

The Frequency of Fluoroquinolone Resistant Enterotoxigenic *Escherichia coli* from Stools of Diarrheic Children in Ebonyi State, Nigeria

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Abstract

Enterotoxigenic *Escherichia coli* (ETEC) is an important cause of acute childhood diarrhea. The evaluation of ETEC in children is important for therapeutic and economic purposes. Hence, this study aimed to evaluate the frequency of ETEC among diarrheic children, their multidrug and fluoroquinolone resistant pattern. A total of twenty diarrheagenic *E. coli* (DEC) isolates were gotten from hundred diarrheal samples using biochemical and molecular methods. Multiplex PCR was used to detect the presence of four different pathological types of DEC. Disk diffusion method was used to determine the antibiotic susceptibility of the organisms. Biofilm formation was detected by thiazoylblue tetrazolium bromide dye in a 96-well plate. Results showed that ETEC represented 30% of the DEC, of which 80% were multidrug and fluoroquinolone resistant. The biofilm production abilities of all the ETEC were found to exist within weak, moderate and strong biofilm producers. We observed a high ETEC frequency and rapid emergence of multidrug/fluoroquinolone resistance, suggesting that it is one of the most important causes of frequent drug resistant diarrhea in children in this region.

1.0 Background

An estimated 4 billion episodes of diarrhea occur each year around the world [1]. Acute infectious diarrhea ranks second amongst the most common cause of death in children in developing countries [2] which account for an estimated 3-4 million deaths annually, the majority of whom are children under two years. The major agents responsible for this diarrheal disease are *enterotoxigenic E. coli* (ETEC), *Vibrio cholerae*, *Shigella sp.* and *rotavirus* [3,4] which are endemic to the developing countries. Amongst all diarrhoeagenic *E. coli* (DEC), ETEC has been reported to be more prevalent in most part of Nigeria [5,6]. The main virulence attributes of ETEC include adherence to epithelial cell surfaces of small intestine by colonization factor antigen (CFA) and elaboration of heat labile (LT) and heat stable (ST) enterotoxins [7], which stimulates intestinal secretions [8,9]. ST binds to and activates a putative intestinal receptor, guanylate cyclase, causing an increase in the intracellular levels of cyclic guanosine monophosphate (cGMP) [10]. LT activates adenylyl cyclase to stimulate cAMP production which activates a cAMP-dependent kinase (PKA). PKA phosphorylates a Cl^- channel (CFTR) and results in stimulation of Cl^- secretion and the inhibition of Na^+ absorption [11]. In Nigeria, limited studies have investigated the microbiology of childhood diarrhea and these have all been carried out in the Southern and North-Central part of the country. The prevalence of ETEC in childhood diarrhea has been reported to be 2.4% in provincial Southwest and 18.0% in North-central Nigeria [12,13].

The fluoroquinolones are potent, broad-spectrum antibiotics that have been used for the treatment of severe or resistant infections since the late 1980s. They are derived from the quinolone family of antibiotics; quinolones are synthetic constructs, developed by modification of 1-alkyl-1,8-naphthyridin-4-one-3-carboxylic acid [14]. Fluoroquinolones differ from quinolones by the replacement of the eighth carbon atom of the backbone with a nitrogen atom and the addition of a fluorine atom at the sixth position, giving them more potent antibiotic action and a broader spectrum of activity [15]. There are four generations of quinolone/fluoroquinolone antibiotics in clinical use; the most commonly prescribed

fluoroquinolones are ciprofloxacin, levofloxacin, and moxifloxacin which are the first generation fluoroquinolones (second generation quinolones) [16]. Fluoroquinolone-resistant lineages identified in African countries were universally resistant to multiple other classes of antibacterial agents [16]. Of importance is the emergence of multidrug resistance in all pathological types of *E. coli* isolated from children with diarrhea [17]. In the last decade, resistance against the commonly used drugs for the treatment of enteric infections including ampicillin, tetracycline, and co-trimoxazole has increased among DEC [18]. DEC resistance to antibiotics used in empiric treatment has led to the use of fluoroquinolone as alternatives in diarrhea patients. Additionally, their use and misuse in the informal sector, by un-licensed providers and through self-medication, is commonplace in Nigeria and in many other parts of Africa [19].

Ebonyi State is one of the smallest and underdeveloped provinces in the South Eastern part of Nigeria with only one teaching hospital, Federal Teaching Hospital, Abakiliki (FETHA). The main goal of this study was to investigate whether ETEC is an important factor in diarrheal children visiting the hospital and to suggest the frequency of fluoroquinolone-resistant ETEC in this region.

2.0 Methods

2.1 Study Population

The samples for this study were collected from Federal Teaching Hospital Abakiliki, Ebonyi State, Nigeria after ethical clearance (Ref. No.: FETHA/REC/VOL.1/2016/386). Stool samples were collected from children with incidence of diarrhea under the age of five (5) years, after obtaining informed consent from their parents/guardians/attendants. The study was limited to children of 0-5 years of age attending FETHA, Abakiliki within the study period.

2.2 Sampling and Isolation of *E. coli*

One hundred diarrheal stool samples were collected and sampled at the Laboratory Unit of Medical Laboratory Department, Federal Teaching Hospital, Abakiliki. The stool samples were cultured within 2-4 hours of collection according to the standard guidelines provided for laboratory diagnosis of enteric pathogens [20]. About 1 g of stool samples were suspended in sterile nutrient broth and incubated at 37° for 24 hours. The isolates from the nutrient broth were inoculated directly on MacConkey's agar plates. The samples were picked up after overnight incubation at 37° and sub cultured in eosin methylene blue (EMB) agar plates at 37° for 24 hours. The following biochemical tests were used on colonies that showed green metallic sheen, typical of *E. coli* isolates: Indole test, Methyl red test, Voges-Proskauer test, Citrate utilization test and Eijkman test (fermentation of lactose and gas production at 44.5° for 24-48 hours). Stocks of each isolate were maintained by cryopreservation for further molecular studies.

2.3 Molecular Identification of *E. coli* by PCR

Genomic DNA extraction: Standard Cetyl-trimethylammonium-bromide/Sodium chloride (CTAB/NaCl) method was used to extract *E. coli* genomic DNA [21]. Colonies were placed in a sterile 1.5 mL eppendorf tubes containing 567 µL TE buffer and lysozyme (20 µL) was added and incubated at 37⁰ for 30 min. About 30 µL 10% SDS was added and incubated for 1 hr. at 56⁰, followed by addition of 100 µL of 5 M NaCl. Incubation was done at 65⁰ for 10 min after addition of 100 µL CTAB/NaCl solution. The mixture was incubated at 65⁰ for 10 min after addition of 100 µL CTAB/NaCl solution. Afterwards, 500 µL of chloroform/isoamylalcohol was added, mixed and centrifuged for 10 min at 4⁰ at 13000 x g. The supernatant was pipetted into a new tube; chloroform/isoamylalcohol was added and centrifuged at room temperature at 15000 x g for 10 min. The supernatant was transferred into a new tube and 240 µL cold isopropanol was added, mixed gently until DNA precipitates. The mixture was centrifuged at 15000 x g for 15 min at 4⁰ to remove isopropanol. The pellet was washed with 1 mL cold 70% ethanol, centrifuged at 15000 x g for 5 min at 4⁰. The ethanol was discarded and the pellet allowed to dry at room temperature and re-suspended in DNase-free water. The DNA concentration was 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 100V. The gels were stained with ethidium bromide and photographed under ultraviolet light using a gel documentation system.

Polymerase Chain Reactions: The primers derived from the DNA sequences flanking the gene encoding the universal stress protein A (*uspA*) of *E. coli* was used for molecular identification; EC1:5'-CCGATACGCTGCCAATCAGT-3': EC2:5'-ACGCAGACCGTAAGGGCCAGAT-3' [21]. PCR was performed in a total reaction volume of 25 µL containing 0.5µL of both forward and reverse primers each, 12.5µL GoTaq Green master mix, 9.0µL nuclease free water (Promega, USA) and 2.5µL of template. The PCR were optimized at the following conditions: (initial template denaturation) 94⁰ for 5 min, (final denaturation) 25 cycles at 94⁰ for 30 s, (Annealing) 50⁰ for 1 min, (extension) 72⁰ for 1min 30 sec and (final extension) 72⁰ 7 min. PCR products, about 884bp were analyzed by gel electrophoresis in 1% agarose gel in TBE buffer at 100V. The gels were stained with ethidium bromide and photographed under ultraviolet light using a gel documentation system.

2.4 Identification of Diarrheagenic *E. coli* Using Multiplex PCR

Detection of virulent markers of diarrheagenic *E. coli* (DEC) was performed in a group of multiplex PCR using their specific primers as previously described by [22]. All the primers and their corresponding virulent genes are shown in table 1. The thermo cycling conditions were programmed using Applied Biosystem, 2720 Thermal Cycler, USA. The PCR was run in batches based on closely related T_m of the primers. The program was as follows: initial denaturation for 5 min at 94⁰C, denaturation at 94⁰C for 30 s, 72⁰C for 1 min 30 s extensions for 25 cycles with final extension of 5 min at 72⁰C. The PCR assay was carried out in 25 µL reaction mixture. The presence of *enterotoxigenic E. coli* (ETEC) was seen by the expression of *eltB* for heat labile, LT-ETEC and/or *eltA* for heat stable, ST-ETEC. *Enteropathogenic* and *enterohaemorrhagic E. coli* (EPEC and EHEC) were seen as the presence of *eaeA*, a structural gene for

intimin found in both organisms. Further distinction was seen as the presence of *bfpA* (a structural gene for the bundle-forming pilus of EPEC) and *vt1*/or *vt2* (Shiga toxin 1 and 2 of EHEC). *Enteroinvasive E. coli* (EIEC) was seen as the expression of *ial* (invasion-associated locus of the invasive plasmid found in (EIEC). *Enteroaggressive E. coli* (EAEC) was seen as the presence of *pCVD* (the nucleotide sequence of EcoR1-Pst1 DNA fragment of pCVD432 of EAEC).

2.5 Serotyping

DEC isolates were serogrouped at National Salmonella and Escherichia Centre, Central Research Institute, Kasauli (H.P) – 173204 India. The serogrouping was done on the basis of the presence of Somatic [O] and Flagellar [H]. Isolates were analyzed for serotype O and H groups using available O (O1 – O185) and H (H1 – H36) *E. coli* antisera. Isolates that did not react with any of the O and H antisera were classified as non typeable (ONT and HNT) and those that were non-motile were denoted (HNM).

2.6 Antibiotic Susceptibility Testing

Antimicrobial susceptibility testing was performed by the Disk Diffusion Method according to the Clinical and Laboratory Standards Institute guidelines [23]. Antimicrobial agents tested were Tetracycline (30 µg), Ampicillin (10 µg), Oxacillin (1 µg), Cefoxitin (30 µg), Amoxicillin /Clavulanic acid (5 µg), Imipenem (10 µg), Ciprofloxacin (5 µg), Methicillin (10 µg), Levofloxacin (5 µg), and Quinipristin/Dalfopristin (15 µg) (Oxoid Ltd, Basingstoke, Hampshire, England). The multi-drug resistance criteria adopted was defined as earlier published[24].

2.7 Biofilm Formation

Formation of biofilms by DEC was evaluated using a method described by Chen *et al* with slight modifications [25]. About 200 µL *E. coli* broth culture was prepared with glucose. The broth was added to 96-well plates and incubated for 24 h at 37⁰C 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 37⁰C, 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.

3.0 Results

3.1 Identification and Isolation of ETEC

Out of thirty samples identified through biochemical tests, about twenty expressed both *E. coli uspA* and DEC virulent genes in a colony PCR. Four samples showed the presence of *eltA* gene for ST-ETEC while two samples showed the presence of *eltB* for LT-ETEC. The presence of pCVD (the nucleotide sequence of EcoR1-Pst1 DNA fragment of pCVD432 of EAEC) gene was seen in four samples for EAEC. Eight samples showed the presence of *ial* gene (invasion-associated locus of the invasive plasmid) found in EIEC while two samples showed the presence of *eaeA/ bfpA* genes, structural genes for intimin and bundle-forming pilus found in both EPEC and EHEC respectively, table 2. Out of the twenty DEC samples, 30% (6/20) were ETEC (4 ST-ETEC and 2 LT-ETEC), 20% (4/20) were EAEC, and 40% (8/20) were EIEC while 10% (2/20) was typical EPEC. EHEC was not found in the samples.

3.2 DEC Serogroups

All the Somatic (O) and Flagellar (H) antigens of the organisms were identified except three of EIEC which are non-motile. The ETEC strains, O8, O6, O25 belonged to O serogroup while H16, H42 belonged to H serogroup. None of the ETEC strains belonged to the classic serogroups associated with this pathological type (Table 3).

3.3 Antibiotic Susceptibility Testing

Zone diameter breakpoint (mm) of ≥ 17 implies susceptibility while ≤ 17 is resistance. All the organisms were resistant to Methicillin/Oxacillin and Ampicillin except 2 ST-ETEC and two EAEC which were resistant to only MET/OX. Only two EAEC were resistant to Cefoxitin (FOX) while other organisms were susceptible. All the ST-ETEC and some ST-ETEC were multidrug resistant. All the organisms are susceptible to Imipeneme, table 4.

All the organisms were resistant to tetracycline and quinupristin-D. Two ST-ETEC, two EAEC, four EIEC and two EPEC were resistant to ciprofloxacin. Out of the 9 samples that were ciprofloxacin (fluoroquinolone) resistant, 2 samples were ST-ETEC, table 5.

3.4 Biofilm Formation

All the DEC samples were found to produce biofilms. Twelve samples corresponding to 6 EIEC, 2 ST-ETEC, 2 LT-ETEC and 2 EAEC were weak biofilm producers. Two ST-ETEC, 2 EIEC, and 2 EPEC were moderate biofilm producers. Two samples of EAEC was a strong biofilm producer, figure 1.

4.0 Discussion

From our study, the detection rate of ETEC among DEC was 30% (6/20). Out of 45% (9/20) fluoroquinolone-resistant DEC samples, 22% (2/9) were ETEC. Similar high ETEC detection rate amongst DEC from children were recorded in Nigeria and elsewhere. Detection rate of 36.3% were recorded in

Kolkata India [26]; in Onitsha, South East Nigeria, 21.57% ETEC strains were detected among DEC isolates [27]. Our result was different from the ones obtained among DEC isolated from infants and children in Dar es Salaam, Tanzania where ETEC was found to be 3.6%, [28]. The PCR assay detected 2.2% ETEC among young children in Hanoi, Vietnam [22]. In Federal Capital Territory, Abuja, Nigeria, 4.0% prevalence of ETEC among DEC was observed [29]. Among adolescents and adults in Hamedan, Western Iran, ETEC detection rate was 17.5% [30]. Again, from our result, ST-ETEC showed greater association with childhood diarrhea than heat-labile, LT-ETEC. This result agreed with the one performed in Southwest Nigeria [12] and other parts of the world [31, 32].

Although none of the ETEC isolated in this study belonged to the classic serotype, serogroup O6 and O8 have also been identified to be associated with ETEC in previous study [33]. This diversity of serotypes seen in this study and the apparent lack of correlation of serotype with virulence category suggests that several clones of diarrhea causing ETEC may be responsible for childhood diarrhea in this area. Okeke *et al*, 2000 also discovered such diversity in serotypes of DEC in Osun State, Nigeria [12].

Most worrisome was that 60% (12/20) of all the DEC including ETEC were found to be both multi-drug and ciprofloxacin (fluoroquinolone) resistant. Out of the 60%, representing 75% (9/12) including ETEC were resistant to both ciprofloxacin and other drugs while the remaining 25% (3/12) resistant to other drugs except ciprofloxacin. Our result agreed with a previous study where most of the DEC strains (67.5%) were resistant to ampicillin and tetracycline with ETEC being significantly more resistant to ciprofloxacin than other DEC group [30]. Such multidrug resistance among ETEC against classical antibiotics like ampicillin and tetracycline was also recorded in Bolivia, although no ciprofloxacin resistance was observed [33]. The relationship between antimicrobial susceptibility pattern of *E coli* from environmental water and clinical diarrheal stool samples in Lagos, Nigeria (LN) further supported our findings, that most of the isolates were resistant to fluoroquinolones (82.5%) [34].

The quinolone resistance in *E. coli* has been associated with mutations in the *gyrA* and *parC* genes [35]. If quinolones are used as a first drug of choice for diarrhea in countries where the use of antibiotics is not regulated, a rapid emergence of resistance will likely occur. Therefore, it is important to continue the surveillance of enteric bacterial pathogens for quinolone 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].

Conclusion

The high detection rate of ETEC in this region suggests that this strain is one of the most common causes of death among diarrheal children. These ETEC were found to be both multi-drug resistant and fluoroquinolone resistant due to availability of over-the-counter antibiotics. The continued use and abuse of these drugs allow for the selection of resistant strains which are easily disseminated. 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 availability of antibiotics is advised.

List Of Abbreviations

ETEC: *Enterotoxigenic Escherichia coli*

PCR: Polymerase chain reaction

LT; Heat labile

ST: Heat stable

DEC: Diarrheagenic *E. coli*

Declarations

Ethics approval and consent to participate

Ethical consent for sample collection was obtained from the Ethics Committee of Federal Teaching Hospital Abakiliki, Ebonyi State, Nigeria with clearance number: (Ref. No.: FETHA/REC/VOL.1/2016/386). Stool samples were collected from children with incidence of diarrhea under the age of five (5) years, after obtaining verbal consent from their parents/guardians/attendants.

Consent for publication

Not applicable

Availability of data and material

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Competing interests

No competing interests are declared by authors.

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Authors' contributions

EE conceived the study, performed molecular experiment and wrote the manuscript. MA co-designed and performed molecular experiment and interpreted data. HC co-conceived the study and co-supervised the design. MN co-supervised the study design and molecular experiment. RA co-designed the work. IO and OS collected samples and performed fecal culture while IR performed biochemical tests. All authors read and approved the final manuscript.

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Tables

Table 1: Primer characteristics used in the multiplex PCR

Primers	Target gene	Sequence	Size (bp)	AT(⁰ C)
LT	<i>eltB</i>	5-TCTCTATGTGCATACGGAGC-3 5-CCATACTGATTGCCGCAAT-3	322	56
ST	<i>estA</i>	5-GCTAAACCAGTAGAGGTCTTCAAAA-3 5-CCCGGTACAGAGCAGGATTACAACA-3	147	56
VT1	<i>vt1</i>	5-GAAGAGTCCGTGGGATTACG-3 5-AGCGATGCAGCTATTAATAA-3	130	56
VT2	<i>vt2</i>	5-ACCGTTTTTTCAGATTTTGACACATA-3 5-TACACAGGAGCAGTTTCAGACAGT-3	298	53
Eae	<i>eaeA</i>	5-CACACGAATAAACTGACTAAAATG-3 5-AAAAACGCTGACCCGCACCTAAAT-3	376	54
SHIG	<i>ial</i>	5-CTGGTAGGTATGGTGAGG-3 5-CCAGGCCAACAATTATTTCC-3	320	53
bfpA	<i>bfpA</i>	5-TTCTTGGTGCTTGCGTGTCTTTT-3 5-TTTTGTTTGTTGTATCTTTGTAA-3	367	56
EA	<i>pCVD</i>	5-CTGGCGAAAGACTGTATCAT-3 5-CAATGTATAGAAATCCGCTGTT-3	630	53

Table 2: Presence of virulent genes in DEC

	<i>eltA</i>	<i>eltB</i>	pCVD	<i>ial</i>	<i>eaeA</i> / <i>bfpA</i>
Sample number (20) 2	4	2	4	8	
DEC pathotype	ST-ETEC	LT-ETEC	EAEC	EIEC	EPEC

Table 3: The presence of Somatic [O] and Flagellar [H] antigens in DEC

DEC	Serogroups
ETEC	ST-ETEC (O8:H9 and O6:H16); LT-ETEC (O25:H42)
EPEC	O125:H16
EAEC	O3:H2, O85:H10
EIEC	O28:NM, O124:H30, O124:NM, O112:NM

Table 4: Beta-lactam antibiotic susceptibility

	ST-ETEC	LT-ETEC	EAEC	EIEC	EPEC
No of samples	4	2	4	8	2
Methicillin/Oxacillin	R	R	R	R	R
Ampicillin	R (2) S (2)	R	R (2) S (2)	R	R
Amoxicillin/Clavulinic Acid	R (2) S (2)	R	R (2) S (2)	R (2) S (6)	R
Cefoxitin	S	S	R (2) S (2)	S	S
Imipinem	S	S	S	S	S

S=Susceptible R=Resistance

Table 5: Non-beta Lactam Antibiotics Susceptibility

	ST-ETEC	LT-ETEC	EAEC	EIEC	EPEC
No of samples	4	2	4	8	2
Ciprofloxacin/Lev	R(2) S(2)	S	R(2) S(2)	R(4) S(4)	R
Quinupristin-D	R	R	R	R	R
Tetracycline	R	R	R	R	R

S=Susceptible R=Resistance

Figures

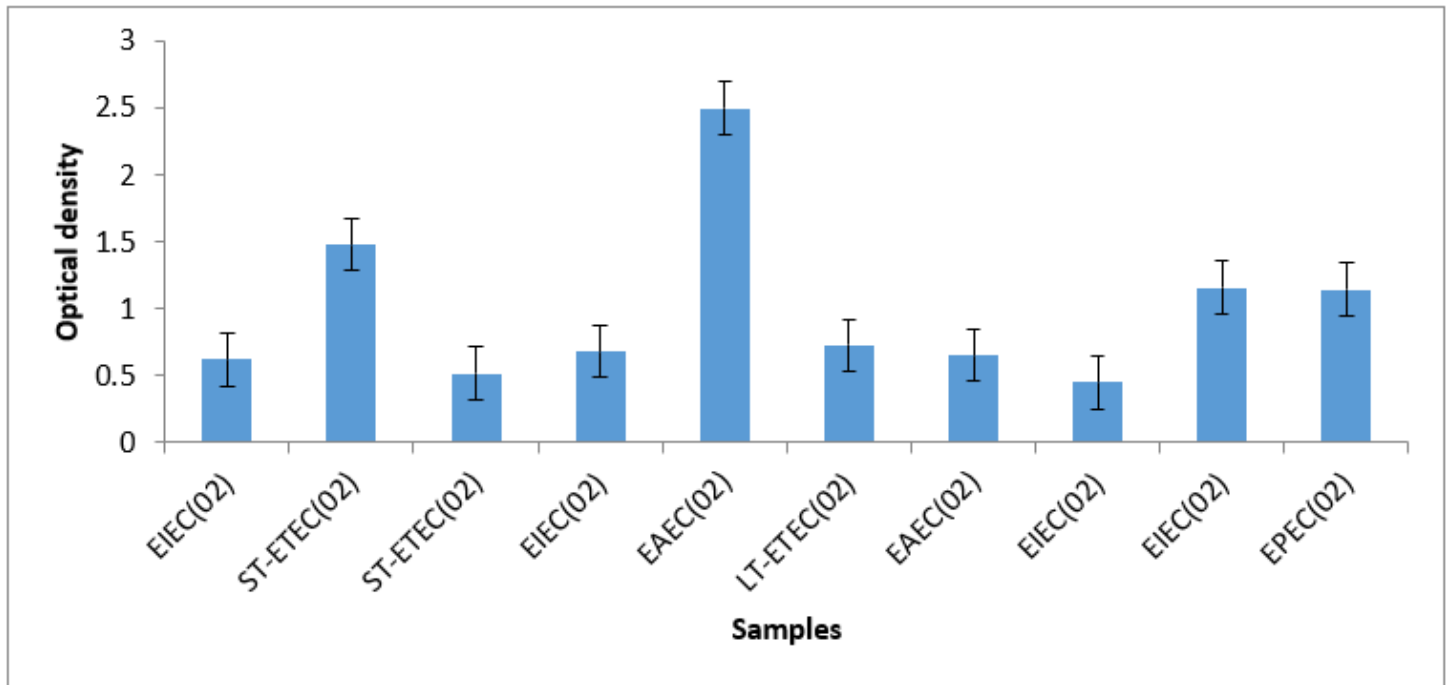


Figure 1

Level of Biofilm formation in DEC All the DEC samples were found to produce biofilms. Twelve samples corresponding to 6 EIEC, 2 ST-EIEC, 2 LT-EIEC and 2 EAEC were weak biofilm producers. Two ST-EIEC, 2 EIEC, and 2 EPEC were moderate biofilm producers. Two samples of EAEC was a strong biofilm producer.