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MULTI-DRUG RESISTANCE AND BIOFILM PRODUCTION AMONG DIARRHEAGENIC *ESCHERICHIA COLI* PATHOTYPES ISOLATED FROM STOOLS OF CHILDREN WITH ACUTE DIARRHEAL DISEASE

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Abstract. *Background.* Diarrheagenic *E. coli* (DEC) is an etiological agent of childhood diarrhea. Resistance against commonly used drugs in the empirical treatment of enteric infections has increased among DEC. Relationship between antibiotic resistance and biofilm formation in microorganisms have been widely reported. This study was aimed to determine the antibiotic resistance and biofilm production pattern among DEC pathotypes isolated from stools of children aged 0–5 years with acute diarrheal disease in Abakaliki, Nigeria. *Materials and methods.* Diarrheal stool samples were obtained from 60 children and *E. coli* were isolated and identified using standard guidelines provided for laboratory diagnosis of enteric pathogens. Molecular identification was done by amplification of *E. coli* universal stress protein A *(uspA)* using polymerase chain reaction (PCR) method. Detection of virulent genes of DEC pathotypes was performed in a group of multiplex PCR using their specific primers. Kirby–Bauer disk diffusion method was used to determine the antibiotic susceptibility patterns of the isolates while biofilms production was detected by thiazolyl blue tetrazolium bromide dye in a 96-well plate. *Results.* DEC was isolated in 40 stools among which EIEC [40% (n = 16)] was commonly detected followed by ETEC [30% (n = 12)], EAEC [20% (n = 8)] and typical EPEC [10% (n = 4)]. Half of EAEC showed the highest multidrug resistance against ampicillin, cefoxitin, ciprofloxacin, levofloxacin, and tetracycline with the strongest biofilm production followed by all the EPEC which were resistant to ampicillin, ciprofloxacin, levofloxacin, and tetracycline with moderate biofilm production. All the LT-ETEC exhibited the least resistance to ampicillin and tetracycline with the weakest biofilm production. *Conclusion.* High frequency of the EIEC pathotype suggests its role as the primary etiological agent of diarrhea in children. Correlation between high drug resistance and biofilm production among the pathotype may indicate that biofilms may provide compatible uptake of resistance genes.

Key words: *diarrheagenic E. coli (DEC), antibiotic resistance, diarrheal stool, multiplex PCR, multi-drug resistance, polymerase chain reaction, biofilms.*

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МНОЖЕСТВЕННАЯ ЛЕКАРСТВЕННАЯ УСТОЙЧИВОСТЬ И ОБРАЗОВАНИЕ БИОПЛЕНОК СРЕДИ ДИАРЕЙНЫХ ПАТОТИПОВ *ESCHERICHIA COLI*, ВЫДЕЛЕННЫХ ИЗ СТУЛА ДЕТЕЙ С ОСТРОЙ ДИАРЕЕЙ

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Резюме. *Актуальность.* Диареагенная кишечная палочка (DEC) является этиологическим агентом диареи у детей. Устойчивость к лекарствам, обычно используемым при эмпирическом лечении кишечных инфекций, среди DEC увеличилась. Широко известна взаимосвязь между устойчивостью к антибиотикам и образованием биопленок у микроорганизмов. Настоящее исследование было направлено на определение устойчивости к антибиотикам и характера биопленкообразования среди патотипов DEC, выделенных из стула детей в возрасте 0–5 лет с острой диареей в Абакалики, Нигерия. *Материалы и методы.* Образцы стула были получены от 60 детей, и с использованием стандартных подходов, предусмотренных для лабораторной диагностики кишечных патогенов, были выделены и идентифицированы *E. coli.* Молекулярную идентификацию проводили путем амплификации участка гена универсального стрессового белка A *(uspA) E. coli* с использованием метода полимеразной цепной реакции (ПЦР). Выявление вирулентных генов патотипов DEC проводили с применением мультиплексной ПЦР со специфическими праймерами. Метод дисковой диффузии Кирби–Бауэра был использован для определения характеристик чувствительности изолятов к антибиотикам, в то время как образование биопленок определяли с помощью красителя тиазолила синего тетразолия бромида в 96-луночном планшете. *Результаты.* DEC была выделена в 40 образцах. Наиболее часто выявлялась энтероинвазивная *E. coli* (EIEC) [40% (n = 16)], затем энтеротоксигенная *E. coli* (ETEC) [30% (n = 12)], энтероагрегативная к *E. coli* (EAEC) [20% (n = 8)] и типичная энтеропатогенная *E. coli* (EPEC) [10% (n = 4)]. Половина штаммов EAEC обнаружила самую высокую множественную лекарственную устойчивость к ампициллину, цефокситину, ципрофлоксацину, левофлоксацину и тетрациклину с самым сильным образованием биопленок. Далее по степени устойчивости к антибиотикам и уровню образования биопленок следовали EPEC, которые были устойчивы к ампициллину, ципрофлоксацину, левофлоксацину и тетрациклину и характеризовались умеренным биопленкообразованием. ETEC с термолабильным токсином LT-ETEC проявляли наименьшую устойчивость к ампициллину и тетрациклину с наименьшим образованием биопленок. *Заключение.* Высокая частота патотипа EIEC свидетельствует о его роли в качестве основного этиологического агента диареи у детей. Корреляция между высокой лекарственной устойчивостью и производством биопленок среди патотипов может указывать на то, что биопленки могут обеспечивать оптимальный уровень захвата генов устойчивости.

Ключевые слова: *диареагенная кишечная палочка (DEC), устойчивость к антибиотикам, диарейный стул, мультиплексная ПЦР, множественная лекарственная устойчивость, полимеразной цепная реакция, биопленки.*

Introduction

Diarrhea is characterized by the passage of watery stools at least two-three times in a 24 h period as a result of gastrointestinal infection majorly caused by a variety of bacterial, viral and parasitic pathogens. Among children less than five years, Nigeria accounts for 11% of 50% global mortalities estimated at 150,000 yearly, with a prevalence rate of 18.8% [2, 18]. Intestinal pathogenic *E. coli* strains also known as diarrheagenic *E. coli* (DEC) is a major etiological agent of pediatric diarrhea [36]. DEC can be transmitted via the fecal-oral route by ingesting food or water contaminated by human or animal feces [1, 11]. 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 [34]. DEC is divided into enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC) and diffusely adherent *E. coli* (DAEC). 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 [32]. The prevalence and other epidemiological features of DEC types in childhood diarrhea vary with geographical area. Resistance against the commonly used drugs for the treatment of enteric infections including ampicillin, tetracycline, and co-trimoxazole has increased among DEC [14], which have led to the use of higher antimicrobials like fluoroquinolones as alternatives. *E. coli* strains form surface communities of biofilm structure that contributes to resistance to different antimicrobial agents and to its pathogenicity. Critical for the formation of biofilm on abiotic material such as food or food-contact surfaces, *E. coli* possess adhesins important for the formation of secreted IgA mediated biofilm within the gut [5].

While the frequency of DEC and its multi-drug resistance in childhood diarrhea have been reported in some parts of Nigeria [11, 12], the correlation between multi-drug resistance and biofilms production among DEC is still lacking. In other parts of the world, relationship amongst antibiotics resistance, distribution of virulence factors and biofilm formation in *E. coli* have been widely reported [6, 9, 13, 28] but scarcely for DEC. Hence, this study was aimed to determine the antibiotic resistance and biofilm production patterns among DEC pathotypes isolated from stools of children aged 0–5 years with acute diarrheal disease.

Materials and methods

Isolation and identification of diarrheagenic E. coli (DEC). Sixty (60) fecal samples were collected from children with incidence of diarrhea under the age of five (5) years at Alex Ekwueme Federal Teaching Hospital, Abakaliki, Nigeria (AE-FETHA). Ethical clearance was obtained from Ethical and Research Committee of the hospital, after which informed consent was obtained from the parents of the children. Fecal samples were processed as described in the standard guidelines provided for laboratory diagnosis of enteric pathogens [7]. *E. coli* was isolated with Eosin Methylene Blue, EMB (a selective media for *E. coli*) and identified with biochemical tests like Indole test, Methyl red test, Voges–Proskauer (VP) test, Citrate utilization test and Eijkman test.

Following extraction of *E. coli* genomic DNA as previously described by Healey and colleagues [30], *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′-CCGATACGCTGCCAATCAGT-3′: EC2:5′-ACG CAGACCGTAAGGGCCAGAT-3′ [16, 21]. 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 were done at the following conditions: 94°C for 5 min, initial template denaturation, 25 cycles at 94°C for 30 s, final denaturation, 50°C for 1 min, annealing, 72°C for 1 min 30 s, extension and 72°C 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 (Fig. S1 in supplementary file).

Detection of virulent genes of DEC pathotypes. Detection of virulent genes of diarrheagenic *E. coli* (DEC) were performed in a group of multiplex PCR using their specific primers as previously described by [23]. All the primers and their corresponding virulent genes are shown in Table S1 (see supplementary file). The thermo cycling 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 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 (Fig. S2 in supplementary file).

Antibiotic susceptibility testing. Antimicrobial susceptibility testing was performed by the Disk Diffusion Method according to the Clinical and Laboratory Standards Institute guidelines, CLSI [10]. Antimicrobial agents tested were tetracycline (30 μg), ampicillin (10 μg), amoxicillin/clavulinic acid (5 μg), imipenem (10 μg), ciprofloxacin (5 μg) and levofloxacin (5 μg) (Oxoid Ltd, Basingstoke, Hamphire, England). The multi-drug resistance criteria adopted was defined as earlier published [19].

Biofilm formation. Formation of biofilms by DEC was evaluated using a method described by [24] with slight modifications. About 200 μL of *E. coli* broth culture was prepared with glucose. The broth was added to 96-well plates and incubated for 24 h at 37°C to allow cell attachment and biofilm formation. The supernatant fluid in each well was aspirated and washed with 0.1 M phosphate buffer saline (PBS). The wells were stained with 100 μL of thiazoylblue tetrazolium bromide for 2 h at 37°C, the staining solutions were aspirated and the wells washed with PBS. About 200 μL DMSO were added to the wells and the amount of stain in each well was determined at 570 nm using micro plate reader (FLUOstar Omega, BMG LABTECH, Germany). Wells containing only 100 μL sterile broth were used as control. Its optical density reading was used as back ground value which was subtracted from the other test values. The method earlier described by Pavlickova et al. [28] was used to group the organisms as weak, moderate and strong biofilm producers.

Results

A total of 60 stool samples from diarrheal children (n = 60) were collected for this study, of which 67% (n = 40) were positive for one or more pathotype of DEC. Virulent *eltA* gene for ST-ETEC was detected in 20% (n = 8) of the isoates while 10% (n = 4) expressed *eltB* for LT-ETEC. Twenty percent (20%) (n = 8) also expressed pCVD (the nucleotide sequence of EcoR1-Pst1 DNA fragment of pCVD432) gene for EAEC while 40% (n = 16) expressed *ial* gene (invasion-associated locus of the invasive plasmid found in EIEC). Ten percent (10%) (n = 4) expressed *eaeA* gene (a structural gene for intimin) and *bfpA* gene (a structural gene for the bundle-forming pilus) found in typical EPEC (Fig.).

The isolates phenotypically exhibited high resistance for tetracycline [36 (90%)] followed by ampi-

cillin [32 (80%)], ciprofloxacin and levofloxacin [20 (50%)], cefoxitin [4 (10%)] and amoxicillin-clav and imipenem [0 (0%)]. Intermediate resistance was seen in amoxicillin-clav [28 (70%)] followed by ampicillin, cefoxitin, tetracycline [4 (10%)] and imipenem, ciprofloxacin, levofloxacin [0 (0%)]. The highest sensitivity was seen in imipinem [40 (100%)] followed by cefoxitin [32 (80%)], ciprofloxacin and levofloxacin [20 (50%)], amoxicillin-clav [12 (30%)], ampicillin [4 (10%)] and tetracycline [0 (0%)] (Table 1).

Fifty percent (50%) isolates of ST-ETEC were resistant to ampicillin, ciprofloxacin and levofloxacin while 100% were resistant to tetracycline. All the LT-ETEC exhibited resistance to ampicillin and tetracycline. Fifty percent (50%) resistance were seen in EAEC against ampicilin, cefoxitin, ciprofloxacin, levofloxacin and tetracycline. In EIEC, all the isolates were resistant to ampicillin, 50% were resistant to both ciprofloxacin and levofloxacin while 75% were resistant to tetracycline. All EPEC were resistant to ampicillin, ciprofloxacin, levofloxacin and tetracycline. Multi-drug resistance were seen in more than 50% of all the pathotypes except LT-ETEC (Table 2).

From Table 3, the level of biofilm production varies across the pathotypes. While 50% of the isolates were moderate biofilm producers, 40% and 10% exhibited weak and strong production respectively. In ETEC, all the LT-ETEC was weak producers while 50% were both moderate and weak producers for ST-ETEC. Half of the EAEC isolates showed both strong and weak biofilm production while all the EPEC isolates were moderate in their production of biofilm. Seventy-five and twenty-five percent of EIEC were moderate and weak producers respectively.

Discussion and conclusion

In this study, the frequency of EIEC [40% (n = 16)] was most common among DEC isolates, followed by ETEC [30% (n = 12)]. This high frequency suggests their role as most common cause of acute childhood diarrhea in this region. In contrast to high EIEC frequency recorded in this study, low frequency (1.2%) of EIEC was seen in the study carried out in south west Nigeria by Okeke et al. [25], in India (1.8%) [8] and Ecuador (3.2%) [37], these suggest that EIEC may play less important role in childhood diarrhea in developing countries. The high ETEC frequency from this study

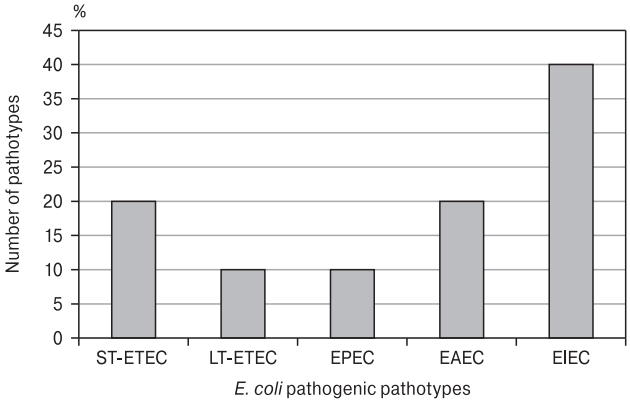

Figure. Distribution of DEC pathotypes

agreed with the one recorded in Onitsha, South East Nigeria where 21.57% ETEC were detected among DEC isolates [24]. Similar high ETEC frequency of 36.3% was recorded amongst hospitalized diarrheal children in Kolkata India [15]. In contrast, the frequency of ETEC obtained among DEC isolated from stools of infants and children in Federal Capital Territory, Abuja, Nigeria, was 4% [17]. The same low frequency was seen in other developing countries; Dar es Salaam, Tanzania (3.6%) [22]; Western Iran (17.5%) [3]; Hanoi, Vietnam (2.2%) [23]. 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. Clinical human ETEC isolates produce enterotoxins; the heat stable toxins (ST) and heat labile toxin (LT) and may produce one or more of several colonization factors (CFs) which mediate adherence to the small intestinal mucosa [31].

Table 1. Antibiogram of DEC isolates (n = 40)

Antimicrobials	Resistant	Intermediate	Sensitivity
AMP	32 (80%)	4 (10%)	4 (10%)
AMC	0 (0%)	28 (70%)	12 (30%)
FOX	4 (10%)	4 (10%)	32 (80%)
IMP	0 (0%)	0 (0%)	40 (100%)
CIP	20 (50%)	0 (0%)	20 (50%)
LEV	20 (50%)	0 (0%)	20 (50%)
TET	36 (90%)	4 (10%)	0 (0%)

Note. AMP — ampicillin, AMC — amoxicillin — clavulinic acid, FOX — cefoxitin, IMP — imipenem, CIP — ciprofloxacin, LEV — levoflxacin, TET — tetracycline.

Table 2. Relationship between DEC pathotypes and antimicrobial resistance

	n	AMP	AMC	FOX	CIP	LEV	IMP	TET
ST-ETEC	8	50% (4)	0% (0)	0% (0)	50% (4)	50% (4)	0% (0)	100% (8)
LT-ETEC	4	100% (4)	0% (0)	0% (0)	0% (0)	0% (0)	0% (0)	100% (4)
EAEC	8	50% (4)	0% (0)	50% (4)	50% (4)	50% (4)	0% (0)	100% (8)
EIEC	16	100% (16)	0% (0)	0% (0)	50% (16)	50% (16)	0% (0)	75% (12)
EPEC	4	100% (4)	0% (0)	0% (0)	100% (4)	100% (4)	0% (0)	100% (4)

Note. AMP — ampicillin, AMC — amoxicillin — clavulinic acid, FOX — cefoxitin, IMP — imipenem, CIP — ciprofloxacin, LEV — levoflxacin, TET — tetracycline.

Table 3. Level of biofilm formation in DEC isolates

	Strong	Moderate	Weak
ST-ETEC	0 (0%)	4 (50%)	4 (50%)
LT-ETEC	0 (0%)	0 (0%)	4 (100%)
EAEC	4 (50%)	0 (0%)	4 (50)
EIEC	0 (0%)	12 (75%)	4 (25%)
EPEC	0 (0%)	4 (100%)	0 (0%)
Total	4 (10%)	20 (50%)	16 (40%)

In this study, EAEC [20% (n = 8)] ranked second to Typical EPEC [10% (n = 4)] 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 [29]. In agreement with our result, EPEC was the least prevalent in Ecuador (0.9%) [37], India 4.79% [8] and Egypt (5.2%) [4]. Different from the result of this study, EAEC was recorded as the most prevalent in Kolkata India (48.2%) [15], Southwest Nigeria (10.3%) [25] and 34.4% in Gwagwalada, Abuja, Nigeria [27].

The DEC isolates were most resistance to tetracycline [36 (90%)] followed by ampicillin [32 (80%)], ciprofloxacin/levofloxacin [20 (50%)] and cefoxitin [4 (10%)]. More than 50% of all the DEC was multi-drug resistant with EAEC showing resistant to 5 different antibiotics. A similar result was also seen in a previous study where most of the DEC isolates (67.5%) were resistant to ampicillin and tetracycline [3]. Such multidrug resistance among DEC isolates against classical antibiotics like ampicillin and tetracycline was also recorded in Bolivia [31]. 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 [26].

From this study, 50% of the DEC isolates were moderate biofilm producers, while 40% and 10% exhibited weak and strong production respectively. Isolates that exhibited strong biofilm production correlates with 50% EAEC with the highest multidrug resistance. Relationship between antimicrobial resistance and biofilm formation among isolates of Gram-negative bacteria species including *E. coli* have been reported [6]. Biofilms are bacterial population firmly crammed by extra-cellular matrix which possesses bacterial secreted polymers such as exopolysaccharides, extracellular DNA, proteins and amyloidogenic proteins. Microbial cells within biofilms have shown 1000 times more antibiotics resistance than the planktonic cells [33]. The antibiotics resistance mechanism of biofilms communities commonly involves the uptake of resistance genes by horizontal gene transfer. Biofilms provides compatible conditions for this horizontal gene transfer which include high cell density, increased genetic competence and accumulation of genetic elements or uptake of resistance genes. Conjugation is the only mechanism of horizontal transfer of resistant genes in biofilms and may confirm the resistance to several antibiotics [20]. This suggests that increased biofilms seen in EAEC isolates increased the chances of horizontal antibiotics resistant gene transfer which may be acquired from other sources. This point to 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 [35].

We observed the presence of four different DEC pathotypes with EIEC and ETEC most commonly encountered. High frequency of EIEC in this region suggests that it is one of the most common causes of diarrhea in children 0–5 years. Most of these DEC isolates are resistant to more than one antimicrobial agent which suggests continued use and misuse of these drugs. Therefore it is important to continue the surveillance of antimicrobial resistance of enteric bacterial pathogens for effective control of childhood diarrheal diseases. The number of drug resistance increased with the strength of biofilm production among the DEC pathotypes. This may suggest that biofilms may provide compatible uptake of resistance genes.

Supplementary files

Supplementary materials are available at:
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Competing interests

No competing interests are declared by authors.

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