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Molecular surveillance of non-O157 Shiga toxigenic *Escherichia coli* in selected chicken abattoirs and retail outlets in Osogbo, Osun State, Nigeria

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ABSTRACT

Three selected chicken abattoirs and two retail locations were studied to determine the frequency of occurrence and profile for Shiga toxin-producing *Escherichia coli* (STEC) strain present in abattoirs and retail (frozen) chicken carcasses in Osun state, Nigeria. Samples were plated on Eosin Methylene Blue agar for the presence of *E. coli*. Furthermore, the isolates were confirmed serologically as non-O157 STEC using a latex agglutination serotyping kit. Multiplex PCR was used to check for specific virulence factors in the isolated *E. coli* strains. The mean colony count results showed that effluent water samples from the Ikirun slaughter slab type abattoir were the highest at 25 cfu/ml. A post hoc comparison showed that this value was significantly higher than that of the slaughtering table at Oluode-1 ($P = 0.04$) and retail chicken meat samples at Igbona ($P = 0.01$). The results show that chicken abattoirs are poor reservoirs of STEC. Moreover, the results from this study showing that the stx_2-producing strains that are more prone to cause hemolytic uremic syndrome are the predominant strain in the study area are worrisome. These results underscore the improper hygiene practices of the abattoir workers combined with inadequate waste management and biological waste disposal systems. It is recommended that regulatory bodies in this locality should focus on ensuring the upgrade of biological waste disposal from these abattoirs in order to limit the spread of potentially virulent pathogens into the runoff and groundwater.

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1. Introduction

Infection of humans by Shiga toxin-producing *Escherichia coli* (STEC) results in various clinical manifestations such as diarrhea, hemorrhagic colitis and (occasionally fatal) hemolytic uremic syndrome (HUS) (1). This bacterial zoonotic agent is a pathogen of a public health concern due to its potential to cause large food- and waterborne outbreaks, as well as its association with the hemorrhagic uremic syndrome (HUS) (2, 3), a leading cause of acute renal failure among children (4).

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Although, most STEC strains associated with human illness belong to serogroup O157, there are more than a hundred of non-O157 serotypes (5), some of which have been associated with large outbreaks of severe illness.

STEC has various virulence factors that are important in pathogenicity. Shiga toxin is the major factor in virulence and there are two toxin forms, stx_1, and stx_2 encoded by stx_1 and stx_2 genes (6). There are three subtypes of the stx1 gene, namely, stx_{1a}, stx_{1c} and stx_{1d}. On the other hand, the stx2 group is divided into seven subtypes, namely, stx_{2a}, stx_{2b}, stx_{2c}, stx_{2d}, stx_{2e}, stx_{2f} and stx_{2g}. The stx2 subtype is associated with more severe HUS syndrome (7).

Shiga toxins bind to the surface of eukaryotic cells, inhibit protein synthesis, thereby causing the death of their hosts (8). A virulence factor coded by the *eae* gene is intimin. Intimin is reported to facilitate bacteria's attachment to intestinal epithelia during colonization leading to lesions and diarrhea (9, 10). Enteropathogenic *E. coli* (EPEC) also possess the intimin virulence factor (11). Enterohaemolysin is another Shiga toxin associated with *E. coli*, this protein toxin damages erythrocyte cell membranes and is used in the detection of Shiga toxin *E. coli* as a surrogate tool (12, 13). While enterohaemolysin activity can be easily visualized in blood agar cultures, PCR amplification of the ehxA gene usually results in confirmation (14, 15). Other *E. coli* strains including O26, O103, O111, O118, O128, O121, O45 and O145 also have the potential to produce disease syndromes and are reported to be enterohaemolysin-positive Shiga toxins producers (16).

Animals are a major potential source of human STEC infection due to their ability to maintain STEC carriage even in the absence of continuous exposure to STEC (i.e. reservoirs or amplifying hosts), including farm and abattoir (slaughter) animals that are frequently exposed to STEC from the environment (17).

Although ruminants, and particularly cattle, are regarded as the main reservoir for STEC (18, 19), there is evidence for non-ruminants, particularly poultry being significant spill-over hosts for STEC. These are animals that are susceptible to colonization by STEC but do not maintain such colonization in the absence of continuous exposure (17, 20- 22).

The role of meat products as vehicles of STEC have been widely reported (23, 24), moreover, STEC strains isolated from animal and food were identified carrying resistance genes against multiple antimicrobial classes, such as aminoglycosides, tetracycline and b-lactams (25). Thus, more information about the prevalence and spread of STEC among animals and food is needed.

The present study aims to investigate the spatial prevalence and virulence characteristics of STEC present in abattoirs, fresh and retail (frozen) chicken carcasses in Osogbo, Osun State, Nigeria. Reports from a recent study (26), showed the presence of non-O157 STEC strains in selected beef abattoirs at most of the study sites sampled within the Osogbo metropolis. In Southwestern Nigeria, poultry and bovine abattoirs are typically spatially separated, the present study provides some basis for comparing the prevalence and virulence characteristics of *E. coli* strains isolated from beef and chicken abattoirs.

This is with a view to providing baseline information necessary to develop best practices needed to limit the spread of STEC and to improve on practices in the local abattoirs, thereby improving public health practice within the study area.

2.Materials and Methods

2.1. Collection of samples

Three chicken abattoirs were targeted for sample collection within 50 km radius of Osogbo, the capital city of Osun state Nigeria, located at 746'N 434'E with a total area size of 47 km^2 (18 sq. miles). Specifically, samples were taken from the 3 busiest batch-type chicken abattoir locations. Two chicken abattoirs in Oluode, hereafter referred to as Oluode-1 and Oluode-2 and another in Ikirun, Osun State, Nigeria were sampled. In addition, 2 retail locations at Akoda-Ede and Igbona markets, Osogbo where processed imported frozen chicken are sold were also sampled. Sterile swab samples were obtained from slaughtering floors, slaughtering tables, butchering knives and worker's hands. The grab method was used in collecting effluent water samples. The isolation of bacterial isolates from the sterile swabs (3cm long and 1cm diameter) moistened with 0.1% peptone water was carried out using the method of Ayoade et al, (26).

2.2. Consent

Approval of the Redeemer's University Research Ethics Committee, Redeemer's University, Ede, Osun State, Nigeria was sought and obtained before the commencement of the research, since consent is required before collection of samples from human subjects.

The research focus was explained to the abattoir workers in the local language (Yoruba) before samples (sterile swabs) were taken from their hands and knives. The right of refusal of each participant to refuse to take swabs from their knives or hands was respected at all times.

2.3. Identification and serotyping of *E. coli* isolates

All analyses commenced immediately after samples were transported to the laboratory and screened for the presence of *E. coli*. The samples were plated directly on selective and differential media, namely, MacConkey (MAC) and Eosin Methylene Blue (EMB) agar. In addition, the isolates were confirmed by growing them at 44°C combined with gas production and indole tests. These isolates were then kept at – 4°C and until needed for genomic DNA extraction and PCR reactions. Moreover, to identify strains of *E. coli* O157, the isolates were tested for their inability to ferment sorbitol on Sorbitol-MacConkey agar. This was followed by a confirmation test by serology using latex agglutination serotyping kit 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.

2.4. DNA Extraction, PCR Amplification and Fragment Purification

In order to extract genomic DNA from 50 randomly selected isolates from a total of 165 distinct isolates obtained in the entire study, approximately 200 mg of the bacteria were re-suspended 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 used in

this study were divided into 2 assays; the first assay comprising 4 sets of primers and the second assay comprising 2 sets of primers, more details about these reactions are noted in Table 1. Five µl aliquot of DNA template including 1 µl each of forward and reverse primers, with 1.5 mM $MgCl_2$ and 16.5 µl of double distilled water was added to the reaction mixture containing illustra™ PuReTaq™ Ready-To-Go™ PCR Beads to make a total of 25 µl PCR reaction mixture. The amplification cycling conditions were 1 min of denaturation at 95°C followed by 2 min of annealing at 65°C for the first 10 cycles, decreased to 60°C by cycle 15; and 1.5 min of elongation at 72°C, increased to 2.5 min from cycles 25 to 35. Electrophoresis of PCR mixtures was carried out on 2% agarose gels and stained with ethidium bromide (30).

2.5. Analysis of *E. coli* cultures by multiplex PCR and sequencing of representative isolates

Multiplex PCR was used in analyzing the cultures of *E. coli* isolates from the sample locations as described earlier, however, since neither the control organism nor the sample isolates showed bands for the assay for the O157 and O111 genes, Sanger sequencing was employed to verify 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 organisms with the highest combination of STEC genes.

Trimming of raw reads from Sanger sequencing with manual base calling where necessary was done using BioEdit Sequence Alignment Editor (version 7.2.6) https://bioedit.software.informer.com). Subsequently, the consensus sequence was then subjected to Basic Local Alignment Search Tool (BLAST) analysis for the purpose of identifying the organism. The sequence was then aligned with other publicly available sequences from GenBank, using the ClustalW algorithm in MEGA X. Evolutionary analyses were conducted in MEGA X (31).

2.6. Statistical analysis

Data were analyzed using version 7 of Epi-Info software (27) and GraphPad Prism version 8 (28). GraphPad Prism version 8 and Microsoft Excel version 16 (29) were used in plotting graphs. χ^2 values were calculated using Yates' correction, Fisher's exact, or Mantel Haenszel tests. Using the ANOVA (Analysis of variance) test, normally distributed, continuous variables *were compared. Tukey Honestly Significant Difference Test (Tukey HSD) was used in making post-hoc* comparisons of multiple parameters, where necessary, with P values of <0.05 taken to indicate significant differences.

3. Results

3.1. Frequency of occurrence of E. coli contamination

A total of 165 pure *E. coli* isolates were obtained from the workers' hands, butchering knives, slaughtering tables, floors, effluent water at the different abattoir locations, and retail chicken meat from sales outlets.

Although hand washing ports for hands cleansing were observed in all the three abattoirs included in this study, many of the abattoir and retail chicken meat outlet workers preferred to wipe their hands with handkerchiefs instead of using the provided handwashing locations. Overall, effluent water samples from Ikirun slaughter slab type abattoir was the highest where the mean colony forming units (cfu)/ml was 25 cfu/ml, followed by slaughtering tables at the same abattoir (16 cfu/cm^2), workers' hands at Oluode-2 batch abattoir (15 cfu/cm^2), slaughtering table at Oluode-1 batch abattoir (10 cfu/cm^2) and chicken meat samples from the Igbona market retail store (15 cfu/cm^2) in descending order from the highest to the lowest (Fig. 1). In a subsequent comparison, the average cfu in samples from the water samples at Ikirun abattoir was significantly higher than that of the slaughtering table at Oluode-1 ($P = 0.04$) and retail chicken meat samples at Igbona ($P = 0.01$).

3.2. Frequency of occurrence of STEC genes

The pure *E. coli* isolates (165) were screened for the presence of six STEC genes. Two of the six genes (O157 and O111) did not amplify in any of the samples obtained from all three study sites (Figure. 2A, B and C). Serotyping assay confirmed these results as none of the strains showed any visible agglutination with the O157 latex reagent. However, visible agglutination was observed as the seroscreen latex reagent detected the 6 common non-O157 STEC.

None of the targeted STEC genes amplified in samples obtained from Oluode-2 slaughtering tables but four of the targeted genes, namely, stx_1, stx_2, *aea* and *hly*A amplified in samples from Oluode-1 and from Ikirun (Fig. 2A and B). Although, five distinct isolates of *E coli* were isolated from retail chicken meat samples from Igbona chicken retail market, two from the cutting tables and three from the chicken meat samples, none of these isolates tested positive for the presence of STEC genes (Fig. 2C). A representative gel picture result is presented (Fig. 3). No *E. coli* isolates were found in samples from Akoda chicken retail market. Moreover, a comparison of pooled data according to sample sources revealed that the frequency of occurrence of distinct *E. coli* isolates was highest at the Ikirun slaughter slab type abattoir (~30 isolates) followed by Oluode-1 (~15 isolates). No distinct colonies of *E. coli* were found in the Oluode-2 batch abattoir (not displayed in Fig. 2), whereas only five distinct colonies of E coli were isolated from samples from Igbona (three from chicken meat samples and two from cutting tables), none of these isolates showed the presence of the targeted STEC genes. In addition, 4 out of the 6 targeted genes were amplified in one isolate (Ik-1A-5e) from workers' hands samples taken from the Ikirun abattoir. This particular isolate was singled out and set aside for molecular identification.

3.3. Sequencing and BLAST analysis of a sample

One of the three isolates from which the maximum number of non-O157 STEC genes were amplified was the isolate with identification number Ik-1A-5e. This isolate was randomly selected for identification. This isolate which was obtained from the abattoir workers' hands from the Ikirun abattoir was sequenced, essentially for identification by targeting the 16S gene and compared with publicly available sequences available on NCBI for phylogenetic analysis (Fig. 4).

Subsequent to BLAST analysis, showed 100% coverage and 97.1% percentage identity with *Escherichia coli* O111 (GenBank Accession number: AP010960) strain from Japan. The Maximum likelihood method and Hasegawa-Kishino-Yano model were used in inferring evolutionary history (32). The tree with the highest log likelihood (-2538.96) is shown (Fig. 4). 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 a superior log-likelihood value. The evolutionary relationship between the *E. coli* 16S nucleotide sequence was closely related to other sequences derived from south-western Nigeria including that obtained from beef abattoirs sampled within the same study area from a recently reported study (with accession number MW463885) and a few other publicly available sequences (Fig. 4). Subsequently, the isolated 16S rRNA sequence was submitted to GenBank and registered with accession number MW463886.

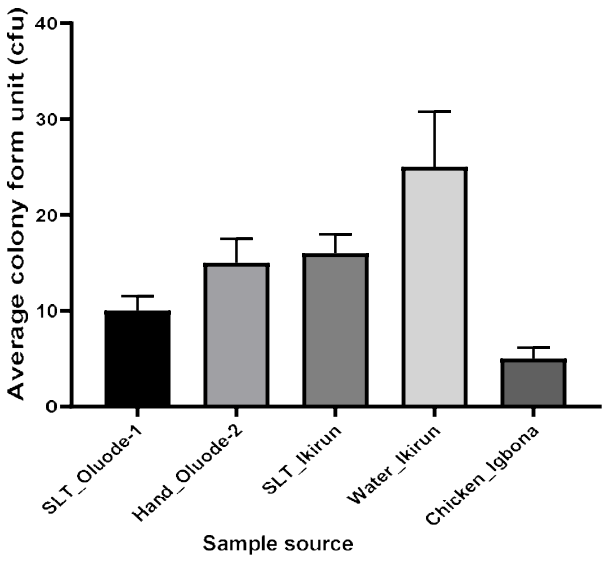


Figure 1. Average colony counts (cfu/ml or cfu/cm^2) of *E. coli* isolates found in the sampled abattoirs and chicken meat retail outlets.

Table 1. The list of PCR Primers used in this study.

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

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

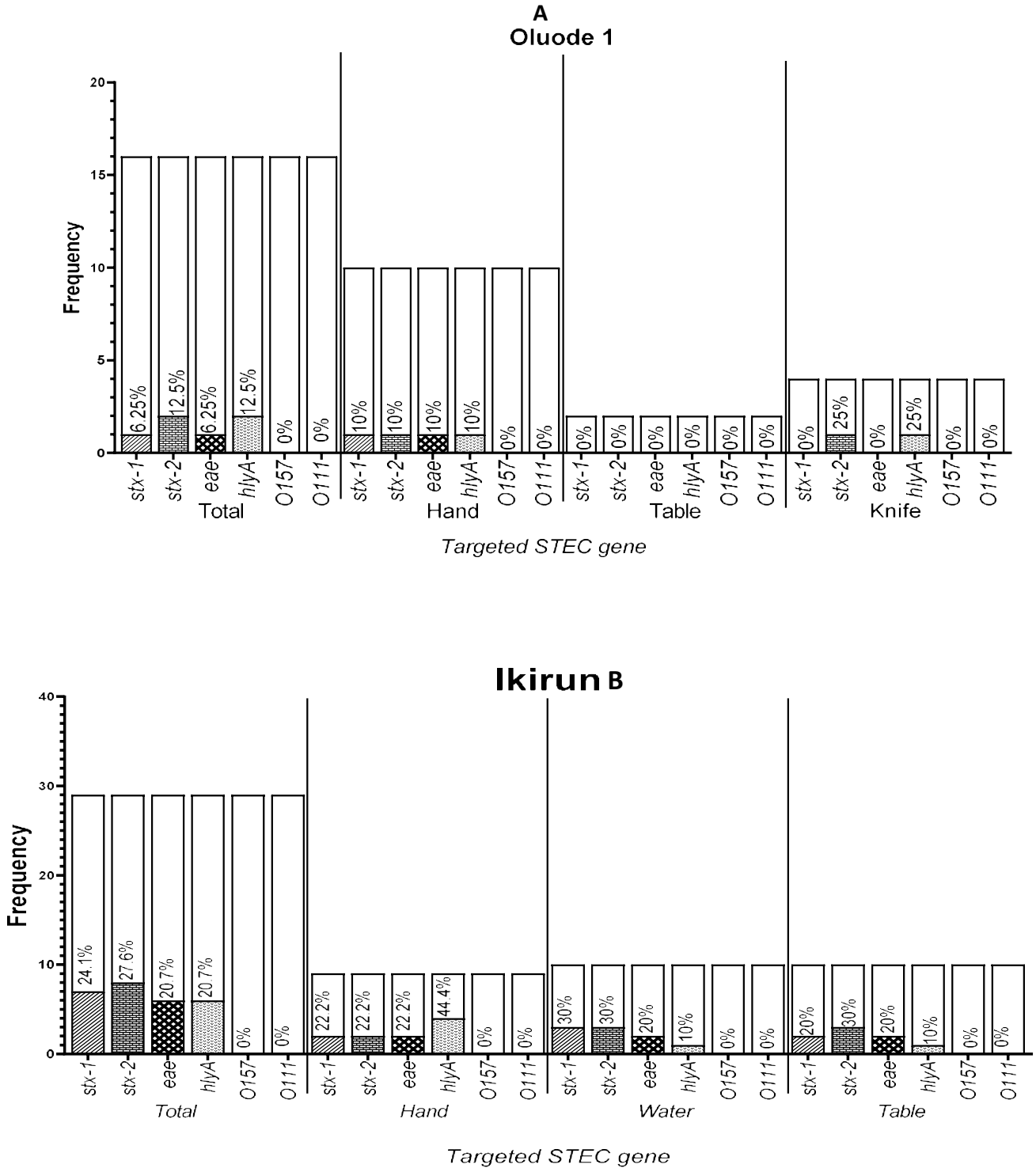
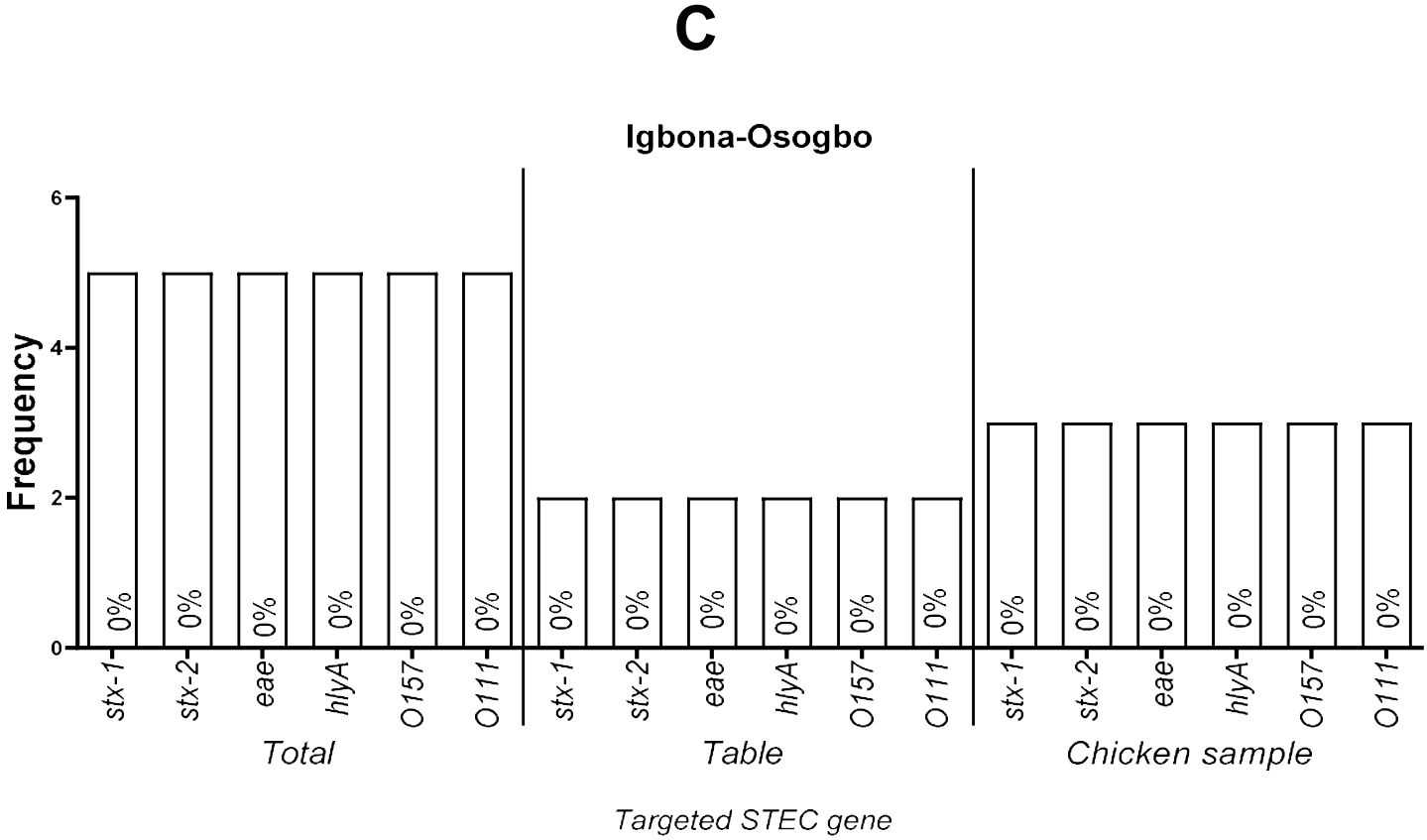


Figure 2. Frequency distribution of targeted STEC genes amplified from *E. coli* colonies isolated from sampled workers' hands, butchering knives, slaughtering tables, floors and effluent water at selected chicken abattoirs and retail chicken meat outlets.



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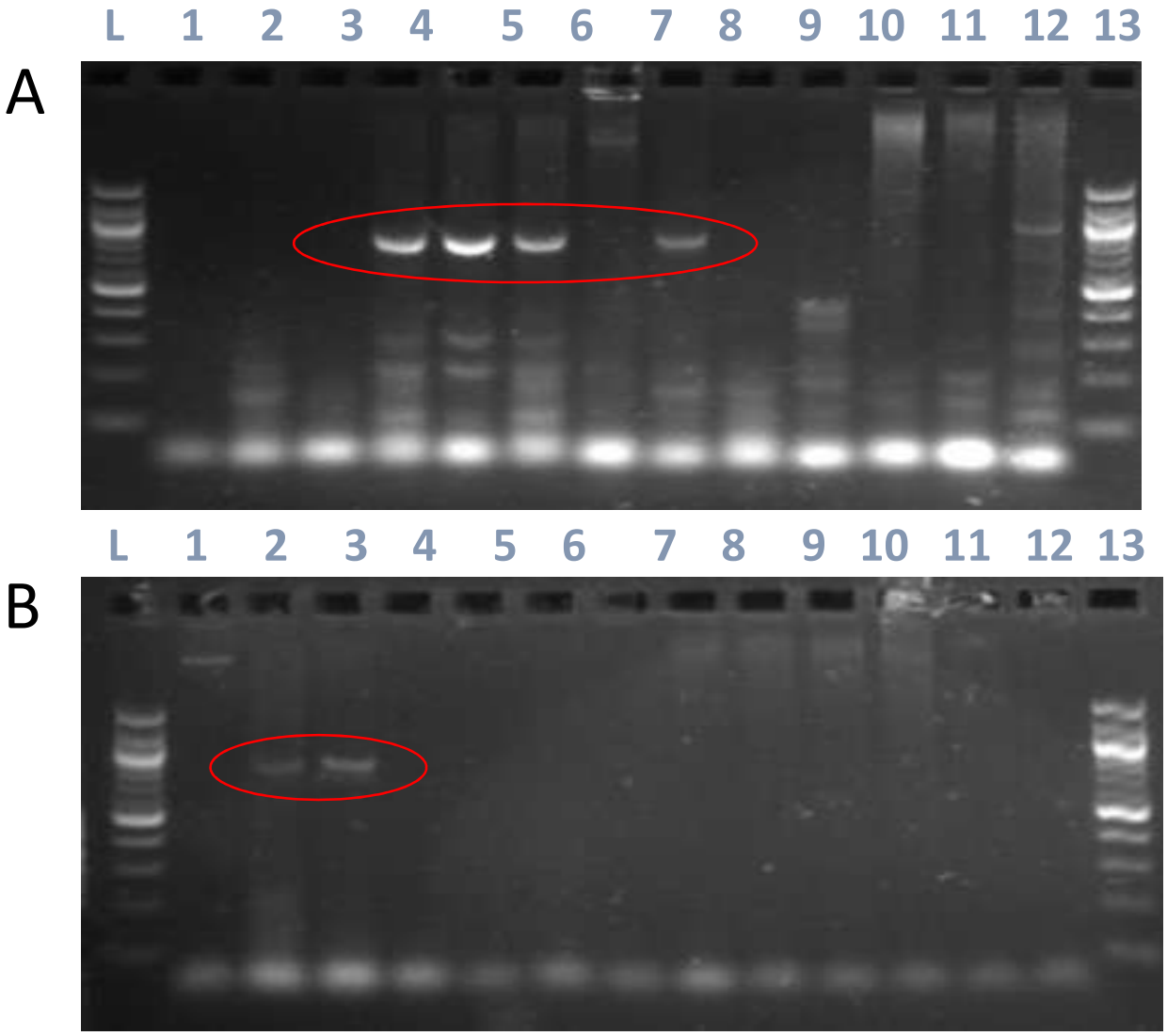

Figure 3: Representative gel picture showing the characterization of *E. coli* strain. Strains were classified based on the visible banding patterns expected mobilities for the various specific PCR products.

Legend: The lane numbers are matched with the corresponding isolate Identification numbers and the sample sources as follows, A1: Ik1W4b (Ikirun, water); A2: Ik3W5d (Ikirun, water); A3: Ik1BST1 (Ikirun, slaughtering table); A4: Ik3W1fa (Ikirun, water); A5: Ik1AST2 (Ikirun, slaughtering table); A6: IGST5 (Igbona, table); A7: OLKN1 (Oluode, knife); A8: IGST1 (Igbona, stock); A9: OLH2c5e (Oluode, hands); A10: OLH2c5a (Oluode, hands); A11: OL1B1ST2 (Oluode, slaughtering table); A12: OL1B1ST1 (Oluode, slaughtering table); A13: OLH2c3b (Oluode, hands); B1: IGST4 (Igbona, Table); B2: Ik3W1fa (Ikirun, water); B3: Ik3W5d (Ikirun, water); B4: OLH2c5e (Oluode, hands); B5: OLKN4 (Oluode, knife); B6: Ik1W2c (Ikirun, water); B7: Ik1W4b (Ikirun, water); B8 Ik3W5d (Ikirun, water); B9: Ik1BST1 (Ikirun, slaughtering table); B10- Ik3W1fa (Ikirun, water); B12: Ik1AST2 (Ikirun, slaughtering tableB; B13: IGST5 (Igbona, table); B14: OLKN1 (Oluode, knife).

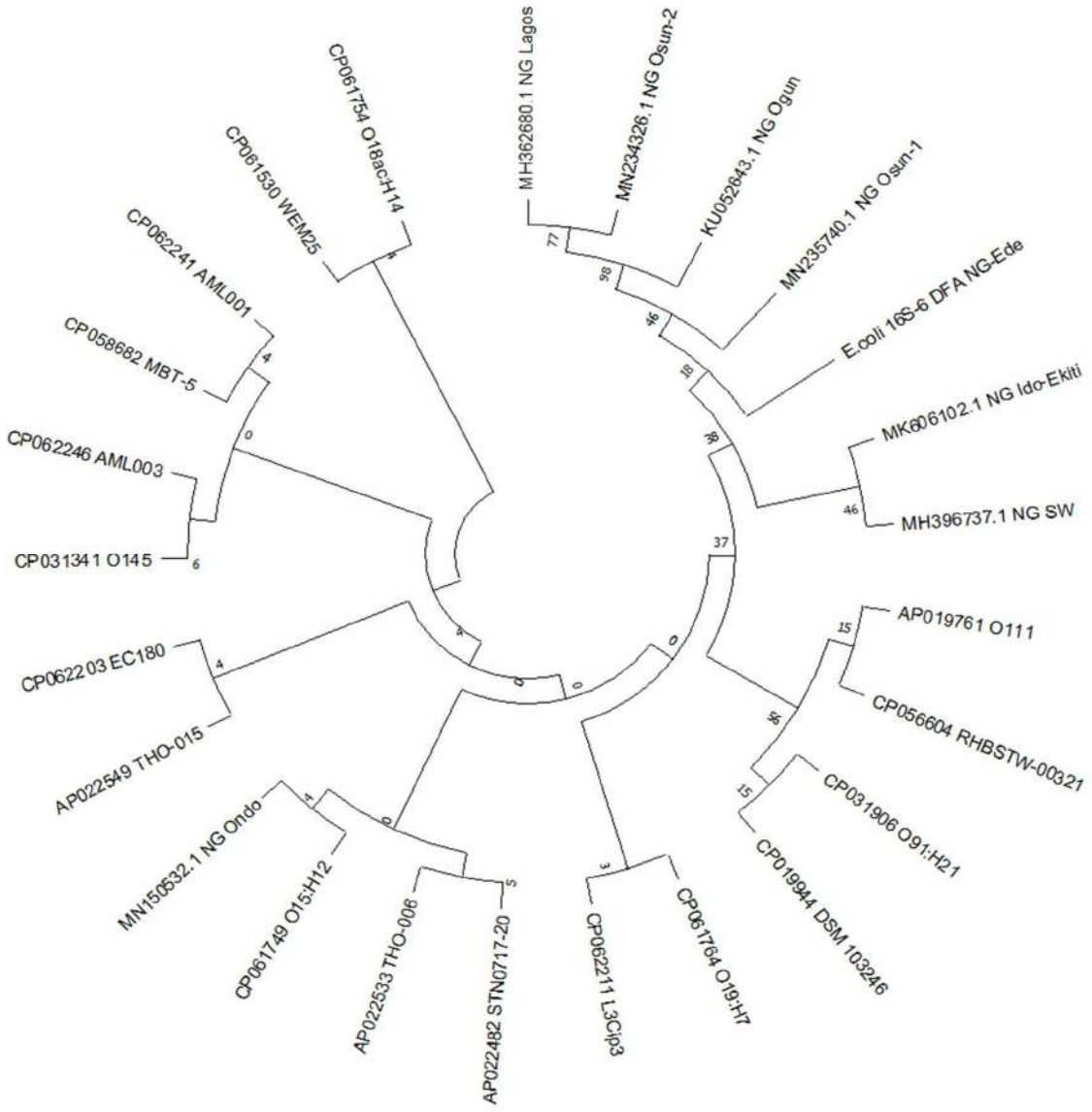

Figure 4. Phylogenetic analysis of *E. coli* sequence from chicken abattoir compared with publicly available sequences.

4. Discussion

Results from the present study are consistent with previous reports that considered poultry as poor reservoirs for STEC and characterized chickens as "spill-over hosts" of STEC (17,22).

Few colony-forming units of *E. coli* and even much fewer numbers of STEC genes-bearing strains were detected from the slaughtering floors, slaughtering tables, butchering knives, worker's hands and retail chicken meat tested from the selected abattoirs in the present study (Fig. 1 and 2) when compared with studies conducted recently on beef abattoirs within the same study area (26). Ruminants generally, and cattle in particular have been identified as a principal reservoir of *E. coli* O157:H7 and other STEC (17, 22). Apart from cattle, some wild birds, pigs, dogs and horses have been classified as "spill-over" hosts for STEC, that is, the condition of being susceptible to colonization by STEC but unable to maintain such colonization in the absence of prolonged exposure (17). The present study confirms that the ability of chicken meat and chicken abattoirs (i.e. abattoirs where chickens are the predominantly processed animal) to serve as a reservoir for STEC may at best be described as sporadic in the absence of sustained exposure. Moreover, the results of the present evaluation confirm that the hygienic practices of the abattoir workers are perhaps the paramount determining factor of the rate of *E. coli* contamination in the abattoir setting than the type of the abattoir, that is, whether it is batch or slaughter slab type. In the present study, all the chicken abattoirs sampled in the study were batch-type abattoirs and it is expected that the trend in the occurrence of *E. coli* and STEC would be similar since the abattoirs are in the same spatial location. The results however showed otherwise; in the present study, the highest colony forming units (cfu)/ml was from effluent water from the Ikirun abattoir, followed by slaughtering tables at the same abattoir, workers' hands at Oluode-2 batch abattoir, slaughtering table at Oluode-1 batch abattoir and chicken meat samples from the Igbona market retail store (Fig. 1). As observed in an earlier study, it seems as if contamination in these abattoirs originate from human activity during meat processing and subsequently through human activities the contaminants spread to the tables and floors and then to the effluent water (26). Results from the present study indicate that the waste management and disposal systems at Oluode-1 and Oluode-2 abattoirs are probably more efficient than those obtainable at the Ikirun abattoir where the colony count for *E. coli* was found to be highest in the effluent water. This observation may be instructive to regulatory bodies in this locality to focus on ensuring the upgrade of biological waste disposal from these abattoirs in order to limit the spread of potentially virulent pathogens into the runoff and groundwater with its dangerous implications to public health.

Only stx1, stx2, eae and hylA were amplified in the *E. coli* colonies, out of the 6 genes targeted in this study. Two of the six targeted genes, namely, O157 and O111 did not amplify in any of the tested samples. While it is somewhat comforting that none of the evaluated *E. coli* isolates exhibited the noxious O157 gene, the frequency of occurrence of the STEC genes data revealed that stx_2 was the predominant gene among the four targeted

genes found in this study. This gene emerged as the most frequently observed in the two abattoirs where STEC genes were found, occurring at the rates of 12.5% and 27.6% at Oluode-1 and Ikirun abattoirs respectively (Fig. 2). A similar trend has been observed in chicken and bovine meat in Nigeria (33) and elsewhere (34). This observation is worrisome because stx_2-producing strains have been reported to be more related to HUS than stx_1-producing strains (35). Isolate Ik-1A-5e recovered from water samples from the Ikirun abattoir amplified 4 out of the 6 STEC genes targeted in the present study and was sequenced for identification purposes only. The sequencing analysis identified this isolate as O113:H21 serotype. The observation that this isolate displayed a closely related evolutionary relationship to other sequences derived from south-western Nigeria including that obtained from beef abattoirs sampled within the same study area from a recently reported study may justify a nationwide study focused on sequencing for STEC genes and probing for the prevalence of O113:H21 serotypes.

Conflict of interests

The authors declare no competing interests.

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