Isolation of Escherichia coli O157:H7 in Poultry by Culture, Serology and Polymerase Chain Reaction Technique in Jos, North Central Nigeria

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SUMMARY
Escherichia coli O157:H7 is an important human pathogen capable of causing food borne infections with severe consequences of haemorrhagic colitis (HC), haemolytic uremic syndrome (HUS) and haemolytic thrombocytopenic purpura. Information about isolation and characterisation of the organism in poultry in North Central Nigeria is lacking. An isolation and characterisation of E. coli O157:H7 was carried out in four hundred and thirty-three (433) samples (167 fresh faeces, 217 cloacal and 49 laryngeal swabs) from intensively reared chicken from 20 farms in Jos, Central Nigeria. Microbiological culture, serology and polymerase chain reaction (PCR) methods were used. Out of the 433 samples, 26 (6%) of the isolates were phenotypically identified as E. coli O157:H7 on cefixime-tellurite Sorbitol MacConkey (CT-SMAC) agar. None of the isolates was positive by serology using Wellcolex* Escherichia coli O157:H7 kit R30959601 (Remel Europe Ltd, Dartford Kent UK) and for rfbEO157 and fliCh7; the defining genes for E. coli O157:H7 serotype on further molecular analysis. Of the 26 isolates, 5 (19.2%) were from fresh faeces while 19 (73.1%) and 2 (7.7%) were from cloacal and laryngeal swabs respectively. Although E. coli O157:H7 was phenotypically found, we could not ascertain if intensively reared chicken in Jos harbour E. coli O157:H7 based on the absence of serotype specific genes rfbEO157 and fliCh7 by PCR analysis.

Key words: Escherichia coli O157:H7, poultry, polymerase chain reaction (PCR), cefixime-tellurite Sorbitol MacConkey (CT-SMAC) agar.

INTRODUCTION
Escherichia coli O157:H7; a member of the verocytotoxin-producing E. coli family has been identified as a food borne pathogen capable of causing serious morbidity and mortality (Wani et al., 2004). Severe diarrhea resulting from infection with E. coli O157:H7 may lead to life threatening complications including haemorrhagic colitis (HC), haemolytic uremic syndrome (HUS) and haemolytic thrombocytopenic purpura (Wang et al., 2014). Over 200 outbreaks of E. coli O157:H7 associated infections have been reported in 30 countries worldwide (Doyle et al., 2006; Mead and Griffin,
Contaminated foods and water are major source of infection especially foods of bovine origin (Nataro and Kaper, 1998). Other vehicles of transmission include contaminated milk and milk products, unpasteurized juice, raw vegetables such as lettuce, radish sprouts, alfalfa sprouts, spinach, cucumber, cabbage and bitter leaf (Michino et al., 1999; Reuben and Makut, 2014; Albarri et al., 2017).

Ruminants especially cattle have been established as major reservoir of E. coli O157:H7 (Witold and Carolyn, 2011). In addition, some earlier studies (Dipineto et al., 2006; Kalin et al., 2012) reported isolation of E. coli O157:H7 in living layer hens and post mortem samples of broiler liver and caecum and from contaminated poultry meat and faeces (Olatoye et al., 2012; Aibinu et al., 2007). Some of the isolates from the aforementioned studies possessed hlyA (enterohaemolysin) and eaeA (intimin) genes which are known virulence factors of human pathogenic strains; bringing to the fore public health concerns of cross transmission (Wang et al., 2002; Rwego et al., 2008). The increasing use of untreated organic poultry manure to fertilize fish ponds, vegetable and crop farms in the area of study coupled with the ability of E. coli O157:H7 to survive in low acidic pH and the requirement for low infective dose (Nataro and Kaper, 1998) to cause an infection warrants an investigation into the role of poultry in the epidemiology and human transmission of E. coli O157:H7. There are limited reports of isolation and characterization of E. coli O157:H7 in poultry especially in Nigeria. In addition, most poultry farms in the study area are located within or near residential quarters and in close proximity to rivulets which serve many purposes to the nearby dwellers including as drinking water or for cooking.

The aim of this study was to determine the presence of E. coli O157:H7 in intensively reared chickens in Jos, North Central Nigeria and to investigate the isolates for the presence of some E. coli O157:H7 virulence associated genes (stx1, stx2, hlyA and eaeA).

### MATERIALS AND METHODS

#### Study Area:
Jos is located in Plateau State, central Nigeria on an elevation of 1,217m (3,993 ft) above sea level between latitude 9° 48’ 00”N and 8° 52’ 00”E. It has a land area of about 8,600km2 or 860,000 hectares and a population of about 900,000 people (based on 2006 national census). Its weather is suitable for poultry, livestock, crop and vegetable farming. Poultry farming is a mainstay of many families both as a hobby and on commercial basis located within and close to residential areas, rivulets and ponds.

#### Sample Collection:
Four hundred and thirty-three (433) samples (167 or 38.6% of freshly voided faeces, 217 or 50.1% cloacal and 49 or 11.3% laryngeal swabs) from apparently healthy intensively reared chickens were collected from 20 farms by a random sampling method from the different geographical locations within Jos between May and October, 2015. Samples were preserved on ice at 4°C in a cooler and transported to the laboratory within 1 to 2hrs.

#### Sample processing:
In the Laboratory, 25g of faecal sample was enriched in 225ml modified tryptone soy broth (mTSB) by supplementation with novobiocin 10mg/L (Oxoid Ltd, Hamshire, England). Each swab sample was also enriched in 9ml of mTSB. A positive control; E. coli O157:H7 (LMG 21756 or ATCC 700728) was reconstituted in nutrient broth (Oxoid CM3) as recommended by the manufacturer (BCCM). Samples were incubated aerobically for 18-24hrs at 37°C. The isolates...
were biochemically screened for indole production, hydrogen sulfide production in triple sugar iron (TSI) agar, citrate utilisation, urease production, acid production on inositol and acid and gas production from glucose and for motility on sulfide indole motility medium. Positive and negative controls were processed using same protocol.

**Serology**
All CT-SMAC negative isolates and non-sorbitol (NSF), non-lactose fermenting (NLF) isolates confirmed as E. coli biochemically were screened serologically using Wellcolex® Escherichia coli O157:H7 kit R30959601 (Remel Europe Ltd, Dartford Kent, UK). Wellcolex® E. coli O157:H7 kit is a rapid latex agglutination test for the presumptive identification of Escherichia coli O157:H7 isolates on laboratory media. The test contains two test reagents. The somatic (O157) antigen reagent which consists of red latex particles coated with antibodies specific for E. coli O157 (O) antigen. When a drop of the reagent is mixed on a card with a suspension of E. coli O157 organisms, positive test is indicated by rapid agglutination of specific IgG and O157 lipopolysaccharide antigen. The H7 test reagent also consists of blue latex coated with antibodies specific for the flagellum antigen; (H7) antigen. The agglutination reaction was controlled by use of normal saline on a drop of emulsified fresh colony grown on nutrient agar (Oxoid). Positive and negative control kits are provided in the Wellcolex® Escherichia coli kit R30959601 (Remel Europe Ltd, Darford Kent, UK). The instruction of the manufacturers on choice of media and laboratory practice was followed strictly.

### DNA Extraction
Three (3) colonies of the non-sorbitol fermenters phenotypically identified as E. coli O157:H7 were sub-cultured into mTSB and incubated for 24hrs at 37 0C. The positive control strain of E. coli O157:H7 was also processed in parallel at the same time. DNA was extracted from 3ml of broth using ZR Fungal/Bacterial DNA KitTM (Catalog No D6005) according to the manufacturer’s instruction. The eluted DNA was stored at -80 0C until ready for PCR analysis. Genes, base sequences and predicted sizes of amplified products for the specific oligonucleotide primers used in this study are shown in Table I.

### TABLE I: Genes, sequences and amplicon sizes of primers used in investigating Escherichia coli O157:H7 in Jos, Nigeria

| Genes  | Sequence (5´ to 3´)                               | Size of PCR amplicon (bp) | Reference          |
|--------|--------------------------------------------------|---------------------------|--------------------|
| 16S rRNA | F- CCCCCTGGACGAAGACTGAC  R- ACCGCTGGCAACAAAAGGATA | 401                       | Wang et al.,(2002) |
| rfbEO157 | F- CTACAGGTGAAATGGATGAAATGG  R- ATTCCTCTTTTCTTCTTGCGG | 327                       | Wang et al.,(2002) |
| fliCh7  | F- TACCATTCGCAAAGCAACTCC  R- GTCGGCAACGTTAGTGATACC | 247                       | Wang et al.,(2002) |

### Multiplex Polymerase Chain Reaction (mPCR)
Primers (Table 1) used for this reaction were as designed by Wang et al., (2002) and supplied by Inqaba SA. E16S rRNA was added as internal control. Positive control used for this reaction was also E. coli O157:H7 non toxigenic strain LMG 21756 while sterile nuclease free water (Biolabs SA) served as negative control. Amplification of bacterial DNA was performed in GeneAmp® 9700 (Applied Biosystems) using 25µl volume of master mix containing 3µl of prepared sample supernatant; 2.5µl of 10X PCR buffer, 1.5µl Magnesium chloride, 0.125µl of each of the four deoxynucleotide triphosphates (dNTPs), 0.3µl fliCh7, 0.9µl rfbEO157, 0.2µl E16SrRNA and 0.25µl Taq polymerase at an initial denaturation temperature of 95 0C for 8mins. This was followed by 30 cycles of denaturation at 95 0C for 30sec; annealing at 58
0C for 30sec and extended at 72 0C for 30sec. Final extension was at 72 0C for 7min. The amplified products were visualized by electrophoresing 10µl of the amplicons in 1X agarose gel (1.5% 1X TBE Electrophoresis Buffer; Fermenters®) stained with 5µl of 10mg/ml ethidium bromide (Promega) at 130 volts for 45min (Bio-Rad): 50 lanes DNA molecular ladder (Fermenters®) was used as marker. The amplified DNA fragments were visualized through U.V. transilluminator (Sigma®) and results were documented using GelDoc™ XRT Image Lab™ software Documentation System (Bio-Rad).

STATISTICAL ANALYSIS
Data were analysed using SPSS version 23 statistical package to test for statistical significance.

RESULTS
Out of 433 combined samples of fresh faeces, cloacal and laryngeal swabs investigated, 26(6%) isolates were phenotypically identified as E. coli O157:H7 by characteristic appearance on CT-SMAC agar, confidence interval (0.46,0.55); (Table II). Of the 26 non-sorbitol fermenting (NSF) isolates, 73%(19) were isolated from cloacal swabs. Faeces and laryngeal swabs had 19.2 %(5) and 7.7%(2) isolates respectively; (Table III).

Serology
All 26 non-sorbitol fermenting (NSF) isolates were negative on Wellcolex® Escherichia coli O157:H7 kit R30959601 (Remel Europe Ltd, Darford Kent, UK).

Multiplex polymerase chain reaction (PCR)
All 26 isolates were amplified at 401bp confirming them primarily as E. coli and none was positive for rfbEO157 and fliCh7. Positive control E. coli O157:H7 (LMG 21756) was amplified to 327bp and 247bp respectively; (Plate 1).

**Table II: Identification of presumptive Escherichia coli O157:H7 by sorbitol fermentation**

| Location (District) | No. of Farms | No. of Samples | NSF(%) |
|--------------------|--------------|----------------|--------|
| Du                 | 5            | 103            | 3(2.9) |
| Zawan              | 4            | 92             | 6(6.5) |
| Kuru               | 4            | 76             | 3(3.9) |
| Vwang              | 3            | 80             | 8(10)  |
| Gyel               | 4            | 82             | 6(7.3) |
| **Total**          | **20**       | **433**        | **26(6)** |

DISCUSSION
Attempts at isolation of E. coli O157:H7 in avian species have yielded varied results globally. Compared to cattle and sheep considered as natural reservoirs of this organism, limited information on characteristics and prevalence of shiga-toxin producing E. coli (STEC) in avian species is available (Nataro and Kaper, 1998). Reports of colonization of chicken cecae, and faecal shedding of E. coli O157:H7 suggested that chicken could serve as host or reservoir host for E. coli O157:H7 (Dipineto et al., 2006).

This study did not yield any confirmed isolate of E. coli O157:H7 in the area studied. All 26 non-sorbitol fermenting (NSF) E. coli isolates which were presumed positive phenotypically were negative by serology and PCR using Wellcolex® Escherichia coli O157:H7 kit R30959601 (Remel Europe Ltd, Darford Kent, UK) and E. coli O157:H7 serotype specific genes
TABLE III: Distribution of Non-sorbitol fermenting *Escherichia coli* in relation to geographic location and source of sample

| Location (District) | No. of Farms | Source of Samples | No. (%) of NSF *E. coli* | Total |
|---------------------|--------------|------------------|--------------------------|-------|
| Du                  | 5            | Faeca 36, Cloaca 46, Larynea 21 | 103 3(2.9) | 3(2.9) |
| Zawan              | 4            | Faeca 33, Cloaca 41, Larynea 18 | 92 3(3.3) | 2(2.2) |
| Kuru                | 4            | Faeca 36, Cloaca 30, Larynea 10 | 76 3(3.9) | 3(3.9) |
| Vwang               | 3            | Faeca 50, Cloaca 30, Larynea 0 | 80 3(3.8) | 5(6.2) |
| Gyel                | 4            | Faeca 50, Cloaca 32, Larynea 0 | 82 1(1.2) | 5(6.1) |

95%CI-Du 0.18, 0.26; Zawan 0.57, 0.66; Kuru 0.50, 0.59; Vwang 0.00, 0.00; Gyel 0.00, 0.00

NSF= non-sorbitol fermenting

95%CI= 95% Confidence interval

rfbEO157 and fliCh7 (Plate 1). The contents of CT-SMAC media inhibits partially or completely growth of other *E. coli* strains (Zadik et al., 1993), hence most other strains were not expected to grow in such media. However, Park *et al.*, 2011 reported false positive results on CT-SMAC medium from non-sorbitol fermenting bacteria such as *Hafnia alvei*, *Proteus spp*, *Providencia spp*, *Aeromonas spp* and *Morganella morganii*. In this study, all 26 NSF isolates were positive for E16SrRNA gene confirming all primarily as *E. coli*. Thus, the phenotypic similarities with *E. coli* O157:H7 on CT-SMAC agar may suggest the presence of other *E. coli* serotypes other than O157 which shares phenotypic characteristics on CT-SMAC (March and Ratnam, 1986).

Related studies in other parts of the world yielded negative findings. In a one year study of 1000 faecal samples in the United Kingdom (UK) by Chapman *et al.*, (1997) they were unable to find *E. coli* O157:H7. In the Czech Republic, Cizek *et al.*, (1999) did not find *E. coli* O157:H7 in 50 pigeons and 20 sparrows respectively. Hajian *et al.*, (2011) and Miri *et al.*, (2014) reported no isolation of *E. coli* O157:H7 in 82 chicken meat samples and 70 chicken nugget samples in Iran. Although, in Ado-Ekiti, South West Nigeria; Oluyege and Famurewa (2015) reported non
isolation of verotoxigenic *E. coli* in intensively reared layer hens and broiler chickens, they found *E. coli* O157:H7 and non O157 in local chickens reared under free range systems which they probably acquired from the environment where ruminants are also kept and grazed.

In contrast to our findings, some positive results of isolation of *E. coli* O157:H7 have been reported by other researchers in poultry and other avian species. Kalin *et al.*, (2012) had a 0.1% and 0.4% isolation of *E. coli* O157:H7 from liver and caecum samples respectively of 1000 chickens investigated in Turkey. All the isolates were positive for *eaeA* gene; a virulence factor responsible for human infections (Wang *et al.*, 2002). Also in Turkey, Akkaya *et al.*, (2006) isolated *E. coli* O157:H7 from 2 of 190 chicken carcase samples. The isolates were positive for *stx1* and *stx2*, an indication that poultry meat can also be a source of infection with *E. coli* O157:H7. Similarly, Dipineto *et al.*, (2006) reported 3.6% (26/720) isolation of *E. coli* O157:H7 in intensively managed living layer hens in Italy. The isolates were positive for *stx2, eaeA* and *hlyA* genes. This finding is significant because of the presence of the virulence factor enterolysin (*hlyA*) which often accompany human pathogenic strains (Dipineto *et al.*, 2002). Akbar *et al.*, (2014) reported 2% isolation of *E. coli* O157:H7 from 152 poultry meat samples in Pakistan. Wang *et al.*, (2014) reported isolation of 1.43% in 140 faecal samples and 1.67% in 60 chicken meat samples in Eastern China. In the UK, Wallace *et al.*, (1996) isolated *E. coli* O157:H7 from faecal samples of gulls.

In Africa, Raji *et al.*, (2006) reported a 9.6% isolation of *E. coli* O157:H7 in free range chicken raised integrated with goats and cattle in Morogoro, Tanzania. This high rate of isolation may be explained by the management system which allowed the chicken access to pastures and environment contaminated by goat and cattle faeces. Kagambega *et al.*, (2012) also reported 6% prevalence of STEC in chicken carcasses slaughtered in Burkina Fasso. In Nigeria, reports on isolation of *E. coli* O157:H7 in avian species is scanty. Aibinu *et al.*, (2007) reported 10% isolation from 50 faecal samples of chicken in Lagos using serological methods. This rate is high compared to 2.5% isolation in free ranged chicken by Oluyege and Famurewa (2015) in Ado-Ekiti, South West Nigeria. Elsewhere, Fuh *et al.*, (2018) reported 6.67% and 1.94% isolation of *E. coli* O157:H7 from faeces in free-ranged and intensively reared chicken in Cross River; South-South Nigeria. The non-isolation of *E. coli* O157:H7 by Oluyege and Famurewa (2015) in intensively managed chicken is in agreement with our findings where all the 26 *E. coli* NSF isolates was negative for *E. coli* O157:H7 by serology and PCR. This result is suggestive that intensively managed chicken may not be carriers or reservoir host for *E. coli* O157:H7 in Jos Central Nigeria. Further research is required to support this finding. The variation in isolation rates observed globally could be attributed to the sensitivity of the methods of isolation. Methods involving enrichment with trypton soy broth (TSB), immunomagnetic separation (IMS) and hydrophobic grid membrane filter-immunoblot procedures and PCR produce a higher isolation rate (Wallace *et al.*, 1997).

**LIMITATIONS**

This research was limited by funds. This reduced our ability to characterize the isolates for virulence genes and full identification of the isolates.

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