Original article

Detection of virulence genes in Shiga toxigenic *Escherichia coli* isolated from diarrhoeic and non-diarrhoeic pediatric patients in Ondo State, Nigeria

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A R T I C L E I N F O

A B S T R A C T

Background: Shiga toxigenic *Escherichia coli* (STEC) is one of the major causes of intestinal infection, bloody and non-bloody diarrhoea and extra-intestinal complications in humans. It is therefore necessary to enumerate the virulence genes present in isolated STEC strains. **Methods:** This research investigated the prevalence of STEC and identified some virulence genes (stx1, stx2, and eaeA) from diarrhoeic and non-diarrhoeic pediatric patients of age 1 to 16 years. A total of 243 stool samples were collected from diarrhoeic and non-diarrhoeic pediatric patients (in ratio 43:200) in four different State Specialist Hospitals in Ondo State, Nigeria. Polymerase chain reaction (PCR) assay was used for detection of Shiga toxin-producing *E. coli*. The virulence genes (stx1, stx2, and eaeA) were detected by PCR assay. **Results:** Out of the 243 samples, 163 (67%) were positive for *E. coli* and specifically 6 of them were positive for *E. coli* O157 (3.7%). Exactly 50% of the 6 *E. coli* O157 contained eaeA while all the strains contained Stx1 and Stx2. The results obtained also revealed that 6 strains that were non-O157:H7 STEC also contained Stx1 and four strains contained Stx2 genes. **Conclusion:** The results obtained from this study revealed that Stx1 and Stx2 genes are of the genes responsible for the virulence of STEC among diarrhoeic and non-diarrhoeic pediatric patients.

Introduction

Escherichia coli (E. coli) is popular for its ability to cause diarrhoea [1]. So far, *E. coli* has remained separated into 6 different classes, which are: enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), diffusely adherent *E. coli* (DAEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and enterohemorrhagic *E. coli* (EHEC or STEC) [2]. Shiga toxin-producing *Escherichia coli* (STEC) are a group of bacteria classified by their ability to produce one or more types of shiga toxin (Stx) [3]. Shiga toxin-producing *Escherichia coli* are

associated with a disease spectrum ranging from diarrhoea and haemorrhagic colitis (HC) to the potentially fatal haemolytic ureamic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) [4]. According to **Rahal et al.** [5], STEC infections are characteristically food-borne and the ability to produce Shiga toxins (Stx1, Stx2 or a variant) is believed to be essential to the pathogenesis of these organisms. Shiga toxins hinder production of protein in the host cell, which causes cell killing. Shiga toxin-producing *Escherichia coli* strains arise as a result of the insertion of one of a group of lysogenic lambdoid

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bacteriophages that holds an Stx1/2-encoding gene into the genome of *E. coli* [5].

The clinical symptoms, pathogenic characteristics and the pathobiology of these organisms and the toxins they produce was reviewed by **Kruger and Lucchesi** [6]. According to **Quinn et al.** [7], over 30% of cows infected with STEC does not show any symptoms. Another study established that it causes mortality in 3-5% of patients with long-term complications in about 30% of patients [8]. Little infectious dose of O157 EHEC increases the virulence and risk of infection [9]. The use of effective antibiotics in treatment of EHEC infection results in the release of verotoxin and also the death of bacteria [10].

The aim of this study is therefore, to isolate and characterize STEC strains and to identify the virulence genes (stx1, stx2 and eaeA) from stool samples of diarrhoeic and non-diarrhoeic pediatric patients in Ondo State, Nigeria.

Materials and Methods

Ethical approval

Ethical approval to conduct this research was obtained from the Health Research Ethics Committee, Ondo State Ministry of Health (OSHREC/20/01/2018/036), while informed consent was obtained from each patient before sampling and strict confidentiality of the participated patients was maintained.

Sample collection

A total of 243 stool samples were collected from diarrhoeic (43) and non-diarrhoeic (200) pediatric patients in State Specialist Hospitals in Akure, Ikare, Ondo and Okitipupa, Ondo State. Patient demographic characteristics including age and gender, socioeconomic and clinical characteristics were recorded.

Preparation and identification of STEC

The stool samples were cultured onto MacConkey's agar (Merck, Germany) and Sorbitol MacConkey agar (Merck, Germany) and then incubated for 24 hours at 37°C. Lactose positive colonies were selected from each sample, streaked onto Eosin Methylene Blue (Merck, Germany) plates and incubated for 18 h at 37°C. Green colonies with a metallic luster identified as *E. coli* colonies were further confirmed using standard biochemical tests such as indole, methyl red, Voges-Proskauer, Indole, H2S production, citrate utilization tests and urease production test [11,12]. Colonies were confirmed as *E. coli* by PCR as described by **Madic et al.** [13]. The *E. coli* isolates were stored in tryptic soy broth (Merck, Germany) which contained 20% glycerol at 70°C for more investigation.

DNA extraction

Bacterial isolates were cultured in trypticase soy agar (Merck, Germany) overnight at 37°C. A single colony was suspended in 100μL of sterile distilled water. The suspension was boiled for 13 min, frozen and centrifuged at 14,000 rpm for 15 min to pellet the cell debris according to **Schoppee and Wamhoff** [14]. The filtrate was then used as a template for polymerase chain reaction (PCR) amplification.

Detection of virulence genes by PCR

Polymerase chain reaction assay was carried out according to the method described by **Schouler et al.** [15]. The set of primers in **table (1)** were used for identification of *E. coli* O157 and the stx1, stx2, eaeA, for the presence of virulence genes. Polymerase chain reaction assay was carried out in a volume of 25 μL, so that 21 μL of Master Mix (CinnaGen, Iran), containing 400 mM of deoxy nucleoside triphosphates, 0.05 U/μL of Taq DNA polymerase, 4 mM of MgCl2, 1 μL of each primer, and 2 μL of the DNA extracted. The PCR was carried out in 35 cycles using the thermocycler (Biorad, USA), One hundred microlitre aliquots of the suspension was then transferred to PCR tubes and heated at 95-99°C for 10 minutes on a thermocycler. Then, the tubes were subjected to the protocol mentioned in **table (2)**. The tubes were centrifuged at 10,000 rpm for 3 minutes to pellet cellular debris. The supernatant was used as the PCR positive control for all PCR assays [16].

DNA electrophoresis

Agarose gel electrophoresis was used to determine the quality and integrity of the DNA by size fractionation on 1.0% agarose gels. Agarose gels were prepared by dissolving and boiling 1.0g agarose in 100ml 0.5X TBE buffer solution and stained with 10μl of 5mg mL-1 ethidium bromide (Merck, SA). The amplified products were visualized and photographed under the BioDoc-It System (UVP Upland, CA 91786, USA). A 100 bp DNA ladder (Promega, USA) was involved on each gel as a molecular size standard. Electrophoresis was done at 80V for 2 hours [16].

Table 1. Characterization of primers for identification of *E. coli* O157 (STEC) and virulence genes [17]. Both stx1 and stx2 primers amplified at 500bp and eae primer amplified at 300bp showing the presence of *E. coli* O157.

Gene target	Primers	Oligonucleotide Sequence (5'-3')	Fragment (bp)	Annealing Temp (°C)
stx1	Stx1-F	AAATCGCCATTCGTTGACTACTTCT	500	56
	Stx1-R	TGCCATTCTGGCAACTCGCGATGCA		
stx2	Stx2-F	CAGTCGTCACTCACTGGTTTCATCA	500	56
	Stx2-R	TGCCATTCTGGCAACTCGCGATGCA		
eaeA	EAE-F	AGGCTTCGTCACTGTG	300	56
	EAE-R	CCATCGTCACCAGAGGA		

Table 2. PCR condition used for identification of virulence genes in STEC strains.

NO	Step	Stx1	Stx2	eaeA
1	Initial denaturation	94°C/3mins	94°C/3mins	94°C/3mins
2	Denaturation	94°C/60sec	94°C/60sec	94°C/70sec
3	Annealing	56°C/60sec	54°C/30sec	56°C/60sec
4	Extension	72°C/120sec	72°C/120sec	72°C/120sec
5	Final extension	72°C/5mins	72°C/5mins	72°C/5mins

Results

Prevalence of pathogenic subtypes and related virulence genes in STEC strains

From the 243 stool samples examined, 163 (67.1%) tested positive for *E. coli* strains, while 6 (2.5%) strains were confirmed as *E. coli* O157. The *E. coli* O157 isolates contained Stx1, Stx2 and eaeA gene. Also, 157 isolates (64.6%) were identified as non-O157 *E. coli*. The presence of virulence genes in the STEC isolates is presented in **table (3)**.

Result of PCR of selected *Escherichia coli* isolate among diarrhoeic and non-diarrhoeic stool

The result of PCR of selected *Escherichia coli* isolate among diarrhoeic and non-diarrhoeic stool is presented in **table (4)**. The *E. coli* isolated from stool samples were identified as *E. coli* strain RTdelA_B_UU3 chromosome, J53 chromosome (complete genome), C600 chromosome (complete genome), DTU-1 chromosome (complete genome), DTU-1 chromosome (complete genome) and K-12 substr. MG1655 strain K-12 chromosome with Stx 1, 2 and eae genes found across the *E. coli* str. except the gene eae absent in *E. coli* strain RTdelA_B_UU3 chromosome.

Distribution of virulence factors in *Escherichia coli* subtype

Table 5 reveals the distribution of virulence factors in *Escherichia coli* subtype, EHEC and EIEC were evenly distributed in all the age groups except EIEC which is absent.

Virulence genes targeted at STEC serogroups

The virulence genes stx1 and eae were found in STEC serotypes O157, 026, 0103 and 0145 while stx2 was only found in STEC serotype O157 (**Table 6**).

Molecular characteristics of *Escherichia coli* isolated from stool samples

The *E.coli* strains isolated from the stool samples were identified as *E. coli* strain RTdelA_B_UU3

chromosome, *E. coli* strain RTdelA_B_UU3 chromosome, *E. coli* strain C600 chromosome, complete genome, *E. coli* strain DTU-1 chromosome, complete genome, *E. coli* strain DTU-1 chromosome, complete genome, *E. coli* str. K-12 substr. MG1655 strain K-12 chromosome (**Plate 1**).

The gel electrophoresis of the isolate using STX 1 to 3 and EAEAF are shown in Plates 2 to 4 as the molecular characterization of the isolated *E. coli* was determined and the *E. coli* possesses the aligned nucleotide sequence.

Table 3. Prevalence of pathogenic subtypes and related virulence genes in STEC strains isolated from patients with diarrhea.

Samples (No)	Subtypes	Number of positive samples	Virulence genes
	E. coli	163 (67%)	-
243	Non-*E. coli* isolates	80 (33%)	-
	E. coli O157	6 (2.5%)	Stx1, stx2, eaeA
	Non-O157 *E. coli*	157 (64.6%)	

Table 4. Result of PCR of selected *E.coli* isolate among diarrhoeic and non-diarrhoeic stool.

| *Escherichia coli* strain	Stool sample	PCR Results	Gene Type
RTdelA_B_UU3 chromosome	Non-diarrhoeic	Positive	Stx 1, Stx 2
J53 chromosome (complete genome)	Non-diarrhoeic	Positive	Stx 1, Stx 2, eae
C600 chromosome (complete genome)	Diarrhoeic	Positive	Stx 1, Stx 2, eae
ME8067 chromosome (complete genome)	Diarrhoeic	Positive	Stx 1, Stx 2, eae
DTU-1 chromosome (complete genome)	Diarrhoeic	Positive	Stx 1, Stx 2, eae
K-12 substr. MG1655 strain K-12 chromosome	Diarrhoeic	Positive	Stx 1, Stx 2, eae

Table 5. Distribution of virulence factors in *E.coli* subtype.

Age of Patients (Years)	*E. coli* Subtypes	No of samples tested positive	% of tested isolates (%)	Virulence genes
<1	EHEC	1	16.67	stx1, stx2
	EIEC	-	-	-
1-5	EHEC	2	33.33	stx1, stx2
	EIEC	2	40	eae
6-10	EHEC	2	33.33	stx1, stx2
	EIEC	2	40	stx1, eae
11-16	EHEC	1	16.67	stx1, stx2
	EIEC	1	20	eae

Table 6. Virulence genes targeted at STEC serogroups.

Virulence genes	O157	026	045	0103	0111	0121	0145
stx1	+	+	-	+	-	-	+
stx2	+	-	-	-	-	-	-
Eae	+	+	-	+	-	-	+

**Plate 1.** Six (6) bacterial isolates amplified by 16s primer.
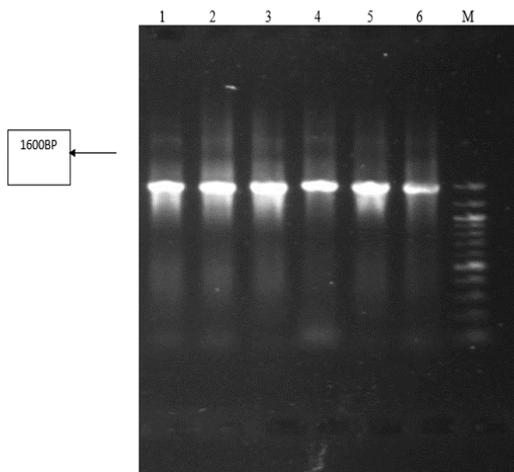
Key: 1 = *Escherichia coli* strain RTdelA_B_UU3 chromosome; 2 = *Escherichia coli* strain RTdelA_B_UU3 chromosome; 3 = *Escherichia coli* strain C600 chromosome, complete genome; 4 = *Escherichia coli* strain DTU-1 chromosome, complete genome; 5 = *Escherichia coli* strain DTU-1 chromosome, complete genome; 6 = *Escherichia coli* strain K-12 substr. MG1655 strain K-12 chromosome; M = Molecular marker.

Plate 2. Gel electrophoresis of Shiga-toxin 1 (STX1) primers from *Escherichia coli*.
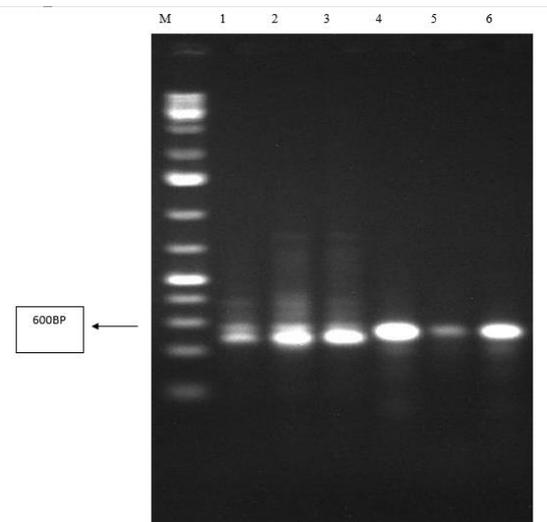

Key: M = Molecular marker; 1-6 = Resistant *Escherichia coli*

Plate 3. Gel electrophoresis of Shiga-toxin 2 (STX2) primers from *Escherichia coli*.
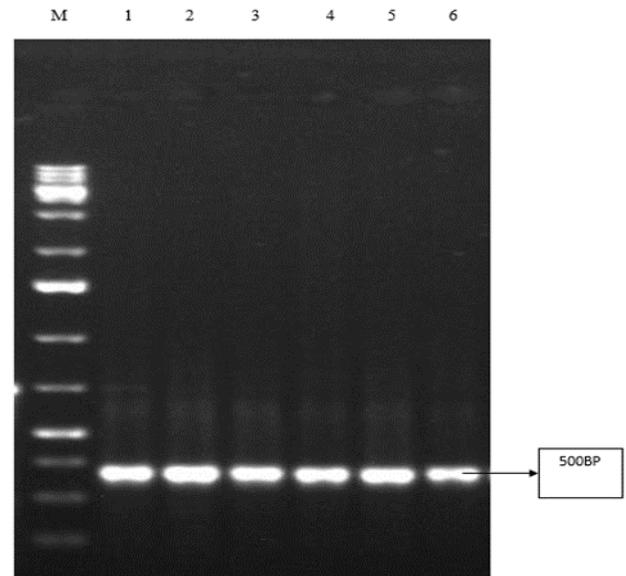
Key: M = Molecular marker; 1-6 = Resistant *Escherichia coli*

Plate 4. Gel electrophoresis of multiple antibiotic-resistant primers from *Escherichia coli*.
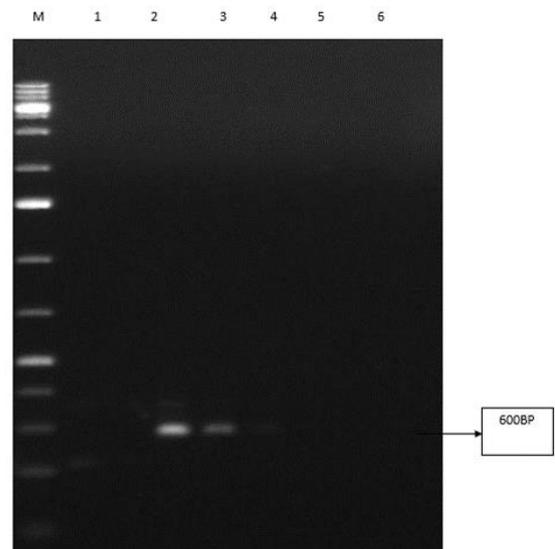
Key: M = Molecular marker; 1 = Unamplified gene, non-resistant *Escherichia coli*; 2-3 = Multiple drug resistant *Escherichia coli*; 4-6 = Unamplified gene, non-resistant *Escherichia coli*.

Discussion

Diarrhoea due to bacterial infections is an important cause of morbidity and mortality in infants and young children in most developing countries including Nigeria [18-20]. Generally, the etiological factors of acute diarrhoea in developing and developed countries are different. In developing countries, Shigella and *E. coli* are the dominant bacteria causing diarrhoea. Studies have shown that STEC strain are responsible for important human diseases including HUS, HC and TTP. In extreme cases, rate of death is closely associated with the presence of the virulence genes. Patients with non-bloody diarrhoea show milder symptoms and are less expected to develop HUS. However, HUS has also been reported in cases of non-bloody diarrhoea.

One hundred and sixty-three (163) out of 243 samples (78%) were positive for *E. coli* strains in stool culture. From the result six *E. coli* strains were identified in **plate (1)**. **Plate 2, 3** and **4** showed *E. coli* strain which harbored stx1 and stx2 gene identified as *E. coli* O157, and eaeA gene. Findings from this current study indicates that STEC strains and non-O157 strains, contributed to 6% of all diarrhoeal infections which were able to produce shiga-like toxins. Prior research studies have shown the low occurrence of *E. coli* O157: H7 strain in people residing in developing countries. The result gotten from this research study showed that only 2.5% of sampled patients were infected with this strain. This result is in agreement with the result of a research study conducted in Iran. **Taghadosi et al**. [21] isolated five (1.3%) STEC strains from 395 diarrheal fecal samples in Iran. **Kalule et al.** [22] reported similar findings of a study carried out in Kenya which indicated the low prevalence (0.2%) of *E. coli* O157: H7 in hospitalized diarrhoeic children.

The presence of *E. coli* from the population of the non-diarrhoeic patients also indicates their role as potent carrier of the organism, which may infect the other vulnerable population of the community [23]. In non-diarrhoeic group, the *E. coli* from both pediatric patients from hospital and the community were detected which is very less than the previous reports in India [24,25]. This might be the possible source of transmission of this pathogen among children through mother, baby sitter or other members of the family [23].

Conclusion

Comparing the stool samples from diarrhoeic patient and non-diarrhoeic pediatric children (<1 to 16years), there was a strong indication of the presence of *E. coli* in stool sample of non-diarrhoeic pediatric children in (<1 to 16years) in Ondo State. It is therefore recommended that:

1. Hygiene education should be addressed for diarrhoeal disease susceptible groups, such as those pre-school children, young generation, old and weak class.
2. There should be periodic enlightenment on hygiene practice and the use of hazard analysis in the preparation and processing of food.

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