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Molecular Characterization of Enterotoxigenic *Escherichia coli*: Effect on Intestinal Nitric Oxide in Diarrheal Disease

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Abstract

This study was aimed to investigate the effect of enterotoxigenic *E. coli* (ETEC)-induced diarrhea on fecal nitric oxide (NO) and intestinal inducible nitric oxide synthase (iNOS) expression in rats. *E. coli* isolates were gotten from infants diarrheal samples. Multiplex polymerase chain reaction (PCR) was used to detect the presence of *eltA* gene for heat-stable (ST) enterotoxigenic *E. coli* and *eltB* for heat-labile (LT) enterotoxigenic *E. coli*. 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. Fecal NO was measured using standard griess reaction system. Reverse transcription PCR was used to investigate the expression of iNOS expression. Although none of the ETECs isolated in this study belonged to the classic serotype, serogroup O6 and O8 were found to be associated with ETECs. Among the three ETECs, two were found to be multidrug resistant. The biofilm production abilities of all the ETECs were found to exist between weak and moderate biofilm producers. Fecal NO was found to be elevated in both LT and ST-induced diarrheal groups but there was no corresponding intestinal iNOS expression. This suggests that the elevated NO could be as a result of up-regulation of constitutive NOS rather than iNOS.

Keywords: Enterotoxigenic *E. coli*; *Escherichia coli*; Nitric oxide; Diarrheal disease

Introduction

Diarrhea is the second leading cause of death in children under the age of five of which about 1.5 million die each year. This number represents nearly one in five child deaths. More of these children die of diarrhea than AIDS, malaria and measles combined and greater percentage of this death due to diarrhea occur in Africa and South Asia of which Nigeria ranks second, with over 151,700 deaths per year [1]. Diarrhea can be defined as having watery stools for at least three times in a day or when it is more frequent than normal in an individual. It is a symptom of gastrointestinal infections that can be caused by bacteria, viruses, protozoa and other pathogens. However, the major organisms that cause most cases of childhood acute diarrhea are Rotavirus, *E. coli*, *Shigella*, *Salmonella*, Campylobacteria and Vibro cholera (during epidemics). These pathogens are transmitted normally from the stool of one person to the mouth of another through different media like food or water [1]. Rotavirus and pathogenic *E. coli* are the most common cause of childhood diarrhea while *Shigella* spp. remains the most important causes of acute bloody diarrhea (dysentery), accounting for about 15% of death in children under 5 years [2].

Escherichia coli are Gram-negative, oxidase-negative, rod-shaped bacterium from the family Enterobacteriaceae [3]. They are commensal bacteria that can be found in intestinal micro flora of a variety of animals including man. Not all the strains of *E. coli* are harmless since some can cause debilitating and sometimes fatal diseases in humans as well as mammals and birds [4]. Pathogenic strains are divided into intestinal pathogens (InPEC) causing diarrhea and extraintestinal *E. coli* (ExPEC) causing a variety of infections including urinary tract infections (UTI), meningitis and septicemia [5]. InPEC strains of *E. coli* also known as diarrheagenic *E. coli* (DEC) is a major etiological agent of pediatric diarrhea, accounting for over 2 million deaths annually [6]. It continues to be the most common cause of infantile morbidity and mortality most especially in developing countries and sub-Saharan Africa [7] particularly in children under 5 years of age [8]. Nigeria records over 50,000 diarrhea-specific mortality among children less than 5 years [3]. DEC can be transmitted via the oral-fecal route by ingesting food or water contaminated by human or animal feces [7]. An altered movement of ions and water in gastrointestinal tract is at the heart of diarrheal diseases. InPEC can alter the balance between fluid-electrolyte absorption and secretion leading to diarrhea [9]. DEC are 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) 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 [6].

Amongst all DEC, ETEC has been reported to be more prevalent in most part of Nigeria [11,12]. The key virulence attributes of ETEC include adherence to epithelial cell surfaces by colonization factor antigen (CFA) and elaboration of heat labile (LT) and heat stable (ST) enterotoxins [6]. ETECs colonize the small intestine and produce the toxic agents heat-labile (LT) and heat-stable (ST) enterotoxinds which stimulate intestinal secretions [13]. Heat-stable enterotoxin (STa) binds to and activates a putative intestinal receptor, guanylate cyclase, causing an increase in the intracellular levels of cyclic guanosine monophosphate (cGMP) [14]. LT's activate 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 [15].

Nitric oxide (NO) is one of the smallest molecules in nature which is synthesized from arginine in a two-step enzyme reaction by nitric oxide synthase (NOS) via the formation of the intermediate N-hydroxy-L-arginine [16]. NOS enzyme exists in three isoforms encoded by distinct genes. Neuronal (nNOS or Type 1) and endothelial (eNOS or Type 3) are both constitutive and calcium dependent. They are present in the neural tissue and in the vascular endothelium, respectively. Inducible NOS (iNOS or Type 2) is both Ca^{++}-independent and induced by bacterial endotoxins and cytokines in macrophages, endothelium, smooth muscle, liver, fibroblast and neutrophils. Constitutive NOS (cNOS) and inducible NOS (iNOS) are responsible for production of NO in physiological and pathophysiological circumstances respectively. NO is implicated in mechanisms maintaining the integrity of the gastric epithelium [16] and has also been shown to be elevated in diarrheal disease. Colonal nitric oxide where shown to be elevated in castor oil and magnesium sulphate-induced diarrhea in wistar rats [17,18]. Metabolites of NO, nitrite and nitrate were significantly elevated in the serum of calves with acute diarrhea [19]. Whether NO and NOS also play a role in ETEC-induced diarrhea remain to be addressed. Hence, this study aims to investigate whether expression of iNOS leads to an elevated fecal NO in ETEC-induced diarrhea.

Methodology

Samples collection

The diarrheal samples for this study were collected and sampled at the Laboratory Unit of Medical Laboratory Department, Federal Teaching Hospital Abakiliki, Ebonyi State, Nigeria after ethical clearance (Ref. No.: FETHA/REC/VOL.1/2016/386). The samples were collected from children (both males and females) with incidence of diarrhea under the age of five years, after obtaining informed consent from their parents/guardians/attendants.

Reagents

All culture media were from Flow Laboratories, U.K. PCR reagents, master mix and kits were purchased from Promega, USA. Standard antibiotics were from Oxoid Ltd, Basingstoke, Hamphire, England. Commasie reagent and Bovine serum albumin were purchased from Sigma Aldrich. All reagents were of analytical grade.

Isolation of *E. coli* strains

Fecal samples were processed according to the standard guidelines provided for laboratory diagnosis of enteric pathogens [20]. The stool samples were cultured within 2-4 h of collection. About one gram of stool sample was suspended in sterile nutrient broth and incubated at 37°C for 24 h. The concentrated isolates from the nutrient broth were inoculated directly on MacConkey's agar plates. After overnight incubation at 37°C, lactose fermenters were picked up and sub cultured into eosin methylene blue (EMB) agar plates and incubated at 37°C for 24 h. Colonies that showed metallic green sheen were further subjected to biochemical tests for confirmation.

Biochemical identification

The biochemical tests that were used to identify the *E. coli* isolates included Indole test, Methyl red test, Voges-Proskauer test, Citrate utilization test and finally confirmed using Eijkman test (fermentation of lactose and gas production at 44.5°C for 24-48 h). Stocks of each isolate were maintained by cryopreservation for onward transportation to Biotechnology Laboratory, COMSATS Institute of Information Technology, Abbottabad, Pakistan for further molecular studies.

Molecular identification of ETEC

Genomic DNA extraction: Standard CTAB/NaCl Method was used to extract genomic DNA [21]. Briefly, colonies were placed in a sterile 1.5 mL eppendorf tubes containing 567 µL TE buffer. Lysozyme (20 µL) was added and incubated at 37°C for 30 min. About 30 µL 10% SDS was added and incubated for 1 h at 56°C, after which 100 µL of 5 M NaCl was added. The mixture was incubated at 65°C for 10 min after addition of 100 µL CTAB/NaCl solution. The DNA concentration was measured in ng/µL using Nanodrop instrument (Colibri Spectrometer, Berthhold Detection System, Germany). DNA quality 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: *E. coli* strains 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}: EC2:5I-ACGCAGACCGTAAGGGCCAGAT-3I [22]. PCR was performed in a total reaction volume of 25 µL containing 12.5 µL GoTaq Green master mix, 9.0 µL nuclease free water (Promega, USA), 0.5 µL of both forward and reverse primers each, 2.5 µL of template. The PCRs were optimized 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 sec (extension) and 72°C 7 min (final extension). PCR products, about 884 bp 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.

Detection of virulent markers of ETEC was performed in a group of multiplex PCR using their specific primers as previously described by [23]. The primers used for LT and ST-ETEC target genes are shown in Table 1. The thermo cycling conditions were programmed using Applied Biosystem, 2720 Thermal Cycler, USA. The program was as follows: initial denaturation for 5 min at 94°C, denaturation at 94°C for 30 s, 56°C for 45 s annealing 72°C for 1 min 30 s extensions for 25 cycles with final extension of 5 min at 72°C. The PCR assay was carried out in 25 µL reaction mixture.

ETEC serotyping

ETEC 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).

Primers	Target gene	Sequence	Size (bp)
LT	*eltB*	5-TCTCTATGTGCATACGGAGC-3 5-CCATACTGATTGCCGCAAT-3	322
ST	*eltA*	5-GCTAAACCAGTAGAGGTCTTCAAAA-3 5-CCCGGTACAGAGCAGGATTACAACA-3	147

Table 1: Primer characteristics used in the multiplex PCR.

ETEC amplicon extraction, purification and sequencing

ETEC Amplicons were extracted and purified by centrifugation using Wizard SV/GEL and PCR Clean-up System, Promega, USA. Following electrophoresis, the DNA bands from gel were excised and placed in a 1.5 mL eppendorf tube. About 10 µL Membrane Binding Solution was added per 10 mg gel slice, vortexed and incubated at 50°C-60°C until completely dissolved. The dissolved gel mixture is transferred into a Mini-column assembly and incubated at room temperature for 1 min. Centrifugation was done at 16,000 xg for 1 min, the flow-through was discarded and Mini-column reinserted into Collection Tube. The sequenced ETEC amplicon yielded 96% and 98% BLAST similarity to 322 bp and 147 bp amplicon sizes for LT and ST-ETEC respectively.

Antibiotic susceptibility testing

Antimicrobial susceptibility testing was performed by the Disk Diffusion Method according to the Clinical and Laboratory Standards Institute guidelines [24]. Antimicrobial agents tested were Ampicillin (10 µg), Tetracycline (30 µg), Imipenem (10 µg), Oxacillin (1 µg), Cefoxitin (30 µg), Amoxicillin /Clavulinic acid (5 µg), Methicillin (10 µg), Levofloxacin (5 µg), Ciprofloxacin (5 µg), and Quinipristin/ Dalfopristin (15 µg) (Oxoid Ltd, Basingstoke, Hamphire, England).

Biofilm formation

Formation of biofilms by ETEC was evaluated using a method described by [25] with slight modifications. Briefly, 200 µL *E. coli* broth culture prepared with glucose were added to 96-well plates and incubated for 24 h at 37°C to allow cell attachment and biofilm formation. After incubation, 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 solution was aspirated and the well washed with PBS. About 200 µL DMSO were added to the wells. The amount of stain in each well was determined at 570 nm using micro plate reader (FLUOstar Omega, BMG LABTECH and 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.

Induction of diarrhea in rats

Animals and diets: Albino rats weighing between 100 to 150 g were obtained from National Institute of Health, Pakistan and grouped into three (A, B, C) with five rats in each group. Group A was control group while groups B and C were test groups. The rats were housed in a temperature and humidity controlled room and allowed to acclimatize for 7 days before start of experiment. Deionized water and food was consumed ad libitum throughout the acclimatization period. Twelve hours to induction, the rats were starved. Diarrhea was induced in the test groups using about 5×10^9 cells/mL; a density of a 4 McFarland Standard in 1 ml normal saline. Test group B was induced with LT-ETEC while C was induced with ST-ETEC. Group A received normal saline.

Sample collection: Diarrhea was induced after 18 h observed as watery stool. Rats were anaesthetizes and sacrificed using chloroform. Fecal samples were collected, weighed and refrigerated in Tris-HCl buffer for nitric oxide (NO) and protein estimation. The colon was collected for RNA extraction. Care and treatment of experimental animals followed approved protocol for animal care guidelines.

Fecal protein estimation: Fecal proteins were estimated using standard Bradford method using micro-plates. The feces were weighed, homogenized and centrifuged in Tris-HCl buffer and the supernatants used for protein estimation. Bovine serum albumin, BSA (Sigma Aldrich) standard were prepared using 2 mg/mL in appropriate diluents used in protein extraction. About 5 µL of each standard or samples were pipetted into the micro wells and 250 µL of Commasie reagent (Sigma Aldrich) were added and mixed with plate shaker for 30 sec. The plates were incubated for 10 min at room temperature. The absorbance was measured using at 595 nm using a plate reader (FLUOstar Omega, BMG LABTECH and Germany). The results were read in triplicates. The average 595 nm measurement for the blanck replicates were subtracted from the reading of other individual standards and sample replicates. A standard calibration curve was plotted using the average blanck-corrected BSA standard against concentrations in mg/mL. The standards were used to determine the protein concentration in the sample. The protein concentrations obtained with Bradford method was compared with the ones obtained using automatic Nanodrop Colibri Spectrometer (Titertek Berthold Detection Systems, GmbH 75173, Germany).

Nitric oxide (NO) determination: Nitric oxide concentration was determined in feces using Griess Reaction System (Promega, USA). The system measures nitrite level which is one of the two primary, stable and nonvolatile breakdown products of NO. About 1ml of a 100 µM nitrite solution was prepared by diluting the provided 0.1M Nitrite Standard 1:1,000 in the buffer used for the experimental samples. Three columns (24 wells) in the 96-well plate were designated for the Nitrite Standard reference curve and 50 µl of the appropriate buffer were dispensed into the wells in rows B–H. About 100 µL of the 100 µM nitrite solution were added to the remaining 3 wells in row A. Immediately, three 6 serial twofold dilutions (50 µl/well) were performed in triplicate down the plate to generate the Nitrite Standard reference curve (100, 50, 25, 12.5, 6.25, 3.13 and 1.56 µM), discarding 50 µl from the 1.56 µM set of wells. Nitrite solution was not added to the last set of wells (0 µM). The final volume in each well was 50 µl, and the nitrite concentration range was 0–100 µM. About 50 µl of each experimental sample was added to wells in duplicate. Using a multichannel pipettor, 50 µl of the Sulfanilamide Solution was added to all experimental samples and wells containing the dilution series for the Nitrite Standard reference curve. The plate was incubated for 5-10

min at room temperature and protected from light and 50 µl of the NED Solution was dispensed to all wells. The plate was again incubated at room temperature for 5-10 min, protected from light. A purple/magenta color began to form immediately. Absorbance was measure within 30 min in a plate reader with a filter between 520 nm and 550 nm. The nitrite concentrations in the samples were determined using the standard curve.

Intestinal inducible nitric oxide (iNOS) expression

Total intestinal RNA extraction: Total intestinal RNA was extracted using standard Guaninidium isothiocynate/Phenol method (Trizol reagent). Briefly, about 100 µL intestinal tissues were homogenized in 1 mL of Trizol using power homogenizer (DAIHAN Scientific Co., Ltd, Korea) at 1000 rpm for 20 sec. The sample was incubated at room temperature for 5 min. About 200 µL chloroform was added, the sample shaken vigorously for 15 sec and incubated for 2-3 min at room temperature. The aqueous phase containing RNA was pipetted into a new tube after centrifuging at 12000 xg for 15 min at 4°C. The concentration of RNA was measured in ng/µL using Nanodrop instrument (Colibri Spectrometer, Berthhold Detection System, Germany) with 260/280 ratio of between 1.6-1.9. The RNA band was visualized in 1% agarose gel-electrophoresis showing two predominant bands of larger ribosomal RNA and smaller tRNA.

cDNA Synthesis: The cDNA was synthesized from total RNA using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV) (Enzynomics, Korea). RNA (0.4 µg) was used to generate first strand complementary DNA. The RNA template (1 µL) was mixed with 1 µL of Oligo (dT) 18 primer, heated at 70°C for 5 min and chilled on ice. The following components were added and incubated at 42°C for 5 min: 2 µL 10X M-MLV RT Buffer, 1 µL M-MLV Reverse Transcriptase, 2 µL dNTP mixture, 0.5 µL RNase Inhibitor, 20 µL Sterile water (RNase free water). The mixture was incubated at 37°C for 60 min and then inactivated by further incubation at 95°C for 5 min.

Reverse transcription polymerase chain reaction: PCR was performed for iNOS and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) from the same complementary DNA samples by using Applied Biosystem, 2720 Thermal Cycler and USA. Oligonucleotide primers for iNOS and GAPDH were: 5^1-ATGGCT TGC CCT TGG AAG TTT CTC-3^1, 5^1-TCC AGG CCA TCT TGG TGG CA AGA-3^1 and 5^1-TCC CTC AAG ATT GTC AGC AA-3^1, 5^1-AGA TCC ACA ACG GAT ACA TT-3^1, which correspond to the rat iNOS and rat GAPDH cDNA respectively [25]. Amplification was initiated by 5 min of denaturation at 95°C for 1 cycle followed by 25 cycled at 95°C for 30 s, 60°C for 45 s, and 72°C for 1 min. After the last cycle of amplification, the samples were incubated for 10 min at 72°C. Reactions without reverse transcriptase (No-RT controls) were run for each sample at the higher RNA input to ensure that signal detected was not from contaminating genomic DNA. The PCR products -- a 574 bp iNOS fragment and a 309 bp GAPDH fragment -were then visualized 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.

Results

Molecular characterization of ETEC strains

The *E. coli* samples that yielded positive results from biochemical tests were subjected to molecular identification using *E. coli* universal stress protein gene (*uspA*). Five (5) samples expressed the gene, Figure 1.

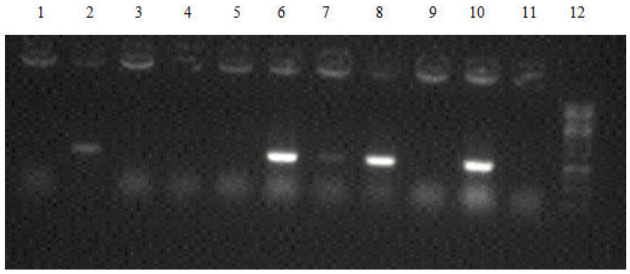

Figure 1: Agarose gel showing the presence of *E. coli uspA* gene following PCR experiment. The gene was seen in five samples, 2, 6, 7, 8 and 10. Line 12 was DNA ladder.

Further identification of ETEC revealed the presence of 2 ST-ETEC and 1 LT-ETEC, Figure 2. 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 2.

DEC	Serogroups
ST-ETEC	O8:H9 and O6:H16
LT-ETEC	O25:H42

Table 2: The presence of Somatic [O] and Flagellar [H] antigens in ETEC.

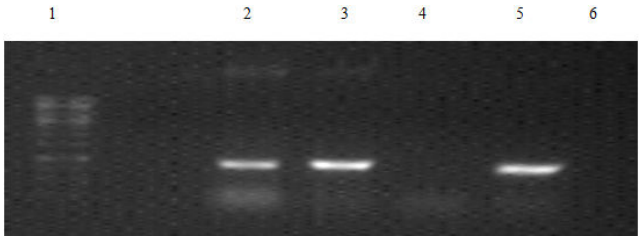

Figure 2: Agarose gel showing the presence of ETEC genes, *eltB* and *eltA*. The presence of ETEC genes were seen in three *E. coli* samples (2, 3 and 5) out of five samples. Two samples, 2 and 3 showed the presence of eltA for ST-ETEC while one sample, 5 showed the presence eltB for LT-ETEC. Line 1 was DNA ladder.

Antibiotic susceptibility

Zone diameter breakpoint (mm) of ≥ 17 implies susceptibility while <17 is resistance. For beta lactam antibiotics (Figure 3), the entire organisms are susceptible to cefoxitin, imipenem and resistant to methicillin/oxacilin. In addition, LT-ETEC and one of the ST-ETECs are resistant to ampicillin and amoxicillin/clavulinic acid. As seen in Figure 4, one of the ST-ETECs is resistant to three non-beta lactam antibiotics used including the fluoroquinolone, ciprofloxacin. The other ST-ETEC and LT-ETEC are both susceptible to ciprofloxacin and resistant to tetracycline and quinupristin D.

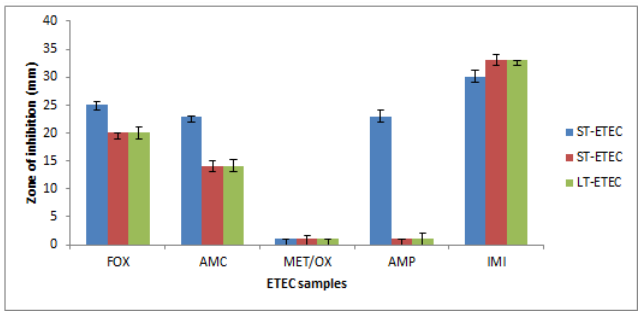

Figure 3: Susceptibility of ETECs to β-lactam antibiotics. The ETECs were susceptible to both IMI and FOX, then resistant to MET/OX. In addition, LT-ETEC and one ST-ETEC were resistant to AMP and AMC while one ST-ETEC was susceptible to both antibiotics. FOX (cefoxitin), AMC (amoxicillin/clavulinic acid), MET (methicillin), OX (oxacillin), AMP (ampicillin), IMI (imipenem).

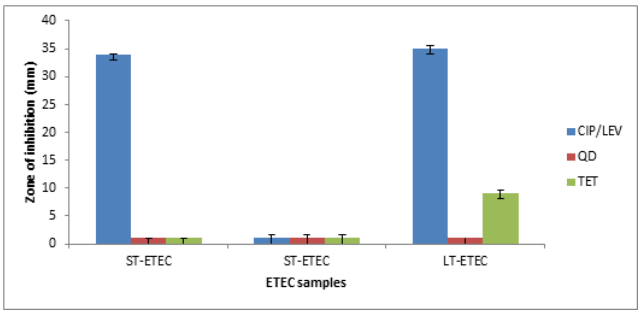

Figure 4: Susceptibility of ETECs to non β-lactam antibiotics. One of the ST-ETECs was resistant to the three drugs while the other is resistant to QD and TET and susceptible to CIP. The LT-ETEC was resistant to QD and TET while susceptible to CIP. CIP (ciprofloxacin), LEV (levofloxacin), QD (quinupristin), TET (tetracycline).

Biofilm formation

All the ETEC samples were found to produce biofilms. One of the ST-ETECs was a strong biofilm producer. The other ST-ETEC and the LT-ETEC were weak and moderate biofilm producers respectively. None of the samples showed a very strong biofilm production, Figure 5.

Fecal protein and nitric oxide concentration

As seen in Table 3, fecal proteins were found to be significantly increased in LT ETEC-induced group when compared to control group. Although there was an increase in fecal proteins of ST ETEC-induced group, but the increase was not significant when compared with the control group. Fecal nitric oxide showed a significant increase in both LT and ST-ETEC induced groups when compared with the control group.

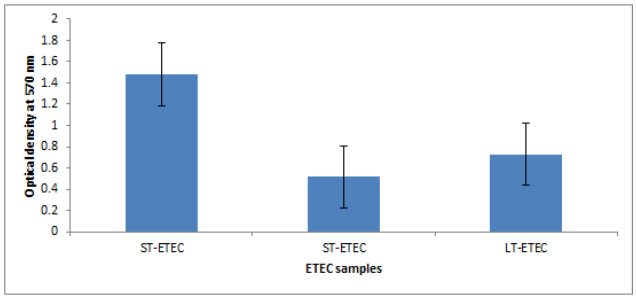

Figure 5: Level of Biofilm formation in ETEC samples. One ST-ETEC showed strong biofilm production while the other showed a weak production. The LT-ETEC showed a moderate biofilm production.

	Control	LT ETEC-induced	ST ETEC-induced
Fecal protein (mg/mL)	2.60 ± 0.52	3.00 ± 0.33[a]	2.85 ± 0.19
Fecal nitric oxide (μM)	54.27 ± 15.44	82.09 ± 22.30[a]	68.81 ± 22.30[a]

Table 3: Fecal Protein and nitric oxide concentrations. [a]Significant increase when compared to control group.

Reverse transcription PCR

The expression of intestinal nitric oxide synthase (iNOS) was not seen in any of the groups. The reference GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene showed in all the groups, Figure 6.

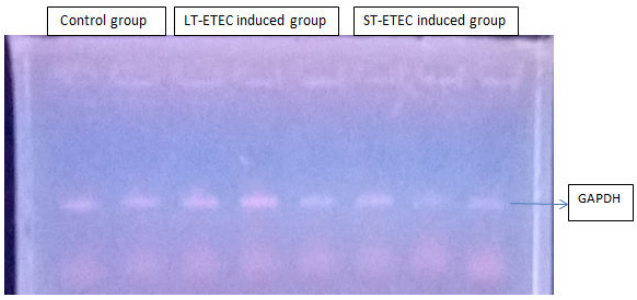

Figure 6: Detection of iNOS gene using reverse transcription PCR. The presence of iNOS was not seen in any of the groups. The housekeeping reference gene, GAPDH was seen in all the groups.

Statistical analysis

All experiments were performed in triplicate. The data were subjected to one-way analysis of variance (ANOVA). A p-value less than 0.05 were considered significant.

Discussion

From our study, the detection rate of ETEC among DEC isolated from children was 27% (3/11). Similar high ETEC rate (36.3%)

amongst hospitalized diarrheal patients in Kolkata India was also recorded [26]. Our result was different from the one obtained among DEC isolated from infants and children in Dar es Salaam, Tanzania where ETEC was found to be 3.6% [27]. The PCR assay also detected 2.2% ETEC among young children in Hanoi, Vietnam [23]. While EPEC was the most prevalent DEC (47.5%) among adolescents and adults in Hamedan, Western Iran, ETEC detection rate was 17.5% [28]. In Nigeria, our result agreed with the one recorded in Onitsha, South East Nigeria where 21.57% ETEC strains were detected among DEC isolates from children [29]. In Federal Capital Territory, Abuja, Nigeria, 4.0% prevalence of ETEC among DEC was observed [30]. The heat stable enterotoxigenic *E. coli* (ST-ETEC) showed greater association with childhood diarrhea than heat-labile, LT-ETEC. This result agreed with the one performed in Southwest Nigeria [31] and other parts of the world [32,33].While the most prevalent DEC differs around the world, ETEC is mostly recorded as the second most prevalent. We also observed such trend in our result.

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 [34]. 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, also discovered such diversity in serotypes of DEC in Osun State, Nigeria [31].

Among the three ETEC, two strains (ST and LT-ETEC) were found to be multidrug resistant (resistance to >3 antimicrobial drugs). The ST-ETEC was resistant to methicillin, ampicillin, amoxicillin/clavulinic acid (beta-lactams), tetracycline, quinupristin and ciprofloxacin (non-beta lactams).The LT-ETEC was resistant to methicillin, ampicillin, (beta-lactams), tetracycline, QD (non-beta lactams). Most worrisome was an ST-ETEC found to be ciprofloxacin (fluoroquinolone) resistant. 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 [28]. Such multidrug resistance among ETEC against classical antibiotics like ampicillin and tetracycline was also recorded in Bolivia, although no ciprofloxacin resistance was observed [34]. 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%) [35].

The quinolone resistance in *E. coli* has mainly been associated with mutations in the *gyrA* and *parC* genes [36]. Strains resistant against ciprofloxacin are likely to have at least one mutation of the *gyrA* gene. 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 quinolone 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 [37].

All the ETEC formed significant levels of biofilms and are resistant against ampicillin, methicillin, quinupristin-dalfopristin and tetracycline. This suggests a possible relationship between biofilm formation and antibiotic resistance in *E. coli*. Similar result has been recorded where some of the *E. coli* cells in the mature biofilms, were resistant to ampicillin [38]. Different mechanisms have been proposed to explain the high resistance of biofilms, which include restricted penetration of antimicrobial agents into biofilms, slow growth due to nutrient limitation, expression of genes involved in the general stress response, and emergence of a biofilm-specific phenotype [38]. Although combinations of these factors are involved in most biofilm studies, it is still difficult to completely understand the mechanisms of biofilm resistance to antibiotics. In another study, biofilm formation was not associated with any virulence determinant, but there was a significant correlation between prevalence of antibiotic resistance in *E. coli* isolates and biofilm formation ability [39].

Intestinal absorption is commonly impaired during acute intestinal infections [40]. The efficiency of protein absorption during diarrhea has not been studied extensively, but marked loss of endogenous protein in stool has been observed [41]. Our result recorded an increase in protein fecal loss in both LT and ST-induced groups when compared with the control group. In a related study, fecal protein loss was observed in more than 50% of children with acute and persistent diarrhea caused by various pathogens [42]. In a more recent publication, acute hemorrhagic diarrhea syndrome (AHDS) is associated with a significant gastrointestinal loss of protein and intestinal inflammation [43].

Several results have suggested that nitric oxide (NO) synthesized by nitric oxide synthase (NOS) modulates diarrheal effects [17,18]. In aloe-induced diarrhea, pre-treatment with NOS inhibitor N(G)-nitro-L-arginine methyl ester (L-NAME) reduced the diarrhea [18]. In endotoxin-induced diarrhea, NO has also been implicated in increased gastrointestinal transit [44]. Our result also showed an increase in fecal NO in both LT and ST-induced diarrhea but did not show a corresponding inducible NOS expression. Although we did not monitor the expression of constitutive NOS, it has been established that an inhibitor of the expression of inducible NO synthase does not affect the increase in the gastrointestinal transit through constitutive NO synthesis [44]. The results suggested that constitutive nitric oxide may be involved in the pathological conditions of endotoxin-induced diarrhea. NO signaling in vertebrates intestine involves activation of soluble guanylate cyclase (GC), leading to the formation of cyclic GMP and resulting in electrolyte and water loss [45].

Conclusion

The detection 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 multi-drug resistance and biofilm producers due to availability of over-the-counter antibiotics. The continued use and abuse of these drugs allows 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. The elevation of NO in fecal sample suggests its role in induction of fluid and electrolyte loss in diarrhea. Although, the expression of inducible NOS was not detected using reverse transcription PCR, the sensitivity of real-time PCR would have confirmed a minute expression of iNOS if used. The result also suggests that constitutive NOS could have been elevated.

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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. AJ performed biochemical identification. All authors read and approved the final manuscript

Competing interests

No competing interests are declared by authors.

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