The frequency of virulent genes and antimicrobial resistance patterns of diarrheagenic *Escherichia coli* isolated from stools of children presenting with diarrhea in a tertiary hospital in Abakaliki, Nigeria

Ebuka Elijah David[1] , Muhammad Arfat Yameen[2], Ikechuku Okorie Igwenyi[3], Arthur Chinedu Okafor[4] ,
Uket Nta Obeten[1], Doris Olachi Obasi[1], Uchenna Regina Ezeilo[1] and Chidinma Nnenne David[5]

1. Department of Chemistry/Biochemistry, Alex-Ekwueme Federal University Ndufu-Alike, Ikwo, Ebonyi State, Nigeria;
2. Department of Pharmacy, COMSATS Institute of Information Technology, Abbottabad, Pakistan; 3. Department of
Biochemistry, Ebonyi State University, Ebonyi State, Nigeria; 4. Department of Microbiology, Edo University, Iyamho, Edo
State, Nigeria; 5. Department of Microbiology, Ebonyi State University, Ebonyi State, Nigeria.
Corresponding author: Ebuka Elijah David, e-mail: david.ebuka@funai.edu.ng
Co-authors: MAY: arfatyameen@gmail.com, IOI: igwenyike@yahoo.com, ACO: arthur.okafor@edouniversity.edu.ng,
UNO: uketgabriel@gmail.com, DOO: obasidoris19@gmail.com, URE: ucmgbenka@gmail.com, CND: nnenne.nnaji@gmail.com
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Abstract

Aim: This study was aimed to determine the virulent genes and antibiotic resistance patterns among circulating diarrheagenic
Escherichia coli (DEC) pathotypes in a tertiary care health center in east of Nigeria.

Materials and Methods: Diarrheal stool samples were obtained from 80 children under 5 years and *E. coli* was isolated
and identified using standard biochemical and molecular methods. Multiplex polymerase chain reaction (PCR) was used
to detect eight virulent genes of DEC. Disk diffusion method was used to determine the antibiotic susceptibility of DEC.

Results: DEC infection was observed in 54 (68%) children among which *ial* gene for enteroinvasive *E. coli* (EIEC)
(40% [n=22]) was commonly detected followed by *eltA/eltB* for enterotoxigenic *E. coli* (ETEC) (30% [n=16]), *pCVD* for
enteroaggregative *E. coli* (EAEC) (20% [n=11]), and *eaeA/bfpA* for typical enteropathogenic *E. coli* (EPEC) (10% [n=5]).
The DEC isolates phenotypically exhibited resistance for ampicillin (AMP) (44 [81%]), followed by ciprofloxacin (CIP)/
levofloxacin (LEV) (28 [52%]), cefoxitin (FOX) (11 [20%]), and amoxicillin-clavulanic acid (AMC) (6 [11%]). About 60%
isolates of stable toxins-ETEC were resistant to AMC, CIP, and LEV while all the labile toxin-ETEC exhibited resistance
to AMP. About 60% (n=6) resistance were seen in EAEC against ampicillin, AMC, FOX, CIP, and LEV. In EIEC, all the
isolates (n=22) were resistant to AMP while 50% (n=11) were resistant to both CIP and LEV. All EPEC (n=5) were resistant
to AMP, FOX, CIP, and LEV.

Conclusion: High frequency of virulent *ial* and *eltA/eltB* genes for EIEC and ETEC, respectively, suggests that they are the
primary etiological agents of diarrhea in children among DEC pathotypes. Resistance of DEC to more than two classes of
antibiotics indicate possible emergence of multidrug resistance.

Keywords: antibiotic resistance, diarrheagenic *Escherichia coli*, diarrheal stool, multidrug resistance, multiplex polymerase
chain reaction, polymerase chain reaction.

Introduction

Escherichia coli are Gram-negative, oxidase-negative, rod-shaped bacteria from the family *Enterobacteriaceae* [1]. *E. coli* are commensal bacteria found in the intestinal microflora of a variety of animals, including man. All the strains of *E. coli* are not harmless since some can cause debilitating and sometimes fatal diseases in humans and birds [2]. Pathogenic strains of *E. coli* are divided into intestinal pathogenic *E. coli* (InPEC) causing diarrhea and

extraintestinal pathogenic *E. coli* (ExPEC) causing a variety of infections, including urinary tract infections, meningitis, and septicemia [3,4]. InPEC strains of *E. coli*, also known as diarrheagenic *E. coli* (DEC), are a major etiological agent of pediatric diarrhea, accounting for over 2 million deaths annually [5]. DEC continues to be the most common cause of infantile morbidity and mortality, most especially in developing countries and sub-Saharan Africa [6], especially in children under 5 years of age [7]. Nigeria reported at least 20.6% diarrhea-specific deaths, representing about 103,000 of the 500,000 annual global pediatric deaths [1]. DEC can be transmitted through the fecal-oral route by ingesting food or water contaminated by human or animal feces [6,8]. Infection with DEC causes an alteration of the movement of ions and water in the gastrointestinal tract by altering the balance between fluid-electrolyte absorption

and secretion leading to diarrhea [9]. DEC is divided into enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli*, entero-invasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli*. The divisions of DEC into groups are based on their specific virulence factors and phenotypic traits. Each pathological type has characteristic virulence determinants that contribute to its pathogenic mechanisms [10]. The prevalence and other epidemiological features of DEC types in childhood diarrhea vary with geographical area [5].

Recently, there is an emergence of multidrug resistance in all pathological types of *E. coli* isolated from children with diarrhea, which makes treatment challenging [11]. In the last decade, resistance against the commonly used drugs for the treatment of enteric infections, including ampicillin (AMP), tetracycline, and cotrimoxazole has increased among DEC [12], which have led to the use of higher antimicrobials like fluoroquinolones as alternatives. In addition, the use and misuse of these empirical drugs in these informal sectors, by unlicensed providers and through self-medication, are commonplace in Nigeria and in many other parts of Africa, which has led to both single drug and multidrug resistance [13]. The frequency of DEC and its multidrug resistance in childhood diarrhea has been reported in provincial Southwest and North Central Nigeria [14,15], but little or no information is available for Southeastern part of Nigeria.

The aim of this study was to investigate the frequency of virulent genes and antimicrobial resistance patterns of DEC isolated from stools of children presenting with diarrhea in Alex Ekwueme Federal Teaching Hospital, Abakaliki (AE-FETHA), Ebonyi State, Nigeria.

Materials and Methods

Ethical approval and informed consent

Ethical clearance (Ref. No.: FETHA/REC/VOL.1/2016/386) was obtained from Ethical and Research Committee of the hospital, after which informed consent was obtained from the parents/guardians/attendants of the children.

Isolation and identification of *E. coli*

Eighty fecal samples were collected from children with incidence of diarrhea under the age of 5 years at AE-FETHA from August to October 2018. Fecal samples were processed according to the standard guidelines provided for laboratory diagnosis of enteric pathogens [16]. A loop full of samples was suspended in sterile nutrient broth and incubated at 37°C for 24 h. The isolates from the broth were inoculated directly on MacConkey's agar plates. The isolates were picked up after overnight incubation at 37°C and subcultured in Eosin Methylene Blue, EMB (a selective media for *E. coli*) agar plates at 37°C for 24 h. Other biochemical tests such as indole test, methyl red test, Voges–Proskauer test, citrate utilization test, and Eijkman test were performed on colonies that showed green metallic sheen, typical of *E. coli* isolates.

Standard cetyltrimethylammonium-bromide/sodium chloride method was used to extract *E. coli* genomic DNA, as previously described by Healey *et al.* [17]. The DNA concentrations were measured in ng/µL using Nanodrop instrument (Colibri Spectrometer, Berthold Detection System, Germany). The quality of DNA was analyzed by electrophoresis in 1% agarose gels in TBE buffer at 100 V. The gels were stained with ethidium bromide and photographed under ultraviolet light using a gel documentation system. *E. coli* isolates were further identified using primers derived from the DNA sequences flanking the gene encoding the universal stress protein A (*uspA*); EC1:5$^{I.}$CCGATACGCTGCCAATCAGT-3^{I}: EC2:5$^{I.}$ACGCAGACCGTAAGGGCCAGAT-3^{I} [18,19]. Polymerase chain reaction (PCR) was performed in a total reaction volume of 25 µL with 12.5 µL GoTaq Green Master Mix, 9.0 µL nuclease-free water (Promega, USA), 0.5 µL of forward and reverse primers each, and 2.5 µL of template. Optimization was done at the following conditions: 94° for 5 min, initial template denaturation, 25 cycles at 94° for 30 s, final denaturation, 50° for 1 min, annealing, 72° for 1 min 30 s, extension, and 72° 7 min, final extension. About 884 bp PCR products were analyzed by gel electrophoresis in 1% agarose gel in TBE buffer at 100 V. The gels were stained with ethidium bromide and photographed under ultraviolet light using a gel documentation system (Figure-S1 in Additional file-1).

Detection of virulent genes in DEC

Detection of virulent genes of DEC was performed in a group of multiplex PCR using their specific primers as previously described by Nguyen *et al.* [20]. All the primers and their corresponding virulent genes are shown in Table-S1 (Additional file-1). The thermocycling conditions were programmed using Applied Biosystem, 2720 Thermal Cycler, USA, in 25 µL reaction mixture as follows: Initial denaturation for 5 min at 94°C, denaturation at 94°C for 30 s, 72°C for 1 min 30 s, and extensions for 25 cycles with final extension of 5 min at 72°C. PCR products were analyzed by gel electrophoresis in 1% agarose gel in TBE buffer at 100 V. The gels were stained with ethidium bromide and photographed under ultraviolet light using a gel documentation system (Figure-S2 in Additional file-1).

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by the disk diffusion method according to the European Committee on Antimicrobial Susceptibility Testing [21] guidelines for *Enterobacteriaceae*. Antimicrobial agents tested were AMP (10 µg), amoxicillin/clavulanic acid (AMC) (5 µg), cefoxitin (FOX) (30 µg), ciprofloxacin (CIP) (5 µg), imipenem (IMI) (10 µg), and levofloxacin (LEV) (5 µg) (Oxoid Ltd., Basingstoke, Hampshire, England).

The multidrug resistance criteria adopted were defined as earlier published by Magiorakos et al. [22]. *E. coli* ATCC 25922 was used as quality control.

Results

Frequency of DEC virulent pathotypes

A total of 80 diarrheal stool samples from diarrheal children (n=80) were collected for this study. Of the 80 children, 68% (n=54) were found to be positive for one or more pathotype of DEC. Virulent *eltA* gene for stable toxins-ETEC (ST-ETEC) was detected in 20% (n=11) of the children while 10% (n=5) expressed *eltB* for labile toxin-ETEC (LT-ETEC). About 20% (n=11) also expressed pCVD (the nucleotide sequence of EcoR1-Pst1 DNA fragment of pCVD432 of EAEC) gene for EAEC while 40% (n=22) expressed *ial* gene (invasion-associated locus of the invasive plasmid found in EIEC). About 10% (n=5) expressed *eaeA* gene (a structural gene for intimin) and *bfpA* gene (a structural gene for the bundle-forming pilus) found in typical EPEC, Figure-1.

Antibiotic susceptibility testing

The DEC isolates phenotypically exhibited resistance for AMP (44 [81%]) followed by CIP and LEV (28 [52%]), FOX (11 [20%]) and AMC (6 [11%]). None of the isolates were resistant to IMI (0 [0%]), Table-1.

About 60% isolates of ST-ETEC were resistant to AMC, CIP, and LEV while all the LT-ETEC exhibited resistance to AMP. About 60% of resistance were seen in EAEC against ampicillin, AMC, FOX, CIP, and LEV. In EIEC, all the isolates were resistant to AMP while 50% were resistant to both CIP and LEV. All EPEC were resistant to AMP, FOX, CIP, and LEV, Table-2. Multidrug resistance was seen in greater number of all the pathotypes except LT-ETEC.

Discussion

In this study, the frequency of EIEC (40% [22/54]) was most common among DEC, followed by ETEC (30% [16/54]), EAEC (20% [11/54]), and EPEC (10% [5/54]). The high frequency of EIEC and ETEC among DEC pathotypes suggests their role as most common cause of acute childhood diarrhea in this region. In contrast to high EIEC frequency recorded in this study, the low rate (1.2%) of recovery of EIEC in the study carried out in Southwest Nigeria by Okeke *et al*. [14], in India (1.8%) [23], and Ecuador (3.2%) [24], suggested that EIEC may play less important role in childhood diarrhea in developing countries. EIEC infection is characterized by the ability of bacteria to invade the human colonic mucosa, conferred by the expression of chromosomal and plasmid-borne genes [3]. High frequencies of ETEC have been recorded in other parts of Nigeria [25] and other developing countries [26]. The high ETEC frequency from this study also agreed with the one recorded in Onitsha, Southeast Nigeria, where 21.57% ETEC strains were detected among DEC isolates [25]. Similar high ETEC frequency (36.3%) among hospitalized diarrheal children in Kolkata, India,
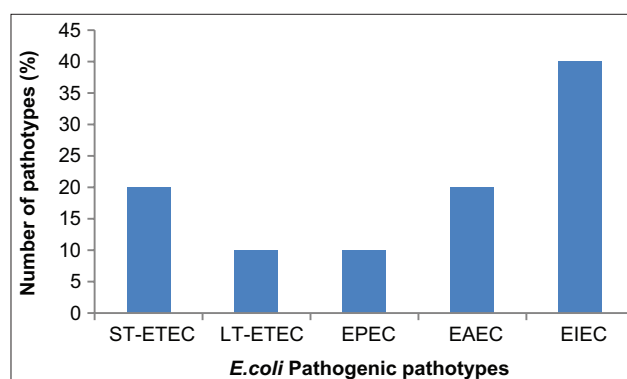
Figure-1: Distribution of diarrheagenic *Escherichia coli* pathotypes.

Table-1: Antibiogram of diarrheagenic *Escherichia coli* isolates (n=54).

Antimicrobials	Resistant (%)	Sensitivity (%)
AMP	44 (81)	10 (19)
AMC	6 (11)	48 (89)
FOX	11 (20)	43 (80)
IMP	0 (0)	54 (100)
CIP	28 (52)	26 (48)
LEV	28 (52)	26 (48)

AMP=Ampicillin, AMC=Amoxicillin-clavulanic acid, FOX=Cefoxitin, IMP=Imipenem, CIP=Ciprofloxacin, LEV=Levofloxacin

was also recorded [26]. Our result was different from the frequency of ETEC obtained among DEC isolated from infants and children in Federal Capital Territory, Abuja, Nigeria, where 4% ETEC frequency was recorded [27] and other developing countries; Dar es Salaam, Tanzania (3.6%) [28]; West Iran (17.5%) [29]; and Hanoi, Vietnam (2.2%) [20]. Consistent with our result, ETEC was not the most prevalent. Clinical human ETEC isolates produce enterotoxins; the heat ST and heat LT and may produce one or more of several colonization factors which mediate adherence to the small intestinal mucosa [30]. In this study, the heat stable ETEC, ST-ETEC showed greater association with childhood diarrhea than heat labile, LT-ETEC. This result agreed with the one performed in Southwest Nigeria [14] and other parts of the world [31,32].

In this study, EAEC ranked second to typical EPEC as the least frequent DEC. When compared to results obtained from other regions, it becomes evident that the prevalence and other epidemiological features of DEC types in childhood diarrhea vary with geographical area [5]. Similar to our result, EPEC was the least prevalent in Ecuador (0.9%) [24], India 4.79% [23], and Egypt (5.2%) [33]. Different from the result of this study, EAEC was recorded as the most prevalent in Kolkata, India (48.2%) [26], Southwest Nigeria (10.3%) [14], and 34.4% in Gwagwalada, Abuja, Nigeria [15].

The DEC isolates were most resistant to AMP, followed by CIP/LEV, FOX, and AMC. Most worrisome is that more than 50% of all the DEC was multidrug and fluoroquinolone (CIP/LEV) resistant. This result is similar with a previous study where most of the DEC

Table-2: Relationship between diarrheagenic *Escherichia coli* virulent pathotypes and antimicrobial resistance.

DEC	*n*	Vir. gene	AMP	AMC	FOX	CIP	LEV	IMP	NODR
ST-ETEC	11	*eltA*	60% (6)	0% (0)	0% (0)	60% (6)	60% (6)	0% (0)	3
LT-ETEC	05	*eltB*	100% (5)	0% (0)	0% (0)	0% (0)	0% (0)	0% (0)	1
EAEC	11	*pCVD*	60% (6)	60% (6)	60% (6)	60% (6)	60% (6)	0% (0)	5
EIEC	22	*ial*	100% (22)	0% (0)	0% (0)	50% (11)	50% (11)	0% (0)	3
EPEC	05	*eaeA* and *bfpA*	100% (5)	0% (0)	100% (5)	100% (5)	100% (5)	0% (0)	4

AMP=Ampicillin, AMC=Amoxicillin-clavulanic acid, FOX=Cefoxitin, IMP=Imipenem, CIP=Ciprofloxacin, LEV=Levofloxacin, NODR=number of drug resistance

isolates (67.5%) were resistant to AMP and tetracycline with ETEC being significantly more resistant to CIP than other DEC group [29]. Such multidrug resistance among DEC isolates against classical antibiotics such as AMP and tetracycline was also recorded in Bolivia, although no fluoroquinolone resistance was observed [30]. Correlation between antimicrobial resistant pattern of *E. coli* from environmental water and clinical diarrheal stool samples in Lagos, Nigeria, further supported our findings that most of the isolates (82%) were resistant to multidrug, including fluoroquinolones [34]. The fluoroquinolone resistance in *E. coli* has been commonly associated with mutations in the *gyrA*, subunit A of gyrase in Gram-negatives [35]. The use of fluoroquinolones as first drug of choice for diarrhea in developing countries where the use of antibiotics is not regulated may lead to a rapid emergence of resistance.. Much of the reasons for these high rates of resistance are related to the fact that, antibiotics, despite not being required for the treatment of acute diarrhea, are widely prescribed for these forms of infections [36]. There is also the difficulty in understanding the complicated dynamics of antimicrobial resistance transmission between humans and animals sharing the same environment. Hence, the need to apply a One Health approach and study environmental reservoirs more closely, rather than focusing only on the resistance that arises following antimicrobial administration [37].

Conclusion

We observed the presence of four different DEC pathotypes with EIEC and ETEC most commonly encountered. High frequency of EIEC and ETEC in this region suggests that it is one of the most common causes of diarrhea in children below 5 years. Most of these DEC are resistant to more than 1 antimicrobial agent. The availability of over-the-counter antibiotics and continued use and misuse of these drugs allow for the selection of resistant isolates. Education of the parents/guardians of these children on the management of diarrheal infections as well as the implementation of more stringent policies governing the empirical treatment of diarrhea by physicians is advised. Therefore, it is important to continue the surveillance of antimicrobial resistance of enteric bacterial pathogens for effective control of childhood diarrheal diseases.

Authors' Contributions

EED: Conceptualization and writing. MAY: Microbial identification and molecular

analysis. IOI: Review and final editing. ACO and UNO: Sample collection and microbial culture. DOO and URE: Interpretation and data analysis. CND: Coordination and formatting. All authors read and approved the final manuscript.

Competing Interests

The authors declare that they have no competing interests.

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Additional File 1

Table-S1: Primer characteristics used in the multiplex polymerase chain reaction.

Primers	Target gene	Sequence	Size (bp)	AT (°C)
LT	*eltB*	5-TCTCTATGTGCATACGGAGC-3 5-CCATACTGATTGCCGCAAT-3	322	56
ST	*estA*	5-GCTAAACCAGTAGAGGTCTTCAAAA-3 5-CCCGGTACAGAGCAGGATTACAACA-3	147	56
VT1	*vt1*	5-GAAGAGTCCGTGGGATTACG-3 5-AGCGATGCAGCTATTAATAA-3	130	56
VT2	*vt2*	5-ACCGTTTTTCAGATTTTGACACATA-3 5-TACACAGGAGCAGTTTCAGACAGT-3	298	53
Eae	*eaeA*	5-CACACGAATAAACTGACTAAAATG-3 5-AAAAACGCTGACCCGCACCTAAAT-3	376	54
SHIG	*ial*	5-CTGGTAGGTATGGTGAGG-3 5-CCAGGCCAACAATTATTTCC-3	320	53
bfpA	*bfpA*	5-TTCTTGGTGCTTGCGTGTCTTTT-3 5-TTTTGTTTGTTGTATCTTTGTAA-3	367	56
EA	*pCVD*	5-CTGGCGAAAGACTGTATCAT-3 5-CAATGTATAGAAATCCGCTGTT-3	630	53
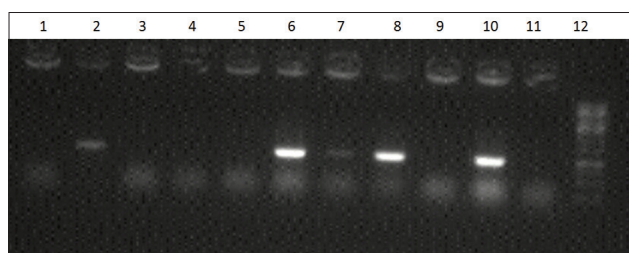
Figure-S1: Representative of molecular identification of *Escherichia coli* strains using *uspA* gene primers. Lines 2, 6, 7, 8, and 10 show the presence of *uspA* genes in the isolates. Line 12 is DNA ladder.
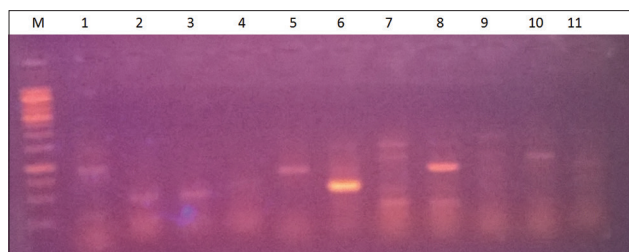
Figure-S2: Representative of multiplex polymerase chain reaction in identification of enterotoxigenic *Escherichia coli* and enteroaggregative *E. coli* using *eltB/eltA* and *pCVD* primers, respectively, *Lane M, marker (1-kb DNA ladder)*; *Lane 1-11, E. coli isolates.*

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