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Phenotypic and Molecular Characterization of *Escherichia coli* Isolated from Donkeys Slaughtered in Abattoirs in Kaduna State, Nigeria

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ABSTRACT

Escherichia coli is a natural inhabitant of the gastrointestinal tract of both humans and animals. There are several strains of which some are harmless while some are pathogenic causing various fatal conditions in both humans and animals. A cross sectional study was conducted to determine the occurrence of E. coli and detection of virulent genes of E. coli isolated from liver, spleen and small intestine of donkeys slaughtered in Maraban Idah, Kaduna State, Nigeria. A total of 384 samples were collected from 128 donkeys from April to August 2018 based on owners' consent. The samples were analyzed using standard methods of cultural enrichment, growth on selective media, biochemical test for identification and isolation of E. coli. Multiplex PCR was then used to detect presence of virulence genes in the E. coli isolates. The isolation rate was 5.2 % with organ distribution of 4.7 %. 4.7 % and 6.3 % for the intestine, liver and spleen respectively. Nineteen (19) of the 20 positive isolates profiled haboured different combinations of virulent genes viz: stx1-rfbO157-hlyA (n=2), stx1-rfbO157-eaeA-hlyA (n=6), stx1-eaeA (n=4), stx1-rfbO157 (n=1), stx1-rfbO157-eaeA (n=3), eaeA-hlyA (n=1), stx1-eaeA-hlyA (n=1) stx1-hlyA (n=1) hlyA (n=1) gene. This study showed that 60% of the isolates had the rfbO157 gene (E. coli O157 serotype), which is of great public health importance. Therefore, proper sanitation and hygienic practices should be ensured during and after slaughtering donkeys.

Key words: Abattoir, Biochemical test, Donkeys, *Escherichia coli*, Multivirulent genes, Multiplex PCR.

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INTRODUCTION

Escherichia coli (*E. coli*) is a gram-negative, rod-shaped, flagellated, nonsporulating, facultative anaerobic bacterium which belongs to the family *Enterobacteriaceae* [1]. The bacterium is classified into several categories based on its virulence factors; Enterotoxigenic *E. coli* (ETEC), Attaching and effacing *E. coli* (AEEC), Enteropathogenic *E. coli* (EPEC), Enterohaemorrhagic *E. coli* (EHEC) and Shiga toxin-producing *E. coli* (STEC or VTEC) [2]. Pathogenic *E. coli* are classified into groups of strains that can cause common diseases using common and remarkable assortments of virulence factors known as pathotypes [3].

Shiga toxin 1 (*stx*1), intimin (*eaeA*), hemolysin (*hlyA*), *rfb*E (O157 antigen), have been known to be amongst the virulence genes targeted when assessing the pathogenicity of various *E. coli* strains [4]. Some strains of *E. coli* are known to particularly produce powerful toxins that can cause intestinal or extraintestinal diseases [3]. These strains include the Verotoxin-producing *E. coli* (VTEC) also known as Shiga toxin-producing *E. coli* (STEC) [5]. The STEC that causes haemorrhagic colitis `and haemolytic uraemic syndrome are called enterohaemorrhagic *E. coli* [6]. It is recognized as an important food borne pathogen [7]. *E. coli* are also classified into serogroups based on the heat stable 'O' and heat-labile surface 'K' or flagellar 'H' antigens [8]. The intestinal pathologies associated with this organism include severe diarrhoea caused by different *E. coli* pathotypes. These intestinal pathologies have the potential to evolve into a Haemolytic uremic syndrome (HUS) in the case of EHEC [3]. Among these pathotypes, EHEC strains of serotype O157:H7 has been established as a zoonotic pathogen with farm animals established as a reservoir for EHEC (O157:H7) [9].

The donkey or ass scientifically is known as *Equus africanus asinus*. It is a domesticated member of the horse family, Equidae [10]. Based on the study carried out by Jesse *et al*. [11] in the North- western part of Nigeria, it was established that there can be intestinal shedding of *E. coli* O157 by donkeys. Previous study on animal-originated human VTEC O157 infection has shown that only cattle and sheep play more role than donkeys in the epidemiology of the disease [12]. Therefore, cattle are known as the main reservoirs of human pathogenic VTEC, there is evidence that sheep, deer, dogs, poultry and goats can also transmit the VTEC strains [13] but there is paucity of information regarding donkeys serving as reservoirs for VTEC strains and other strains of *E. coli*. Thus, the aim of this study was to determine the occurrence of *E. coli* and detection of virulent genes of *E. coli* isolated from liver, spleen and small intestine of donkeys slaughtered in Maraban Idah, Kaduna State, Nigeria.

MATERIALS AND METHODS

Study Design

This was a cross sectional study in which tissue samples were collected from slaughtered donkeys at Maraban Idah, Kagarko Local Government Area using convenience sampling based on availability. Samples were collected only from animals whose owners consented to the study. For each donkey sampled, sections of the liver, spleen and intestine were collected at the point of evisceration in the abattoir, thus three (3) tissue samples from each donkey giving a total of 384 tissue samples were collected from 128 donkeys from April to August 2018.

Sample Collection

A piece (10g) of the tissue sample was collected

from the whole organ of each sampled donkey immediately after slaughter, placed in polythene bags, properly labeled and kept in a cooling box containing ice packs and transported within an hour to the Kaduna Poultry Laboratory, Barnawa Kaduna for analysis. The samples were taken once every week, usually on the market day of the week when so many donkeys are been slaughtered.

Laboratory Procedures

Tissue samples were macerated and 1g of each tissue sample was weighed and suspended in a sample bottle containing 9 ml of buffered peptone water and incubated at 37^{o}C for 24 hours. A loop full of the inoculum was analyzed using standard methods of selective plating using Eosine Methylene Blue (EMB). Isolates with greenish metallic sheen on EMB were subjected to biochemical tests such as Triple Sugar Iron (TSI) Agar, Urease, Citrate, Indole, Methyl Red, Voges Proskauer and Motility agar for proper identification and isolation of *E. coli* as described by Cheesbrough [14].

DNA Extraction

Following biochemical tests, the positive isolates obtained were sub-cultured on nutrient agar slants incubated at 37^{o}C for 24 hours. The positive isolates were subjected to DNA extraction prior to the multiplex polymerase chain reaction. DNA extraction was carried out using *AccuPrep*® Genomic DNA Extraction Kit (K-3032) and manufacturer's instruction were strictly adhered.

Primers

The sets of primer pairs that were used for this study are as shown in Table 1. The primers were obtained from Bioneer (Imam 5007854) USA.

Table 1: Primer sequences for gene identification of *Escherichia coli* isolates from slaughtered donkeys in Maraban Idah, Kagarko Local Government Area of Kaduna state, Nigeria.

Primers	Sequence: F-(5'-3') R-(3'-5')	Target Genes	Product size (bp)	Reference
LP30	**F**CAGTTAATGTCGTGGCGAAGG	*stx*1	384	Cebula
LP31	**R**CACCAGACAATGTAACCGCTG			*et al.,* [15]
PF8	**F** CGTGATGATGTTGAGTTG	*rfb*O157	420	Maurer
PR8	**R** AGATTGGTTGGCATTACTG			*et al.,* [16]
Int-Fc	**F** GGGATCGATTACCGTCAT	*eae*A	837	Batchelor
Int-Rc	**R** TTTATCAGCCTTAATCTC			*et al.,* [17]
HlyA-F	**F**GCATCATCAAGCGTACGTTCC	*hly*A	534	Schmidt
HlyA-R	**R**AATGAGCCAAGCTGGTTAAGCT			*et al.,* [18]

Multiplex Polymerase Chain Reaction

Multiplex PCR was carried out on the isolates using the protocol described by Henegariu *et al*. [19] at the DNA Laboratory at Agwan Sarki, Kaduna. The procedure for PCR amplifications involves pool of all the positive isolates by taking 0.1 µl of each isolate into a tube. 0.1 µl of the pool of the isolates were suspended in 25 µl of sterile DNase and RNase-free deionized water (ICN Biomedicals, Costa min (Thermomixer; Eppendorf, Mesa, CA, USA) and heated at 99°C for 10 min (Thermomixer; Eppendorf, Hamburg, Germany). The suspensions were then centrifuged at 12000 revolutions for 1 minute to pellet the cellular debris. The supernatant (5 for 1 µl) was subsequently used as a source of DNA templates. The DNA amplification was performed in 200 μl microtubes using a 50 μl reaction mixture consisting the DNA template, 1×*Taq* m buffer (10 mm Tris–HCl, pH 8·8, 50 ml KCl and 0·08% Nonidet P40), 2 *Taq* DNA polymerase (MBI Fermentas, Vilnius, Lithuania), 200 μm of dNTPs, 5 ml of $MgCl_2$, and 5.0 μl of each primer, and water to the final volume of 50 μl. All amplifications were run in a thermocycler (PTC-100; M.J Research, Watertown, MA, USA) under the following conditions: initial DNA denaturation at 94°C for 5 minutes followed by 30 cycles of 94°C for 1 minute, 53°C for 1 minute and 72°C for 1 minute. The final extension step was performed at 72°C for 5 minutes.

Gel Electrophoresis

The amplified PCR products were visualized using standard gel electrophoresis in a 1·5 % agarose gel stained with ethidium bromide (5 µ/ml) for 1minute. The gels were photographed under ultraviolent light using the Gel Doc 2000 documentation system (Bio-Rad, Hercules, CA, USA).

Data Analysis

Data collected was analyzed using SPSS software (version 20). Chi-square was used to test for association between *E. coli* and organs sampled from each donkey slaughtered. $P \leq 0.05$ was considered to be statistically significant. The PCR results were interpreted as presence or absence of virulence gene.

RESULTS

From the 384 tissue samples collected from 128 donkeys at the slaughter slab from the different selected internal organs, 20 were positive for *E. coli* with an isolation rate of 5.2 %. The isolation rate of *E. coli* varied among the different internal organs: 4.7% in the intestine, 4.7 % in liver and 6.3 % in spleen as shown in Table 2. The highest rate of isolation was obtained from the spleen but statistical analysis of data obtained showed that there was no significant association (p=0.810) between the occurrence of *E. coli* and the type of organ sampled. Table 3 shows *E. coli* isolates from selected internal organs of donkeys at the slaughter slab using biochemical tests. The results obtained from the processed internal organs were typical characteristics of *E. coli* with greenish metallic sheen on Eosine Methylene Blue (EMB) using standard methods of selective plating. The isolates were Indole positive, Methyl Red positive, Voges-Proskauer negative, Citrate negative, motile in motility medium and gas production with no Urease production.

E. coli Virulence Genes

Nineteen (19) of the isolates were found to have more than one virulence gene; *stx*1 gene was present in 18(90 %) isolates demonstrated by the presence of a 348 bp PCR product, *rfb*O157 gene was present in 12(60 %) isolates demonstrated by the presence of a 420 bp PCR product, *eae*A was found in 15(75 %) isolates demonstrated by the presence of a 837 bp PCR product and *hly*A gene was found in 12(60 %) isolates demonstrated by the presence of a 534 bp PCR product as show in Table 4.

Gene Profiles Observed in the E. coli Isolates

After subjecting the positive samples from the slaughtered donkeys to multiplex Polymerase Chain Reaction 19 isolates were observed to have more than one of the virulence genes present while some had all the four virulence genes present. The various multi-virulence genes observed were: *stx*1-*rfb*O157-*hly*A (10%), *stx*1-*rfb*O157-*eae*A-*hly*A (30%), *stx*1-*eae*A (20%), *stx*1-*rfb*O157 (5%), *stx*1-*rfb*O157-*eae*A (15%), *eae*A-*hly*A (5%), *stx*1-*eae*A-*hly*A (5%) and *stx*1-*hly*A (5%). Only one isolate was observed to have only one virulence gene which was the *hly*A as represented in Table 5.

Table 2: *Escherichia coli* isolation rate from slaughtered Donkeys in Maraban Idah, Kagarko Local Government Area of Kaduna State, Nigeria

Organ	Number of Samples	Number Positive	Isolation rate (%)
Liver	128	6	4.7
Spleen	128	8	6.3
Intestine	128	6	4.7
Total	**384**	**20**	**5.2**

(χ^2= 0.422; p= 0.810)

Table 3. Isolates of *E. coli* from selected internal organs of donkeys at the slaughter slab in Maraban Idah, Kagarko Local Government Area of Kaduna State, Nigeria using biochemical tests.

Organ	Number Positive	TSI	C	U	I	M	MR	VP	Organism isolated
Liver	6	Acid/Acid plus gas	-	-	+	+	+	-	*Escherichia coli spp*
Spleen	8	Acid/Acid plus gas	-	-	+	+	+	-	*Escherichia coli spp*
Intestine	6	Acid/Acid plus gas	-	-	+	+	+	-	*Escherichia coli spp*

TSI- Triple Sugar Iron (TSI) Agar, U- Urease, C- Citrate, I- Indole, MR- Methyl Red, VP-Voges Proskauer, M- Motility agar

Table 4: Virulence genes present in *E. coli* isolates obtained from slaughtered Donkeys in Maraban Idah, Kagarko Local Government Area of Kaduna state, Nigeria

Sample	*E. coli* Isolates	Presence of Virulence Genes (%)			
		***Stx*1**	***Rfb*O157**	***eae*A**	***hly*A**
Intestine	6	6(100)	4(67)	4(67)	4(67)
Liver	6	6(100)	5(83)	5(83)	1(17)
Spleen	8	6(75)	3(37.5)	6(75)	7(88)
Total	**20**	**18(90)**	**12(60)**	**15(75)**	**12(60)**

Table 5: Multi -virulent gene occurrence patterns in *Escherichia coli* isolates from slaughtered donkeys in Maraban Idah, Kagarko Local Government Area of Kaduna State, Nigeria.

S/NO	Gene occurrence Patterns	Number and percentage of occurrence (%)
1	***stx*1-*rfb*O157-*hly*A**	2(10)
2	***stx*1-*rfb*O157-*eae*A-*hly*A**	6(30)
3	***stx*1-*eae*A**	4(20)
4	***stx*1-*rfb*O157**	1(5)
5	***stx*1-*rfb*O157-*eae*A**	3(15)
6	***eae*A-*hly*A**	1(5)
7	***stx*1-*eae*A-*hly*A**	1(5)
8	***stx*1-*hly*A**	1(5)
9	*hly*A	1(5)

DISCUSSION

Isolates obtained from the processed internal organs had typical characteristics of *E. coli* similar to those described by Cheesbrough [14] with greenish metallic sheen on Eosine Methylene Blue (EMB), and were Indole positive, Methyl Red positive, Voges-Proskauer negative, Citrate negative, motile in motility medium and produce gas with no Urease production. The isolation rate of *E. coli* recovered in donkeys from this study is of public health significance because some strains are pathogenic. The high isolation rate from the spleen in this study could be as a result of an

ongoing clinical infection or may be associated with product contamination during evisceration which has important implication from the point of view of both meat and public health [20]. The spleen being the organ with the highest isolation rate in this study, differs from the result obtained in India on ducks where the highest isolation rate was recovered from the intestine (53.19 %) followed by the liver (37.14 %), spleen (30.23 %) [21]. This disparity may be due to variation in the host sampled and point of sampling.

There are virulence markers for various *E. coli* pathotypes; shigatoxin (*stx*) in STEC/EHEC, intimin (*eae*A) in EPEC and STEC/EHEC, haemolysin (*hly*A) in STEC/EHEC [22]. In this study, the *stx*1 gene was the gene possessed by most of the isolates. Though Jesse *et al*. [11] reported that *E. coli* 0157 serotypes (31.67%) were shed by a moderately large proportion of working donkeys in north-western Nigeria using antigen-specific latex-agglutination test. The *stx*1 genes have been known to code for the shiga toxins which are responsible for inhibition of protein synthesis, death of host cells and the development of haemolytic colitis and haemorrhagic uremic syndrome [23]. The virulence gene *stx1* targeted in this study was based on previous study that all of the *E. coli* O157:H7 contains *stx1* or/and *stx2*, which is a virulence gene encoding a family of related toxins called Shiga (Stx). Generally, strains possessing the *stx1* or/and *stx2* gene has been proven to be *E. coli* O157:H7 [7] [24]. The gene *eae*A was found in 75% of the isolates. This gene codes for intimin which is responsible for the intimate adherence of the organism to the enterocyte membranes that triggers the attaching and effacing lesions [25] produced by EPEC and EHEC. The gene *hly*A known as the plasmid encoded enterohaemolysin was found in 60% of the isolates. This gene serves as a virulence marker for EHEC strains and its closely related to the O157; H7 strain [26]. The gene *rfb*O157 was found in 60% of the isolate. This gene has been known to code for O157 *E. coli* [2]. The *rfb*O157 gene, which separates O157 from non-O157 *Escherichia coli* serotypes, is the fifth gene in a 12-rfb gene cluster and has been used for O157 identification [27]. The isolation rate of *E. coli* in this study was 5.2%. Unlike other previous studies which reported the isolation rate of *E. coli* as 2% from cattle slaughtered at beef cattle slaughtering plant for meat in Ethiopia according to a report given by Rosa *et al*. [28]. The isolation rate of *E. coli* O157 in this study was 60% which disagrees with a previous study carried out in Kano State on fresh and roasted beef, where the isolation rate was 53% in fresh beef and 25% in roasted beef according to a report given by Dahiru *et al*. [29]. The differences in the prevalence of *E. coli* among the different studies could be as a result of disparity in sampling scheme, species, sample types, detection protocol, geographic location, environmental and seasonal factors [30]. Nineteen (19) of the *E. coli* isolates in this study were observed to be multi-virulent with most of the isolates possessing *stx*1-*rfb*O157-*eae*A-*hly*A (30%). This could be as a result of significant association observed between both *hly*A and *eae*A, either independently or with the shiga toxin genes and this supports the suggestion of synergy between the adhesion intimin (*eae*A), enterohaemolysin (*hly*A) and Shiga toxins (*stx*1) genes [31]. *E. coli* O157:H7 has been reported to have significant virulence determinants which are shiga toxins encoded by the *stx1* gene, intimin encoded by the *eae*A gene and plasmid encoded enterohaemolysin encoded by the *hlyA* genes [32] which are the genes possessed by most of the isolates in this study. *E. coli* O157 is of great public health importance because of its zoonotic ability to

cause various fatal conditions which includes diarrhoeal diseases, peritonitis, colitis, bacteremia, infant mortality, and urinary tract infections [3] and cancer [33]. The multi-virulent *E. coli* strains recovered in this study is in accordance with a study carried out by [34]. Therefore, it can be said that the *E. coli* strains present in the internal organs of donkeys could be multi-virulent and this multi-virulence has been associated with outbreaks of diarrhoea, hemorrhagic colitis, and haemolytic-uremic syndrome in humans. Most clinical signs of disease arise as a consequence of the production of Shiga toxin 1 (*Stx*1), Stx2 or combinations of these toxins including other major virulence factors such as enterohaemorrhagic *E. coli* hemolysin (EHEC *hlyA*), and intimin, which are of the *eaeA* gene that is involved in the attaching and effacing adherence phenotype and *rfb*O157, which encodes the *E. coli* O157 serotype [35]. This study indicates the possibility that slaughtered donkeys in Maraban Idah, Kagarko Local Government Area in Kaduna State are habouring enteropathogenic, shiga toxin producing *E. coli* and or enteroheamorrhagic strains of *E coli* which is of public health significance due to the fatal effect of these strains/pathotypes in causing conditions ranging from asymptomatic carriage to uncomplicated diarrhoea, bloody diarrhoea, haemolytic uremic syndrome (HUS), thrombocytopenia, haemolytic anaemia, and acute renal failure in humans [36] and it has been established that most outbreaks and sporadic cases of bloody and non-bloody diarrhoea and HUS have been attributed to strains of the STEC serogroups including O157, O26, O103, O111, O145, O45, O91, O113, O121 and O128 [37].

Conclusion

In conclusion, 90 % of the isolates possessed *stx*1 gene, 12 (60 %) had *rfb*O157 gene, 15 (75 %) had *eae*A gene and 12 (60 %) had *hly*A gene. Nineteen (19) of the isolates showed multi-virulence with *stx*1-*rfb*O157-*eae*A-*hly*A having the highest percentage of 30 %. This study showed the presence of pathogenic *E coli* strains having various multi-virulent gene combinations in the liver, spleen and intestine from Donkeys slaughtered. Also 60% of the isolates possessed the *rfb*O157 gene which indicates O157 *E. coli* serotype.

Hence, people handling donkeys as well as donkey products should always maintain good personal hygiene before handling anything edible and there should be public enlightenment on the dangers of handling or eating undercooked donkey products to avoid transfer of various zoonotic microorganisms harboured by these animals.

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