Original article

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

Joy Efe Olanrewaju*1, Anthony Kayode Onifade 1
Department of Microbiology, Federal University of Technology, PMB 704, Akure, Ondo State.

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ABSTRACT

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), enteroinvasive *E. coli* (IEC), 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 uremic 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
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 do 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 diarrheic and non-diarrheic 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 diarrheic (43) and non-diarrheic (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 further 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  | AAATCGCCATTCTGGACTACTTCT        | 500           | 56                  |
|             | Stx1-R  | TGCCATTCTGGCAACTCGCGATGCA       |               |                     |
| stx2        | Stx2-F  | CAGTCGTCACTGACTGTTCATCA         | 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       |
|--------------|---------------------------|----------------------------|-----------------------|
| 163 (67%)    | *E. coli*                 |                            | -                     |
| 80 (33%)     | Non-*E. coli* isolates    |                            | -                     |
| 6 (2.5%)     | *E. coli* O157            |                            | Stx1, stx2, eaeA      |
| 157 (64.6%)  | Non-O157 *E. coli*        |                            |                       |

**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, EIEC        | 1                             | 16.67                    | stx1, stx2            |
| 1-5                     | EHEC, EIEC        | 2                             | 33.33                    | stx1, stx2, eae       |
| 6-10                    | EHEC, EIEC        | 2                             | 33.33                    | stx1, stx2, stx1, eae |
| 11-16                   | EHEC, EIEC        | 1                             | 16.67                    | stx1, stx2, eae       |

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 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, complete genome; 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 strains 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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References

1- Jenkins C. Enteroaggregative Escherichia coli. Escherichia coli, a Versatile Pathogen 2018; 27-50.
2- Govindarajan DK, Viswalingam N, Meganathan Y, Kandaswamy K. Adherence patterns of Escherichia coli in the intestine and its role in pathogenesis. Medicine in Microecology 2020; 5: 100025.
3- Ion F, Zhang P, Meng J, Xie Q, Li Y, Pan Y, et al. Characterization of Shiga-toxin producing Escherichia coli (STEC) isolated from retail raw meats in Southeast China. Food Control 2021; 126: 108061.
4- Hwang SB, Chelliah R, Kang JE, Rubab M, Banan-MwineDaliri E, Elahi F, et al. Role of Recent Therapeutic Applications and the Infection Strategies of Shiga Toxin-Producing Escherichia coli. Frontiers in cellular and infection microbiology 2021; 11: 450.
5- Rahal EA, Fadlallah SM, Nassar FJ, Kazzi N, Matar GM. Approaches to treatment of emerging Shiga toxin-producing Escherichia coli infections highlighting the O104: H4 serotype. Frontiers in cellular and infection microbiology 2015; 5: 24.
6-Kruger A, Lucchesi PM. Shiga toxins and stx-phages: highly diverse entities. Microbiology 2015; 161(3): 451–462.
7-Quinn PJ, Markey BK, Carter ME, Donnelly WJC, Leonard FC. Veterinary microbiology and microbial disease. Blackwell science 2020.
8-Jenssen GR, Vold L, Hovland E, Bangstad HJ, Nygård K, Bjerre A. Clinical features, therapeutic interventions and long-term aspects of hemolytic-uremic syndrome in Norwegian children: a nationwide retrospective study from 1999–2008. BMC Infectious Diseases 2016; 16(1): 1-10.
9-Schroeder CM, Meng J, Zhao S, DebRoy C, Torcolini J, Zhao C, et al. Antimicrobial resistance of *Escherichia coli* O26, O103, O111, O128, and O145 from animals and humans. Emerging Infectious Diseases 2002; 8(12): 1409–1414.
10-Goldwater PN, Bettelheim KA. Treatment of enterohemorrhagic *Escherichia coli* (EHEC) infection and hemolytic uremic syndrome (HUS). BMC medicine 2012; 10(1): 1-8.
11-Brenjchi M, Jamshidi A, Farzaneh N, Bassami MR. Identification of shiga toxin producing *Escherichia coli* O157: H7 in raw cow milk samples from dairy farms in Mashhad using multiplex PCR assay. Iranian Journal of Veterinary Research 2011; 12(2): 145-149.
12-Cheesbrough M. District Laboratory Practice in Tropical Countries. IJMS 2018; 1(1): 65-68.
13-Madic J, Vingadassalon N, de Garam CP, Marault M, Scheutz F, Brugere H, et al. Detection of Shiga toxin-producing *Escherichia coli* serotypes O26: H11, O103: H2, O111: H8, O145: H28, and O157: H7 in raw-milk cheeses by using multiplex real-time PCR. Applied and environmental microbiology 2011; 77(6): 2035-2041.
14-Schoppee Bortz PD, Wamhoff BR. Chromatin immunoprecipitation (ChIP): revisiting the efficacy of sample preparation, sonication, quantification of sheared DNA, and analysis via PCR. PloS one 2011; 6(10): e26015.
15-Schouler C, Schaeffer B, Bree A, Mora A, Dahbi G, Biet F, et al. Diagnostic strategy for identifying avian pathogenic *Escherichia coli* based on four patterns of virulence genes. Journal of clinical microbiology 2012; 50(5): 1673-1678.
16-Zahraa JJ, Akeel HA, Amin SB. Isolation and characterization of enteroaggregative *Escherichia coli* among the causes of bacterial diarrhea in children. Tikrit Journal of Pure Science 2015; 20(4): 1813-1662.
17-Rey J, Sanchez S, Blanco JE, De Mendoza JH, De Mendoza MH, Garcia A, et al. Prevalence, serotypes and virulence genes of Shiga toxin-producing *Escherichia coli* isolated from ovine and caprine milk and other dairy products in Spain. International journal of food microbiology 2006; 107(2): 212-217.
18-Adegunloye DV. Carrier rate of enteric bacteria associated with diarrhea in children and pupils in Akure, Ondo State, Nigeria. Academic Journal 2005; 4: 3-6.
19-Akingbade OA, Akinjinni AA, Olasunkanmi OL, Okerentugba PO, Onajobi BI, Okonko IO. Bacterial Organisms Isolated From Children with Diarrhoea In Abeokuta, Nigeria. Stem Cell 2013; 4(4): 5-9.
20-Chukwu MO, Akebe LA, Eunice U, Larry CO, John BD. Antibiotic Resistance Profile and Clonality of *E. coli* Isolated from Water and Paediatric Stool Samples in the North-West, Province South Africa. Journal of Pure and Applied Microbiology 2019; 13(1): 517-530.
21-Taghadosi R, Shakibaie MR, Alizade H, Hosseini-Nave H, Askari A, Ghanbarpour R. Serogroups, subtypes and virulence factors of shiga toxin-producing *Escherichia coli* isolated from human, calves and goats in Kerman, Iran. Gastroenterol Hepatol Bed Bench 2018; 11: 60-67.

22-Kalule JB, Keddy KH, Nicol MP. Characterization of STEC and other diarrheic *E. coli* isolated on CHROMagar™ STEC at a tertiary referral hospital, Cape Town. BMC microbiology 2018; 18(1): 1-8.

23-Karuppasamy C, Tapan KD, Indu S, Surajit D, Nachimuthu SK, Lalsanglura R. Prevalence of multi drug resistant enteropathogenic and enteroinvasive *Escherichia coli* isolated from children with and without diarrhea in Northeast Indian population. Annals of Clinical Microbiology and Antimicrobials 2017; 16: 49.

24-Wani SA, Asifa N, Iffiat F, Irfan A, Yoshikazu N, Khursheed Q, et al. Investigation of diarrhoeic faecal samples for enterotoxigenic, Shiga toxin-producing and typical or atypical enteropathogenic *Escherichia coli* in Kashmir, India. FEMS Microbiology Letters 2006; 261: 238-244.

25-Hegde A, Ballal M, Shenoy S. Detection of diarrheagenic *Escherichia coli* by multiplex PCR. Indian Journal of Medical Microbiology 2012; 30(3): 279–28.