National Microbiology Laboratory (Canada) for assisting with serotyping and phage typing, including Dr. Celine Nadon, Rafiq Ahmed, Helen Tabor, and Dr. Matthew Gilmour. We thank the laboratory staff of Laboratoire de Contrôle des Eaux et Denrées Alimentaires de l'Institut

Pasteur de Tunis in Tunis, Tunisia. We also thank the staff of EU-Reference Laboratory for *E. coli* Istituto Superiore di Sanità, Rome, Italy for their valuable input into this study.

References

- Dupont A, Somer F, Zhang K, Repnik U, Basic M, Bleich A, Kuhnel M, Backhed F, Litvak Y, Fulde M, Rosenshine I, Hornef MW (2016) Age-dependent susceptibility to enteropathogenic *Escherichia coli* (EPEC) infection in mice. *PLoS Pathog* 12: e1005616.
- Ochoa T, Contreras C (2011) Enteropathogenic *E. coli* (EPEC) infection in children. *Curr Opin Infect Dis* 24: 478-483.
- Kotloff KL, Nataro JP, Blackwelder WC, Nasrin D, Faraq TH, Panchaligam S, Wu Y, Sow SO, Sur D, Breiman RF, Faruque AS, Zaidi AK, Saha D, Alonso PL, Tamboura B, Sanogo D, Onwuchekwa U, Manna B, Ramamurthy T, Kanungo S, Ochieng JB, Omoro R, Oundo JO, Hossain A, Das SK, Ahmed S, Qureshi S, Quadri F, Adegbola RA, Antonio M, Hossani MJ, Akinsola A, Mandomando I, Nhampossa T, Acaios S, Biswas K, O'Reilly CE, Mintz ED, Berkeley LY, Muhsen K, Sommerfelt H, Robins-Brown RN, Levine MM (2013) Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. *Lancet* 382: 209-222.
- Moon HW, Whipp SC, Argenzio RA, Levine MM, Giannella RA (1983) Attaching and effacing activities of rabbit and human enteropathogenic *Escherichia coli* in pig and rabbit intestines. *Infect Immun* 41: 1340-1351.
- Vallance B, Finlay B (2000) Exploitation of host cells by enteropathogenic *Escherichia coli*. *Proc Natl Acad Sci USA* 97: 8799-806.
- Jerse AE, Yu J, Tall BD, Kaper JB (1990) A genetic locus of enteropathogenic *Escherichia coli* necessary for the production of attaching and effacing lesions on tissue culture cells. *Proc Natl Acad Sci USA* 87: 7839-7843.
- Celli J, Deng W, Finlay B (2000) Enteropathogenic *Escherichia coli* (EPEC) attachment to epithelial cells: exploiting the host cell cytoskeleton from the outside. *Cell Microbiol* 2: 1-9.
- Mora A, Blanco M, Blanco JE, Dahbi G, López C, Justel P, Alonso MP, Echeita A, Bernárdez MI, González EA, Blanco J (2007) Serotypes, virulence genes and intimin types of Shiga toxin (verocytotoxin)-producing *Escherichia coli* isolates from minced beef in Lugo (Spain) from 1995 through 2003. *BMC Microbiol* 7: 13-21.
- Okeke I (2009) Diarrheagenic *Escherichia coli* in sub-Saharan Africa: status, uncertainties and necessities. *J Infect Dev Ctries* 3: 817-842. doi:10.3855/jidc.586.
- Kenny B, DeVinney R, Stein M, Reinscheid DJ, Frey EA, Finlay BB (1997) Enteropathogenic *Escherichia coli* (EPEC) transfers its receptor for intimate adherence into mammalian cells. *Cell* 91: 511-520.
- Zhang WL, Kohler B, Oswald E, Beutin L, Karch H, Morabito S, Caprioli A, Suerbaum S, Schmidt H (2002) Genetic diversity of intimin genes of attaching and effacing *Escherichia coli* strains. *J Clin Microbiol* 40: 4486-4492.
- Oswald E, Schmidt H, Morabito S, Karch H, Marches O, Caprioli A (2000) Typing of intimin genes in human and animal enterohemorrhagic and enteropathogenic *Escherichia coli*: characterization of a new intimin variant. *Infect Immun* 68: 64-71.

13. Stone K, Zhang H, Carlson I, Donnenberg M (1996) A cluster of fourteen genes from enteropathogenic *Escherichia coli* is sufficient for the biogenesis of a type IV pilus. *Mol Microbiol* 20: 325-337.
14. Pelayo J, Scaletsky ICA, Pedroso M, Sperandio V, Giron JA, Frankel G, Trabulsi L (1999) Virulence properties of atypical EPEC strains. *J Med Microbiol* 48: 41-49.
15. Kaper JB (1996) Defining EPEC. In: Proceedings of the International Symposium on Enteropathogenic *Escherichia coli* (EPEC). *Rev Microbiol* 27: 130-133.
16. Trabulsi L, Keller R, Tardelli Gomes T (2002) Typical and atypical enteropathogenic *Escherichia coli*. *Emerg Infect Dis* 8: 508-513.
17. Giron JA, Ho AS, Schoolnik GK (1991) An inducible bundle-forming pilus of enteropathogenic *Escherichia coli*. *Science* 254: 710-713.
18. Scaletsky ICA, Silva ML, Trabulsi LR (1984) Distinctive patterns of adherence of enteropathogenic *Escherichia coli* to HeLa cells. *Infect Immun* 45: 534-536.
19. Scaletsky ICA, Aranda KRS, Souza TB, Silva NP (2010) Adherence factors in atypical enteropathogenic *Escherichia coli* strains expressing the local adherence-like pattern in Hep-2 Cells. *J Clin Microbiol* 48: 302-306.
20. Scaletsky ICA, Pedroso MZ, Oliva CAG, Carvalho RLB, Morais MB, Fagundes-Neto U (1999) A localized adherence-like pattern as a second pattern of adherence of classic enteropathogenic *Escherichia coli* to HEP-2 cells that is associated with infantile diarrhea. *Infect Immun* 67: 3410-3415.
21. Jenkins C, Lawson AJ, Cheasty T, Willshaw GA, Wright P, Dougan G, Frankel G, Smith HR (2003) Subtyping intimin genes from enteropathogenic *Escherichia coli* associated with outbreaks and sporadic cases in the United Kingdom and Ireland. *Mol Cell Probes* 17: 149-156.
22. Knutton S, Shaw R, Phillips AD, Smith HR, Willshaw GA, Watson P, Price E (2001) Phenotypic and genetic analysis of diarrhea-associated *Escherichia coli* isolated from children in the United Kingdom. *J Pediatr Gastroenterol Nutr* 33: 32-40.
23. Nair GB, Ramamurthy T, Bhattacharya MK, Krishnan T, Ganguly S, Saha DR, Rajendran K, Manna B, Ghosh M, Okamoto K, Takeda Y (2010) Emerging trends in the etiology of enteric pathogens as evidenced from an active surveillance of hospitalized diarrhoeal patients in Kolkata, India. *Gut Pathog* 2: 4-17.
24. Santana S, Diaz N, Fiori PL, Francisco M, Sidat M, Cappuccinelli P, Rappelli P (2013) Genotypic and phenotypic features of enteropathogenic *Escherichia coli* isolated in industrialized and developing countries. *J Infect Dev Ctries* 7: 214-219. doi: 10.3855/jidc.3054.
25. Shukry S, Zaki AM, DuPont HL, Shoukry I, el Tagi M, Hamed Z (1986) Detection of enteropathogens in fatal and potentially fatal diarrhea in Cairo, Egypt. *J Clin Microbiol* 24: 959-962.
26. Ifeanyi CI, Ikeneche NF, Bassey BE, Al-Gallas N, Ben Aissa R, Boudabous A (2015) Diarrheogenic *Escherichia coli* pathotypes isolated from children with diarrhea in the Federal Capital Territory Abuja, Nigeria. *J Infect Dev Ctries* 9: 165-174. doi: 10.3855/jidc.5528.
27. Sambrook J, Fritsch EF, Maniatis T, Cold Spring Harbor Laboratory (1989) Molecular cloning: a laboratory manual, 2nd ed. In Sambrook J, Fritsch EF, Maniatis T, editors. *Eukaryotic cells: Laboratory manuals*. New York: Cold Spring Harbor Laboratory Press. 125-128.
28. Gunzburg S, Tornieporth N, Riley L (1995) Identification of enteropathogenic *Escherichia coli* by PCR-based detection of the bundle-forming pilus gene. *J Clin Microbiol* 33: 1375-1377.
29. Rüssmann H, Kothe E, Schmidt H, Franke S, Harmsen D, Caprioli A, Karch H (1995) Genotyping of Shiga-like toxin genes in non-O157 *Escherichia coli* strains associated with haemolytic uremic syndrome. *J Med Microbiol* 42: 404-410.
30. Schultsz C, Pool G, Van Ketel R, De Wever B, Speelman P, Dankert J (1994) Detection of enterotoxigenic *Escherichia coli* in stool samples by using nonradioactively labeled oligonucleotide DNA probes and PCR. *J Clin Microbiol* 32: 2393-2397.
31. Tornieporth NG, John J, Salgado K, De Jesus P, Latham E, Melo N, Gunzburg ST, Riley LW (1995) Differentiation of pathogenic *Escherichia coli* strains in Brazilian children by PCR. *J Clin Microbiol* 33: 1371-1374.
32. Morabito S, Karch H, Mariani-Kurkdjian P, Schmidt H, Minelli F, Bingen E, Caprioli A (1998) Enterotoxigenic, Shiga toxin-producing *Escherichia coli* O111:H2 associated with an outbreak of hemolytic-uremic syndrome. *J Clin Microbiol* 36: 840-842.
33. Savarino SJ, Fasano A, Watson J, Martin BM, Levine MM, Guandalini S, Guerry P (1993) Enterotoxigenic *Escherichia coli* heat-stable enterotoxin 1 represents another subfamily of *E. coli* heat-stable toxin. *Proc Natl Acad Sci USA* 90: 3093-3097.
34. Vial PA, Mathewson JJ, DuPont HL, Guers L, Levine MM (1990) Comparison of two assay methods for patterns of adherence to HEP-2 cells of *Escherichia coli* from patients with diarrhoea. *J Clin Microbiol* 28: 882-885.
35. Schmidt H, Plaschke B, Franke S, Rüssmann H, Schwarzkopf A, Heesemann J, Karch H (1994) Differentiation in virulence patterns of *Escherichia coli* possessing *eae* genes. *Med Microbiol Immunol Berl* 183: 23-31.
36. Schmidt H, Rüssmann H, Karch H (1993) Virulence determinants in nontoxigenic *Escherichia coli* O157 strains that cause infantile diarrhea. *Infect Immun* 61: 4894-4898.
37. O'Brien AD, Newland J, Miller S, Holmes R, Smith H, Formal S (1984) Shiga-like toxin-converting phages from *Escherichia coli* strains that cause hemorrhagic colitis or infantile diarrhoea. *Science* 226: 694-696.
38. Mariani-Kurkdjian P, Denamur A, Milon B, Picard H, Cave N, Lambert-Zechovsky C, Loirat P, Goulet P, Sansonetti PJ, Elion J (1993) Identification of a clone of *Escherichia coli* O103:H2 as a potential agent of hemolytic-uremic syndrome in France. *J Clin Microbiol* 31: 296-301.
39. Blanco JE, Blanco M, Blanco J, Mora A, Balaguer L, Cuervo L, Balsalobre C, Muñoz F (1997) Prevalence and characteristics of enteropathogenic *Escherichia coli* with the *eae* gene in diarrhoeic rabbits. *Microbiol Immunol* 4: 77-82.
40. Vargas M, Gascón J, Casal C, Schellenberg D, Urasa H, Kahigwa E, Ruiz J, Vila J (2004) Etiology of diarrhea in children less than five years of age in Ifakara, Tanzania. *Am J Trop Med Hyg* 70: 536-539.
41. Clinical and Laboratory Standards Institute (2006) Performance standards for antimicrobial susceptibility testing. Sixteenth Informational Supplement. Wayne, PA: CLSI.
42. Clinical and laboratory standard Laboratory Standards Institute (2012) Performance standards for antimicrobial susceptibility testing. Twenty-Second Informational Supplement. Wayne, PA: CLSI.

43. Cravioto A, Tello A, Navarro A, Ruiz J, Villafán H, Uribe F, Eslava C (1991) Association of *Escherichia coli* HEp-2 adherence patterns with type and duration of diarrhoea. *Lancet* 337: 262-264.
44. Nunes B, Saridakis O, Irino K (2003) Genotypic and phenotypic characterization of attaching and effacing *Escherichia coli* (AEEC) which were isolated from children with and without diarrhoea Londrina, Brazil. *J Med Microbiol* 52: 499-504.
45. Ochoa TJ, Barletta F, Contreras C, Mercado E (2008) New insights into the epidemiology of enteropathogenic *Escherichia coli* infection. *Trans R Soc Trop Med Hyg* 102: 852-856.
46. Pelayo J, Scaletsky I, Pedroso M, Sperandio V, Giron J, Frankel G (1999) Virulence properties of atypical EPEC strains. *J Med Microbiol* 48: 41e.
47. Jensen C, Ethelberg S, Olesen B, Schiellerup P, Olsen KEP, Scheutz F, Nielsen J, Neimann J, Høgh B, Gemer-Smith P, Molbak K, Krogh KA (2007) Attaching and effacing *Escherichia coli* isolates from Danish children: clinical significance and microbiological characteristics. *Clin Microbiol Infect* 13: 863-872.
48. Xu Y, Bai X, Zhao A, Zhang W, Ba P, Liu K, Jin Y, Wang H, Guo Q, Sun H, Xu J, Xiong Y (2016) Genetic diversity of intimin gene of atypical enteropathogenic *Escherichia coli* isolated from human, animals and raw meats in China. *PLoS One* 11: e0152571.
49. Pitondo-Silva A, Nakazato G, Falcao JP, Kinue Irino K, Martinez R, Darini AC, Hernandez RT (2015) Phenotypic and genetic features of enteropathogenic *Escherichia coli* isolates from diarrheal children in the Ribeirão Preto metropolitan area, São Paulo State, Brazil. *Acta Pathol Microbiol Immunol Scand* 123: 128-135.
50. Memariani M, Najari-Peerayeh S, Salehi T, Khalil S, Mostafavi S (2015) Occurrence of SHV, TEM and CTX-M β -lactamase genes among enteropathogenic *Escherichia coli* strains isolated from children with diarrhoea. *Jundishapur J Microbiol* 8: e15620.
51. Malvi S, Appannanavar S, Mohan B, Kaur H, Gautam N, Bharti B, Kumar Y, Taneja N (2015) Comparative analysis of virulence determinants, antibiotic susceptibility patterns and serogrouping of atypical enteropathogenic *Escherichia coli* versus typical enterotoxigenic *Escherichia coli* in India. *J Med Microbiol* 64: 1208-1215.
52. Dhanashree B, Mallya SP (2012) Molecular typing of enteropathogenic *Escherichia coli* from diarrheagenic stool samples. *J Clin Diagn Res* 6: 400-444.
53. Sudershan R, Naveen K, Bharathi K, Kashinath L, Bhaskar V, Polasa K (2014) *E. coli* pathotypes and their antibiotic resistance in young children with diarrhoea in Hyderabad, India. *Int J Curr Microbiol App Sci* 3: 647-654.
54. Dembélé R, Bonkougou IJO, Konaté A, Tchamba GB, Bawa, HI, Bako E, Bagré TS, Kagambèga A, Zongo C, Traor, AS, Barro N (2015) Serotyping and antimicrobial resistance of enteropathogenic *Escherichia coli* and enterohemorrhagic *E. coli* O157 isolated from children under five years of age with diarrhea in rural Burkina Faso. *African J Microbiol Res* 9: 1053-1059.
55. Shamki JA, Al-Charrakh AH, Al-Khafaji JK (2012) Detection of ESBLs in enteropathogenic *E. coli* (EPEC) isolates associated with infantile diarrhea in Kut City. *Med J Baby* 9: 404-412.
56. Shakya P, Barrett P, Diwan V, Marothi Y, Shah H, Chari N, Tamhankar AJ, Pathak A, Lundborg CS (2013) Antibiotic resistance among *Escherichia coli* isolates from stool samples of children aged 3–14 years from Ujjain, India. *BMC Infect Dis* 14: 2-6.
57. Franicek R, Sobieszczanska B, Turniak M, Kasprzykowska U, Krzyanowska B, Jermakow K, Mokracka-Lataika G (2012) ESBL-producing *Escherichia coli* isolated from children with acute diarrhea - antimicrobial susceptibility, adherence patterns and phylogenetic background. *Adv Clin Exp Med* 21: 187-192.
58. Vignoli R, Varela G, Mota MI, Cordeiro NF, Power P, Ingold E, Gadea P, Sirok A, Schelotto F, Ayala JA, Gutkind G (2005) Enteropathogenic *Escherichia coli* strains carrying genes encoding the PER-2 and TEM-116 extended-spectrum beta-lactamases isolated from children with diarrhea in Uruguay. *J Clin Microbiol* 43: 2940-2943.
59. Khoshvaght H, Haghi F, Zeighami H (2014) Extended spectrum beta-lactamase producing Enterococci *Escherichia coli* from young children in Iran. *Gastroenterol Hepatol Bed Bench* 7: 131-136.

Corresponding author

Casmir Ifeanyi Chukwu Cajetan Ifeanyi
 Department of Veterinary Microbiology
 Faculty of Veterinary Medicine
 University of Abuja
 Abuja Airport Road, Kilometer 23
 P.M.B 117, Abuja, Nigeria
 Phone: +2348030750990
 Email: ifeanyi.casmir@gmail.com

Conflict of interests: No conflict of interests is declared.