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SHORT COMMUNICATION

Detection of Verocytotoxin-Producing *Escherichia Coli* in Raw and Fermented (Nono) Milk in Sokoto Metropolis, Sokoto State, Nigeria.

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SUMMARY

Verocytotoxin-producing *Escherichia coli* (VTEC) also called Shiga toxin producing *Escherichia coli* (STEC), are food borne organisms which cause fatal disease in human. The bacteria are frequently found in cattle gastrointestinal tract with high potential of contaminating animal products such as milk, meat, and cheese. A cross-sectional study was conducted to investigate the prevalence of VTEC and to detect the presence of *stx*1, *stx*2, and *eaeA* genes in raw and fermented milk sold within sokoto metropolis.Milk samples were analysed using bacterial culture, biochemical test and PCR for molecular identification. Bacteriological culture and biochemical characterization produced *Escherichia coli* with detection rate 0f 16.0% (38/238). Molecular identification of isolates by Polymerase chain reaction revealed 47.4% (18/38) detection rate of VTEC. The total prevalence of VTEC in the study was 7.6% (18/238), with proportion of raw and fermented milk were 12.5% (13/104) and 3.7% (5/134). There was no association (P≥ 0.05) between VTEC and different milk types. Amplification of isolate by mPCR using specific primers *stx*1, *stx*2, and *eaeA* gene confirmed that (94.4% (17) harboured *stx*2 and 1 (5.6%) harboured *stx*1 and *eaeA* genes.The study established prevalence of VTEC in raw and fermented milk consumed within sokoto metropolis, and presence of *stx*1, *stx*2 and *eaeA* gene. These findings indicate potential faecal contamination of the milk with VTEC in raw and fermented milk. Veterinary services in the state should educate farmers on the importance of farm hygiene and enlighten the Hausa/Fulani nomad and public on the danger associated with the consumption of contaminated milk.

Key Words: Escherichia coli; Fermented; Milk; Nigeria; Raw; Sokoto; Verocytotoxin

INTRODUCTION

Verocytotoxin-producing *Escherichia coli* (VTEC) are members of a set of pathogenic *Escherichia coli* strains and are significant food borne pathogen associated with serious disease outbreak globally (Kosi *et al*., 2015). The outbreak of VTEC was first reported in 1982 in U.S.A, from individuals with severe abdominal cramps and bloody diarrhea after being eating hamburgers in a food restaurant (CDC, 1982). Verocytotoxin-producing *Escherichia coli* are the major zoonotic food-borne organisms causing several illnesses in both humans and animals (Kumar *et al*., 2014). And it has been associated with life threatening conditions among human such as haemolytic uremic syndrome (HUS), thrombotic

thrombocytopenic purpura (TTP), and haemorrhagic colitis (HC) (Lupindu *et al*., 2018). The bacteria have the ability to produce a potent cytotoxins called Shiga toxins (*Stx*), which are the most virulence factor during pathogenesis of diseases caused by the VTEC (Perera *et al.*, 2015). The *stx2* is more significant than *stx1* in terms of causing severe diseases in human and importantly linked with increased risk of haemolytic uremic syndrome in Verocytotoxin-producing *E. coli* infection (Yakubu *et al.*, 2018).

Ruminant animals are the major source of VTEC with cattle as the main principal reservoir (Lupindu *et al*., 2018, Perera *et al*, 2015). Non ruminant animals such as pig and pigeons are also reported to becomes reservoirs of VTEC, and these may harbour and shed VTEC while asymptomatic (Ateba & Bezuidenhout., 2008). Raw milk can be contaminated with animal faeces and become good source of human infection if consumed without proper pasteurization (Enem *et al*., 2015). However, people are vulnerable to HUS after direct contact with faeces or consumption of animal products that are contaminated such as meat, milk and milk products (Lupindu *et al*., 2018). Raw and fermented milk are some of animal products reported of transmitting pathogenic organism, and these products are prepared and sold by Fulani who control over 80% of Nigeria cattle production (Johnsen *et al*., 2001).

In the study area and some parts of Nigeria, milk and milk products are commonly consumed without pasteurization and there is increase health concern over the unhygienic method of milking which was commonly hand milking by livestock farms. These can present considerable risk of milk-borne disease in human populace. The study was aimed to determine the prevalence of Verocytotoxin-producing *Escherichia coli* and to detect the presence of stx1, *stx*2, and *eaeA* genes in raw and fermented milk sold within Sokoto metropolis.

MATERIALS AND METHODS

Study area: The research was carried out in Sokoto metropolis, capital city of Sokoto State Nigeria located on latitude 13^{o}N of the equator and longitude 4^{o} 8'E and 6^{o} 54'E east of Greenwich Meridian in Northwestern Nigeria. It consists of four local government areas, which are, Dange Shuni, Sokoto North, Sokoto South, and Wamakko. The State shares border with Niger republic to the north, Kebbi State to the South, and Zamfara State to the East. The state has estimated projected population of about 5,271,037 peoples (NBS, 2018). Hausa and Fulani are the main indigenous tribes. Sokoto state was rated second in livestock population in Nigeria, with about 3 million cattle, 3.85 million sheep, 0.8 million camels (Yakubu *et al.*, 2018).

Study Design: A cross sectional study was conducted, the study population were raw milk collected from individual dairy cattle, fermented milk from milk vendor and dairy cattle within Sokoto metropolis. The inclusion criteria used were all dairy cattle in selected farms and all fermented milk (nono) sold in selected markets. The exclusion criteria used were all mastitis dairy cattle in selected farms and all non-fermented milk sold at selected market.

Sample size determination:

N=Z2p(1-q) /d2 (Thrushfield; 2007)

Where,

N=Sample

Z= the standard normal deviate corresponding to a two-sided level of significant of 5% (1.96)

p=Expected prevalence

q= 1-prevalence

d= is the desired level of precision (usually at 5% for single proportion) = 0.05

Based on previous prevalence of 9.6% for fermented milk (Ivbade *et al.* 2014) and 7.3% for raw milk (Enem *et al.*, 2015); the estimated sample size for fermented milk was 134 samples and for raw milk was 104 samples.

Sample Collection: Dairy cow farms and milk selling points (market) within Sokoto metropolis were identified for the collection of raw and fermented milk (nono). Total of 238 of raw and fermented milk samples were collected from 8 dairy farms and 8 markets within 4 local government areas (LGAs) comprising of 24 raw and 34 fermented milk from each of the following LGAs, Dange Shuni, Sokoto North, Sokoto South, and Wamakko. Multistage sampling techniques was used, all the four LGAs were selected; simple random sampling was used to select two farms and two markets from each LGAs by balloting and selection of dairy cattle and fermented milk vendors was done based on proportional to the size of farms and markets. To collect the samples, simple random sampling was used; lactating cows and fermented milk hawkers were assigned numbers and randomly selected without replacement. Ten milliliters (10mls) of both raw and fermented milk were collected in a sterile screw cap bottles from eight different farms and market within the metropolis. All the samples were transported in an ice pack to the Veterinary Public Health Laboratory, Faculty of Veterinary Medicine Ahmadu Bello University Zaria, Nigeria.

Culture and biochemical test: Five milliliters (5mls) of raw and fermented milk samples were enriched with modified trypticase soya broth, supplemented with 10 mg/L of acriflavine to reduce the growth of Gram-positive organism for isolation of *E. coli*. All samples were incubated at 37^0C for 24 hours. Spread plating technique was used to inoculate enriched samples into Eosin Methylene Blue agar (Oxoid, UK). The plates were incubated at 37ºC for 24 hours and bacterial colonies that showed greenish metallic sheen on Eosin Methylene Blue agar were suspected to be *Escherichia coli*. All suspected colonies were subjected to a conventional biochemical test and isolate that were positive for and methyl red and indole test but negative for Voges Proskaur, sulphide and citrate utilization test; produce acid and gas on TSI media following incubation at 37ºC for 24 hours were identified as *E. coli*. The suspected *E. coli* isolates were subsequently confirmed using commercially prepared biochemical test kit Microbact ^{TM} GNB 12E identification kits (Oxoid). The kit was inoculated with the emulsified *E. coli* suspension, incubated at 37^OC for 24 hours and results was read as described by the manufacturer.

***E. coli* O157:H7 screening using CT-SMAC:** CT-Sorbitol MacConkey agar was used to screen for E. coli O157:H7. Suspected E. coli O157:H7 isolates were placed on CT-SMAC to test for the fermentation of sorbitol. Colonies of suspected VTEC were removed from EMB agar and plated on nutrient agar for the formation of pure culture. The real isolated colonies were then streaked on CT-SMAC. The inoculated plates were then incubated at 37^{0c} for 24hr. *Escherichia coli* O157:H7 serotype cannot ferment sorbitol but the non-*E. coli* O157:H7 serotype can.

Serological test for suspected *E. coli* O157:H7 isolates: Pure colonies of *E. coli* from Eosin methylene blue agar were picked and inoculated into tubes containing Tryptone Soya broth and incubated at 37^OC nightlong. A suspension was made from overnight growth and used for testing agglutination of O157 antigen. Latex agglutination test was used following

manufacturer's instructions. A single drop suspension was distributed on the ring of the reaction card. The latex test was distributed onto a ring on the reaction card, by dropping it on the side of the ring. The suspension was then mixed with the latex test and spread to cover the reaction area using a loop. Later the card was jiggled for a minute and agglutination was observed. **DNA extraction:** Boiling method was used with slight modification (Junior *et al.*, 2016). Bacterial colonies of each isolate were suspended in a 1.5 ml nuclease free tubes containing 100 microliter of nuclease free water. Tubes were transferred into a water bath and boiled at 100 ^{O}C for 30 minutes. The tubes were centrifuged at 1300 rpm, for 5 minutes. The supernatants were transferred into new Eppendorf tubes, 100µl of isopropanol was added into the mixture; tubes were inverted 5 times and incubated at -20^{O}C for 15 minutes. The tubes were centrifuged at 1400 rpm for 5 minutes again; supernatant was carefully discarded without disturbing the pellet. The pellets were re-suspended in 50µl of nuclease free water and stored at -20^{O}C.

Molecular identification: Molecular characterization of *stx*1, *stx*2, and *eaeA* were done on serologically identified isolates by PCR using precise primers (Table i). Five microliters of template were subjected to 1% agarose gel electrophoresis for quality control, the electrophoresis machine was programmed at 100 volts for 10 minutes. The gel was visualized for the presence of the extracted DNA using UV transilluminator in a gel documentation device. The primer was reconstituted by resuspending the lyophilized primers using manufacturer recommended volume of nuclease free water to give a stock concentration of 100mM. Furthermore, working concentration of 10mM was prepared by diluting 10 microliters stock primer to 90 microliters of nuclease free water in a 1.5 ml nuclease free tubes. The tubes were placed on ice, 5 microliter of template DNA was dispensed into allocated 0.2 ml microtube. PCR cocktail was prepared containing the following for each reaction; Qiagen Toptaq Master mix 12.5 mM, 0.5 microliter of each STX1 F, STX1 R, STX2 F, STX2 R, ENT A1 and A2, and 4.5 microliter of nuclease free water. 20 microliters of cocktail were dispensed into tubes already containing 5 microliter of template DNA to give a 25mcroliter reaction mixture. The tubes were moved into an applied biosystem 9700 thermocycler (Bio-Rad) and the cycling conditions were programmed as follows. Initial denaturation at 95^{O}C for 3 minutes, followed by 35 cycles of denaturation at 95^{O}C for 30 seconds, annealing at 63^{O}C for 1.30 minute following an extension for 2 minutes, and a final extension for 2 minutes at 72^{O}C.

TABLE I: Primer sequence and respective product size

Gene	Primer	Primer sequence	Size (bp)	Reference
*Stx*1	F	ATAAATCGCCATTCGTTGACTAC	180	Paton and Paton
	R	AGAACGCCCACTGAGATCATC		1998
*Stx*2	F	GGCACTGTCTGAAACTGCTCC	255	Paton and Paton
	R	TCGCCAGTTATCTGACATTCT		1998
eaeA	*eaeA*1	GACCCGGCACAAGCATAAGC	384	Paton and Paton
	*eaeA*2	CCACCTGCAGCAACAAGAGG		1998

Data analysis: Data was entered and coded into the Microsoft Excel 2017, and analysed using SPSS software for appropriate analysis. Univariate analysis was conducted for frequency and proportion. Bivariate analysis was also conducted, using Chi square test to measure the association between *E. coli* isolation and different milk types and association between Verocytotoxin-producing *Escherichia coli* and different milk types.

RESULTS

A total of 41 (17.2%) *E. coli* were isolated on Eosin methylene blue agar and 38 (92.7%) were confirmed by biochemical test (Table ii). The overall prevalence of *Escherichia coli* isolated was 16.0% with an isolation rate of 19.2% and 13.4% for raw and fermented milk respectively. Statistical analysis using Chi-square showed a non-significant association between *Escherichia coli* isolated and different milk type (x^2=1.467, OR= 1.534, 95% CI: 0.765).

TABLE II: Frequency of *Escherichia coli* isolated from raw and fermented milk within Sokoto Metropolis (n=238)

Type of sample	Number of sample positive for *Escherichia coli*	Number of sample negative for *Escherichia coli*
Raw milk (n=104)	20	84
Fermented milk (n=134)	18	116
Total	38	200

n=Number of samples

This Shows the frequency of *Escherichia coli* isolated from raw and fermented milk using Eosin methylene blue agar and biochemically characterized by conventional and commercial biochemical test (Microbact ™ GNB 12E (oxoid)). Raw milk had the highest frequency of 20 while fermented milk has 18.

TABLE III: Frequency of Verocytotoxin-producing *Escherichia coli* (VTEC) detected from raw and fermented milk by PCR within Sokoto Metropolis (n=238)

Type of sample	Number of sample positive for VTEC	Number of sample negative for VTEC
Raw milk (n=20)	13	7
Fermented milk (n=18)	5	13
Total	18	20

n= number of samples

Molecular identification by PCR amplification of Shiga toxin (*stx)* and intimin (*eaeA*) gene on isolated *Escherichia coli* showed 18 out of 38 were positive for Verocytotoxin-producing *Escherichia coli* (Table iii) and (Figure 1-3).

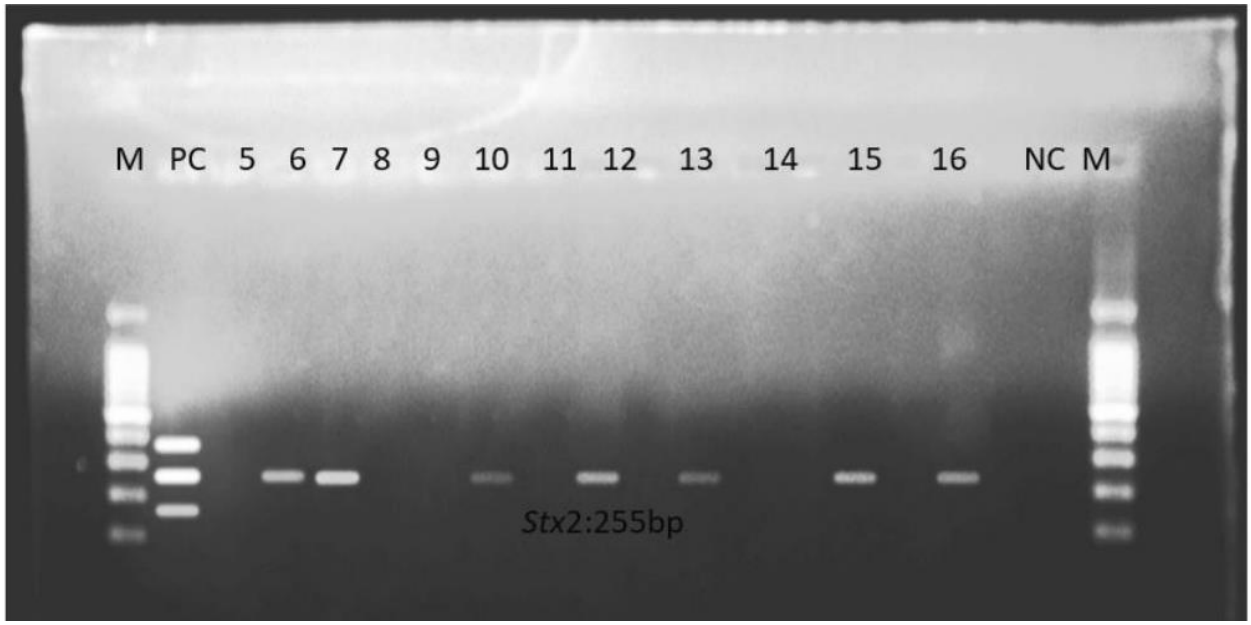

Fig 1: Agarose gel electrophoresis result for PCR detection of *stx*2 (255bp) gene in *Escherichia coli* O157:H7. Lane M: 100bp ladder (BioLabs), PC: positive control, lane: 5 to 16: positive samples, and NC: negative control

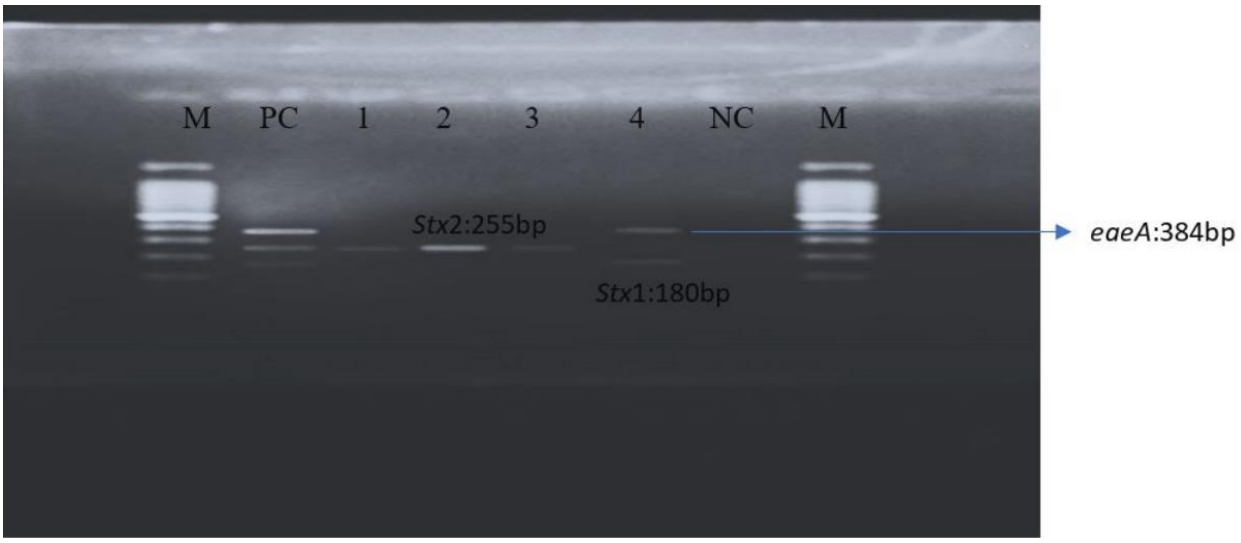

Fig 2 : Agarose gel electrophoresis result for PCR detection of *stx*1 (180bp), *stx*2 (255bp), and *eaeA* (384) gene in *Escherichia coli* O157:H7. Lane M: 100bp ladder (BioLabs), lane1: positive control, lane: 2 to 6: positive samples, and lane7: negative control.

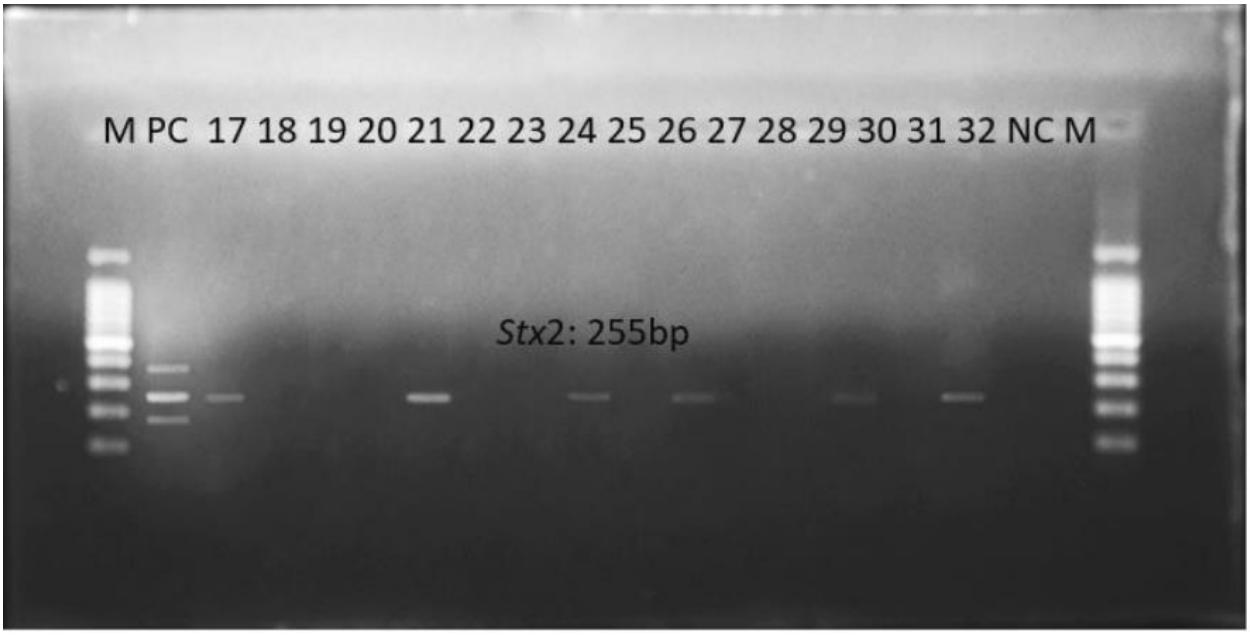

Fig 3 : Agarose gel electrophoresis result for PCR detection of *stx*2 (255bp) gene in *Escherichia coli* O157:H7. Lane M: 100bp ladder (BioLabs), PC: positive control, lane: 17 to 32: positive samples, and NC: negative control.

Chi-square test showed no significant association between Verocytotoxin-producing *Escherichia coli* detected and different milk types P≥0.05 (Table iv).

TABLE IV: Bivariate analysis of Verocytotoxin-producing *Escherichia coli* detected and different milk type in Sokoto metropolis

Type of sample	**Number of samples positive for VTEC**	**Number of samples negative for VTEC**	**Odds ratio**	**95% CI**
Raw milk (n=20)	13	7	1.68	0.64-4.41
Fermented milk (n=18)	5	13		

n= number of samples

The overall prevalence of Verocytotoxin-producing *E. coli* was 7.6% with positive proportion of 12.5% and 3.7% for raw and fermented milk respectively. However, out of 13 Verocytotoxin-producing *Escherichia coli* detected from raw milk, Sokoto North local government had the highest proportion of 19.2%. For fermented milk; out of 5 detected, Dange Shuni and Wamakko local government had the highest proportions of 5.9% each.

Molecular characterization of *stx*1, *stx*2, and *eaeA* gene, 17 (94.4%) out of 18 Verocytotoxin-producing *Escherichia coli* detected harboured *stx*2 and 1 (5.6%) harboured *stx*1 and *eaeA* gene (Table v).

TABLE V : Occurrence of VTEC associated virulence genes in raw and fermented milk samples in Sokoto metropolis

Type of sample	Virulence genes		
	***Stx*1**	***Stx*2**	***eaeA***
Raw milk (n=13)	0	12	0
Fermented milk (n=5)	1	5	1
Total	**1**	**17**	**1**

n= number of samples

This shows virulence genes detected from Verocytotoxin-producing *Escherichia coli* by PCR. Out of the 18 verocytotoxin-producing *Escherichia coli* detected 17 produced Shiga toxin 2 (*stx*2), while one isolate produced Shiga toxin 1(*stx*1) and intimin gene (*eaeA)*.

DISCUSSION

Escherichia coli O157 is one of the most common organism of foodborne disease in human and have been isolated from dairy and beef cow at all stages of production (Bélanger *et al.*, 2011). The shedding of the organism is intermitted and can be difficult to detect (Bavaro, 2012). This study confirmed the presence of VTEC in raw and fermented milk in Sokoto metropolis. The prevalence of VTEC in raw milk from this study is higher than 0.9% and 2.0% found in Abuja (Enem *et al.*, 2015) and Ogun State (Ivbade *et a*l. 2014) respectively. And lower than the 9.4% reported in Sokoto (Yakubu *et al.* 2018). . Detection of VTEC in raw milk in this study may be due to contamination of milk by poor farms hygiene and unhygienic hand milking practices observed by almost all the farms. Previous study found out that bacterial contaminants from hides and faeces of cow can also lead to presence of VTEC in raw milk (Elder *et al.*, 2000). Verocytotoxin-producing *Escherichia coli* have been detected in the faeces and hide of ruminants especially cattle (Stephan *et al.* 2008). In this study Fermented milk has 3.7% prevalence of VTEC which is higher than 1.6% prevalence reported in study conducted in Abuja (Enem *et al.*, 2015) and lower than 9.6% in Ogun state, (Ivbade *et al*., 2014) another study was conducted in selected cities in Nigeria which found 6.4% prevalence of VTEC in fermented milk (Enabulele & Nwankiti, 2016). These differences could be due to difference in the levels of hygiene along fermented milk processing and marketing chain between the places. Contamination of fermented milk could either be before the process fermentation or at the stage of transportation. The isolation of Verocytotoxin-producing *Escherichia coli* from both raw and fermented milk in this study signifies potential faecal shedding of the pathogen and subsequent contamination of the milk. Cattle are known to be the major reservoirs of the organism and can actively shed the organism in faeces without manifestation of clinical signs (Leedom, 2006; Neher *et al.*, 2015);Thus, there is high tendency of milk contamination following unsanitary collection from disease animals or herds with carrier of the organism.

Predominance of *stx*2 (94.4%) in this study is related to the studies conducted in Ogun State Nigeria, India and Switzerland, where frequency of *stx*2 was more against other gene (Stephan *et al.*, 2008; Vendramin *et al.*, 2014; Enem *et al.*, 2015). Higher frequencies of *stx*2 positive isolate can be of a public health concern as the strains that express *stx*2 were most likely associated with HUS in human (Enabulele & Nwankiti, 2016). Production of this toxin is an

indicator for severe outcome in the infected patient (Vendramin *et al.*, 2014). The non-detection of *stx*1 gene in the remaining isolate may have been brought by instability of the phages conveying *stx* gene, as loss of *stx* gene in sequential cultures is common after long storage/culturing of the bacteria (Law, 2000). Verocytotoxin-producing Escherichia coli strains apart from toxin production also produce other virulence factors that may increase the severity of human infection (Paton and Paton, 1998). The *eaeA* gene is well known virulence factor not only for Enteropathogenic *Escherichia coli* (EPEC) and Enterohaemorrhagic *Escherichia coli* (EHEC) but also atypical EPEC. In this study *eaeA* gene was detected in one isolate (5.56%) from raw milk (table 3), which could be classified as atypical EPEC (Paton and Paton, 1998). This finding was similar to study in Egypt which detect one *eaeA* gene (0.90%) in raw milk (Vendramin *et al.*, 2014). All the isolates found from this study were harbouring both the highly pathogenic and less pathogenic genes of Shiga toxin. Based on these findings highly pathogenic VTEC strain of zoonotic origin is circulating in dairy products within sokoto metropolis, and consumption of such products may cause serious disease outbreak and lead to human death which of serious public health concern.

CONCLUSION

The study established prevalence of VTEC in raw and fermented milk consumed within sokoto metropolis, and presence of *stx*1, *stx*2 and *eaeA* gene. These findings indicate potential faecal contamination of the milk with VTEC in raw and fermented milk. Veterinary services in the state should educate farmers on the importance of farm hygiene and enlighten the Hausa/Fulani nomad and public on the danger associated with the consumption of contaminated milk.

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Conflicts of Interest

The authors declare no conflict of interest

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