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Molecular Detection of Virulence Genes and Antibiotic Resistance Patterns of Escherichia coli O157:H7 Isolated from Raw Beef Sold in Abeokuta, South-West Nigeria

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Abstract
Escherichia coli O157:H7 is an important food-borne pathogen that can cause diarrhea, haemorrhagic colitis and haemolytic uremic syndrome. This study was conducted to investigate the prevalence, virulence genes and antibiotic resistance patterns of E. coli O157:H7 in raw beef meat sold in Abeokuta, South west Nigeria. One hundred and twenty samples of raw beef meat were collected from four abattoirs and examined for the presence of E. coli O157:H7. The virulence genes (stx1, stx2, eaeA and hlyA) were detected in E. coli O157:H7 isolates by polymerase chain reactions. The antibiotic resistance patterns of the isolates were determined using Kirby-Bauer disc diffusion method. Of 120 samples analyzed, 8 (6.67%) were contaminated with E. coli O157:H7, with highest prevalence rate (2.5%) found in beef samples collected from Rounder abattoir. The virulence genes (stx 1 and stx 2 genes) were detected in 7 (87.5%) of E. coli O157:H7 isolates while no eaeA and hlyA genes were found. All the E. coli O157:H7 isolates were highly resistant to tetracycline, ampicillin, erythromycin and chloramphenicol and sensitive to ciprofloxacin and streptomycin. The results of this study revealed that raw beef meat could be potential vehicles of transmitting multi-drug resistant, shiga toxin-producing E. coli O157:H7 to humans.

Keywords: Pathogen, E. coli O157:H7, virulence genes, antibiotic-resistance, beef meat

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Introduction

The threat posed by enterohaemorrhagic Escherichia coli diseases spread via contaminated and improperly cooked meat has been well recognized (Elmali et al., 2005). Infections caused by Escherichia coli O157:H7 have been a significant public health problem world-wide causing human diseases including diarrhea, haemorrhagic colitis (HC), haemolytic uremic syndrome (HUS) and thrombotic thrombocytopenia purpura (Smith et al., 2003). The natural reservoirs of this pathogen include cattle, sheep and goats; and the modes of transmission of the infections are animal to person, waterborne, foodborne and person to person. However, the organism has also been isolated from other animal meat products such as chicken, pork and lamb (Ateba and Mbewe, 2011). Consumption of improperly cooked contaminated beef meat as well as raw milk of bovine origin have been found to be one of the methods of transmitting this organism to humans and these products have been implicated in the outbreaks of E. coli O157:H7 infections (Dontorou et al., 2003; Oksuz et al., 2004; Abong'O and Momba, 2009). Other foods such as unpasteurized goat's milk, cheese, meat sandwiches, lettuce, unpasteurized apple cider and apple juice have also been implicated in causing outbreaks of E. coli O157:H7 (Rahimi et al., 2011).

Escherichia coli O157:H7 causes hemolytic uremic syndrome, haemorrhagic colitis and other infections by secretion of shiga toxins which are encoded by the genes stx1 and/or stx2, as well as other virulence factors such as intimin (encoded by bacterial eaeA gene) and enterohaemolysin (encoded by E-hlyA) (Bidet et al., 2005; Abu-Ali et al., 2009; El-Jakee et al., 2009; Zhang et al., 2015). These virulence mechanisms are genetically coded for chromosomal, plasmid and bacteriophage DNA. Intimin is known to be responsible for attachment of the bacteria to the intestinal epithelial cells, causing attaching and effacing (A/E) lesions on the intestinal mucosa. Enterohaemolysin has also been reported to cause enterocyte and leukocyte lysis in cattle.

In addition, studies have shown an increasing antibiotic resistance of Escherichia coli O157:H7 in cattle which may pose serious threat to humans because such antibiotic-resistant strains can be transmitted to humans through the consumption of contaminated beef. However, with the advent of polymerase chain reaction (PCR) technique, it is now possible to determine the genes encoding for the virulence factors in bacterial strains.

To the best of our knowledge, there have been no published reports on the prevalence of antibiotic resistant E. coli O157:H7 in raw beef sold in Abeokuta,. Therefore, the objectives of the present study were: (1) to determine the prevalence of Escherichia coli O157:H7 in raw beef sold in Abeokuta, (2) to assess the frequency of four virulence genes (stx1, stx2, eaeA and hlyA genes) in the isolated strains, and (3) to determine the antibiotic resistance patterns of the isolates.

Materials and Methods

Sample collection: In this study, a total of 120 samples of raw beef were collected randomly from four abbatoirs in Abeokuta (Odo-eran, Aladesanmi, Lafenwa and Rounder). Before collecting the muscle meat samples, the external surfaces were disinfected with 70% alcohol to reduce surface contamination. Using sterile scissors and forceps, pieces of the muscles were collected into sterile universal bottles and immediately transported in an ice box to the laboratory for further processing.

Isolation and identification of Escherichia coli O157:H7: Isolation of E. coli O157:H7 from raw beef samples was carried out using the method described by Rahimi et al. (2011) with little modifications. Briefly, 2.0g of each meat sample was thoroughly homogenized in 18.0ml of sterile tryptone soya broth supplemented with 20mg/L novobiocin and incubated at 37^{o}C for 24 hours. The enrichment broth cultures were streaked onto Sorbitol-MacConkey agar (Oxoid, U.K). The plates were then incubated at 37^{o}C for 24 hours. Non-sorbitol fermenting colonies were picked and sub-cultured on sorbitol-MacConkey agar plates. The sorbitol negative colonies were Gram-stained and biochemically characterized using Analytical Profile Index (API) identification kit (API 20E test strips; bioMerieux, France). The colonies were then serologically typed for O157:H7 antigens by slide agglutination test using polyvalent and monovalent anti-E. coli O and H sera.

Molecular detection of virulence genes in E. coli O157:H7 isolates: This was carried out at Biotechnology Centre, Federal University of Agriculture, Abeokuta, Nigeria. The genomic DNA of the isolates was extracted using ZYMO (ZR) Fungal/Bacterial Genomic DNA extraction kit (Zymo Research, U.S.A.) following the manufacturer's instructions. The concentrations and purities of the genomic DNA were determined using NanoDrop Lite Spectrophotometer (Thermo Scientific). The presence of four virulence genes (stx1, stx2, hlyA and eaeA) in E. coli O157:H7 isolates was detected by polymerase chain reaction (PCR). Amplifications of the genes were achieved by employing the specific oligonucleotide primers which were synthesized by Integrated DNA Technologies (IDT) Inc, U.S.A. The sequences and annealing temperatures of the primers are shown in Table 1.

Table 1: Sequences of the oligonucleotide primers used for amplification of DNA from E. coli O157:H7 isolated from raw beef

Target gene	Specificity	Sequences	Melting temperature (^oC)	Annealing temperature (^oC)
stx1	shiga toxin1	F: 5'- ACA CTG GAT GAT CTC AGT GG-3'	54.0	48.3^oC
		R: 5'-CTG AAT CCC CCT CCA TTA TG- 3'	52.5	
stx2	shiga toxin2	F: 5'-CCA TGA CAA CGG ACA GCA GT-3'	57.9	55.9^oC
		R: 5'-CCT GTC AAC TGA GCA CTT TG- 3'	63.8	
eaeA	Intimin	F: 5'-GTG GCG AAT ACT GGC GAG ACT-3	60.4	54.0^oC
		R: 5'-CCC CAT TCT TTT TCA CCG TCG-3'	57.5	
hlyA	Hemolysin	F: 5'-ACG ATG TGG TTT ATT CTG GA-3'	51.3	45.4^oC
		R: 5'-CTT CAC GTG ACC ATA CAT AT-3'	49.4	

Polymerase chain reactions were performed in a total reaction volume of 10µl containing 1.5µl of template DNA (1µg), 5.0µl of 2×PCR master mix (Norgen Biotek Corporation, Canada) which contains Taq DNA polymerase, dNTPs, reaction buffer, $MgCl_2$, KCl and PCR enhancer/stabilizer; 1.0µl of forward primer (2.5µM), 1.0µl of reverse primer (2.5µM) and 1.5µl of nuclease-free water. PCR reactions were carried out in a TC-412 Thermocycler employing the following amplification conditions. Initial denaturation step of 95^oC for 2 minutes, followed by 35 amplification cycles each consisting of denaturation at 94^oC for 1 min, annealing for 60 seconds and extension or elongation at 72^oC for 2 minutes. Reactions were terminated at final extension of 72^oC for 10 minutes. The amplified products were analyzed by electrophoresis on a 1% (w/v) agarose gel, stained with ethidium bromide in the presence of a 1kb PCR sizer ladder (Norgen Biotek Corporation, Canada). Electrophoresis was performed at 80V for 60 minutes.

Antibiotic – resistance test: Antibiotic resistance patterns of E. coli O157:H7strains were carried out by the Kirby-Bauer disc diffusion method using Mueller-Hinton agar. The antibiotic impregnated discs used were ampicillin (10µg), ciprofloxacin (10µg), gentamicin (10µg), amoxicillin (30 µg) streptomycin (30 µg), tetracycline (15µg), kanamycin (30 µg), erythromycin (15µg) and chloramphenicol (30 µg). Zones of inhibition were measured after 24h of incubation. The strains were classified as 'resistant (R)', 'intermediate sensitive (I)' or 'sensitive (S)' using standard recommendations of Clinical and Laboratory Standards Institute (CLSI, 2009).

Results

In the present study, 120 samples of slaughtered cow meat were investigated to determine the prevalence of Escherichia coli O157:H7 strains among the raw beef sold in Abeokuta. It was found that 8 out of 120 meat samples were positive for Escherichia coli O157:H7 representing 6.7% prevalence (Table 2). The highest percentage (2.5%) of Escherichia coli O157:H7 was found in raw meat samples collected from Rounder abbatoir, followed by samples from Lafenwa and Aladesanmi while the samples collected from Odo-eran had the least percentage (0.8%) of E. coli O157:H7 (Table 2).

Table 2: Prevalence of Escherichia coli O157:H7 in raw beef sold in Abeokuta, Southwest Nigeria

Source of samples	Number of samples	Non-sorbitol fermenters	Number of confirmed E. coli O157:H7
Odo-Eran	30	18	1(0.8%)
Lafenwa	30	21	2(1.7%)
Aladesanmi	30	18	2(1.7%)
Rounder	30	21	3(2.5%)
Total	120	78	8(6.7%)

Table 3 shows the distribution of virulence genes among Escherichia coli O157:H7 isolated from raw beef samples sold in Abeokuta. Stx1 and stx2 genes were detected in 7 out of 8 E. coli O157:H7 isolates. The genes were detected in all E. coli O157:H7 isolates from raw beef samples from Odo-eran, Lafenwa, Aladesanmi and two out of the three E. coli O157:H7 isolates from Rounder abattoir. However, none of eaeA and hlyA genes were detected in all E. coli O157:H7 isolates. The gel image showing amplified products of stx1 and stx2 genes are shown in Figures 1 and 2 respectively. From the Figures, it was discovered that the amplified fragment size of stx1 gene was 320bp fragment, while stx2 gene was 600bp.

Table 3: Distribution of virulence genes among Escherichia coli O157:H7 isolated from raw beef meat sold in Abeokuta, Southwest Nigeria

		stx1	stx2	eaeA	hlyA
Source of samples	No of E. coli O157:H7	Virulence genes			
Odo-Eran	1	1	1	0	0
Lafenwa	2	2	2	0	0
Aladesanmi	2	2	2	0	0
Rounder	3	2	2	0	0
Total	8	7	7	0	0

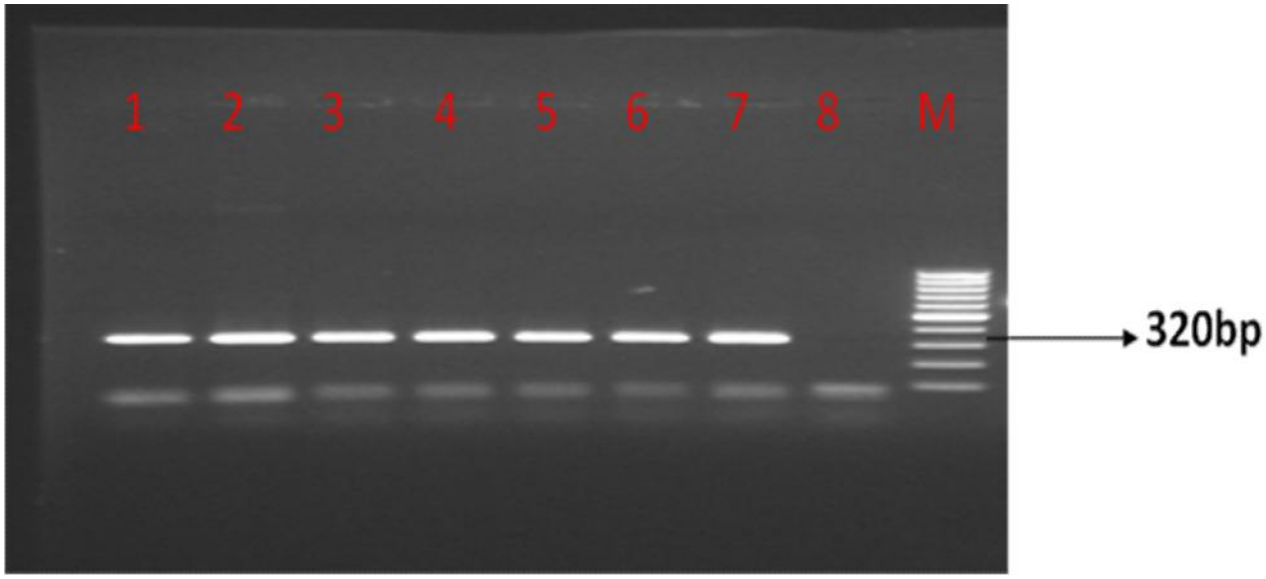

Figure 1: Gel electrophoresis of amplified products of stx1 genes in Escherichia coli O157:H7 strains isolated from raw beef sold in Abeokuta.

M: PCR sizer ladder, 1: E. coli O157:H7 isolate from Odo-eran abattoir, 2-3: E. coli O157:H7 isolates from Lafenwa abattoir, 4-5: E. coli O157:H7 isolates from Aladesanmi abattoir; 6-8: E. coli O157:H7 isolates from Rounder abattoir

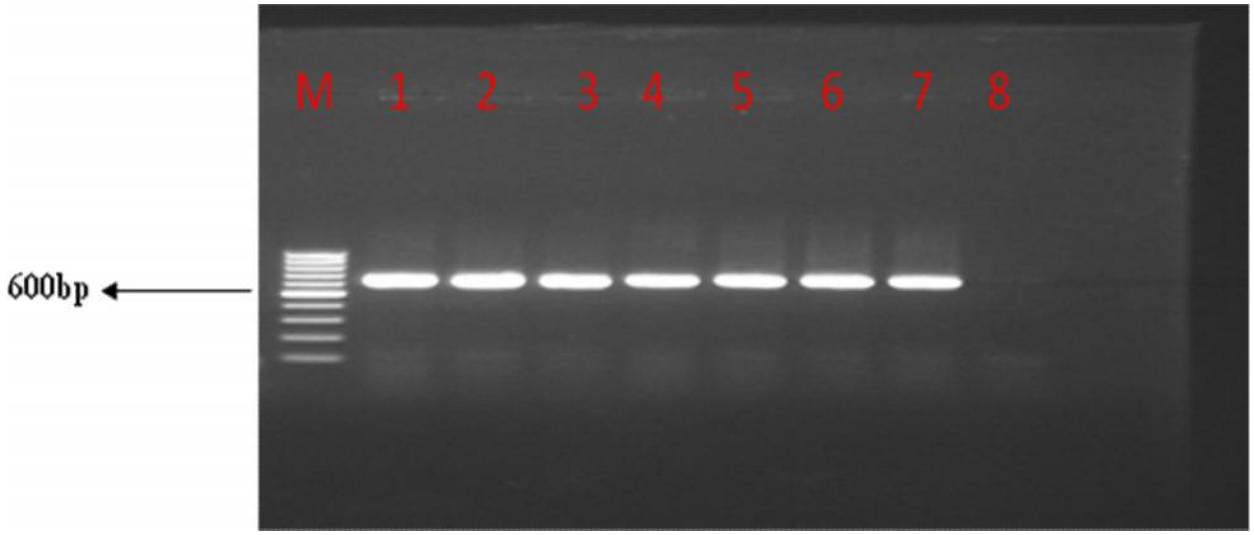

Figure 2: Gel electrophoresis of amplified products of stx2 genes in Escherichia coli O157:H7 strains isolated from raw beef meat sold in Abeokuta.

M: PCR sizer ladder, 1: E. coli O157:H7 isolate from Odo-eran abattoir, 2-3: E. coli O157:H7 isolates from Lafenwa abattoir, 4-5: E. coli O157:H7 isolates from Aladesanmi abattoir; 6-8: E. coli O157:H7 isolates from Rounder abattoir

Table 4 summarizes the resistance patterns of Escherichia coli O157:H7 isolated from raw beef sold in Abeokuta to nine antibiotics tested in this study. All the isolates were resistant to tetracycline, ampicillin, chloramphenicol and erythromycin (100%), 5(62.5%) of the isolates were resistant to kanamycin and none of the isolates was resistant to streptomycin and ciprofloxacin.

Table 4: Antibiotic resistance patterns of Escherichia coli O157:H7 isolated from raw beef sold in Abeokuta

Antibiotics	E. coli O157:H7 isolates (n=8)
Ampicillin	8 (100%)
Ciprofloxacin	-
Gentamicin	2 (25%)
Amoxicillin	2 (25%)
Streptomycin	-
Tetracycline	8 (100%)
Kanamycin	5 (62.5%)
Erythromycin	8 (100%)
Chloramphenicol	8 (100%)

Discussion

Escherichia coli O157:H7 is the most studied strain among all the pathogenic strains of E. coli because it is a leading cause of many human food-borne infections such as diarrhea, haemorrhagic colitis and haemolytic uremic syndrome. This strain can be transmitted to humans through consumption of contaminated foods including beef. In the present study, 6.70% of the raw beef samples collected from four abattoirs in Abeokuta was found to be contaminated with Escherichia coli O157:H7, which was higher than the frequency (3.5%) found in ground beef samples analyzed in Sao Paulo, Brazil by Bergamini et al. (2007). Seven of eight Escherichia coli O157:H7 strains harbor both shiga toxin 1 (stx1) and shiga toxin 2 (stx2) genes while none of the isolates carries intimin (eaeA) and hemolysin (hylA) genes. Thus, those seven isolates could be classified as Shiga toxin-producing Escherichia coli (STEC), but not enterohaemorrhagic. The predominance of shiga toxin-producing Escherichia coli O157:H7 could become a serious risk to public health since this strain carrying stx1 and stx2 genes have been associated with such serious illness as haemolytic uremic syndrome (HUS) (Liua et al., 2007). However, the results of this study revealed that consumption of improperly cooked beef as well as cross-contamination of

raw beef with other foods or food utensils could be major sources of STEC strains.

The presence of the intimin gene (eaeA) in STEC strains has been reported in some O groups such as O26, O103, O157 and O111 (Sandhu et al., 1996). The absence of eaeA in any of the E. coli O157:H7 strains isolated in this study could probably be related to their H-groups. Similar reports were obtained by Mazaheri et al. (2014). The negative result of this study may also be related to the variability of the eaeA gene among E. coli strains. Although, shiga toxin-producing E. coli carrying intimin gene are frequently associated with severe infections, outbreaks of HUS by intimin negative STEC have also been reported by Paton and Paton (1998). The present study therefore revealed that there is need for a strict surveillance of Escherichia coli O157:H7 in meat because shiga toxin-producing Escherichia coli can survive in foods for long periods and can easily contaminate other foods or food utensils during meat processing.

In an attempt to determine the antimicrobial resistance patterns of E. coliO157:H7 isolates, all the isolates showed resistance to four antibiotics tested (ampicillin, tetracycline, erythromycin and chloramphenicol). These isolates are considered to be multi-drug resistant since they showed resistance to more than three antibiotic classes (β-lactam, tetracycline, macrolides, phenicols and aminoglycoside). The results of the antibiotic resistance patterns of E. coli O157:H7 obtained in this study are similar to the report of Mazaheri et al. (2014) who found that all STEC isolated from lettuce samples in Tehran were all resistant to tetracycline and ampicillin. However, the percentage of multi-drug resistance found in this study is higher than the previous report of Momtaz et al. (2012) who found multi-drug resistance in 64.91% of E. coli isolated from slaughtered commercial chickens in Iran. The resistance patterns observed in this study should be considered not only for its effects on human health, but also as a potent source of transferring the antibiotic-resistant genes to other important pathogenic serotypes through horizontal gene transfer between bacteria through plasmids or transposons, and thereby contributing to the increase of the resistant genes in the environment.

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