Molecular Surveillance of Shiga Toxigenic *Escherichia coli* in selected beef abattoirs in Osun State Nigeria

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Abstract

Shiga toxigenic strains of *E. coli* (STEC) known to be etiological agents for diarrhea were screened for their incidence/ occurrence in selected abattoirs and retail meat sources in Osogbo metropolis of Osun State, Nigeria using a randomized block design. Samples were plated directly on selective and differential media and confirmed serologically using latex agglutination serotyping kit, then, multiplex PCR analysis was used to screen for the presence of specific virulence factors. The results showed a percent occurrence of STEC at the sampled sites ranging from 25.8–46.3%. None of the strains showed any visible agglutination with the O157 latex reagent. Sequence analysis of PCR products was performed on a representative isolate showing the highest combination of virulence genes. This sequence was subsequently submitted to GenBank with accession number MW463885. The sequence showed 100% coverage and 96.46% percentage identity with *Escherichia coli* O113:H21 (GenBank Accession number: CP031892.1) strain from Canada. From evolutionary analyses, the strain from Nigeria, sequenced in this study, is evolutionarily distant when compared with the publicly available sequences from Nigeria. Although no case of *E. coli* O157 was found within the study area, percent occurrence of non-O157 STEC as high as 46.3% at some of the sampled sites is worrisome and requires regulatory interventions in ensuring hygienic practices at the abattoirs within the study area.

1. Introduction

Shiga toxigenic strains of *E coli* (STEC) are widely recognized as major pathogens for public health problems in developing countries and represent the leading etiological agent of diarrhea[1,2]. Serotype O157 has been the most reported STEC in humans and has been found to be responsible for most infections, sporadic cases and outbreaks of bacterial enteritis in humans, globally. On the other hand, there is increasing evidence that non-O157 STEC are acquiring greater importance as they are frequently associated with sporadic outbreak of both mild and severe STEC disease in humans globally[3,4].

Many of the STEC strains are found in the gastrointestinal tracts of domesticated farm animals, hence these form the principal source of human infections. The most noxious *E coli* strains are those that are able to produce putative accessory virulence factors such as intimin (encoded by eaeA) and the plasmid-encoded enterohemolysin, encoded by enterohemorrhagic *E. coli* (EHEC) hlyA. Moreover, *E coli* strains producing Shiga toxin type 2 (Stx2, encoded by stx2) appear to be more commonly responsible for serious complications such as HUS than those producing only Shiga toxin type 1[5]. On the other hand, Stx production is not restricted to serotype O157 strains, as over 100 STEC serotypes have been isolated from humans with diarrheal illness[4].

Contaminated raw meat is one of the main sources of food-borne illnesses. The risk of the transmission of zoonotic infections is also associated with contaminated meat[6]. While meat is usually consumed well-done in Nigeria, thereby limiting infections from meat consumption, contamination of water bodies from abattoir waste constitutes significant environmental and public health hazards[6, 7, 8]. Bacteria from abattoir waste discharged into water columns can subsequently be absorbed to sediments, and when the bottom stream is disturbed, the sediment releases the bacteria back into the water columns presenting long-term health hazards[9]. In Nigeria, numerous abattoirs dispose of their effluents directly into the streams and waterways without any type of treatment and the butchered meat is washed by the same water[10].

The incidence of Shiga toxigenic *E coli* varies by country, where such data is available. Shiga toxigenic *E coli* infections have been reported for most parts of the world, including a number of African countries[4], however, specific incidence data are not always collected or readily available in most sub-saharan African countries, especially in Nigeria.

There are two main types of abattoirs available within the study area, namely, the slaughter slab and the batch systems. Slaughter slabs are the most commonly found in Nigeria. They are usually established and operated by municipal and local authorities. These operate in well-built areas and conform to a good extent with the WHO guidelines for abattoirs. On the other hand, the batch type of slaughter system are those where animals are killed and processed sometimes on bare floor or on corrugated roofing sheets placed on the floor. These are usually located in abandoned buildings or under the shade of trees and open exposed grounds that a butcher might find suitable for the business[11].

The present surveillance work is aimed at screening for the incidence/ occurrence of Shiga Toxigenic *Escherichia coli* in selected abattoirs and retail meat sources in Osogbo metropolis in Osun State of Nigeria, using molecular and serotyping methods.

The work is expected to positively inform on best practices in the local abattoirs, and enhance effective planning, implementation and evaluation of public health practice within the study area.

2. Results

2.1 Percent occurrence of E. coli contamination

Out of a total of 147 pure isolates of *E. coli* obtained in the entire study from the workers' hands, butchering knives, slaughtering tables, floors and effluent water at the different abattoir locations, the highest number of isolated organisms were obtained from the Sekona-1 slaughter slab-type

abattoir followed by Almaleek batch abattoir and Sekona-2 slaughter slab-type abattoir in descending order of percent occurrence with actual values ranging from 46.3%, 27.9% and 25.8% respectively (Table 2). When typical contamination sites, namely, workers' hands, butchering knives, slaughtering tables and floors were compared for degree of contamination for the selected abattoir types, results showed that the Sekona-2 slaughter slab consistently had the lowest contaminants followed by the Almaleek batch type abattoir and Sekona-1 slaughter slab in increasing degree of contamination (Table 3).

Table 2
Percent occurrence of E coli isolates found in the various meat abattoir types

Location	Abattoir type	*Total number of pure isolates
Almaleek	Batch	41 (27.9)**
Sekona – 1	Slaughter slab	68 (46.3)
Sekona-2	Slaughter slab	38 (25.8)
Total		147(100)

*This number represents the total number of distinct isolates obtained from the workers' hands, butchering knives, slaughtering tables and slaughtering floors. **Percentage of the total number of isolates observed for all sample sites shown in parentheses.

Table 3
Total colony counts (TCC of *E coli* colonies expressed in log10cfu/ml) sampled from workers' hands, butchering knives, slaughtering tables, floors and effluent water at selected abattoirs.

Sample Location	Abattoir Type	Workers' Hands	Butchering Knives	Slaughter Tables	Slaughtering Floors	Effluent Water	Total
Almaleek	Batch	15(10)	2(1.5)	17(11)	4(2.7)	8(5.5)	46(31)
Sekona-1	Slaughter Slab	11(8)	2(1.5)	13(9)	25(17)	12(8)	63(43)
Sekona-2	Slaughter Slab	12(8)	3(2)	3(2)	12(8.1)	8(5.5)	38(26)
Total		38(26)	7(5)	33(22)	41(28)	28(19)	147(100)

**Percentage of the TCC values are shown in parentheses.

2.2 Banding patterns from the multiplex assays of pcr products

Figures 1a and b show the banding patterns of pcr products of representative samples from the multiplex assay for the presence of *stx1*, *stx2*, *eae* and *hly* genes when these were run on 2% agarose gel. The banding pattern was varied, indicating that some of the isolates possess up to four of the virulence genes, while some others did not show any evidence of possessing any of these genes. Conversely, none of the samples showed any bands for the multiplex assay for the presence of *O157* and *O111* genes (Figs. 3a and b- supplementary file). These results were confirmed through serotyping as none of the strains showed any visible agglutination with the O157 latex reagent. On the other hand, they caused a visible agglutination with the seroscreen latex reagent for detecting the 6 common non-O157STEC and were therefore identified as belonging to any of the non-O157 serotypes.

Furthermore, the results of the banding pattern revealed that all the sampled locations exhibited the presence of *E coli* strains possessing a combination of the *stx1*, *stx2*, *eae* and *hly* genes (Table 4). However, the only isolate with 4 out of the total of 6 genes considered was Se1-5-W5 (Table 4).

This isolate which was obtained from the effluent water from the Sekona abattoir was sequenced and the phylogenetic analysis of the sequencing result is presented in Fig.
2.

Table 4

Grouping of *E coli* isolates based on the banding pattern of pcr products on 2% gel electrophoresis. The expected band sizes were stx 1–180; stx 2- 255; eae- 384; hlyA- 534; O157–259 and O111–406 bps. Where genes were indicated based on banding pattern, a positive mark (+) was used, where absent, negative (-) mark was inserted.

S/N	Isolate ID	Source	Stx-1	Stx-2	eae	hlyA	O157	O111
1	Alh-A3-3	Almaleek- Hands	-	-	-	-	-	-
2	Alh-B3-5	„	+	+	+	-	-	-
3	Alh-A3-5	„	+	+	+	-	-	-
4	Alh-C3-1	„	+	+	+	-	-	-
5	Alh-B5-4	„	+	+	+	-	-	-
6	Alh-A3-4	„	+	+	+	-	-	-
7	Se2h-C1-1	Sekona- Hands	+	-	-	-	-	-
8	Se1h-A5-1	„	-	-	+	-	-	-
9	Se1h-C3-4	„	-	+	+	-	-	-
10	Se1h-A1-4	„	-	+	+	-	-	-
11	Se1h-A1-4	„	+	-	+	-	-	-
12	Se1h-B3-5	„	+	-	-	-	-	-
13	Se1FA3-4	Sekona- Floor	-	+	+	-	-	-
14	Se2FC1-2	„	+	-	+	+	-	-
15	Se2FC1-5	„	-	-	+	-	-	-
16	Se1F3-1	„	+	-	+	-	-	-
17	Se2FA3-3	„	-	-	-	-	-	-
18	Se1EC1-3	„	-	-	-	-	-	-
19	Se1FA3-1	„	-	-	-	-	-	-
20	Se2FC5-3	„	-	-	-	-	-	-
21	Se1FC1-3	„	-	-	-	-	-	-
22	AlFA-3	Almaleek- Floor	-	-	-	-	-	-
23	AlfC5-3	„	-	+	+	-	-	-
24	AlFC3-4	„	-	+	+	-	-	-
25	AlF-3-2	„	+	+	+	-	-	-
26	Al-3-W3	Almaleek- Water	+	+	+	-	-	-
27	Al-3-W4	„	-	+	+	-	-	-
28	Al-1-W2	„	-	-	+	-	-	-
29	Se2-3-W3	Sekona- Water	-	-	-	-	-	-
30	Se2-5-W1	„	-	-	-	-	-	-
31	Se2-3W1	„	+	+	+	-	-	-
32	Se1-5-W5	„	+	+	+	+	-	-
33	ALTB-1	Almaleek- Table	-	+	+	-	-	-
34	AlTC-5	„	-	+	+	-	-	-
35	AlTA-5	„	+	-	-	-	-	-
36	AlTC-1	„	-	-	-	-	-	-

S/N	Isolate ID	Source	Stx-1	Stx-2	eae	hlyA	O157	O111
37	ALTB-5	„	-	-	-	-	-	-
38	Se2TC-3-4	Sekona- Table	-	+	+	-	-	-
39	Se1TC-3-3	„	-	+	+	-	-	-
40	Se2-TB-1-4	„	-	-	-	-	-	-
41	Se1TB-3-1	„	-	-	-	-	-	-
42	Se1TA-3-2	„	-	-	-	-	-	-
43	Alk-B3-1	Almaleek- Knife	-	-	-	-	-	-
44	AlkB3-2	„	-	-	-	-	-	-
45	Se2K-C3-2	Sekona- Knife	-	-	+	-	-	-
46	SeikA-3-2	„	-	-	+	-	-	-
47	Se2K-BC-2	„	-	-	-	-	-	-
48	SeiKB5-5	„	-	-	-	-	-	-
49	Se2K-A3-3	„	-	-	-	-	-	-
50	Al-1-W1	Almaleek-Water	-	-	-	-	-	-

2.3 Sequencing and BLAST analysis of representative sample

Following BLAST analysis, the sequence showed 100% coverage and 96.46% percentage identity with *Escherichia coli* O113:H21 (GenBank Accession number: CP031892.1) strain from Canada. From evolutionary analyses, the strain from Nigeria, sequenced in this study, is evolutionarily distant when compared with the publicly available sequences from Nigeria (Fig. 2). The isolated 16S rRNA sequence was subsequently submitted to GenBank and registered with accession number MW463885.

3. Discussion

The present study investigated the prevalence of Shiga-toxigenic *E. coli* from selected beef abattoirs in Osun State. The results revealed a higher prevalence of these in batch-type abattoirs where butchering of meat is done on concrete floor under unhygienic conditions than in the slaughter slabs (Tables 2). Moreover, when typical contamination sites, namely, workers' hands, butchering knives, slaughtering tables and floors were compared for degree of contamination, the batch-type abattoirs still showed the highest degree of contamination than the slaughter slabs (Table 3). These results agree with previous reports showing that cattle are the primary source of Shiga-toxigenic strains of *E. coli*[15, 16, 17]. The present results are consistent with reports from similar studies where prevalence rates of up to 67% of STEC have been reported in cow hides, a human delicacy in the study area[18]. Similarly, several authors have reported high prevalence rates of STEC and non-STEC E. coli in meat sampled from Nigerian abattoirs such as Ojo et al, (2010) and Kabiru et al, (2015)[19,20].

Moreover, results from the present study showing that none of the isolates tested positive for the presence of O157 and O111 genes (Figs. 3a and b- supplementary file; Table 4) is not surprising since even in studies where these have been reported in south-western Nigeria, the incidence rates has been found to be very low. For example, in nearby Ado-Ekiti, [21] a prevalence rate of 4.1% was reported in healthy cattle which is considerably lower than 14% non-O157 STEC strains recovered from cattle feces in Alberta, Canada[22]. The present study adds to the body of evidence that ruminants, particularly cattle represent the most important reservoir and source of human infection as a result of exposure to animal manure by contamination of food and water.

In addition, the present study shows the absence of O157 STEC strains within the study area (Table 4), this is in contrast to reports from Lagos (which falls within the south-western geographical zone of Nigeria) where the isolation of *E. coli* O157:H7 from some food animals including goats have been reported [23]. On the other hand, the presence of non-O157 STEC strains at most of the study sites sampled in this study remains worrisome and a source for concern from the stance of public health.

The results of the BLAST analysis of the sequencing of a representative *E. coli* strain from this study showing 100% coverage and 96.46% percentage identity with *Escherichia coli* O113:H21 (GenBank Accession number: CP031892.1) strain from Canada is unusual but not far-fetched. It is well known that behavioral and demographic factors affect human-livestock bacterial transmission rates when it comes to *E. coli* transmission. For example, the results of a study investigating the transmission of *E. coli* between livestock and humans in rural Uganda showed that transmission rates between humans in 2 study locations tended to share genetically similar *E. coli* strains and the study provides evidence

that close contact between people and livestock can lead to high rates of *E. coli* transmission between species[24]. The high similarity of the representative strain sequenced in the present study with strains from Canada perhaps may be a case of human to animal transmission when human travelers carrying *E. coli* from Canada discharge through sewage, grazing cattle may come in contact with this through untreated sewage, however, this should be further investigated and may form the focus of further studies.

Results from the present study records no case of *E. coli* O157 within the study area, however, percent occurrence of non-O157 STEC was as high as 46.3% at some of the sampled sites (Tables 2, 3 and 4). Up until now, zoonotic E. coli transmission was assumed to be more problematic in developed nations with highly industrialized agricultural systems, however, our results show that this may not be the case and the lack of reporting of high numbers of occurrence of *E.coli* transmissions may be due to lack of a surveillance system. Our study underscores the importance of developing a Laboratory-based Enteric Disease Surveillance (LEDS) system in Nigeria to stem the spread of noxious enteric diseases.

4. Materials And Methods

4.1 Sample collection

Samples were obtained from 3 different sites of abattoirs within 50 km radius of Oshogbo, the capital city of Osun state Nigeria. Oshogbo is located at coordinates: 7°46'N 4°34'E and has a total area size of 47 km^2 (18 sq. miles). Sterile swab samples were obtained from slaughtering floors, slaughtering tables, butchering knives and worker's hands. Composite effluent water samples were collected using the grab method.

All samples were transported immediately to the laboratory for analyses. The samples were screened for the presence of members of the family *Enterobacterioacae*, specifically *E coli*, and these were targeted for use as indicators of microbial contamination.

4.2 Consent

The purpose of the study was explained to the abattoir workers at each location with translation provided in the local language (Yoruba) and informed consent was obtained before taking sterile swabs of the workers' hands and knives. Each participant's right to refuse taking of swabs from their knives or hands was respected at all times.

This research was conducted according to the approved guidelines set by the Redeemer's University Research Ethics Committee, Redeemer's University, Ede, Osun State, Nigeria.
This research was approved by the Redeemer's University Research Ethics Committee, Redeemer's University, Ede, Osun State, Nigeria.

4.3 Identification and serotyping of E coli isolates

The inocula were plated directly on selective and differential media, namely, MacConkey (MAC) and Eosin Methylene Blue (EMB) agar. Furthermore, gas production and indole tests when isolates were grown at 44°C were used in order to confirm the isolates as *E coli*. Subsequently, the confirmed *E coli* isolates were stocked at – 4°C and until used for downstream analyses such as genomic DNA extraction. The strains of E. coli O157 were identified culturally on the basis of inability to ferment sorbitol on SMAC agar and confirmed serologically as O157 by latex agglutination serotyping kit (Dryspot E. coli O157 latex test) for E. coli O157 (Oxoid, Basingstoke, UK) and (Dryspot E. Coli serocheck and seroscreen latex test) for the detection of six non-O157 serotypes O26, O91, O103, O111, O128 and O145.

4.4 DNA Extraction, PCR Amplification and Fragment Purification

Genomic DNA was extracted from 50 randomly selected isolates from the entire study, approximately 200 mg of the bacteria were resuspended in 200 µL of PBS then, Quick DNA™Fungal/Bacterial Miniprep kit was used in extracting the DNA by following manufacturer's instructions. The multiplexed PCR reactions were sectioned into 2 assays, namely assay 1 (comprising 4 sets of primers) and assay 2 (comprising 2 sets of primers), see Table 1 for more details. An aliquot of 5 µl of the extracted DNA was used as a template in a 25 µl PCR reaction mixture containing illustra™ PuReTaq™ Ready-To-Go™ PCR Beads, 1µl each of forward and reverse for each of the primers, including 1.5 mM $MgCl_2$ and 16.5µl of double distilled water. Samples were subjected to 35 PCR cycles, each consisting of 1 min of denaturation at 95°C; 2 min of annealing at 65°C for the first 10 cycles, decrementing to 60°C by cycle 15; and 1.5 min of elongation at 72°C, incrementing to 2.5 min from cycles 25 to 35. PCR reaction mixtures were electrophoresed on 2% agarose gels and stained with ethidium bromide[12].

Table 1
The PCR Primers

Primer	Sequence (5'–3')	Specificity[1]	Amplicon size (bp)
Assay 1			
stx1F	ATAAATCGCCATTCGTTGACTAC	nt 454–633 of A subunit coding region of *stx*	180
stx1R	AGAACGCCCACTGAGATCATC		
stx2F	GGCACTGTCTGAAACTGCTCC	nt 603–857 of A subunit coding region of *stx*2 (including *stx*2 variants)	255
stx2R	TCGCCAGTTATCTGACATTCTG		
eaeAF	GACCCGGCACAAGCATAAGC	nt 27–410 of *eaeA* (this region is conserved between EPEC and STEC)	384
eaeAR	CCACCTGCAGCAACAAGAGG		
hlyAF	GCATCATCAAGCGTACGTTCC	nt 70–603 of EHEC *hlyA*	534
hlyAR	AATGAGCCAAGCTGGTTAAGCT		
Assay 2			
O157F	CGGACATCCATGTGATATGG	nt 393–651 of *rfbE*O157:H7	259
O157R	TTGCCTATGTACAGCTAATCC		
O111F	TAGAGAAATTATCAAGTTAGTTCC	nt 24–429 of ORF 3.4 of *E. coli* O111 *rfb* region	406
O111R	ATAGTTATGAACATCTTGTTTAGC		

[1] nt, nucleotide; ORF, open reading frame[12].

4.5 Analysis of E coli cultures by multiplex PCR and sequencing of representative isolates

Cultures from the *E coli* isolates obtained from the sample locations were analyzed by PCR as described earlier, but since neither the control organism nor the sample isolates showed bands for assay 2, that is, the assay for the O157 and O111 genes, Sanger sequencing was employed to verify the veracity of the results from the multiplex assays. To achieve this, isolates with similar banding patterns for the multiplex assays were grouped together and Sanger sequencing was applied to the representative organism that displayed the highest number and diversity of virulence genes.

Raw reads from Sanger sequencing were trimmed using BioEdit Sequence Alignment Editor (version 7.2.6) (https://bioedit.software.informer.com) with manual base calling where necessary. The resulting consensus sequence was then subjected to BLAST (Basic Local Alignment Search Tool) analysis for the identification of the organism. Alignment of the sequence, with other publicly available sequences from GenBank, was carried out using the ClustalW algorithm in Geneious Prime (version 2020.2.4). Evolutionary analyses were conducted in MEGA X [13].

The evolutionary history was inferred by using the Maximum Likelihood method and Hasegawa-Kishino-Yano model[14]. The tree with the highest log likelihood (-3908.74) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. This analysis involved 25 nucleotide sequences. Evolutionary analyses were conducted in MEGA X [13].

Declarations

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Author contributions

F.A., J.O., O.F., C.H and P.E. conceived the experiments. F.A, J.O, T.O., Tes.O. and O.O conducted the experiments. F.A, J.O, T.O., Tes.O. and O.F. analyzed the samples. F.A, J.O, T.O., Tes.O. and O.O analyzed the results and prepared the figures. F.A., and Tes.O. wrote the manuscript. All authors reviewed and accepted the final version of the manuscript.

CONFLICT OF INTEREST

None declared.

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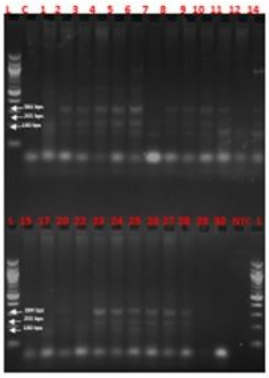
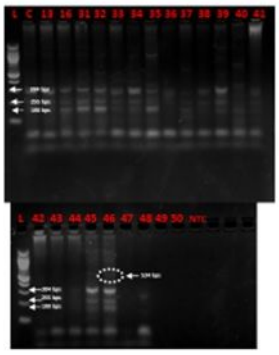
Figures



Characterization of *E coli* strains by multiplex PCR assay strains were classified based on the visible banding patterns expected mobilities for the various specific PCR products (see Table 1). The grouping pattern based on the bands shown by each *E coli* sample is shown in Table 4.



Characterization of *E coli* strains by multiplex PCR assay strains were classified based on the visible banding patterns expected mobilities for the various specific PCR products (see Table 1). The lone sample with faint band showing at 534 bps was selected for sequencing, see sequencing result on Fig. 3. The grouping pattern based on the bands shown by each *E coli* sample is shown in Table 4.

Figure 1

Banding patterns of pcr products

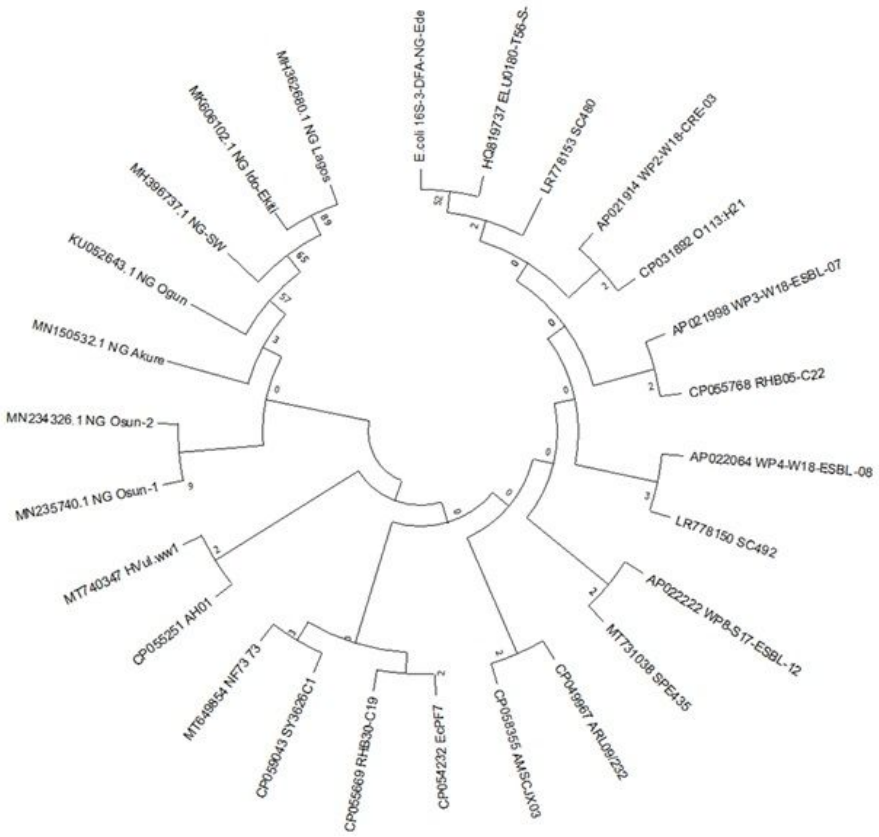


Figure 2

Phylogenetic analysis of E. coli sequence from Beef abattoir compared with publicly available sequences.

Supplementary Files

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