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Epidemiological Investigation, Serotypes and Distribution of Verocytotoxigenic *Escherichia coli* (VTEC) in Raw Milk and Milk Products in Uyo, Nigeria

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Abstract

Food borne diseases are of great concern globally especially in the developing countries where poor sanitation is applied during collection and processing of milk from animals. The epidemiological investigation, serotypes and distribution of verocytotoxin (VTI and VT2)-producing *Escherichia coli* in raw milk and milk products were determined using structured questionnaire, Cefixime tellurite-sorbitol MacConkey agar, agglutination kits and VTEC-RPLA Toxin detection Kit. Out of 27 milkers, 7.4 % had primary education, 22.2 % washed the milk utensils with cold water and soap, 11.1 % washed their hands before milking, while 7.4 % milkers washed the udder of the animals before milking. All the yoghurts had the product names; 85.7 % had NAFDAC numbers; 80.0% had Batch Numbers, while 71.4 % had Manufacturer s' Addresses. The unpasteurized milk samples had *E. coli* 0157 and non 0157 *E. coli* counts (CFU.ml^{-1}) ranging from 4.0 x 10^2 to 1.7 x 10^3 and 6.0 x 10^2 to 2.0 x 10^3 , respectively, while *E. coli* 0157 and non 0157 *E. coli* counts of milk products were between 1.0 x 10^2 and 1.0 x 10^3 CFU.ml^{-1}. *E. coli* 0157 had the highest percentage occurrence (38.3%), while *E. coli* 0145 had the lowest percentage occurrence (2.1%). More than 38.3% of the *E. coli* serotypes produced VT2, while ≥ 12.8% were VT1 producers. The occurrence of VTEC in the unpasteurized milk shows that the milkers should be enlightened on the necessary sanitary practices to adopt during milking and also post-pasteurization contamination of milk products should be avoided.

Key Words: Verotoxigenic, Escherichia coli, Milk, Yoghurt, Nono, Serotypes.
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Introduction

Verocytotoxigenic *Escherichia coli* (VTEC) or Shigatoxigenic *Escherichia coli* (STEC) are rod shaped, Gram negative, facultative anaerobe, lactose fermenter and non-endospore forming pathogens of animals and humans (Dwight *et al.*, 2004; Akinjogunla *et al.*, 2009). These enteric pathogens with an estimated infectious dose of < 50 organisms are regarded as the most common food-borne zoonotic pathogens causing several disease conditions in humans (Tilden *et al.*, 1996; Kumar *et al.*, 2014). The serotype of a VTEC is based on the 'O' antigen determined by the polysaccharide portion of cell wall lipopolysaccharide and the 'H' antigen by the flagella protein (Griffin and Tauxe, 1991).

Ruminants are considered an important source of VTEC with cattle being regarded as the

primary reservoir (Blanco *et al*., 1996; Perera *et al*., 2015). In some countries, direct consumption of raw milk is much frequent and more popular than consumption of pasteurized milk and milk products (yoghurt and nono) for it is presumed especially by the rural populace, that raw milk and its by-products have nutritional advantages over the pasteurized milk (Altalhi and Hassan, 2009). Although milk is an extremely nutritious food, it can likewise serve as an excellent growth medium for a broad range of microorganisms such as *E. coli*. Fresh raw milk obtained from a healthy animal normally contained a microbial load (< 10^3 CFU/ml), but the microbial load might increase up to 100 times fold if stored for some time at normal temperature (Pitkala *et al*., 2004). Inadequate cooling of milk, improper udder preparation methods, unhygienic milking equipment and water used for cleaning purposes are considered as the sources of milk contamination (Harding, 1995; Altalhi and Hassan, 2009).

Humans may acquire STEC/VTEC infections primarily from consumption of undercooked beef, raw milk, meat, dairy products, unpasteurized fruit juices and water contaminated with faeces of animals (Nataro and Kaper, 1998; Kumar *et al*., 2014). Food borne diseases are of great concern around the world in the developing countries where poor sanitation is applied during collection and processing of milk from cattle, cows, goats and buffaloes. Verocytotoxigenic *E. coli* (VTEC) O157 is a predominant cause of haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS) in humans worldwide (Adwan *et al.,* 2002; Borgattaa *et al*., 2012). The non- O157 *E. coli* serotypes which have emerged as a significant cause of human diseases are *E. coli* 026, 0111, 0121, 045 and 0145 (Tarr and Neil, 1996) and some of them are linked to cattle (Blanco *et al*., 1997).

Production of verocytotoxin (VT) is the major determinant of the virulence of *E. coli* serotypes and two major types of VT (VT1 and VT2) have been recognized (Paton and Paton, 1998). These two toxins are genetically and immunologically distinct with only about 55 to 60 % genetic and amino acid sequence relatedness (Lee *et al*., 2007). The verocytotoxins inhibit cellular protein synthesis, leading to death of the affected cells (Paton and Paton, 1998). The toxins have a profound effect on the endothelial cells of blood vessels, thus causing endothelial damage (Paton and Paton, 1998). Consequently, this study aimed at determining the serotypes and distribution of VTEC in raw milk and milk products in Uyo, Nigeria

Materials and Methods

Collection of Samples

The cow milk (n=29) and goat milk (n=47) samples were collected directly from cows and goats using sterile, wide-mouth sample containers by the Hausa / Fulani cattle rearers residing in Uyo, while nono (n=42) and yoghurt (n=35) samples were purchased from the hawkers. All the samples were properly labelled, immediately kept in ice packed flask (4 ^oC) and transported to Microbiology Department, University of Uyo, for bacteriological analysis.

Epidemiological Investigation

An epidemiological investigation was conducted using a well-structured questionnaire to obtain information on the hygienic milking practices by milkers (respondents) such as milk utensils used for milking, cleaning frequency of milk utensils, washing of milk utensils, hand washing by the milkers, udder washing and towel used for udder drying. The information on the socio-demographic characteristics of the milkers was also obtained.

Isolation of E. coli 0157 and Non- *0157 E. coli from Raw Milk and Milk Products*

One (1) ml of each serially diluted raw milk and milk products was inoculated onto each plate of Sorbitol MacConkey agar supplemented with Cefixime Tellurite in triplicates and incubated aerobically overnight at 37 °C. After incubation, a loopful of each colourless colony (presumptive *E. coli* 0157) and pink colony (presumptive non-0157 *E. coli*) obtained was streaked onto Eosin Methylene Blue (EMB) agar plates and aerobically incubated overnight at 37 °C. The greenish metallic sheen colonies on EMB plates were streaked onto nutrient agar slants and incubated overnight at 37 °C. The morphological and biochemical identifications of the *E. coli* were carried out using conventional methods (Cheesbrough, 2006).

Serological Identification of E. coli 0157 and Non-0157 E. coli

The presumptive colonies of *E. coli* O157 were serologically confirmed using Dry Spot *E. coli* O157 latex agglutination test kits (Oxoid, UK), while the non - O157 *E. coli* serotypes: O26, O125, O103, 0111, O128 and O145 were determined using the Dryspot *E. coli* Seroscreen Latex Test Kits (Oxoid, UK). Each *E. coli* (24-hr old) was emulsified in a drop of sterile normal saline / phosphate buffered saline on the small circle at the base of the test ring reaction area. The suspension was well mixed using a loop and placed onto the circle on the appropriate test card. The test card was gently hand rocked and observed for agglutination within 1-2 mins. Agglutination indicated positive reaction and identified the *E. coli* serotypes.

Detection of Verocytotoxins Producing E. coli Serotypes

The production of verocytotoxins (VT1 and VT2) by *E. coli* serotypes was detected using a VTEC-RPLA Toxin detection Kit (Oxoid, TD0960A). Each *E. coli* was inoculated onto each plate of Brain Heart Infusion Agar slope (10 ml) and incubated at 37^{o}C for 18 hrs. After incubation, a loopful of each colony was suspended in 0.85 % NaCl solution (1ml) containing polymyxin B and incubated for 30 mins at 37 ^{o}C. The suspension was centrifuged at 4,000 rpm for 20 mins and the supernatant was collected for the assay. A 25 µL of diluent was dispensed into 24 wells in three rows of V-bottom micro-titre plate. With 25 µL of the supernatant obtained above, a 1:2 serial dilution was made in each row from the first well to the seventh. The eight (last) well was left containing only the diluents. Thereafter, 25 µL of latex VT2 was added to all the eight wells in the second row and 25 µl latex control was added to all the eight wells in the third row. The micro-titre plate was covered with a lid, left undisturbed on a vibration-free surface at room temperature for 20 hrs, then the contents of each well were mixed by agitating using hand ; each well was examined for agglutination against a black background.

Results

The socio-demographic characteristics of the milkers (respondents) are presented in Table 1. Of the 27 milkers, 19 (70.4 %) were males, while 8 (29.6 %) were females; 16 (59.3 %) of the milkers did not know their ages, while 11 milkers were within ≤ 20 yrs and ≥ 51yrs. Twenty three (23) milkers had no formal education, 2 (7.4 %) attended primary education, 2 (7.4 %) attended secondary education, while none had university education. Fifteen (55.6 %) milkers were employed as herders, while 12 (44.4 %) owned the cows / goats (Table 1). The results showed that 33.3 % milkers used plastic cups and plates for collection of milk from the cows and goats, while 59.3 % milkers used plastic bottles only (Figs 1 and 2). All the milkers (n=27) cleaned the milk utensils; 22.2 % milkers washed the milk utensils with cold water and soap, while 77.8 % milkers washed the milk utensils with cold water only. Of the 27 milkers, 11.1 % washed their hands before milking, 37.0 % washed their hands after milking, and 51.9 % milkers did not wash their hands. Only two (2) milkers washed the udder of the animals before milking and also cleaned the udder with a towel (Table 2).

The records of the physical examination of packaged yoghurts are presented in Table 3. Of the 35 milk products (yoghurts) collected, 25 (71.4 %), 33 (94.3 %) and 30 (85.7 %) had NAFDAC numbers, production dates and expiry dates, respectively. All the yoghurts had the product's names; 80.0% had Batch Numbers, 71.4 % had Manufacturer's Addresses, while 94.3 % had the Volumes of their Contents (yoghurt) written on the packages (Table 3).

The results of the *E coli* 0157 and non - 0157 *E coli* loads of the raw milk and milk products are presented in Table 4. The cow milk had the minimum *E coli* 0157 count of 4.0 x 10^2 CFU/ml and maximum *E coli* 0157 count of 1.7 x 10^3 CFU/ml; the goat milk had the minimum *E coli* 0157 count of 5.0 x 10^2 CFU/ml and maximum *E coli* 0157 count of 1.2 x 1 0^3 CFU/ml, the nono had the minimum *E coli* 0157 count of 1.0x 10^2 CFU/ml and maximum *E coli* 0157 count of 7.0 x 10^3 CFU/ml, while the yoghurts had the minimum *E coli* 0157 count of 1.0 x 10^2 CFU/ml and maximum *E coli* 0157 count of 5.0 x10^3 CFU/ml

(Table 4). The goat milk had the highest mean (mm ± S.D) non 0157 *E coli* count of 1.1 ± 1.0 $x10^3$ CFU/ml, followed by cow milk with 9.0 ± 4.8 $x10^2$ CFU/ml, nono with 5.7 ± 2.5 $x10^2$ CFU/ml, while yoghurts had the lowest mean (mm ± S.D) non 0157 *E coli* count of 3.8 ± 2.8 $x10^3$ CFU/ml (Table 4). The occurrences of 59 *E. coli* isolated from the raw milks and milk products are as follows: 13/29 (44.8 %) from cow milk; 22/47 (46.8 %) from goat milk; 15/42 (35.7 %) from nono, while 9/35 (25.7 %) were obtained from yoghurts (Table 5). Out of the fifty-nine (59) *E. coli* isolates from the raw milk and milk products, 47 (99.7 %) were typable *E. coli*, while 12 (20.3%) were non- typable *E. coli*. The highest number of typable *E. coli* (n=19) was obtained from the goat milk, followed by cow milk with n=11 , nono had n=11 typable *E. coli,* while the typable *E. coli* obtained from yoghurts was n=6 (Table 7).

Out of the 47 *E. coli* serotypes obtained, *E. coli* 0157 had the highest percentage occurrence (38.3 %), followed by *E. coli* 0125 (19.1 %), while *E. coli* 0145 had the lowest percentage occurrence (2.1 %). The percentage occurrence of *E. coli* 0111, *E. coli* 026, *E. coli* 0103 and *E. coli* 0128 from the raw milk and milk products was 8.5 %, 14.9 %, 12.8 % and 4.3 %, respectively (Table 5). Out of the 47 *E. coli* serotypes, 12.8 % *E. coli* serotypes produced only verocytotoxin VT1, 38.3 % *E. coli* serotypes produced only verocytotoxin VT2, while 14.8 % *E. coli* serotypes were both verocytotoxin VT1 and VT2 producers (Table 6). There was no statistically significant difference between the verocytotoxin- and non-verocytotoxin- producing *E. coli* serotypes (p: 0.81; $\chi 2$: 2.99).

Table 1: Socio-demographic Characteristics of Milkers (Respondents)

Demographic Information	Categories	No (%) of Milkers
Gender	Male	19 (70.4)
	Female	8 (29.6)
Age (yrs)	≤ 20	3 (11.1)
	21-30	5 (18.5)
	31-40	2 (7.4)
	41-50	1 (3.7)
	≥ 51	0 (0.0)
	Don't Know	16 (59.3)
Level of Education	No Formal Educ.	23 (85.2)
	Primary School	2 (7.4)
	Secondary School	2 (7.4)
	Tertiary Institution	0 (0.0)
Ownership of Cow/Goat	Owner / Herding	12 (44.4)
	Employed as Herder	15 (55.6)

Table 2: Milking Containers Used and Sanitary Practices of Milkers (Respondents)

	Responses of Milkers	
Variables	Number	Percentage
Milk utensils used for milking		

(a) Plastic cup / plate	9	33.3
(b) Plastic bottles	16	59.3
(c) Others	2	7.4
Cleaning Frequency of milk utensils		
(a) Before every use	5	18.5
(b) After every use	9	33.3
(c) Before and after use	13	48.1
Washing of milk utensils		
(a) Cold water and soap	6	22.2
(b) Water only	21	77.8
(c) Warm water and Soap	0	0.0
Hand washing by the milkers		
(a) Before milking	3	11.1
(b) After milking	10	37.0
(c) No washing	14	51.9
Udder washing		
(a) Before milking	2	7.4
(b) No washing	25	92.6
Towel Used for Udder Drying		
(a) Common towel	0	0.0
(b) Just with hand	2	7.4
(c) No washing and drying	25	92.6

Fig 1: Collection of goat milk using plastic bottle by a milker

Fig 2: Collection of cow milk using plastic plate by a milker

Table 3: Physical Examination of Yoghurt Containers for Labelling Compliance

Parameters	No of Yoghurts Collected	Compliance Displayed	
		Yes No (%)	No No (%)
NAFDAC Number	35	25 (71.4)	10 (28.6)
Production Date	35	33 (94.3)	2 (5.7)
Expiry Date	35	30 (85.7)	5 (14.3)
Batch Number	35	28 (80.0)	7 (20.0)
Manufacturer's Address	35	25 (71.4)	10 (28.6)
Product's Name	35	35 (100)	0 (0.0)
Volume	35	33 (94.3)	2 (5.7)

Key: NAFDAC: National Agency for Food and Drug Administration and Control;
Values in parenthesis represent percentages

Table 4: Mean *E. coli* 0157 and Non-*E. coli* 0157 Counts of Raw Milk and Milk Products

Samples	Number of Samples Collected	CFU/ml					
		E. coli 0157			Non-*E. coli* 0157		
		Min	Max	mean ± S. D	Min	Max	mean ± S. D
Cow	29	4.0×10^2	1.7×10^3	$8.6 \pm 4.5 \times 10^{2b}$	6.0×10^2	1.9×10^3	$9.0 \pm 4.8 \times 10^{2b}$
Goat	47	5.0×10^2	1.2×10^3	$7.4 \pm 2.4 \times 10^{2b}$	9.0×10^2	2.0×10^3	$1.1 \pm 1.0 \times 10^{3c}$
Nono	42	1.0×10^2	7.0×10^2	$3.7 \pm 2.0 \times 10^{2a}$	2.0×10^2	1.0×10^3	$5.7 \pm 2.5 \times 10^{2ab}$
Yoghurt	35	1.0×10^2	5.0×10^2	$2.3 \pm 1.9 \times 10^{2a}$	1.0×10^2	9.0×10^2	$3.8 \pm 2.8 \times 10^{2a}$

Key: S.D: Standard deviation; Min: Minimum; Max: Maximum; mean within the column followed by the different superscript letters are significant as determined by Duncan multiple range test ($P < 0.05$), CFU: Colony Forming Units

Table 5: Occurrence of *E. coli* Isolated from Raw Milk and Milk Products

Sample		No Collected /Analyzed (%)	Nos. Positive of *E coli* (%)	Percentage among Positive Samples
Raw Milk	Cow milk	29 (19.0)	13 (44.8)	22.0
	Goat milk	47 (30.7)	22 (46.8)	37.3
Milk Products	Nono	42 (27.4)	15 (35.7)	25.4
	Yoghurt	35 (22.9)	9 (25.7)	15.3
	Total	153 (100)	59 (38.6)	100

Table 6: Occurrence of Typable and Non-typable *E. coli* Isolated from Raw Milk and Milk Products

Sample	No. of *E. coli* isolated	Typable *E. coli* No (%)	Non-typable *E. coli* No (%)
Cow milk	13	11 (84.6)	2 (15.4)
Goat milk	22	19 (86.4)	3 (13.6)
Nono	15	11 (73.3)	4 (26.7)
Yoghurt	9	6 (66.7)	3 (33.3)
Total	59	47 (79.7)	12 (20.3)

Table 7: Occurrence of *E. coli* Serotypes Isolated from Raw Milk and Milk Products

Bacterial Isolate	Serotypes	Cow Milk No (%)	Goat Milk No (%)	Nono No (%)	Yoghurt No (%)	Total No (%)
E. coli (n=47)	0157	5 (45.5)	7 (36.8)	3 (27.3)	3 (50.0)	18 (38.3)
	0125	2 (18.2)	4 (21.1)	2 (12.2)	1 (16.7)	9 (19.1)
	0111	1 (9.0)	2 (10.5)	1 (9.0)	0 (0.0)	4 (8.5)
	026	2 (18.2)	2 (10.5)	1 (9.0)	2 (33.3)	7 (14.9)
	0103	0 (0.0)	3 (15.8)	3 (27.3)	0 (0.0)	6 (12.8)
	0128	1 (9.0)	0 (0.0)	1 (9.0)	0 (0.0)	2 (4.3)
	0145	0 (0.0)	1(5.3)	0 (0.0)	0 (0.0)	1 (2.1)
	Total	11(100)	19 (100)	11 (100)	6 (100)	47 (100)

Table 8: Occurrences of Verocytotoxins VTI- and VT2- Producing *E. coli* Serotypes from Raw milk and Milk Products

Serotypes	No of Isolates	Verocytotoxin Producers			Non-Verocytotoxin Producers	χ^2	p-value
		VTI	VT2	VT1 / VT2	Total		
		No (%)	No (%)	No (%)	No (%)		
0157	18	2 (11.1)	7 (38.9)	5 (27.8)	4 (22.2)		
0125	9	1 (11.1)	3 (33.3)	1 (11.1)	4 (44.4)		
0111	4	0 (0.0)	2 (50.0)	0 (0.0)	2 (50.0)		
026	7	1 (14.3)	3 (42.9)	0 (0.0)	3 (42.9)	2.99	0.81
0103	6	1 (16.7)	2 (33.3)	1 (16.7)	2 (33.3)		
0128	2	0 (0.0)	1 (50.0)	0 (0.0)	1 (50.0)		
0145	1	1 (100)	0 (0.0)	0 (0.0)	0 (0.0)		
Total	47	6 (12.8)	18 (38.3)	7 (14.9)	16 (34.0)		

Discussion

The level of hygienic practices of the milkers and the milking processes obtained via the administration of questionnaires revealed that 33.3 % milkers used plastic cups and plates for collection of raw milks, while 59.3 % milkers used plastic bottles only. The use of plastic containers for collecting raw milk in this study corroborated the work of Duguma and Geert (2015) who reported that 92.6 % milkers in Jimma collected milk using plastic containers. The occurrence of more male milkers (70.4 %) than female milkers (29.6 %) in this study substantiated the findings of Yitaye *et al*. (2008) who reported more male milkers than female milkers in Northwest Ethiopia but this differed from the results of Bereda *et al*. (2012) who reported that dairying offered more opportunities for females than males and made them to be closely involved in the dairy management in Ezha District of the Gurage Zone.

Twenty-three (23) milkers had no formal education, 7.4 % had primary education, and 7.4 % had secondary education, while none had University Education. Our findings agreed with the reports from Southwest Ethiopia by Bereda *et al*. (2014) where majority of the household heads (milkers) were between illiterate and primary school. The non-usage of towel to clean and dry udders of cows /goats after milking in this study differed from the findings of Zelalem and Faye (2006) who reported that in the Central Highlands of Ethiopia, dairy producers used common towels for drying udders. Duguma and Geert (2015) reported that only 13 % milkers in Southwestern Ethiopia used towel to dry the udders of the animals and this differed from this study as none of the milkers used towel for drying and cleaning the udders.

The absence of NAFDAC registration number and other relevant information on some packages of the yoghurts indicated that they might not be duly registered and approved by the government regulating agency. The unavailability of manufacturers' addresses on the packages may presumably make the producers untraceable in case of disease outbreaks resulting from the consumption of the products. The percentage occurrences (≤ 44.8 %) of *E. coli* in these samples were in accordance with Fadel and Ismail (2009) and Okonkwo (2011) who reported > 20 % *E. coli* in milk and milk products. The isolation rate of *E. coli* O157 in the raw cow milk (38.3%) in this study was higher than 11 % obtained by Sancak *et al*. (2015). The *E. coli* 0157 had the highest percentage occurrence (38.3%), followed by *E. coli* 0125, while

E. coli 0145 had the lowest percentage occurrence in the samples. The high occurrence of *E. coli* 0157 obtained in this study was in consonance with the reports of Doyle *et al*. (2015).

The latex agglutination screening of *E coli* serotypes from raw milk and milk products showed that 12.8% *E. coli* serotypes produced verocytotoxin VT1, 38.3% *E. coli* serotypes produced verocytotoxin VT2, while 14.8% *E. coli* serotypes were both verocytotoxin VT1- and VT2- producers. In this study, the milk products had verocytotoxigenic *E. coli* O157 and these findings were in agreement with reports from Canada and United States by Morgan *et al*. (1993) and Dorn (1995) in which VTEC O157 were isolated from nono and yoghurts. The VTEC O157 infections have been associated with the consumption of yoghurt (Morgan *et al*., 1993). The occurrence of VTEC O157 in raw milk and nono in this study is indicative of cross infection from apparently healthy dairy cows to the dairy products especially as they may not have been properly pasteurized. The isolation of VTEC 0125, 0111, 026 and 0145 in raw milk was similar to the findings of Muehlherr *et al*. (2003) who obtained 12 VTEC strains belonging to the non - O157 VTEC from goat milk. This result indicated that goats can be a reservoir of non - O157 VTEC and the consumption of raw goat milk or milk products can pose health risk to consumers, especially in the light of the fact that the goat milk is recommended for children allergic to cow milk and also for persons with decreased immunity. The occurrence of VTEC in the unpasteurized milk shows that the milkers should be enlightened on the necessary sanitary practices to adopt during milking and also post-pasteurization contamination of milk products should be avoided.

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