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Prevalence and Characterization of Verotoxigenic *Escherichia coli* O157 Isolated from Local Chicken in Morogoro, Tanzania

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Abstract: The role of the chicken as a source of verotoxin producing *Escherichia coli* O157 was studied in Morogoro, Tanzania. Intestinal contents of slaughtered chickens (n = 120) in restaurants selling chicken dishes in Morogoro were analyzed for STEC including *E. coli* O157. The isolates were tested for shiga toxin (stx1 and stx2), enterohaemolysin (*ehly A*) and attachment and affacing (*eae A*) genes by Polymerase Chain Reaction (PCR). Eleven of 120 samples examined 9.6% were positive for *E. coli* O157. Other bacterial agents such as *Proteus* sp., *Pseudomonas* sp. and coliforms were isolated in this study; all VTEC isolates produced both verotoxins (ST1 and ST2). Based on the Reverse Passive Latex Agglutination Assay (RPLA). The isolates were subjected to PCR for the detection of stx1, stx 2, eae A and *ehly* A genes. None of the *E. coli* O157 strains isolated showed the presence of stx 1, stx2, *Ehly* A and eae A genes by PCR. The antibiotic susceptibility tests showed that the isolates were susceptible to most of the antimicrobial agents tested. This study is the first attempt to investigate VTEC O157 prevalence in chickens in Tanzania. The chicken can be considered as an important carrier of VTEC in Tanzania but its pathogenicity for humans in this environment is questionable based on the presence of low virulence factors in these isolates. There is a need for further studies to elucidate on the role of chickens in the maintenance and transmission of VTEC O157 to humans in Tanzania.

Key words: Chicken, tanzania, PCR, verotoxigenic producing *Escherichia coli* O157

INTRODUCTION

Verotoxigenic *E. coli* (VTEC) are a major cause of food-borne disease in humans. Since it was first described[1], Escherichia *coli* VTEC O157 have been linked to a range of disorders in humans such as Haemorrhagic Colitis, (HC) Haemolytic Uremic Syndrome (HUS) and Thrombotic Thrombocytopenic Purpura (TTP)[2]. Although O157:H7 serotype is the predominant VTEC in many parts of the world, it is now recognized that VTEC strains belong to a broad range of O:H serotypes[3]. The pathogencity of these bacteria is mainly mediated by shiga toxins (Stx1, Stx2 and their variants) encodes by stx1 and stx2 genes and products of the Locus of Entreocyte Effacement (LEE), the pathogencity island, with the *eae A* gene that encodes for the intimin protein involved in the intimate adhesion of bacteria to enterocytes and the production of Attaching and Effacing (AE)[4, 5]. Observed that a high proportion of VTEC strains had a novel haemolytic phenotype conferred by the production of enterohaemolysin called EHEC-Hly and coded by the *ehly A gene*. VTEC can potentially enter the human food chain from a number of animal sources, most commonly by the contamination of meat with faeces or intestinal contents after slaughter, particularly during evisceration[6]. There are many reports of isolation and characterization of VTEC from cattle and sheep, which are considered as the main reservoirs[7-9]. However, goats, horses, dogs and deer also harbour these bacteria[10,11]. With regard to poultry, a limited number of surveys have been published on the prevalence and characteristics of VTEC O157 in the live chickens and on processed poultry meat[12-14]. In Tanzania, no reports are available on the prevalence and molecular characteristics of VTEC in avian species. The aims of this study was, therefore, to determine the prevalence of VTEC O157 in poultry intestinal contents, especially in chickens at slaughter and in restaurants as potential factors for carcass contamination and to investigate their virulence genetic profiles including *stx, eae A* and *Ehly A*.

MATERIALS AND METHODS

Sample collection: A total of 120 intestinal content swabs were obtained from chickens slaughtered for used in the

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restaurants in the town of Morogoro. These birds were clinically healthy. These were raised on free-range, in integration with goats and cattle that exist in the farm typical of village production system. Most of the chickens were left to scavenge during the day and were provided with simple housing at night 95.2% of the owners. Only small amounts of supplementary feeds were occasionally given and minimal health care was provided. The swabs were placed into aliquots of 10 mL of modified Tryptose Soya Broth (mTSB) (Oxoid, UK) and immediately transported to the laboratory and processed within 2 h after collection. After mixing the swabs were incubated at 37°C for 24 h.

Preliminary screening of the samples: The samples were first screened by inoculating them onto the surface of Cefixime-tellurite Sorbitol-MacConkey agar (CT-SMAC) with 5-bromo-4-chloro-3 iodo-'β-D-glucuronide (BCIG) (Oxiod, UK). After an incubation period of 24 h, suspected (*E.coli* O157) colonies (mixed cultures of non sorbitol fermenting with pink or purple colonies) were then picked from the CT-SMAC-BCIG and inoculated onto mTSB broth for Immunomagnetic Separation (IMS)[15].

Isolation of O157:H7 VTEC by IMS: After 8 h of incubation of suspected colonies from preliminary screening of the samples on CT-SMAC-BCIG agar was inoculated onto mTSB. The aliquots of 5 mL of each broth culture was used for Immunomagnetic Separation (IMS) using magnetic beads coated with antibody to O157 (Dynal), according to the instructions of the manufacturer. The concentrates were inoculated onto CT-SMAC and the plates incubated at 37°C for 20 h. The β-glucuronidase activity was accessed using CT-SMAC containing BCIG (Oxiod, UK) (2). Presumptive O157:H7 VTEC isolates (those with a typical *E. coli* metallic sheen on Eosine Methylene Blue (EMB); and isolates that gave agglutination with *E. coli* latex test kit (Oxoid) and were BCIG negative was confirmed to be *E. coli* by using biochemical tests for lactose and sucrose fermentation in Triple Sugar Iron agar (TSI, Difco) slants, indole production, methyl red and Voges Proskauer reactions, citrate utilization (IMViC tests) and pink mauve on CHROM^R agar TM O157[16].

Serotyping of *E. coli*: O and H grouping was carried out by bacterial agglutination with antiserum against *E. coli* groups O157 and H7. Strains that gave clumping with 4% saline were defined as rough. The O157 and H7 antigens were tested with the *E. coli* O157 antigendetection kit Oxoid, Hampshire, England[17].

Verotoxin production: The ability of the isolate to produce Stx1 and/or Stx2 was determined by a reverse latex agglutination test (Vertox F; Deka Seiken, Tokyo, Japan) according to the manufacturer's instructions[18].

Antimicrobial sensitivity: Antimicrobial susceptibility testing was done using the selected antimicrobial agents for the treatment of diarrhoea in humans. An 8 to 12 h broth culture was prepared for eleven isolate obtained from the chickens in Morogoro. Using a sterile cotton swab, an entire surface of dried Muller-Hinton agar plate with 4 mm of agar depth, was streaked uniformly with the swab, previously dipped in the test *E. coli* culture suspension, after squeezing off extra fluid on the wall of the tube. The inoculated plate was allowed to dry for 5 min and appropriate antibiotic disks from commercial sources were applied using sterile forceps and then incubated at 37°C overnight[19]. The antibiotic disks used were: amoxyslav, cephtriaxone, norfloxacin, ofloxacin, nitrofurantoin, nalidixic acid, gentamicin, Cephalexin and sulphamethoxazole supplied by Hi-media Laboratory Ltd., India. Streptomycin, chloramphenicol, oxytetracyclines and neomycin were from Oxoid UK. The inhibition zones were interpreted by measuring the diameter of the zone of inhibition. For analytical purposes, isolates that were moderately sensitive were taken as fully sensitive.

Polymerase Chain Reaction (PCR). Primers: For PCR analysis, the primer sequences selected for the amplification of the stx1, stx2, eae A and *EhlyA* genes matched the sequences of the corresponding genes encoding stx toxin, *eae A* gene of EPEC and haemolysin, *Ehly A* in the GenBank/EMBL database libraries. The oligonucleotides used as primers were purchased from Synthengen®, USA.

Processing of samples for PCR: The bacterial isolates were cultured on Sorbitol MacConkey agar at 37°C for 24 h. A loopful of bacterial culture from the agar plate was suspended in 200 μL of sterile distilled water in Eppendorf micro centrifuge tube and boiled in a water bath at 80°C for 20 min and then centrifuged at 12,000 rpm. The supernatant served as the DNA source[20].

PCR protocol: All reactions were performed using USA Technologies Rapid Cycler® MJ Research brand thermolcycler. The PCR mixture consisted of 1 μL of 10X PCR buffer, 0.2 μL of Taq polymerase, 5.98 μL of double deionized DNA free water, 0.8 μL of 2.5 mM $MgCl_2$, 0.02 μL of 0.2 mM (dATP, dCTP, dGTP and dTTP), 0.5 μL of 100 pmole (each) of the Stx-specific primer pair and 1 μL

Table 1: Primers nucleotide sequence, primer size and region amplified on the DNA

Primer	Primer sequences	Length	Amplified region
Stx1	5'ACCCTGTAACGAAGTTTGCG3'	140bp	215 to 234
Reversed	5'ATCTCATGCGACTACTTGAC3',		1089 to 1109
Stx2	5'ATCCTATTCCCGGGAGTTTACG3'	584bp	288 to 307
Reversed	5'GCGTCATCGTATACACAGGAGC 3'		747 to 766
Eae A	5'CCCGAATTCGGCACAAGCATAAGC3'	800bp	2366 to 2386
Reversed	5'CCCGGATCCGTCTCGCCAGTATTCG3'		2776 to 2754
EhlyA	5'GGTGACGCAGAAAAAGTTGTAG 3'	1551bp	1054 to1074
Reversed	5'TCTCGCCTGATAGTGTTTGGTA3'		1869 to 1849

Table 2: The programme used in the present study

	Reaction temperature (reaction time, sec) for 30 cycles each				
	Denaturation	Annealing	Extension	Size of products (bp)	References
Stx1	94(60)	55(60)	72(60)	140	29
Stx2	94(60)	55(60)	72(60)	584	29
Eae A	94(60)	53(60)	72(90)	800	22
EhlyA	94(30)	57(60)	72(90)	1551	38

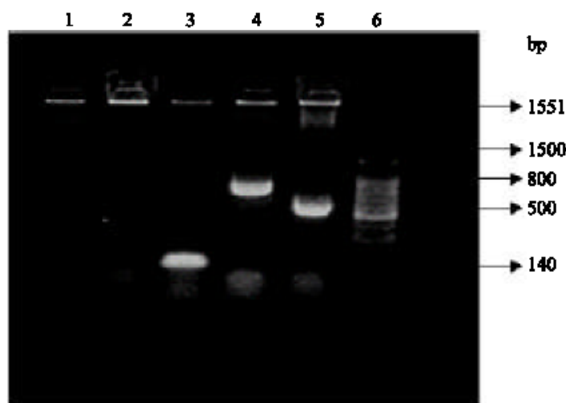

Fig. 1: DNA profiles of virulence genes associated with *Escherichia coli* O157:H7 isolated from poultry. Lane 6: shows the molecular marker of 100 bp DNA ladder size, lane 5: shows Stx 2 gene (584 bp), lane 4: shows the *eae A* gene (800 bp), lane 1: shows *Ehly A* gene (1551 bp), lane 3: shows the Stx1 genes (140 bp) of positive samples of cattle isolated from the same area. Lane 2: shows negative results. None of the poultry isolates showed any of these products

of DNA in a final volume of 10 µL. In the PCR assays, DNA was amplified by stx 1, *ehly* A, stx2 and *eae A* primers separately. The PCR was started with 0.2 of 5 U of Taq polymerase (Promega^R, USA). And the PCR mixture was overlaid with mineral oil and run in the thermalcycler[21,22]. The PCR primers and programs used in the present study were as shown in Table 1 and 2.

Electrophoresis of amplified products: Aliquots of 10 µL of PCR amplified products were analysed by electrophoresis in 1.5% agarose gels (SeaKem; FMC Bio-products, Rockland, Maine USA). The gels were stained with ethidium bromide (0.5 µg mL^{-1}). 1XTris-borate/EDTA electrophoresis buffers were used and the electrophoresis was run at 100 V for 30 min. The gels were then photographed under UV transillumination. A 100 bp molecular weight marker (Promega® USA) was run with each gel. Positive samples were identified based on the presence of bands of appropriate sizes for stx 1, stx2, *ehly A* and *eae A*.

RESULTS

Isolation rate of *Escherichia coli*: A total of 78 *E. coli* colonies were isolated from intestinal contents of healthy chickens (n = 120) from Morogoro. The result showed that *E. coli* was the predominant isolate, in this study, representing 65% of the total faecal sample collected. The isolates exhibited three distinct colonial morphology on CT-SMAC-BCIG. The non-sorbitol fermenting and glucuronidase negative isolates accounted for 14%, while the sorbitol positive and glucuronidase positive isolates, which appeared as purple colonies on CT-SMAC-BCIG, accounted for 37% Table 3. The sorbitol positive and glucuronidase negative isolates, which appeared as pink colonies accounted for 49% Table 3. Other bacteria, such as *Pseudomonas* sp., were isolated in 0.83% of chicken samples Table 4. This was based on colonial morphology, sorbitol negative and glucuronidase negative properties of Verotoxigenic *E. coli*, which appeared as colourless on Sorbitol MacConkey agar with BCIG. This was further confirmed on the basis of a greenish metallic sheen produced on EMB and pink mauve on CHROM® agar TM O157. The isolation rate of verotoxigenic *E. coli* based on colonial morphology from chickens was 9.16%.

Serotyping of *Escherichia coli*: The serotyping of *E. coli* O157 was demonstrated using the Oxoid latex agglutination kit. All the isolates from chicken agglutinated with *E. coli* O157 latex Table 5. Three of the isolates from chickens autoagglutinated with O157 latex. All of the Presumptive verotoxigenic *E. coli* isolated from chickens were therefore *E. coli* O157 on the basis of the agglutination with the Oxoid *E. coli* O157 latex test kit. Three of the chicken isolates were rough strains, on the basis of autoagglutination with 4% saline. The presence of verotoxin was demonstrated with reverse passive latex verotoxin agglutination kit from Denka Seiken Japan. All the *E. coli* isolates from humans and animals were verotoxigenic on the basis of agglutination with this test.

Table 3: Characterization of *Escherichia coli* from humans and animals in Tanzania

Sample origin	Location	Colonial morphology on SMAC-CT after IMS NSF	SF	P	Total number of isolates	Total samples
Chicken	Morogoro restaurant	11	38	29	78	120

NSF = Non-sorbitol fermenting and gluruonidase negative *Escherichia coli* (colourless), SF = Sorbitol Fermenting and glucuronidase negative *Escherichia coli* (pink), P = Sorbitol fermenting and glururonidase positive *Escherichia coli* (purple)

Table 4: Distribution of other enteric pathogens from chickens in Tanzania

Bacterial species	Number
Pseudomona sp.	1
Proteus sp.	11
Coliforms	22
S. dysenteriae	0
Total	34

Table 5: Antibiotic sensitivity pattern of *Escherichia coli* O157

Antimicrobial agent	Chicken (n = 11) Resistant
Amoxyslav	81.81%
Nitrofurantoin	0
Nalidixic acid	0
Norfloxacin	0
Gentamicin	0
Ofloxacin	0
Sulphamethoxazole	36.37%
Cephtriaxone	0
Streptomycin	0
Neomycin	54.55%
Chloramphenicol	0
Oxytetracycline	0
Cephalexin	0

Antibiotic susceptibility patterns: Of the 13 antimicrobial agents tested against eleven verotoxigenic *E. coli* isolates, resistance was recorded against sulphonamide, neomycin and amoxyslav (either alone or combined with other antibiotics). Resistance to neomycin was noted in 55% of chicken isolates and was the second most frequently found resistance pattern. The highest frequency of resistance to antimicrobial drugs of isolates recovered from chicken was observed for the amoxyslav, 81.18%. However, 55% of the isolates were resistant to neomycin Table 5.

Molecular characterization: Eleven VTEC O157 isolates from this study were used for PCR investigation. None of the isolates from the chicken VTEC O157 strains were positive for stx1, stx2, *eae A* and *Ehly A* gene tested.

DISCUSSION

In the present investigation, although eleven 9.2% of 120 samples belonged to O157 serogroup and none of these was positive for stx, *eae A*, *ehly* Agenes. This is in agreement with 9.2% of VTEC reported by Pilipcinec *et al.*[14] in Slovakia, where cloacal swabs from poultry were examined for the presence of verotoxigenic strains of *E. coli* O157:H7. Abdul-Raouf *et al.*[23] in a study in Egypt reported that *E. coli* O157:H7 was isolated in 4% of chicken samples obtained from slaughterhouses, supermarkets and household. In the USA, Doyle and Schoeni[24] isolated *E. coli* O157:H7 from 1.5% of poultry samples. In Ivory Coast, Dadie *et al.*[25] reported two isolates of O157:H7 in poultry; and in France, Vernozy-Rozand *et al.*[26] reported a 1.6% VTEC O157:H7 isolation from poultry samples. Baran and Gulmez[27] reported isolation of *E. coli* O157:H7 in 2% of the samples from chicken giblets in Costa Rica. It was reported that colonization of chicken giblets with VTEC O157:H7 was worrying because this bacterium is capable of surviving and multiplying even when giblets are stored between 0, 6 and 12°C[28]. Since chicken giblets are consumed well cooked, the importance of this finding is based on the potential of cross contamination during processing, handling and marketing of the product.

Other workers have reported contrasting results whereby no VTEC O157:H7 was isolated from chickens. Thus, Blanco *et al.*[29] in Spain did not isolate VTEC from any of the 100 cloacal samples from broiler chickens. Beutin *et al.*[30] did not isolate *E. coli* O157:H7 from healthy chickens in Germany. In Canada, Irwin *et al.*[31] did not isolate VTEC O157:H7 from chickens. Griffin and Tauxe[32] did not recover this bacterium from raw chicken meat. Similarly, Heuvelink *et al.*[33] in Netherlands investigated meat samples originating from poultry and wild birds and were found to be negative of VTEC O157:H7. Read *et al.*[34] did not recover any VTEC O157:H7 from 200 chicken samples that were randomly selected from a meat processing plant in the south western Ontario area. In another study by Chapman *et al.*[35] in the UK, no *E. coli* O157 were isolated from any of 1000 chickens screened. The other *E. coli* isolates were identified based on colonial morphology on SMAC-BCIG and EMB. They appeared as Sorbitol fermenting and glucuronidase positive (purple colonies) and Sorbitol fermenting and glucuronidase negative (pink colonies). The differences in the results of Stx assay using VTEC-Screen SEIKEN and PCR may be attributed to the fact that VTEC-Screen SEIKEN has the ability to cross-react with the variant of Stx 1 and Stx 2 whereas PCR primer Stx 1 and Stx 2 are specific for these genes[36]. The losses of Stx 1and Stx 2 prior to testing by PCR have been documented[37-39]. This may be plausible explanation for the difference between the result of VTEC-RPLA and PCR obtained in this study. This is in agreement with the findings of Morabito *et al.*[13] who detected stx genes in

stool enrichment culture collected from feral pigeons in Italy and Parreira and Gyles[40] who reported stx genes in avian pathogenic *E. coli* strains in Canada. Schmidt *et al.*[41] also reported the isolation of VTEC strains from faeces of feral pigeons, which contained a new stx 2 variants gene designated stx 2f. In the present study, none of the 11 *E. coli* strains isolated from chickens possessed *eae A* or *Ehly A*. This disagreed with the finding of other workers who also reported the presence of the *eae A* gene in *E. coli* isolates from gulls, pigeons and chicken[42]. Kobayashi *et al.*[42] reported that all *E. coli* isolates from broiler chickens, 33 pigeons and 86 gulls carrying *eae A* gene lacked *Ehly* A gene. There are no reports available in Tanzania to compare with the present study. None of the *E. coli* O157 strains of 11 hickens showed the presence of stx or any other genes tested by PCR. This is in agreement with recent reports of Wani *et al.*[43], who reported that, none of three (0.7%) VTEC O157 isolated from chickens in India was positive for stx or any other genes tested. Some data suggest that toxin types could be important in determining the probability of developing HUS. Studies have shown that naturally occurring VT2 sequence variation may have a direct effect on the capacity of a given VTEC to cause disease[4]. The findings of the present study are consistent with the concept that VTEC have an array of properties that contribute to the ability of the organisms to cause disease[44].

Some properties that were not examined in this study, such as serine-protease (*esp*), bundle forming pili (*bfp*), catalase peroxidase (*ktp*) and variant of stx, *eae A*, *EhlyA*[45] probably contribute to the virulence of O157 VTEC and need to be investigated. Host factors are probably important in the outcome of exposure to VTEC. The isolates that have many or all of the VTEC virulence factors are likely to induce disease in most individuals after a low dose of bacteria is ingested. Less virulent VTEC may cause disease only after ingestion of larger doses and/or in individuals who are highly susceptible due to impairment of specific or nonspecific defenses. Thus, there is probably a virulence continuum and it may not be possible to draw a clear-cut line of distinction between pathogenic and non-pathogenic VTEC. Of the 11 *E. coli* O157 isolated and characterized in this study, approximately half displayed resistance to one or more antimicrobials, including sulfonamides, neomycin and amoxyslav. This data is in accord with previous studies suggesting that the use of these three drugs has been the key factor in the emergence of antimicrobial-resistant *E. coli*[46, 47]. Even though antibiotic treatment is not used for *E. coli* O157:H7 infections in humans or food animals, we found that some of the isolates were resistant to one or more antibiotics. One plausible explanation for this resistance is the relative ease with which resistance factors are exchanged among promiscuous bacteria[48]. Another possibility is that cattle are being treated with antibiotics for other conditions, thereby selecting for resistant populations of *E. coli* O157. This study is the first attempt to investigate the presence of O157 in chickens from Tanzania. The isolation of VTEC O157:H7 from local chickens in Tanzania suggests that contact with humans or cattle faeces may be the source of transmission to chickens. Considering the free-range system under which the local chickens are raised in Tanzania, for instance, contact with humans and animals faecal material is bound to be common. Most reports from developed countries focus on commercial broilers, which are raised in intensive system of management. The transmission of poultry O157:H7 VTEC to humans, in Tanzania, may depend on gross contamination of food and food products during processing of chicken intestinal contents, since poultry meats are consumed well cooked. The extent to which poultry play a role in the epidemiology of human O157 VTEC infection needs further confirmation due to absence of virulence factors which *eae, ehly A* associated with pathogenic strains for humans in the chicken strains isolated in this study.

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