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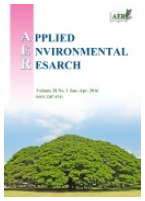

Virulence Factors and Antibiotic Resistance of *Escherichia coli* O157:H7 Isolated from Ogun River, Abeokuta Metropolis, Nigeria

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Abstract

Although *Escherichia coli* (*E. coli*) is a harmless gut microbe in man, some strains of this bacterium are pathogenic due to the acquisition of virulence factors. The aim of this study is to investigate *E. coli* O157:H7 strains isolated from Ogun River, Abeokuta metropolis, for virulence factors and antibiotic resistance. Water samples were collected bimonthly from six different locations over a period of six months. The samples were cultured on Sorbitol MacConkey Agar and *E. coli* O157:H7 isolates were confirmed through serological characterization using the latex agglutination test. The presence of virulence genes (*stx1, stx2, eae,* and *hylA*) in the isolates was analyzed through polymerase chain reaction (PCR). Further, antibiotic susceptibility of the isolates was tested using the disc diffusion method. The PCR analysis revealed that the five *E. coli* O157:H7 strains isolated possessed Shiga toxin 1 (*stx1*), Shiga toxin 2 (*stx2*), and Haemolysin (*hlyA*) genes. Additionally, the isolates were resistant to Augmentin, Ceftriaxone, Nitrofurantoin, Gentamycin, Amoxicillin, and Pefloxacin. This study shows that *E. coli* O157:H7 strains are present in Ogun River and that these strains possess multiple virulence factors and are resistant to multiple drugs.

Keywords: *Escherichia coli*; Virulence factor; Shiga toxin; Antibiotic resistance; Ogun River

Introduction

E. coli is an important member of the Enterobacteriaceae family. It forms a part of the normal flora of the gastrointestinal tract of humans and some animals [1]. This bacterium has an fecal-oral lifestyle and is therefore used as an indicator of environmental fecal contamination in the assessment of food and water quality [2]. Acquisition of virulent factors confers some *E. coli* strains with pathogenic properties.

Based on the production of virulence factors and their toxigenicity, *E. coli* can be grouped into six classes. These are Enterohemorrhagic *E. coli*

(EHEC), Enterotoxigenic *E. coli* (ETEC), Enteropathogenic *E. coli* (EPEC), Enteroinvasive *E. coli* (EIEC), Enteroaggregative *E. coli* (EAggEC), and Diffusely adherent *E. coli* (DAEC) [3].

E. coli O157:H7, also known as Shiga toxin-producing *E. coli* (STEC), is an enterohemorrhagic *E. coli* (EHEC). Unlike other harmless intestinal strains, *E. coli* O157:H7 strains are pathogenic and may cause severe illnesses in humans. These strains are primarily attributable for diarrhea (watery and bloody), hemolytic uremic syndrome, hemorrhagic colitis, and thrombotic thrombocytopenic purpura [4]. They are highly infectious and even only a few cells (less than 10 cells) can cause diseases [5].

The pathogenic feature of *E. coli* O157:H7 is a consequence of the acquisition of multiple virulence factors. Major virulence factors are Shiga toxins (*stx*), which inhibit protein synthesis in eukaryotic cells, and Intimin (*eae*), which is responsible for the intimate attachment of STEC to intestinal epithelial cells [6]. Haemolysin (*hly*) is another virulence factor produced by *E. coli* O157:H7 and is responsible for tissue damage and release of host nutrients [7].

According to Ferens and Hovde [8], the major reservoirs of *E. coli* O157:H7 are ruminant animals, especially cattle and sheep. These *E. coli* strains do not cause diseases in these animals (carriers), and the carriers excrete the bacteria in their feces, which can contaminate soil and water bodies. *E. coli* O157:H7 can survive for long periods in water and are transmitted to humans primarily through the consumption of contaminated food and water [9].

Studies have revealed incidences of antibiotic resistance in *E. coli* O157:H7 isolated from surface water, abattoir effluents [10], and raw beef [11] in Abeokuta. The use of antibiotics is paramount in the treatment of diseases caused by *E. coli*. However, antibiotic resistance among certain strains of this bacterium has resulted in longer and more severe illnesses. Therefore, this study aims to detect virulence factors and antibiotic resistance in *E. coli* O157:H7 isolated from Ogun River in Abeokuta metropolis.

Materials and methods

1) Study area

The study was carried out in Abeokuta metropolis. Abeokuta is the capital city of Ogun State, Nigeria. It is located in the sub-humid tropical region of southwestern Nigeria. Abeokuta has a prevailing tropical climate with mean annual rainfall and temperature of about 1,270 mm and 28 °C, respectively, while its estimated mean annual potential evaporation is 1,100 mm [12].

This city is underlain by a crystalline pre-Cambrian Basement complex of igneous and metamorphic origin, which is noted for its poor groundwater-bearing ability. The city is primarily drained by Ogun River, which passes through Abeokuta, dividing the city into two parts, and has a dendritic drainage pattern. The study region covers a geographical area of 1,256 km^2 and has a population of about 605,461. Major occupations of the indigenous people are farming, production of local textiles (Adire), pottery, and fishing [12].

2) Sample collection

Figure 1 shows the map of the sampling locations within Abeokuta metropolis. Water samples were collected from Ogun River at six different locations from January to June 2017. These are Enugada (Location A), a municipal area with intense human activities (washing clothes, farming, and locust bean production); Alowonle (Location B), downstream of Location A without human settlement but with urban run-off; Opako (Location C), 200 m downstream of Location B with intense dredging activity; Location D, 200 m downstream of Location C

with abattoir effluent discharge into the river; Bridge (Location E), 200 m downstream of Location D with a waste dump site; Obada (Location F) 300 m downstream of Location E with a rocky terrain and fecal contamination during dry seasons (Table 1).

Samples were collected bimonthly between 7:00 am and 11:00 am in sterile 1-L bottles. The bottles were opened 15 cm below the water surface, allowed to fill, closed under water, and then transported to the laboratory in an ice bag for immediate analyses.

3) Isolation and characterization of *E. coli* O157:H7

E. coli O157:H7 strains were isolated using the method described by Ngwa et al. [6] with some modifications. Water samples were serially diluted three-fold and 1 mL of the dilution was inoculated on sterile Sorbitol MacConkey Agar plates. These plates were then incubated at 37 °C for 24 h, after which non-sorbitol-fermenting colonies were selected and sub-cultured.

4) Serological characterization of the isolates

The non-sorbitol-fermenting *E. coli* were serotyped using commercially prepared Salmonella polyvalent O and H antisera for *E. coli* O157:H7. The isolates were emulsified in a drop of 0.85 % normal saline on a clean slide to form a smooth suspension, to which a drop of antiserum was added and mixed gently. The slide was rocked for 2 min and checked for agglutination. Agglutination indicated positive result, while the absence of agglutination indicated negative result [9].

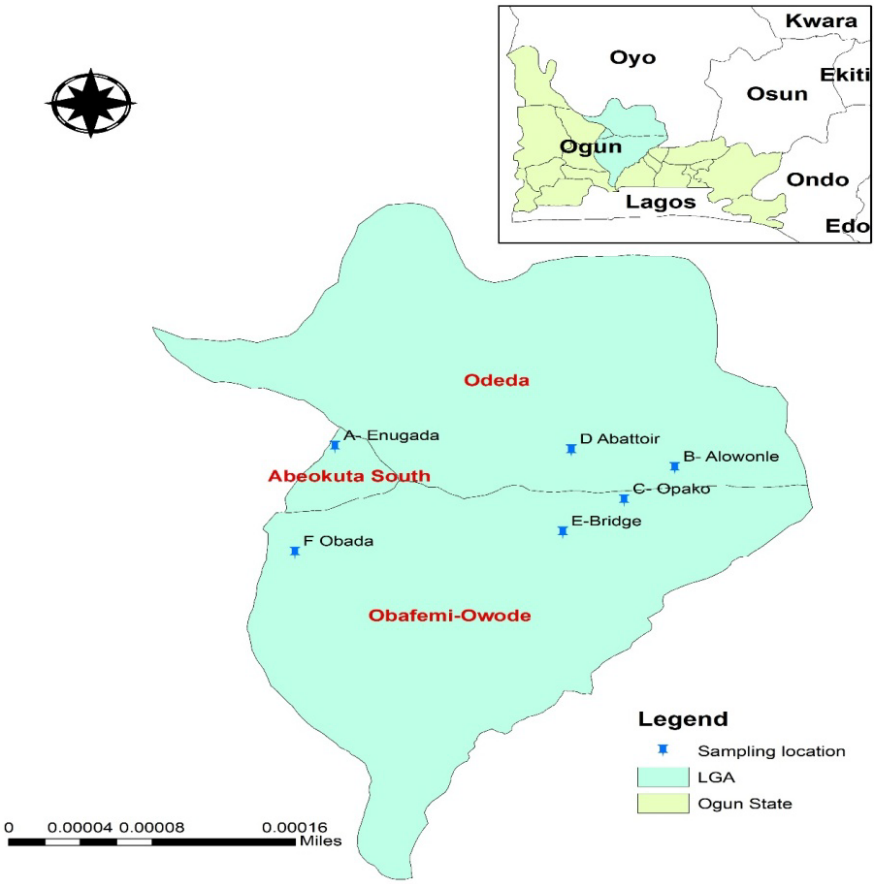

Figure 1 Map of the sampling locations.

Table 1 Description of the sampling locations along Ogun River in Abeokuta metropolis

Sample	Location	GPS location	Area description
A	Enugada	N070°9.196 E003°19.114	Intense human activities
B	Alowonle	N070°6.285 E003°24.916	Urban run-off
C	Opako	N070°6.147 E003°24.752	Dredging
D	Abattoir	N070°5.421 E003°24.581	Abattoir effluent
E	Bridge	N070°4.128 E003°20.793	Dumping Site
F	Obada	N070°3.102 E003°16.164	Dredging

Note: All locations are within Abeokuta metropolis.

5) Antibiotic susceptibility pattern of the isolates

The antibiotic susceptibility test was carried out on Mueller-Hinton Agar using the Kirby-Bauer disc diffusion method. Sterile Mueller-Hinton Agar plates were inoculated with 1 mL standardized cultures of the isolates (0.5 McFarland standard turbidity equivalent of $1.5x10^6$ CFU mL^{-1}). Subsequently, antibiotic-impregnated discs were placed on the surface of the agar plates using sterile forceps. Amoxycilin (25 µg), Ofloxacin (5 µg), Ceftriazone (30 µg), Gentamycin (10 µg), Pefloxacin (5 µg), Cotrimoxazole (25 µg), Ciprofloxacin (10 µg), Augmentin (30 µg), Tetracycline (30 µg), and Nitrofuraton (200 µg) antibiotic discs (Fondiscs laboratory) were used. These plates were incubated at 35 °C for 18 h and zones of inhibition were measured. The results were interpreted with reference to criteria provided by the Clinical and Laboratory Standard Institute (CLSI).

6) Detection of virulence genes in *E. coli* O157:H7

Specific oligonucleotide primers were designed for detecting four virulence genes, *stx1, stx2, eae,* and *hylA*, in the *E. coli* O157:H7 isolates. The sequences, names, and annealing temperatures of the primers used are listed in Table 2.

A 1 mL aliquot of an overnight bacterial broth culture was centrifuged at 14,000 rpm for 30 s and the pellets formed were collected. The cells were re-suspended in 250 µL sodium dodecyl sulfate. Proteinase K (12 µL) was added to the cell suspension, which was then vortexed and incubated at 55 °C for 30 min. Subsequently, 500 µL of a binding solution was added to the lysate and vortexed; 750 µL of this mixture was applied to the spin column assembly and centrifuged for 1 min at 8,000 rpm. The flow-through was discarded, after which a wash solution (500 µL) was added to the column and centrifuged for 1 min at 14,000 rpm. The column was spinned for 2 min to allow it to dry thoroughly, and then the collection tube was discarded. The spin column (with DNA bound to it) was assembled with a 1.7 mL elution tube, and 200 µL of the elution buffer was added to the center of the column bed. The column was then centrifuged for 1 min at 6000 rpm. Further, the elution buffer was centrifuged at 14,000 rpm for another 2 min to collect the total elution volume. The washed and air-dried DNA pellet was dissolved in DNA-grade water and stored at -20 °C until it was used for polymerase chain reaction (PCR) [13].

Table 2 Oligonucleotide primers used for amplification of DNA extracted from *E. coli* O157:H7 isolates

Primer designation	Sequences	Specificity	Annealing temperature
stx1-F	5'- ACA CTG GAT GAT CTC AGT GG-3'	Shiga toxin 1	48.3 °C
stx1- R	5'-CTG AAT CCC CCT CCA TTA TG- 3'		
stx2-F	5'-CCA TGA CAA CGG ACA GCA GT-3	Shiga toxin 2	55.9 °C
stx2-R	5'-CCT GTC AAC TGA GCA CTT TG- 3'		
eaeA- F	5'-GTG GCG AAT ACT GGC GAG ACT-3'	Intimin	53.0 °C
eaeA- R	5'-CCC CAT TCT TTT TCA CCG TCG-3'		
hly A- F	5'-ACG ATG TGG TTT ATT CTG GA-3'	Haemolysin	45.4 °C
hly A- R	5'-CTT CAC GTG ACC ATA CAT AT-3'		

The amplification reaction was performed using 1.5 µL of template DNA (1.0 µg); 5.0 µL of 2x PCR master mix (Norgen Biotek Corporation, Canada), which is composed of Taq DNA polymerase, dNTPs, reaction buffer, $MgCl_2$, KCl, and PCR stabilizers; 1.0 µL of forward primer (2.5 µM); 1.0 µL of reverse primer (2.5 µM); and 1.5 µL of nuclease-free water (total reaction volume of 10.0 µL). Amplification was carried out in a thermo cycler. The operating conditions were initial denaturation at 94 °C for 2 min, followed by 36 amplification cycles of denaturation at 94 °C for 30 s, annealing for 30 s, and extension at 72 °C for 2 min. Reactions were terminated at the final extension step carried out at 72 °C for 5 min. The PCR products were visualized through electrophoresis on 1 % (w/v) agarose gels at 100 V for 1 h, after staining with ethidium bromide in the presence of a 1 kb ladder.

Bi-directional sequences obtained with forward and reverse primers were edited and aligned, to generate a consensus sequence, using BioEdit sequence Alignment Editor (version 7.1.9). These consensus sequences were subsequently aligned with sequences deposited in the National Centre for Biotechnological Information (NCBI) gene bank using the Basic Local Alignment Search Tool (BLAST) to establish the identities of the bacterial isolates.

7) Data analysis

All the data obtained were subjected to Analysis of Variance (ANOVA) using SAS software. Means were separated using the Student Newman Keuls (SNK) method at $p < 0.05$. A dendogram was constructed using GelCompar software (version 6.6.11) to show the genetic relatedness of the five isolates with *E. coli* O157:H7 (NC 002695.1) as the standard.

Results and discussion

1) Isolation and characterization of *E. coli* O157:H7

Five *E. coli* O157:H7 strains were isolated from the 64 samples collected during this study. One was isolated from Location B, which is associated with urban run-off, two were isolated from Location D, which is associated with abattoir effluents, and the remaining two were isolated from Location E, which is associated with waste dumping (Table 3). Figure 2 shows a dendogram that represents the phylogenetic relationships of the isolated *E. coli* O157:H7 strains. This dendogram reveals that all the five isolates were generally identical, with dissimilarity degrees of 56, 100, 66, and 96 respectively compared to the standard.

In this study, samples collected from locations exposed to abattoir effluents and dumping sites accounted for 80 % of the isolated *E. coli*

O157:H7. This is because abattoir effluents are usually discharged into the nearest surface-water bodies, which in turn contaminate the receiving water body. Further, water bodies receive run-off from dumping sites. Different waste materials at dumping sites such as fecal matter from cattle, sheep, and humans contribute significantly to the contamination of the receiving water bodies. This finding agrees with the fact that cattle and sheep are the major reservoirs of *E. coli* O157:H7. In a similar study, Tanaro et al. [14] isolated *E. coli* O157:H7 strains from surface water near cattle feedlots. Adebowale et al. [10] also reported the isolation of *E. coli* O157:H7 strains from abattoir effluents and the receiving surface-water body. Further, Balogun et al. [15] reported the isolation *E. coli* O157:H7 and other coliforms from Ogun River.

2) Antibiotic susceptibility pattern of the *E. coli* O157:H7 isolates

Table 4 shows the antibiotic susceptibility pattern of the *E. coli* O157:H7 isolates. All the isolates were completely resistant to Augmentin, Ceftriaxone, Nitrofurantoin, Gentamycin, Amoxicillin, and Pefloxacin. However, isolates 2 and 4 were sensitive to Ciprofloxacin with zones of inhibition measuring 9.0 mm and 1.0 mm, respectively. Further, isolates 5, 1, and 4 were sensitive to Tetracycline with zones of inhibition measuring 8.0 mm, 6.0 mm, and 3.0 mm, respectively. Ofloxacin resulted in zones of inhibition measuring 5.0 mm, 4.0 mm, and 2.0 mm against isolates 5, 4, and 1, respectively. There were significant differences ($p < 0.05$) between the susceptibilities of the isolated *E. coli* O157: H7 strains to the tested antibiotics.

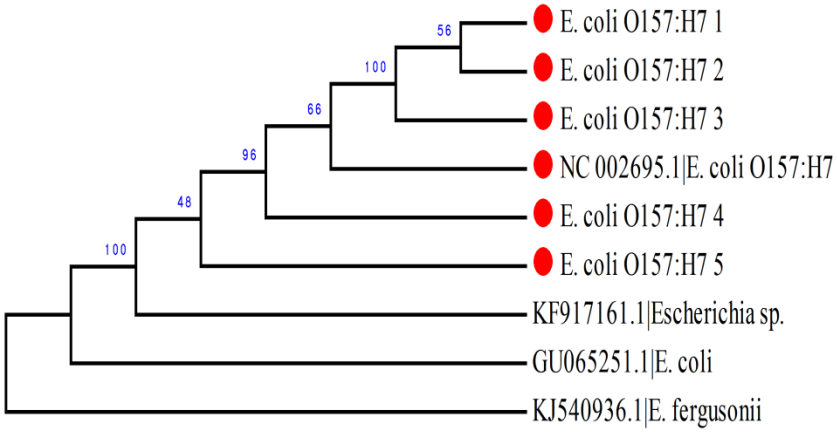

Figure 2 Dendogram of the isolated *E. coli* strains.

Table 3 Number of *E. coli* O157:H7 isolates obtained from each sampling location

Sample	Location	Area description	Number of isolates
A	Enugada	Intense human activities	0
B	Alowonle	Urban run-off	1
C	Opako	Dredging	0
D	Abattoir	Abattoir effluent	2
E	Bridge	Dumping Site	2
F	Obada	Dredging	0
Total			5

The *E. coli* O157:H7 strains isolated in this study were multi-drug resistant as indicated by their resistance to six of the ten antibiotics tested. This result is in agreement with that of Ibrahim et al. [16] who reported that *E. coli* O157:H7 strains were completely resistant to five antibiotics including Amoxicillin, Gentamycin, and Chloramphenicol. Further, in another study, Oloyede et al. [11] reported that *E. coli* O157:H7 strains isolated from raw beef sold in Abeokuta were resistant to Ampicillin, Tetracycline, Kenamycin, Erythromycin, and Chloramphenicol.

3) Detection of virulence genes in the *E. coli* O157:H7 isolates

Table 5 shows the occurrence of different virulence genes in the isolates. All the isolates possessed *stx*1 (Shiga toxin 1), *stx2* (Shiga toxin 2), and *hlyA* (haemolysin) genes, but did not have the *eae* (Intimin) gene.

Rigobelo et al. [17] detected *stx1*, *stx2,* and *eae* genes in *E. coli* O157:H7 isolated from diarrheic calves. In another study, Ateba and Mbewe [4] reported the occurrence of *stx1*, *stx2*, *hlyA,* and *eae* genes in *E. coli* O157:H7 isolated from beef, pork, water, and human and animal feces. Further, Oloyede et al. [11] reported the presence of *stx1* and *stx2* genes in *E. coli* O157: H7 isolated from raw beef sold in Abeokuta, but *eae* and *hlyA* genes were absent.

Table 4 Antibiotic susceptibility pattern of the *E. coli* O157:H7 isolates

	Zone of Inhibition (mm)									
Isolate code	**Augmentin**	**Ceftriaxone**	**Nitrofurantoin**	**Gentamycin**	**Cotrimoxazole**	**Ofloxacin**	**Amoxicillin**	**Ciprofloxacin**	**Tetracycline**	**Pefloxacin**
1	0.0 ±0.0^{a}	0.0 ±0.0^{a}	0.0 ±0.0^{a}	0.0 ±0.0^{a}	0.0 ±0.0^{a}	2.0 ±1.2^{a}	0.0 ±0.0^{a}	0.0 ±0.0^{a}	6.0 ±1.16^{a}	0.0 ±0.0^{a}
2	0.0 ±0.0^{a}	0.0 ±0.0^{a}	1.0 ±0.3^{b}	0.0 ±0.0^{a}	7.0 ±0.4^{a}	0.0 ±0.0^{a}	0.0 ±0.0^{a}	9.0 ±1.3^{bc}	0.0 ±0.0^{a}	3.0 ±0.9^{bc}
3	0.0 ±0.0^{a}	0.0 ±0.0^{a}	0.0 ±0.0^{a}	0.0 ±0.0^{a}	0.0 ±0.0^{a}	0.0 ±0.0^{a}	0.0 ±0.0^{a}	0.0 ±0.0^{a}	0.0 ±0.0^{a}	0.0 ±0.0^{a}
4	0.0 ±0.0^{a}	0.0 ±0.0^{a}	0.0 ±0.0^{a}	0.0 ±0.0^{a}	0.0 ±0.0^{a}	4.0 ±1.6^{ab}	0.0 ±0.0^{a}	1.0 ±0.2^{a}	3.0 ±1.8^{b}	0.0 ±0.0^{a}
5	1.0 ±0.2^{a}	0.0 ±0.0^{a}	0.0 ±0.0^{a}	0.0 ±0.0^{a}	0.0 ±0.0^{a}	5.0 ±1.2^{b}	0.0 ±0.0^{a}	0.0 ±0.0^{a}	8.0 ±3.1^{bc}	0.0 ±0.0^{a}

Note: Values are represented as Mean of triplicate reading ± Standard error of mean
Values with the same subscript on each row are significantly different ($p < 0.05$)

Table 5 Occurrence of virulence genes in the *E. coli* O157:H7 isolates

Isolate	**Source**	**Location**	**Virulence genes**			
			stx1	***stx2***	***hlyA***	***eae***
1	Abattoir effluents	Abattoir	+	+	+	-
2	Dumping site	Bridge	+	+	+	-
3	Abattoir effluents	Abattoir	+	+	+	-
4	Dumping site	Bridge	+	+	+	-
5	Urban run-off	Alowonle	+	+	+	-

Note: + indicates presence and - indicates absence of the particular virulence gene

Conclusion

This study revealed that the discharge of abattoir effluents and dumping of refuse into surface waters can affect the levels of *E. coli* O157:H7 strains in flowing rivers. These bacteria may in turn infect humans who depend on rivers for their domestic water needs. As these pathogenic bacteria harbor virulence genes and are multi-drug resistant, they are significant in terms of public health.

Therefore, it is recommended that environmental regulatory bodies in Nigeria device strategies to enforce the existing laws concerning the discharge of effluents and refuse into flowing rivers. Such efforts will go a long way in achieving a significant reduction in the contamination of surface waters, thereby ensuring their suitability for domestic and industrial use.

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