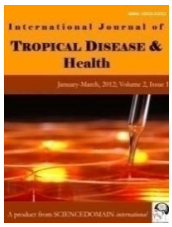

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Shiga Toxin and Non-Shiga Toxin-Producing *Escherichia coli* O157 from Cattle, Goats and Chickenin Ado-Ekiti, South West, Nigeria

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Authors' contributions

Authors AOO and OF designed the study. Author AOO performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors AOO and OF managed the analyses of the study and the literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

Background: Illnesses due to contaminated food, particularly food of animal origin, are perhaps the most widespread health problem in the contemporary world.
Aims: To detect Shiga toxin-producing *Escherichia coli* (STEC) in food animals in Ado-Ekiti, Nigeria and the possible risk to human health.
Study Design: Non-repeat faecal samples from various animals and poultry birds were examined for STEC.
Place and Duration of Study: Department of Microbiology, Ekiti State University, Ado-Ekiti, between January 2010 and December 2011.
Methodology: We investigated 722 non-repeat faecal samples from animals and poultry birds for the presence of STEC using bacteriological, serological, and tissue culture techniques. Detection of virulence genes was performed by PCR.
Results: Overall, 316 isolates of *E. coli* were recovered from 62.3% cattle, 19.6% local chicken, 10.1% goats, 4.1% broiler, 2.9% layers, and 0.9% cockerels. Of the non-sorbitol fermenting *E. coli*

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phenotype selected from the isolates, 13.3% were presumptively identified as O157 serotype based on inability to ferment sorbitol on sorbitol MacConkey agar (SMAC).
Serotyping using commercial kits capable of detecting O157 and non-O157STEC confirmed 6.6% of these as O157 comprising 4.1% from cattle and 2.5% from local chicken. Only 4.7% of the strains were serologically confirmed as non-O157 of which 0.9% was from cattle, 3.2% from goat and 0.6% from local chicken. Verocytotoxicity test and the presence of virulence genes *stx*1, *stx*2 and *eae* assayed by PCR showed the complete absence of virulence genes in the 13 serologically confirmed strains of O157 from cattle. The virulence gene *stx*1 was detected only in non-O157 strain from goat and local chickens.
Conclusion: This study has shown that the prevalence of *E. coli* O157 is low in food animals in the study area compared to reports from the developed countries. Furthermore, our study is the first to report the isolation of non-O157STEC in goat, a very common domestic animal, in the study area.

Keywords: Shiga toxin; non-shiga-toxin; Escherichia coli; STECO157; non-O157STEC; food animals.

1. INTRODUCTION

Shiga toxin (Stx)-producing *Escherichia coli* (STEC) has been reported as one of the most important causes of foodborne infections and emerging issues worldwide [1-8]. STEC, also known as verocytotoxin-producing *E. coli* (VTEC) is a foodborne organism that has been associated with sporadic cases and outbreaks of bacterial enteritis in humans [4,9-14]. STEC infections in humans have been acquired through consumption of food or water contaminated directly or indirectly with cattle faeces [15]. Most infections due to STEC in humans have been attributed to serotype O157. Albeit, non-O157STEC is increasingly being recognized to be of considerable importance as it is frequently associated with sporadic outbreak of both mild and severe STEC disease in humans globally [2,16-18].

Diseases caused by STEC are characterized by abdominal pain and bloody diarrhoea, and 5 to 15% of those infected with serotype O157 develop haemolytic uremic syndrome (HUS), a potentially life-threatening condition consisting of haemolyticanaemia, thrombocytopenia and kidney failure caused by *Stx* [19]. STEC is the primary aetiologic agent of HUS, and *E. coli* O 157:H7 is the prevalent serotype detected [2,12,17,19-21].

Shiga toxins are members of a family of highly potent bacterial toxins responsible for severe clinical symptoms and are the main virulence marker for STEC [5,22]. Stx belongs to a defined protein subfamily, the RNA N-glycosidases that can be classified into two antigenic groups: Shiga toxin 1 (Stx 1) and Shiga toxin 2 (Stx 2). Stx 1 is a rather homologous group with three variants (Stx 1, Stx1c and Stx1d). Stx 2 group is more heterogenous and comprises several subtypes [23,24,25]. Other virulence factors that may play a role in the pathogenicity of Shiga toxin include intimin (encoded by the *eae* A gene), which is required for intimate adherence of these pathogens and formation of attaching and effacing (A/E) lesion [5,23]. Stx production is not restricted to serotype O157 strains as over 100 STEC serotypes have been isolated from humans with diarrhoeal illness [2,21,22].

It has been estimated that *E. coli* O157:H7 causes two thirds of STEC infections in humans in the United States with the other one-third of cases attributed to non-O157STEC population [26]. Furthermore, studies in Europe indicate that non-O157STEC infections occur more frequently than do STECO157 infections [2,13]. In resource-poor countries, STEC strains belonging to serotype O157 are also the most common causes of human diseases. However, there is yet no report on the role played by non-O157 strains of STEC in these areas.

Healthy cattle and small ruminants are the main reservoir of the organism while direct and indirect contact with animals is one of the transmission routes by which infection is acquired [5,27-29]. Person-to-person transmission by faecal-oral route occurs in families and institutional settings such as schools and nurseries. Ruminant animals carry a diversity of STEC and isolation rates have been reported to be generally higher in sheep than cattle [30]. However, cattle appear to be a major natural reservoir and an important source of human infection [31,32]. STECO157 strains have also been isolated from other domestic animals including sheep, goats, horses, pigs, geese and turkeys [33]. In low income countries, the common transmission routes include animal-to-human, waterborne and person-to-person [34]. Faecal contamination is

one source of environmental contamination and is responsible for the presence of these pathogens in the environment [29,35]. Transmission of STECO157 to man occurs mainly by consumption of contaminated food, including undercooked beef and meat products, unpasteurized milk and ready-to-eat products including cooked meats and vegetables that have been contaminated [13,21,36].

Although, meat is generally consumed well done in Southwest, Nigeria, cattle grazing is often carried out around residential areas in the locality. At times, grazing is done near riverbeds and flowing streams, which serve as sources of drinking water particularly in most villages, thereby contaminating the environment and water sources. Moreover, cattle faeces are used ignorantly and/or deliberately as manure in vegetable farms and gardens. Furthermore, cross-contamination of meat products with bacteria often occurs during slaughter and production. Consumption of chicken and other foods contaminated either through faecal contact or processing failure is the most important cause of indigenous foodborne outbreaks [29,32,37]. Moreover, faeces are washed off and water sources including rivers, streams, rivulets and wells meant for domestic use, are contaminated during heavy rainfall. Foods and vegetables meant for human consumption may subsequently become contaminated [7,38].

Although considerable progress has been made in understanding the ecology of STEC in farm animal hosts and the modes of transmission to humans, it still remains unclear if all STEC present in animal reservoirs present a risk to human health. Substantial gaps in knowledge about the epidemiology of Shiga toxin-producing *E. coli* exists in the study area, Ekiti State.

The aim of this study was to determine the prevalence of Shiga toxin-producing *E. coli* O157 in known reservoirs of the organisms such as cattle, goats and chicken in Ekiti State, Nigeria as a prelude to assessing the presence of these pathogens in the environment and their possible risk to human health in the study area.

2. MATERIALS AND METHODS

2.1 Collection of Faecal Samples from Poultry Sources, Goats, and Cattle

A total of 722 faecal samples from various sources were examined in this study. Of these 361 were from cattle, 49 from goats, 19 from cockerels, 141 from local chickens, 100 from broilers and 52 from layers. The samples were collected with culturette.

Faecal samples were also collected from cattle slaughtered at two central abbatoirs in Ado-Ekiti and samples were transported to the laboratory immediately and processed within one hour of collection.

2.2 Processing of Faecal Samples

Faecal samples were inoculated directly onto Eosin Methylene Blue (EMB) agar (Fluka 70186), streaked to obtain discrete colonies and incubated at 37ºC for 24-48 h. The plates were examined for colonies with characteristic green metallic sheen, which is the distinguishing feature of *E. coli* on EMB agar. A maximum of 3 distinct colonies of *E. coli* were picked per sample and stored as slants on Nutrient agar. All colonies showing green metallic sheen on EMB agar were streaked onto Sorbitol MacConkey agar plates (SMAC; Oxiod, Basingstoke, Hampshire, UK) and incubated at 37ºC for 24 hours.

2.3 Identification and Serotyping of *E. coli*

The bacterial isolates from the various sources were identified on the bases of their cultural, morphological and biochemical characteristics as described by Barrow and Feltham [39].

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 agglutiation 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.

2.4 Cytotoxicity Assay

The cytotoxicity of *E. coli* on vero cells (African Green Monkey kidney cell) was determined as described by Clarke et al. [40]. The vero cells were grown in a 24-well microtitre plate in Eagle's minimal essential medium (MEM) supplemented with 10% fetal calf serum and incubated at 37ºC until a confluent monolayer growth had formed. All *E. coli* strains tested were grown overnight in Brain Heart Infusion broth and

the supernatant was clarified by membrane filtration with 0.22 µm size Millipore filter. The clarified supernatant was diluted fivefold (1/5 to 1/25) and added to $2.5x10^5$ cells/ml of confluent vero cells in a 24-well microtiter plates. The plates were incubated in a CO_2 environment for 72h. The cells were examined for the cytotoxic activity and the strain was considered cytotoxic if there was >50% cytotoxic effect on the vero cells.

2.5 Detection of Virulence Genes Stx1, Stx2 and Eae by PCR

The chromosomal DNA used in this study was extracted as described by Aranda et al. [41]. Overnight culture of 12 previously identified *E. coli* strains from poultry (3), cattle (6), and goat (3) were harvested from MacConkey agar and suspended in 250 µl of sterile water, incubated at 100ºC for 5 min to release DNA and centrifuged. The supernatant was used in the PCR assay. The primers used (VT1-A, VT1-B, VT2-A, VT2-B, EAE-2 to amplify the virulence genes were capable of detecting s*tx1* and s*tx2* and *eae*. Amplification was performed using a 50 µl volumes containing 10 µl of the prepared sample supernatant; 150 ng oligonucleotide primers; 0.2mM (each) dATP, dGTP, dCTP and dTTP buffer solution (10mMTrisHCl (pH 8.8); 1.5 mMMgCl2; 50mMKCl) and 1U of *Taq* polymerase (Roche Diagnostics GmbH, Germany).The conditions for the PCR were 94ºC for 2 minutes for initial denaturation of DNA within the sample followed by 35 cycles of 94ºC for I minute (denaturation), 55ºC for 1 minute (primer annealing) and 72ºC for 1 minute (DNA synthesis). This was performed in a thermal cycler (FT GENE5D, Techne, Cambridge, England). For every reaction, a negative (sterile distilled water) and a positive (reference strain ATCC 25929) controls and a molecular size marker were used to determine the size of amplified fragments. Base sequences and predicted sizes of amplified products for the specific primers used in the study are shown on Table 1.

3. RESULTS

3.1 Isolation of *Escherichia coli* from Poultry and Animal Sources

A total of 316 strains were isolated from 62 (19.6%), 13 (4.1%), 9 (2.85%) and 3 (0.9%) samples of local chicken, broiler, layers and cockerels respectively. *Escherichia coli* was also isolated from 32 (10.1%) of goats and 197 (62.3%) of cattle faecal samples (Table 2).

3.2 Isolation and Serotyping of Non-sorbitol Fermenting Phenotype of *E. coli* Isolated from Cattle, Local Chicken and Goats

Of the 316 samples which yielded growth of *E. coli*, 42 (13.3%) were non-sorbitol fermenters. Of the 42 non-sorbitol-fermenting strains tested, only 21 made up of 13 (4.1%) from cattle and 8 (2.5%) from local chicken caused visible agglutination within one minute with the latex reagent for O157. None of the strains from broilers, layers and cockerels showed any visible agglutination with the O157 latex reagent. Furthermore, a total of 15 (4.7%) strains made up of 3 (0.9%) from cattle, 2 (0.6%) from local chicken and 10 (3.2%) from goats 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 (Table 3).

3.3 Detection of Verocytotoxin by Tissue Culture

Only 5 strains from cattle and 2 from local chicken had cytotoxic effect on over 50% of the tissue cell monolayer after 24 hours of growth. Out of the 5 *E. coli* strains that showed cytotoxic effect, 2 were O157 serotype and three were non-O157. Also, the 2 strains from local chicken were non-O157 (Table 4).

Table 1. Nucleotide sequence of primers used

Gene	Primer	Oligonucleotide sequence	Fragment size (bp)
stx1	VT1-A	5'- CGCTGAATGTCATTCGCTCTGC	302
	VT1-B	5'- CGTGGTATAGCTACTTCACC	
stx 2	VT2-A	5'- CTTCGGATCCTATTCCCGG	516
	VT2-B	5'CTGCTGTGACAGTGACAAAACGC	
Eae	EAE-1	5'-GGAACGGCAGAGGTTAATCTGCAG	775
	EAE-2	5'-GGCGCTCATCATAGTCTTTC	

Table 2. Isolation of *Escherichia coli* from poultry and animal sources in Ado-Ekiti

Sources	Number examined (%)	Number positive for *E. coli* (%)
Local chicken	141 (19.5)	62 (19.6)
Broilers	100 (13.9)	13 (4.1)
Layers	52 (7.2)	9 (2.9)
Cockerels	19 (26.3)	3 (0.9)
Goat	49 (6.8)	32 (10.1)
Cattle	361 (50.0)	197 (62.3)
Total	722	316 (43.8)

Table 3. Serotyping of *E. coli* isolated from various animal sources in Ado-Ekiti

Source of isolation	Number isolated	Non-sorbitol fementation on SMAC (%)	Serotyping	
			E. coli O157	Non-O157 *E. coli*
Local chicken	62	11 (3.5%)	8 (2.5)	2 (0.6)
Broilers	13	0	0	0
Layers	9	0	0	0
Cockerels	3	0	0	0
Goats	32	0	0	10 (3.2)
Cattle	197	31 (9.8%)	13 (4.1)	3 (0.9)
Total	316	42 (13.3%)	21 (6.6)	15 (4.7)

Table 4. Detection of Shiga toxin by cytotoxicity effect on Vero cells and virulence genes by PCR

Source	Serotype examined	No of strains examined	No of strains with >50% cytotoxic effects	Detection of virulence genesby PCR		
				*stx*1	*stx*2	*Eae*
Cattle	O157	13	2	+	-	-
Cattle	Non-O157	3	3	+	-	-
Goats	Non-O157	10	0	+	-	-
Local chicken	Non-O157	2	2	-	-	-
Total		28	7			

+ Present; - Not detected

3.4 Detection of Virulence Genes (*Stx*1, *Stx*2, *Eae*) by PCR

Three out of the selected 12 strains whose genes were amplified by PCR showed the presence of detectable bands with molecular sizes ranging between 302 and 303 bp. This molecular size of 302 bp corresponds to the molecular size of *stx*1 used in the experiment. Of the three strains two were non-O157 isolates from goats, one from cattle and none from poultry. None of the other isolates from cattle investigated harboured any of the virulence genes examined.

4. DISCUSSION

Shiga toxin-producing *E. coli* (STEC) are essentially zoonotic enteric pathogens, commonly associated with human gastroenteritis globally and cattle and small ruminants are considered an important animal reservoirs for STEC [33]. The present study investigated animal reservoirs for STEC in Ado-Ekiti, Nigeria.

A total of 21 (6.6%) and 15 (4.7%) of O157 and non-O157 *E. coli* strains were respectively isolated from the faeces of various domestic animals in this study. Strains of O157 were isolated from 8 (2.5%) local chicken and 13 (4.1%) cattle while non-O157 was isolated from local chicken (0.6%), cattle (0.9%) and (3.2%) goats (Table 2). The finding in this study agrees with earlier reports that cattle are the primary source of O157strain of *E. coli* [34,42,43]. Various prevalence rates of *E. coli* O157 ranging from 0% to 70% in dairy calves [44], 66.6% in sheep, 56.1% in goats, 7.5% in pigs, 13.8% in cats and 4.8% in dogs and < 0.7% in chickens have also been reported [45]. The low incidence

of O157 serotype in this study may explain why infection with *E. coli* O157 in humans is not common in the study area. Furthermore, this finding corroborates the report of a low incidence of *E. coli* O157 infections in humans in Nigeria [46]. However, studies in other countries have reported a strong seasonal influence on the occurrence of *E. coli* O157 in cattle [47] and prevalence rates have been reported to vary with method of detection, geographical location [48-50,51], season of the year and age group in the animal reservoir [9]. Moreover, the finding of Ramoneda et al. [9] indicated that even under good manufacturing practices and stringent European standards, the total absence of STEC may not be absolutely guaranteed. However, our method of detection using SMAC, which was indirect may not have been very selective and sentive enough.

Result from this study also showed a prevalence of 4.1% of *E. coli* O157 in healthy cattle and 3.2% non-O157 *E. coli* strains in healthy goats (Table 3). These are slightly higher than a similar study in an Irish abattoir McCann et al. [52]. However, the fact still remains that while non-O157 *E. coli* is carried by other domestic animals such as goats, serogroup O157 remains the most dominant *E. coli* strain in the study area. This agrees with the findings of McCann et al. [52] who also reported a prevalence of 2.6% for O157STEC from cattle faeces and a prevalence ranging between 0.8-1.8% for non-O157STEC in an Irish abattoir and Renter et al. [53] recorvered 14% non-O157STEC strains in cattle faeces in Alberta, Canada. Khandaghi et al. [28] reported the recovery of non-O157STEC strain O26 from bovine faeces in Iran.

Ruminants, especially cattle constitute a vast reservoir of STEC, and it is not surprising that human infection can frequently be traced to contamination of food or water with manure. The carriage of the pathogens by cattle can be targeted as an area in which interventions may reduce contamination of food and the environment with pathogenic STEC shed by healthy cattle. More than 470 serotypes of STEC have been reported from humans [54] and most of these being serotypes that have been identified in cattle. In North America, cattle are the most significance reservoir of STEC, but in countries such as Australia, sheep are of greater significance [55].

Our study is the first published report of the isolation of non-O157STEC from goat in the study area, hence the role of non-O157 in STEC-associated disease may need to be further established in the study area. The significance of non-O157STEC as enteric pathogens is probably underestimated because there are no simple laboratory methods to detect and isolate these organisms. However, high incidence rates of non-O157STECserogroups have been reported under conditions of enhanced surveillance [35,56,57]. Reports have shown that global "hot spots" exist including Argentina, Australia and Germany in which non-O157STECserogroups dominate over O157 serogroups, [58]. For example, in Germany, non-O157STEC account for up to 80% of STEC-associated diarrhoeal illnesses [2,10].

Production of Shiga toxins is the critical virulence factor in STEC diseases. There is an evidence of an association of *stx2* with a higher risk of developing HUS and the presence of both *eae* and *stx*2 in a STEC isolate is considered to be a predictor of HUS [59]. Furthermore, *stx*2 is about 1,000 times more toxic for human renal microvascular endothelial cells than *stx*1 [60]. In this study, only virulence gene *stx1* was detected both in O157 and non-O157STEC isolated from cattle and non-O157 from goat (Table 4). This may probably explain the low severity of the infection and possibly lack of awareness, serious concern and attention for the organisms in the study area [61]. In a previous study, Smith et al. [62] reported the isolation of *E. coli* O157:H7 from some food animals including goats in Lagos State, Nigeria. Adefarakan et al. [63] on the other hand, reported the isolation of *E. coli* that lack of *stx* and *eae* genes from apparently healthy ram and goat.

Although several other reports outside Africa cited goats as potential sources of *E. coli* O157:H7 infection [64-66] none was isolated from goats in our present study. Outbreak of *E. coli* O157:H7 associated with unpasteurized goat milk has been described in Canada [67]. This study did not focus on the detection of the H antigen which may probably have eliminated the dectection of *E. coli* O157:H among the isolates and a limitation of the study. However, the role of goats as potential reservoirs of non-O157STEC in the study area is established in this study. The presence of *stx*1 detected in isolates from goat in this study is the first report of the presence of virulence associated-genes in *E. coli* isolates from animal origin in Nigeria. The fact that some of the serologically confirmed O157 strains isolated from cattle in this study did not harbour

any of the virulence genes shows that carriage of the virulence genes by O157STEC is not automatic.

Chicken is one of the most common animal protein sources consumed by the Nigerian population; they are readily reared at homes thereby many individuals come in close contact with them and their faeces than other animals which are reared in farms. A previous study in Nigeria also reported isolation of *E. coli* O157 from poultry even though they appeared healthy [46]. Therefore, irrespective of serotype, carriage of the combination of *stx, eae,* and EHEC-*hly*A may be a good indicator for the pathogenic potential of STEC strains.

Ruminants have been identified as the major reservoir of *E. coli* O157:H7 and also appear to be a reservoir of non-O157:H7STEC [68] although non-O157STEC have also been detected in non-ruminant animals [69]. STEC has been isolated from a variety of domestic animals. However, it is believed that in many cases they are present as transient bacteria that the animals acquired from feeds or water probably contaminated with faecal materials from ruminants. This may be a possible reason why non-O157 *E. coli* was isolated from local chicken which are free-range domestic animals but was absent in categories of poultry chicken which are caged and do receive medical attentions. However, STECO157 has been isolated in life layer hens in Italy.

5. CONCLUSION

This study has shown that the prevelence of *E. coli* O157 is low both in cattle (6.6%) and local chicken (2.5%) in the study area compared to that observed in developed countries. Furthermore, our study is the first to report the isolation of non-O157STEC in goats, a very common domestic animal, in the study area.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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