



ISOLATION, IDENTIFICATION, CHARACTERIZATION OF SHIGA TOXIN-PRODUCING ESCHERICHIA COLI SEROTYPES AND DETECTION OF STX1 AND STX2 IN CHICKENS IN MAIDUGURI

¹FALMATA A ABADAM, FUSAM S
KUBURI³, YUSUF A ISA ²ISA A GULANI AND
MUSTAPHA A ISA,

¹*Department of Food Science and Technology
Ramat Polytechnic Maiduguri, Borno State.*

²*Department of Veterinary Microbiology
University of Maiduguri, Borno State.*

³*Department of Veterinary Medicine,
University of Maiduguri, Borno State, Nigeria*

⁴*Department of Microbiology University of
Maiduguri, Borno State.*

INTRODUCTION

Escherichia coli (E. coli) are group of bacteria whose members are typically non-pathogenic normal micro flora of the intestinal tract of humans and animals (Gyles, 2006). However, certain of these bacterial species have acquired genes that enable them to cause intestinal disease. The E. coli that causes enteric diseases have been divided into patho-type based on their virulence factors and mechanism by which they cause disease. One of these pathogens, called Shiga toxin-producing E. coli refers to those strains of E. coli that produce at least one member of a class of potent cytotoxin called Shiga toxins (Gyles, 2006).

Abstract

This study was carried out in Maiduguri to isolate, identify and characterize Shiga toxin-producing Escherichia coli (STEC) in broiler and village chickens' faeces using conventional microbiology culture, and phenotypical characterization with biochemical test. Three hundred (300) (150 each of broiler and village chickens) faecal samples were collected at random from chickens brought for dressing to the Maiduguri Monday Market Chicken slaughter slab. Out of the 150 faecal samples collected from each of broiler and village

Keywords: *Village and broiler chickens, Shiga toxin producing Escherichia coli STEC, stx1, stx2, O157, non O157 E coli and Maiduguri*

chickens, a total of 83 E. coli isolates were obtained comprising 36(24%) from broiler chickens and 47(31.3%) from village chickens respectively. The E. coli positive isolates were then serotyped based on their somatic 'O' antigen using latex agglutination test for O157 STEC and dry spot polyvalent Sero-check for Non-O157 STEC, out of which 19

(12.7%) and 26 (17.3%) were O157 STEC; while 8(5.3%) and 9(6%) were non-O157 STEC from broilers and village chickens respectively. The remaining E. coli isolates 9(6%) from broiler chickens and 12(8%) from village chickens were untypable using the conventional sero kits. No stx1 and stx2 genes were detected using ELISA in all the positive E.coli O157 and non O157 samples detected. In conclusion, there was presence of E.coli O157 and non O157 in chickens in Maiduguri

During the past two decades, an increasing number of food-borne illness outbreaks have been traced to consumption of undercooked ground beef and other beef products contaminated with Shiga toxin-producing E. coli O157. Shiga toxin E. coli also refers to as Verocytotoxin-producing E. coli (VTEC). These STEC or VTEC are the causes of major, potentially fatal zoonotic food-borne illnesses, whose clinical spectrum includes diarrhoea,

haemorrhagic colitis (HC) and haemolytic uremic syndrome (HUS) (Karmali, 2003). Infections caused by STEC are considered a public health problem in both developed and developing countries because of the severity of the disease they cause and the global nature of the food supply chain (Brando et al., 2008). Sources of food STEC infections in humans include food of animal origin such as meat (especially ground beef), unpasteurised contaminated milk and other vehicles that have been contaminated with STEC like fresh-pressed apple cider, yogurt, and vegetables such as lettuce and other leafy greens. Water borne transmission and contact with infected animals are two routes that are becoming increasingly recognized. In addition to large wide spread outbreaks in the United States, outbreaks of STEC infection have been documented in at least 14 countries in a variety of settings including households day care centres, schools, restaurant, nursing homes and prisons (Karmali, 2003). The STEC severe gastro enteritis may cause life threatening HUS, the most serious complication of STEC infection. Most patients with HUS in developed countries have evidence of exposure to Shiga toxin-producing *E. coli* (O'Brien et al., 1998). Haemolytic uremic syndrome (HUS) is a leading cause of acute renal failure in children and occurs in about 6% of patients with STEC infection which occurred in Washington DC (Griffin, 1998,) up to 40% of patients with HUS develop long term renal dysfunction and about 3-5% of patient die during the acute phase of the disease (Karmali, 2003).

Although the main virulence factor of STEC is the production of one or more types of Shiga toxin (stx1 and stx2 or both) adherence to the intestinal epithelium and colonization of the gut are also important components of the disease. These STECs are not typically invasive and are restricted to the lumen of the gut, in some circumstance Shiga toxin (stx) produced within the intestinal tract is able to cross epithelial border and enter the blood stream (Brando et al., 2008). Both stx1 and stx2 are capable of crossing epithelial borders via an energy requiring process, and the toxin that moves across the border retains its biologic intestinal as well as systemic dysfunction (Brando et al., 2008). While the route that the toxin uses to pass across epithelial cell barriers is not well understood, it appears

to take a transcellular route. This notion is based on the observation that toxin movement is energy dependent and directional with greater toxin movement in the apical-basolateral direction vice-versa (Acheson et al., 1998).

Apart from the main virulence factors of STEC (stx), two additional markers also play role in the pathogenesis of HC and HUS. An outer membrane protein (intimin) encoded by the eae gene and enterohaemolysin encoded with hlyA gene (Paton and Paton 1998; Karmali et al., 2010). This genetic virulence characteristic is often used in epidemiological studies to correlate between strains from various sources (Askari et al., 2010). Molecular sub-typing techniques used in ten of 19 human incidents in Scotland showed that STEC O157 isolate from cattle and humans cases were indistinguishable (Synge et al., 1994). The STEC isolates from animals have been implicated as cause of diarrhoea and haemorrhagic colitis in humans (Gyles and Fairbrother 2004; Radostits et al., 2007; Islam et al., 2008). In Nigeria, studies conducted in different parts of Nigeria reported the isolation of E. coli O157:H7, non O157 and other pathogenic E. coli strains from human patients with diarrhoea, diarrhoeic calves and from human and cattle faeces. (Tekdek et al., 1995; Olorunshola et al., 2000; Okeke et al., 2003 ; Moses et al. 2005).

Rahimi et al. (2010) reported the prevalence of STEC O157:H7 in animals during processing in Iran. However, STEC are becoming the causes of serious public health concern in the world and are associated with food-borne outbreaks leading to the life threatening disease. The prevalence of STEC serotypes O157, O26, O91, O103, O111, O128 and O145 which are associated with public health risk has not been reported in chickens in Maiduguri. This study was conducted to isolate and characterize Shiga toxin producing E. coli (STEC) from chickens in Maiduguri, Nigeria

MATERIALS AND METHODS

The Study Area

This study was carried out in Maiduguri the capital of Borno State, and a commercial nerve centre of the North-eastern region of Nigeria. Maiduguri is located in the semi-arid zone of Borno state with an area 69,436km²and

lies between latitude 10-13°N and longitude 12-15°E (Google Maps, 2016). It lies within the savannah and Sahel vegetation and receives little rainfall. The area falls within the tropical continental north, with dry season of between four to seven months (November to May), followed by a short wet season from early June to late October. (Gisilambe, 1990). Borno State shares boundary with Chad to the north east, Cameroon to the east and Adamawa state to the south west. According to the census the population is estimated to be 4,558,668 and ranked 12th out of the 36 state in the country (Gisilambe, 1990).

Experimental Design and Sampling Technique

Samples were collected from two different sources; Broiler chickens and Village chickens. The samples were collected randomly using convenient sampling technique.

Sample collection

A total of three hundred chicken (300) faecal samples were collected at random from chickens brought for dressing to the Maiduguri Monday Market chicken slaughter house. One hundred and fifty (150) each of broiler chickens and village chickens were sampled. All samples were collected in sterile well labelled universal containers and were transported to the University of Maiduguri Veterinary Medicine laboratory on ice pack to avoid degeneration prior to analysis

Experimental Design and Sampling Technique

Samples were collected from two different sources; Broiler chickens and Village chickens. The samples were collected randomly using convenient sampling technique.

Identification of Micro organism

Gram staining

The Gram's stained glass slides were examined under the oil immersion object lens (X100) of a light microscope and pinkish rods were observed.

All the E. coli isolates obtained from the broiler and village chickens were pinkish rod shaped

Biochemical Test

Indole test

All the isolates obtained from broiler and village chickens were subjected to indole test. The isolates were incubated in peptone water for 48 hours at 37°C and Kovac's reagent was added which produced a red coloured ring compound indicating indole positive. All the E. coli isolates obtained from the broiler and village chickens were indole positive

Methyl red Test

The E. coli isolates obtained from broiler and village chickens were incubated in peptone water for 48 hours at 37°C. When 5 drops of methyl red reagent was added a distinct (bright) red colouration was observed indicating methyl red positive. All E. coli isolates obtained from the broiler and village chickens were methyl red test positive

Carbohydrate test

All the isolates were subjected to carbohydrate (glucose, lactose and sucrose) tests and gave colour change from a very slight orange to pink with the production of acid indicating a positive reaction. All the E. coli isolates obtained from broiler and village chickens were lactose positive

Serotyping of E. coli

Identification of E. coli O157

Presumptive E.coli colonies on nutrient agar (CM3, Oxoid) were picked and streaked on to sorbitol MacConkey agar cultures (SMAC) (CM813, Oxoid) and then incubated for 24 hours at 37°C. Escherichia coli O157 generally produce colourless colonies (Non-sorbitol fermenting colonies). When cultured on this media, E. coli distinguished from other Shiga toxin-producing Escherichia coli (STEC) serogroups and other micro-flora.

Detection of E.coli O157 Sero Type

The E.coli isolates were serotyped base on their somatic 'O' antigen. Oxoid latex agglutination test were conducted according to manufactures instructions. The component of the kit are; a test latex, which consist of blue latex particles sensitized with specific rabbit antibody reactive with the 0157 somatic antigen, control latex which consist of blue latex particles sensitized with pre-immune rapid globulin, positive control which consist of a suspension of inactivated E. coli 0157 cells in buffer, negative control suspension which consists of E. coli 0116 cells in buffer and disposable reaction card (Saline, wire loop and Bunsen burner required but not in the kit).

Test Procedures

A drop of the test latex was dispensed on to the edge of the a cycle on the reaction card and a drop of normal saline was also dropped on to the other edge of the cycle on the reaction card ensuring that the test latex and saline do not mix. Using a sterile wire loop, a portion of the colony to be tested was carefully emulsified in the saline drop ensuring that the resulting suspension was moved. After which, the test latex and the suspension were mixed together and sprayed to cover the reaction area using a sterile wired loop. The card was rocked in a circular motion to observe agglutination within sixty seconds. If positive, the portion of the colony is re-tested with control latex reagent to ensure that the isolate is not an auto-agglutinating strains.

Detection of Non 0157 STEC

Oxoid dry spot sero check agglutination test were used according to manufacturer's instructions. The E.coli isolates were sero typed based on their somatic 'O' antigen.

Materials

Materials provided in the kit included test cards sensitized with specific (blue latex) rabbit antibody reactive with the relevant serogroups of E.coli (as indicated on the test card) (dried unto card) (test reaction area). A blue

latex particle sensitized with Non-reactive rabbit globulin (control reaction area).

Pouches contain test card and moisture absorbent sachet. There are 3 test and 3 control reaction areas in each test card a positive control sticks, negative control sticks, a bottle of phosphate buffered saline (PBS) mixing, paddles and plastic pouch clips.

Test procedure

A drop of PBS was added to the small ring in both test and control area ensuring that the liquid does not mix with the dried latex reagents. Using a sterile loop, colony from the culture media plate was picked and applied to the control reaction area. The colonies were emulsified in the PBS to obtain a slightly turbid suspension ensuring that the resulting suspension is smooth. Using a sterile loop or paddle provided, the suspension was mixed into the dried latex spots until completely suspended and sprayed to cover the reaction area. The test card was picked and rocked in a circular motion for 60 seconds to observe agglutination under normal lightening condition.

Detection of Shiga Toxin E. coli.

Enzyme Immuno Assay using Oxoid Prospect Shiga toxin E. coli (STEC) microplate assay were used to confirm the presence of stx1 and stx2 in the preserved faecal samples according to manufactures instructions. ProSpecT Shiga Toxin E.coli (STEC) Microplate Assay

The ProSpecT™ Shiga Toxin E.coli (STEC) microplate assay was used to qualitatively detect Shiga Toxin (stx1 and stx2) in aqueous extracts of faecal specimens and broth enriched faecal cultures. The test was used as an aid in the diagnosis of enterohemorrhagic E.coli infections.

Principle of the test

ProSpecT Shiga Toxin E.coli (STEC) Microplate Assay is a solid phase enzyme immunoassay for detection of Shiga Toxins. Diluted specimens are added to break-away microplate wells on which rabbit polyclonal anti-Shiga Toxins 1 and 2 are bound. If toxin is present, it is 'captured' by the antibody. The wells are incubated and then washed to remove unbound

material. The enzyme conjugate (monoclonal mouse anti-Shiga Toxins 1 and 2 labelled with horseradish peroxidase enzyme) was added. The wells are incubated and then washed to remove unbound enzyme conjugate. In a positive reaction, the enzyme bound to the well by the toxin converts the substrate to a coloured reaction product. Colour development was detected visually and spectrophotometrically. In a negative reaction, there was no toxin or insufficient amount of toxin present to bind the enzyme conjugate to the well and no coloured reaction product develops.

Results

Growth on culture Media

Out of the total 300 faecal samples collected from broiler and village chickens, 110 (73.33%) and 91 (60.7%) were positive for lactose fermenting organisms respectively with button-like pinkish colonies on MacConkey agar (Appendix plates 1). On EMB agar, 47 (24%) and 36 (31.3%) of the previously lactose fermenting colonies obtained from broiler and village chickens respectively gave greenish metallic sheen confirming E.coli

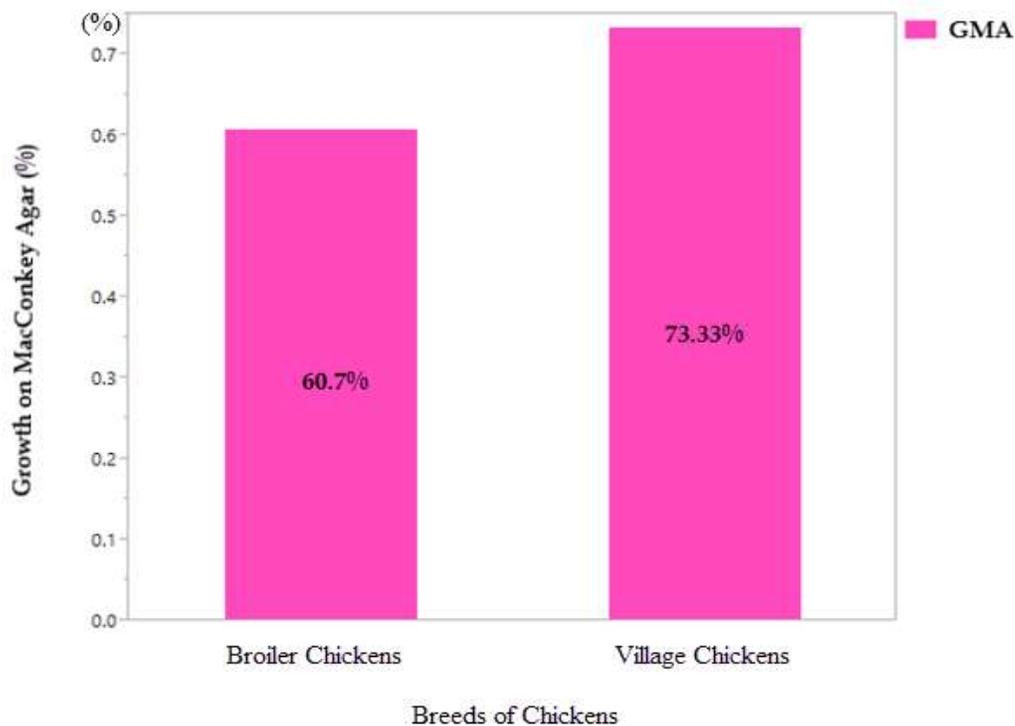


Figure 4. 1: Frequency of growth of Lactose Fermenting Bacteria on MacConkey Agar from faecal samples of Broiler and village chickens in Maiduguri

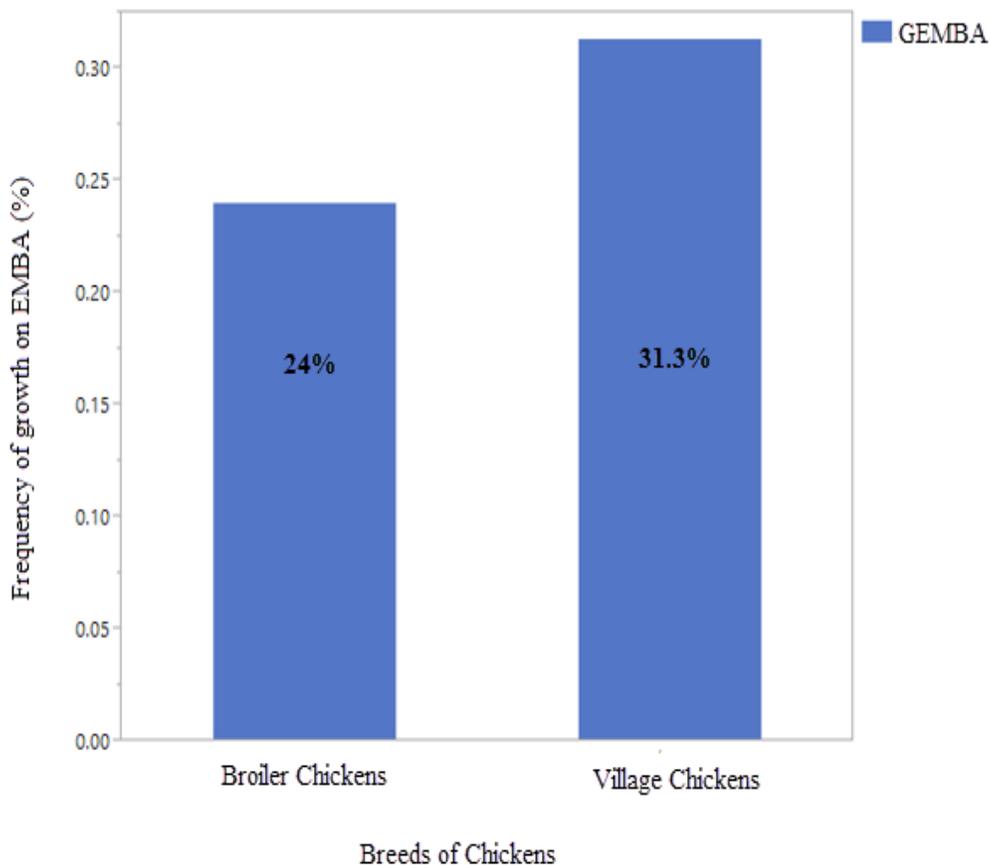


Figure 4. 2: Frequency of growth on Eosin Methylene Blue Agar (EMBA) of lactose Fermenting Bacteria obtained from faecal samples from Broiler and village chickens in Maiduguri

STEC Serotype

STEC in Broiler and village chickens

The results of the latex agglutination for O157 STEC and the polyvalent dry spot sero check for Non-O157 STEC are shown in Figure 4.3. The broiler chickens showed 19 (12.5%) and 8 (5.3%) positives for O157 and non-O157 STECs respectively. The village chickens had 26 (17.3%) and 9 (6%)

positive for O157 and non-O157 STECs respectively. The remaining 12 (8%) and 9 (6%) of the E.coli isolates obtained from broiler and village chickens cannot be serotyped with the conventional kit.

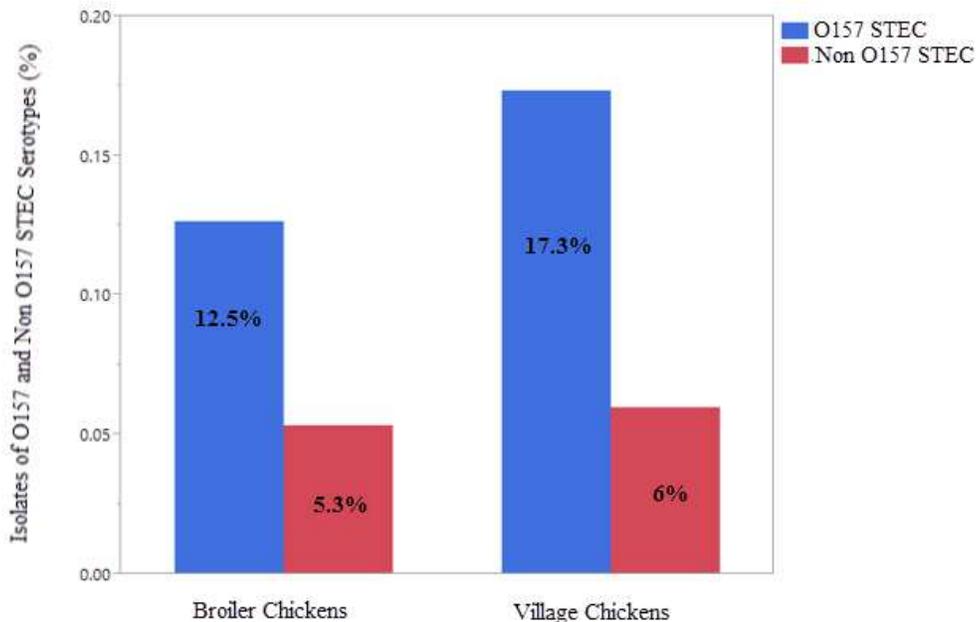


Figure 4.3: O157 and Non-O157 STEC serotypes isolated from Broiler and Village Chickens in Maiduguri

Enzyme immuno Assay for detection of Shiga Toxins

The results from the findings of enzyme immuno assay of Shiga toxin showed no yellowish colouration which indicates the absence of stx1 and stx2 from the faecal samples collected.

Discussion

E. coli is used as an indicator of faecal contamination of water bodies, vegetables, milk, and meat products. The ingestion of the E. coli contaminated foods can lead to detrimental health hazards. In this study, high percentage prevalence of O157 STEC serotypes were found among the E. coli isolated from broiler and village chickens with higher distribution of O157 STEC in village chickens. These findings disagree with the earlier reports by Blanco et al., (1998); Raji et al. (2007); Geidam et al. (2012) who reported that there is an apparent paucity of pathogenic E. coli in poultry environment. The inability to type the remaining E. coli isolates appear to

agree with the earlier reports by Blanco et al. (1998); Raji et al. (2007); Geidam et al. (2012) which stated that many *E. coli* strains cannot be typed. However, there was low prevalence of Non-O157 STEC serotypes isolated in broiler and village chickens compared to the O157 STECs in this study. The non-O157 serotypes isolated in this study are similar to those isolated in faeces of Camels, Cattles and humans in Maiduguri (Mosses et al., 2005; Sakuma, 2014).

Investigation on STEC serotypes O157 and Non-O157 (O26, O91, O103, O111, O128 and O145) STEC showed out of the 300 faecal samples collected at random from broiler and village chickens in Maiduguri, there was high prevalence of Shiga toxin-producing *E. coli* serotypes which has relatively higher prevalence of STEC in village chickens compared with the STEC obtained from broiler chickens.

Ruminants have been identified as the major reservoir of *E. coli* O157 and also appear to be a reservoir Non-O157 STEC (Wieler, 2003) although Non-O157 STEC has been detected in non-ruminant animals (Makino et al., 2000). 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 faecal materials from ruminants. This maybe the possible reason why STEC was isolated more in village chickens which are free-range domestic animals than in the broilers.

The findings of the present study showed the absence of the virulence genes *stx1* and *stx2* in the faecal samples of broiler and village chickens in Maiduguri using an enzyme immuno assay. This could be that the faecal samples had insufficient amount of toxins to bind with the enzyme conjugate in the well to produce the coloured reaction or the STEC strains isolated in the faecal samples of the broiler and village chickens in Maiduguri are not *stx* producers. However, the findings agrees with the findings of Kobayashi et al. (2002) who reported absence of STEC in 199 broiler chickens 32 pigeons and 86 gulls in Finland. Schroeder et al. (2003) also failed to isolate any STEC in retail chicken and turkey obtained from Washington DC USA. The result agrees with the findings of Persson et al. (2004) who reported that not all O157 strains are *stx* producers.

Conclusion

The findings of this study revealed that Shiga toxin-producing *E. coli* sero groups are found in the faecal samples of broiler and village chickens in Maiduguri with relatively higher prevalence of the O157 STEC strains in Village chickens. However, the virulence genes *stx1* and *stx2* were absent when detected with ELISA in this study. This study is the first to report the isolation of O157 and Non-O157 STEC serotypes in chickens in Maiduguri.

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