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SCREENING FOR VIRULENCE GENES IN *ESCHERICHIA COLI* O157:H7 OBTAINED FROM DRINKING WATER FROM IKARA, KADUNA STATE, NIGERIA.

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ABSTRACT

Eighty (80) sources of drinking water comprising boreholes (24), streams (3), wells (29), pipe-borne (5) and 19 sachet water samples were collected between March 2014 and February 2015. *Escherichia coli* (*E.coli*) O157:H7 was isolated by enrichment in Tryptone soy broth at elevated temperature and streaking on Eosin Methylene Blue agar. Typical green colonies with metallic sheen were Gram stained, after which biochemical tests and streaking on cefuxime-tellurite sorbitol MacConkey agar was done, followed by serological tests and by partial sequencing of the 16S rRNA gene were carried out to confirm the identity of the isolates. The isolates were screened for the presence of virulence genes (stx1, stx2, eaeA, hlyA) using the polymerase chain reaction (PCR) technique with specific primers. Three samples (2 wells and 1 stream) were found to be contaminated with *E. coli* O157:H7. None of the isolates possessed the *stx1* gene, one carried the *eaeA* gene (229bp), while all three showed amplicons for the *stx2* and *hlyA* genes (1181 and 534bp respectively). The 16S rRNA sequences were deposited in the National Center for Biotechnology Information GenBank under accession numbers KX602652, KX602653 and KX602654. The isolation of this organism possessing virulence genes from drinking water is of public health significance and therefore, more attention needs to be paid to drinking water of Ikara, Kaduna state.

Keywords: Drinking water, *Escherichia coli* O157:H7, PCR, virulence genes

INTRODUCTION

In Nigeria, as with other developing countries, there is relative scarcity of potable water that can be used for drinking and other domestic purposes (WHO and UNICEF, 2010). The quality of drinking water is of importance to human health. It has been estimated that about 3.4 million people die due to the effects of water-borne diseases, and children form a large percentage of this figure (UNICEF, 2008). Most bacterial pathogens usually get to drinking water through feacal contamination, either through sewage or other sources of contamination.

Escherichia coli (*E. coli*) is a normal flora of the gastrointestinal tract of animals and humans. However, some strains such as the O157:H7 strain possess virulence genes such as the intimin gene (*eaeA*), the shiga toxin genes (*stx1* and *stx2*), haemolysin gene (*hlyA*) among others (Mora *et al.* 2007). This organism has been associated with intestinal disease such as bloody and non-bloody diarrhea, with complications like haemorrhagic colitis and haemolytic uraemic syndrome which is a life-threatening condition (Kaper *et al.* 2004). *E. coli* O157:H7 has been isolated from vegetables, well water, meat, abattoirs, fruits and so on (Franiczek *et al.* 2006; Tijjani *et al.* 2006 and Olukosi *et al.* 2008).

In this report, we examined the prevalence of *Escherichia coli* O157:H7 in drinking water from Ikara, Kaduna state, Nigeria, and screened the isolates for the presence of some virulence genes.

MATERIALS AND METHODS

Study Location

Ikara is the headquarters of Ikara Local Government Area of Kaduna North senatorial district of Kaduna state in the North-Western part of Nigeria. The town is about 75 km North-east of Zaria. The people are predominantly of the Hausa and Fulani ethnic groups and the population of the town is about 194,723 people (NPC, 2006). The sources of drinking water available at the time of sampling in the town

included boreholes and wells mainly, a few areas had pipe-borne water and there were a few streams.

Sample Collection

The samples were collected randomly, based on availability at the time of sampling which was between March 2014 to February 2015.

The borehole, well, stream and pipe borne water samples were aseptically collected in sterile containers while the sachet water samples were purchased from selling points and were all transported to the laboratory of the Department of Microbiology, Ahmadu Bello University, Zaria in ice boxes for analysis within 6 hours of collection.

Isolation of E. coli **O157:H7**

Escherichia coli O157:H7 was isolated from the water samples using the method of LeJejune *et al* (2001). Twenty (20) millilitres of each water sample were inoculated into duplicate flasks containing 20 ml of sterile double strength tryptone soy broth. The tubes were incubated at 44 ◯C for 24 hours. After this time, a loopful of the enrichment culture was streaked on plates of EMB agar and incubated at 44 ◯C for 24 hours. Mixed cultures were re-streaked for purity on plates of sterile EMB agar and then colonies that were observed to be shiny green with dark centres were transferred to nutrient agar slants and Gram stained before storage for further identification.

Biochemical characterization of the isolates

The presumptive *E. coli* isolates were subjected to a number of conventional biochemical tests which include indole, citrate, methyl red, Voges-Proskaeur, motility, Triple sugar iron, and urease tests (Cheesebrough, 2006). The Microgen *Enterobacteriaceae* GN A ID kit was used for further biochemical characterization of the organisms using the Microgen ID computer software, version 1.2.5.26.

The isolates that were confirmed to be *E. coli* on the basis of their Gram reaction and biochemical profiles were streaked on cefuxime-tellurite Sorbitol MacConkey agar and incubated at 37 °C for 18 hours. Those that were sorbitol negative (colourless colonies) were tested with the *E. coli* O157:H7 latex agglutination kit (Microgen, England) for confirmation of *E. coli* O157:H7. Those that showed agglutination with the kit were recorded as positive *E. coli* O157:H7 (Renter *et al.,* 2003).

DNA Extraction

A single colony of pure culture of each isolate selected for the polymerase chain reactions (PCR) were inoculated into 10 ml of Luria-Bertani (LB) broth medium and incubated at 37 °C overnight. The overnight culture was streaked on nutrient agar to obtain pure colonies. The colonies were picked into Eppendorf tubes and the cells were lysed in 400 µl of lysis buffer. Exactly 40 µl of proteinase was added to 200 µl of the isolates and mixed. The tubes were incubated at 65 °C for 10 minutes. After this, 400 µl of phenol chloroform was added and the tubes were vortexed to mix. They were centrifuged at 13,000 rpm for 10 minutes, after which the upper layer was pippetted into freshly labelled tubes. To the new tubes, 400 µl of chloroform was added and the tubes were vortexed and centrifuged again.

To the upper layer, 1 ml of 100% ethanol and 40 µl of 3 M sodium acetate were added and the tubes were stored at -20 °C overnight. After the incubation period, the tubes were centrifuged at -4 °C for 1 hour after which the ethanol was discarded. Then, 400 µl of 70% ethanol was added and the tubes were centrifuged at 13,000 rpm for 10 minutes. The ethanol was then discarded and the DNA was air-dried. Then, 50 µl of DNase-free water was added and the DNA was stored at -20 °C for further use.

Primer design

The oligonucleotide primer sequences used in this study were as used by various researchers as indicated in table 2.1. They were designed by Inqaba Biotech, South Africa, except the 16S rRNA primer which was manufactured by Integrated DNA Technologies, USA. They were all diluted following the manufacturer's instructions to produce working solutions for the PCR (Moyo *et al.*, 2007; Bitrus *et al.*, 2011).

Table 2. 1: Primers used in this study

Primer	Primer sequence (5′-3′)	Size (bp)	Tm(°C)	Reference
hlyA	GCA TCA TCA AGC GTA CGT TCC AAT GAG CCA AGC TGG TTA AGC T	534	61.5	Paton and Paton, (1998)
eaeA	TGA TAA GCT GCA GTC GAA TCC CTG AAC CAG ATC GTA ACG GC	229	61.5	Moyo *et al.,* (2007)
stx1	ACA CTG GAT GAT CTC AGT GG CTG AAT CCC CCT CCA TTA TG	614	60.4	Jalil *et al.,* (2011)
stx2	ATGAAGTGTATATTATTTAAATGGGTAC TCACAGATAAAACACTCTCCAGG	1181	64.0	Ding *et al.,*(2011)
16SrRNA	AGA GTT TGA TCA TGG CTC AG AAG GAG GTC ATC CAA CCG CCA	1500	56.0	

PCR for the detection of virulence markers stx1, stx2, hlyA, and eaeA genes in the E. coli **isolates**

Specific primers sets were used to detect Stx1, Stx2, hlyA and eaeA genes in the *E. coli* O157:H7 isolates in separate PCR reactions. The PCR reaction mixture of 10 µl contained 5 µl of master mix (Promega, USA), 1 µl of nuclease free water, 3 µl of template DNA and 0.5 µl of each primer mix. A tube containing all the above except the DNA template was also included to serve as a negative control.

PCR amplification included the initial denaturation at 94 °C for 5 minutes. This was followed by denaturation at 94 °C for 20 seconds; annealing was done at the respective temperature for each primer (60.4, 61.0, 61.59 and 61.53 °C) for 30 seconds and 36 cycles; and extension at 72 °C for 45 seconds. The final extension was at 72 °C for 5 minutes and then a hold temperature at 4 °C. Five microlitres of the PCR product was electrophoresed in 2% agarose gel (Bioline) containing 5 µl of 10 mg/ml ethidium bromide at 100 V for 45 minutes. A 1kb plus DNA marker was used as molecular size marker. DNA amplifications were examined under ultraviolet (U.V) transilluminator and results documented (Paton and Paton, 1998; Moyo *et al*., 2007; Bitrus *et al*., 2011; Ding *et al.,* 2011; Jalil *et al*., 2011).

Sequencing of the 16SrRNA gene was carried out with a sequencing machine (Applied Biosystems, HITACHI 3130 x 1 Genetic Analyzer). The sequences were analyzed with the Finch TV and BIOEDIT (version 7.2.5.0) software after which the basic local alignment search tool (BLAST) was carried out on the National Centre for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov) to identify the organism.

RESULTS AND DISCUSSION

Table 3.1 : Isolation frequency of E. coli **O157:H7 from water sources of Ikara LGA**

Water source (n)	E. coli **O157:H7 isolated n (%)**
Borehole (24)	0 (0)
Pipe-borne water(5)	0 (0)
Sachet water (19)	0 (0)
Well water (29)	2 (6.9)
Stream (3)	1 (33.3)
Total- 80	**3 (3.75)**

Table 3.2: Sources of E. coli **O157:H7 possessing virulence genes**

Isolate s/no	**Identity**	**Source**	**Virulence genes detected**	**GenBank accession number**
196	*E. coli* O157:H7	Well water	*Stx2, hlyA*	KX602654
198	*E. coli* O157:H7	Well water	*Stx2, hlyA, eaeA*	KX602653
207	*E. coli* O157:H7	Stream water	*Stx2, hlyA*	KX602652

The 3.75% isolation rate of *E. coli* O157:H7 though low, is a very significant finding because of the pathogenicity of this organism (Table 3.1). The WHO states that *E. coli* should not be found at all in drinking water, therefore, we can safely conclude that by this standard, the affected water sources are unfit for drinking. Most of the wells in the study area had previously been observed to lack covers and casings (Olukosi *et al*., 2008), so this could be a reason why the wells could easily get contaminated from run-offs during rainfall. Also, being a semi-rural area, there was the presence of animals such as sheep, goats and cows grazing around. A previous study on well water of Zaria metropolis reported a 2.5% isolation rate of the organism (Olukosi *et al.,* 2008).

The genes that were observed to be present in the isolates are a sign that they are most likely pathogenic (Barkocy-Gallagher *et al*., 2004). The *stx1* gene was not observed in any of the isolates, while the *stx2* gene was observed in all three (Table 3.2). The progression of disease caused by *E.coli* O157:H7 in humans is largely dependent on a number of bacterial virulence factors including s*tx1, stx2,* haemolysin (*hlyA*), and intimin (*eaeA*) genes among others (Wang *et al*., 2008), with stx2 being the most important of them all (Kawano *et al*., 2008). In human, Shiga toxins are the major virulence factors of STEC responsible for haemorrhagic colitis (HC) and haemolytic uremic syndrome (HUS). Within the human disease-associated strains, those producing *stx2* appear to be more commonly responsible for serious complications. Previous studies have shown that *stx1* genotype is one of the important factors of clinical outcome of *E. coli* O157:H7 infection and that pathogenicity for humans was higher in the *stx2* genotype strains (Kargar and Homayoon, 2015). It is also possible that the *stx1* gene was lost during culturing of the isolates (Mazaheri *et al.,* 2005).

The intimin gene (*eaeA*) was observed in just one *E. coli* O157:H7 isolate (Table 3.2). This gene has been shown to be responsible for the organism's intimate attachment to epithelial cells of the intestine, giving rise to attaching and effacing lesions in the intestinal mucosa. However, it has been observed from other studies that many human STEC isolates including those from patients with haemolytic uraemic syndrome and haemorrhagic colitis did not contain *eaeA*. This indicates that this gene might not be essential for human infection (Blanco *et al.,* 2006).

The enterohemolysin (Ehly), also called enterohemorrhagic *E. coli* haemolysin (EHEC-HlyA), encoded by the *hly* gene is another virulence factor of *E. coli*. This gene has been shown to be responsible for the production of enterohemolysin which gives the organism the ability to cause severe disease as it has been observed in isolates that caused hemolytic uraemic

syndrome (Paton and Paton, 1998; Pradel *et al*., 2001; Grauke *et al*., 2002).

CONCLUSION

The occurrence of *E. coli* O157:H7 carrying virulence genes is an indication that the water sources from which they were isolated are unfit for human consumption except with further treatment. More focus has to be given to drinking water in Ikara, Kaduna state of Nigeria so as to prevent health hazards of waterborne diseases. The populace could also be educated on the need to drink water from safe sources, or at least boil water before drinking. The government also needs to provide potable sources of drinking water for the general public.

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