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

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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 faecal 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 LeJeune *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-
Proskauer, 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 pipetted
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*).
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>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence (5'-3')</th>
<th>Size (bp)</th>
<th>Tm(°C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>hlyA</td>
<td>GCA TCA TCA AGC GTA CGT TCC AAT GAG CCA AGC TGG TTA AGC T</td>
<td>534</td>
<td>61.5</td>
<td>Paton and Paton, (1998)</td>
</tr>
<tr>
<td>eaeA</td>
<td>TGA TAA GCT GCA GTC GAA TCC CTG AAC CAG ATC GTA ACG GC</td>
<td>229</td>
<td>61.5</td>
<td>Moyo et al., (2007)</td>
</tr>
<tr>
<td>stx1</td>
<td>ACA CTG GAT GAT CTC AGT GG CTG AAT CCC CCT CCA TTA TG</td>
<td>614</td>
<td>60.4</td>
<td>Jalil et al., (2011)</td>
</tr>
<tr>
<td>stx2</td>
<td>ATGAAGTGTATATTATTTAAAATGGGTAC TCACAGATAAAACACTCTCCAGG</td>
<td>1181</td>
<td>64.0</td>
<td>Ding et al., (2011)</td>
</tr>
<tr>
<td>16SrRNA</td>
<td>AGA GTT TGA TCA TGG CTC AG AAG GAG GTC ATC CAA CGG CCA</td>
<td>1500</td>
<td>56.0</td>
<td></td>
</tr>
</tbody>
</table>
The 3.75% isolation rate of \textit{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 \textit{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 \textit{E. coli} O157:H7 in humans is largely dependent on a number of bacterial virulence factors including stx1, 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 \textit{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 \textit{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 \textit{E. coli} haemolysin (EHEC-HlyA), encoded by the hly gene is another virulence factor of \textit{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 haemolytic uraemic

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

<table>
<thead>
<tr>
<th>Water source (n)</th>
<th>E. coli O157:H7 isolated n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Borehole (24)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Pipe-borne water(5)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Sachet water (19)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Well water (29)</td>
<td>2 (6.9)</td>
</tr>
<tr>
<td>Stream (3)</td>
<td>1 (33.3)</td>
</tr>
<tr>
<td><strong>Total- 80</strong></td>
<td><strong>3 (3.75)</strong></td>
</tr>
</tbody>
</table>

**Table 3.2: Sources of \textit{E. coli} O157:H7 possessing virulence genes**

<table>
<thead>
<tr>
<th>Isolate s/no</th>
<th>Identity</th>
<th>Source</th>
<th>Virulence genes detected</th>
<th>GenBank accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>196</td>
<td>\textit{E. coli} O157:H7</td>
<td>Well water</td>
<td>stx2, hlyA</td>
<td>KX602654</td>
</tr>
<tr>
<td>198</td>
<td>\textit{E. coli} O157:H7</td>
<td>Well water</td>
<td>stx2, hlyA, eaeA</td>
<td>KX602653</td>
</tr>
<tr>
<td>207</td>
<td>\textit{E. coli} O157:H7</td>
<td>Stream water</td>
<td>stx2, hlyA</td>
<td>KX602652</td>
</tr>
</tbody>
</table>
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.

REFERENCES


