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Assessment of Bovine Raw Milk Obtained from Selected Farms in Zaria Environs Nigeria for Toxigenic Strain of *Escherichia Coli*

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Abstract: The presence of E. coli in food or water became accepted as indicative of recent faecal contamination and the possible presence of pathogens. The study therefore aims at detecting the presence of E.coli and E. coli O157 and an indication of quality assessment of the raw milk samples sold to the open market. A total of 199 composite milk samples and 13 bulk milk samples were collected from selected farms in Zaria environs and analyzed for total aerobic and coliform counts using standard cultural methods. The total bacterial and were more than 105 in 95.48% while total coliform counts was more than 100 cell/ml in 47.24% of the composite milk analysed. All the bulk samples collected had 100% bacterial and 69.2% coliform contamination. The cleaned teats had a mean of 3.03±2.13log10cfu/ml which was not significantly different (t=1.574, p=0.117) when compared to the count obtained from the teats that were unclean (3.12±1.97log10cfu/ml). However, total coliform counts of composite milk samples from animals with cleaned teats had a mean of 1.42±1.05log10cfu/ml was significantly lower (t=6.418, p-0.001) than the counts of milk obtained from cows with unclean teats (2.78±1.95log10cfu/ml) The incidence of Escherichia coli in the milk samples was 7.5%, when isolates were tested for enterotoxin production using the VET-RPLA kit, 14.3% of the isolates were found to possess the heat-labile toxin. All the isolates were found to be susceptible to Ceftriaxone and Ammox-Clav (n=15; %=100), one isolate was resistant to gentamycin, ciprofloxacin and chloramphenicol (n=15, 6.7%). Eight isolates were found resistant to nalixidic (n=15, 53.3%) and nine were found to be resistant to Sulphamethazole and Trimethoprim (n=15, 60%).

Keywords: *EschErichia coli*; Enterotoxin; Composite milk; Bulk milk; Unclean teats; Coliform contamination.

1. Introduction

Milk is a liquid and is therefore amenable to sampling, monitoring and hence meaningful control resulting in a significant awareness of food safety and quality issues even at primary production [1]. Cow's milk is produced on an industrial scale and is by far the most commonly consumed form of milk [2]. Throughout the world, there are more than 6 billion consumers of milk and milk products; the majority of them in developing countries [2]. Milk composition has a dynamic nature, and the composition varies with stage of lactation, age, breed, nutrition, energy balance and health status of the udder [3].

Cows, like humans, are natural reservoir of some bacteria. Many of these bacteria are not harmful to humans, but some are even though the cows are not affected and appear healthy [4]. Raw and processed milk are well-known good medium that supports the growth of several microbes with resultant spoilage of the product which becoming unsafe to consumers [5, 6]. Microbes may gain entry into raw milk directly from dairy cows experiencing sub-mastitis [7]. Some diseases causing organisms (pathogens) can be shed through cow faeces and may contaminate the outside of the udder and teats, from the farm environment particularly the water source [8, 9] and utensils used for the storage of milk on farm and during transportation [9], among these are *Escherichia coli*. Consumption of unpasteurized milk is the most frequently reported cause of outbreaks of infection [10]. Foodborne illnesses from dairy product consumption include infections with *Salmonella enterica*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Camphylobacter jejuni* and *Escherichia coli* O157:H7 [11, 12]. The presence of food-borne pathogens in unpasteurized raw milk either directly or indirectly increases the risk of ingestion and transmission of food- borne pathogens and ingestions of potentially harmful toxins [5]. Some diseases causing organisms (pathogens) can be shed through cow faeces and may contaminate the outside of the udder and teats, from the farm

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environment particularly the water source [8, 9] and utensils used for the storage of milk on farm and during transportation [9], among these are *Escherichia coli*. The presence of *E. coli* in food or water became accepted as indicative of recent faecal contamination and the possible presence of pathogens [13]. Thus, incidence of *E. coli* in milk and eventually milk products would pose serious problems especially the enterotoxigenic strains. The study therefore aims at detecting the presence of *E.coli* and *E. coli* O157 and an indication of quality assessment of the raw milk samples sold to the open market.

2. Methodology

2.1. Study Area

The study was carried out in Giwa, Sabon-Gari and Soba local Governments located in Northern Nigeria and bordering Zaria in Kaduna State. The vast Sahel savannah environment provides a good grazing field devoid of the forest regions that are often infested with tsetse fly and transmit sleeping sickness. The area has an annual rainfall lasting from May to October and the occupations of the locals are farming and animal husbandry with several herds maintained in extensive and intensive farms. These locations often serve as the source of most of the fresh milk sold in Zaria metropolis.

2.2. Sample Collection

A total of one hundred and ninety-nine composite milk samples were collected from 13herds that had a minimum of 10 cows located in various locations. The milk samples were collected from all milking cows selected randomly in the herds. A total of 10 mls of milk sample was collected from all the teats on each animal into sterile containers. Prior to collection, the animal teats were disinfected using a paper towel containing disinfectant. Subsequently, the first two streams were voided to prevent contamination with the body flora of the cows. Composite milk samples were also collected from animals without disinfecting their teats. This is to have an idea of the raw milk released into the market by farmers. Also, one sample of milk was drawn from the bulk tank of each farm sampled. All the samples were transferred into a cool box and transported to the Laboratory for processing.

2.3. Sample Processing

a) Enumeration of Total Bacterial Load in the Raw Milk Sample

Enumeration of the total bacterial load was done using the Nutrient agar (NA) by the spread plate technique. From the composite milk, 0.1ml was inoculated on the Nutrient agar plates and spread out aseptically on the surface of the agar medium using a sterile bent glass rod after flame sterilization. A serial dilution was done for the milk samples drawn from the bulk tank. From dilution10^{-7}-10^{-8}, 0.1ml was inoculated on the Nutrient agar plates and spread out aseptically on the surface of the agar medium. The plates were inverted and incubated at 37°C for 24 h. Colonies formed were counted and recorded.

b) Enumeration of Total Coliform Count in the Raw Milk Sample

Enumeration of the total coliform count was done using the Eosin Methylene Blue Agar (EMB) by the spread plate technique. From the composite milk, 0.1ml was inoculated on the EMB Agar plates and spread out aseptically on the surface of the agar medium using a sterile bent glass rod. A serial dilution was done for the milk samples drawn from the bulk tank. From dilution10^{-7}-10^{-8}, 0.1ml was inoculated on the EMB agar plates aseptically and incubated at 37°C for 24 h. All plates were counted and the average counts were calculated. They were expressed as Colony Forming Units/ml of the sample (CFU/ml). The colonies with greenish metallic sheen dark centers were presumptively considered *Escherichia coli* and subjected to biochemical characterization.

c) Biochemical Characterization

All isolates with metallic sheen dark centers that stained red with Gram reaction were subjected to biochemical characterization by conducting the following tests: Production of hydrogen sulphide (H_2S), Indole, Motility, Citrate utilization, Methyl Red (MR), Voges-Proskauer (VP) test. The suspected *E. coli* isolates from the conventional biochemical test were confirmed using the Microgen GNA biochemical test identification system (UK) in accordance with the manufacturer's instructions.

d) Isolation of *E. coli* O157

The confirmed *E. coli* isolated was streaked on Sorbitol MacConkey Agar (Oxoid Ltd., Cambridged, UK) containing cefixime 0.5mg/liter and potassium tellurite 25mg/liter and incubated at 24 h. The sorbitol negative colonies exhibiting colourless were regarded as presumptive *E. coli* O157 colonies while colonies with characteristics pink color are regarded as non *E. coli* O157 colonies.

e) Detection of Enterotoxin in the *E. coli* Isolates

The *Vibro cholera* enterotoxin and *Escherichia coli* heat labile enterotoxin test kit (Oxoid UK Ltd) by reverse passive latex agglutination was used. The *Escherichia coli* isolates were inoculated into 5ml of sterile Mundell's medium and incubated at 37°C for 24 h. To the overnight broth culture Polymyxin B was added to a concentration of 10,000units/ml and incubated for 4 h. After incubation, the culture broth was centrifuged at 3000rpm for 20 minutes

at 4°C and the supernatant of the sample was used as the test sample. The toxin control provided was reconstituted by using 0.5ml diluent and this was used to confirm the correct working of the test latex. The microtitre plate was arranged in such that each row consists of eight (8) wells. Each sample uses 2 rows of the microtitre plates. Using a pipette, 25µl of diluent was dispensed into the wells of the 2 rows except for the first well in each row 25µl of the test sample was dropped into the first and the second well in each row. Using a pipette and starting from the second well of each row, 25µl was picked up and performing a doubling dilution along each of the two rows. The dilution was stopped at the 7th well to leave the last well containing diluent only. To each of the well on the first row, 25µl of the sensitized latex was added and also to each of the well on the second row, 25µl of the latex control was added. The content in each well was mixed by agitating using hand and then the plates were covered with lid and incubated at room temperature for 24 h after which each well in each row was observed for agglutination against a black background.

3. Results

It was observed that 95.48% of the composite milk sample had bacterial counts above the acceptable level indicating bacterial contamination. Similarly, 105 samples representing 52.76% had coliform level above the acceptable levels. A similar trend was observed for the bulk milk where all the total bacteria recorded for all the samples were above the acceptable levels and 69.2% of the bulk milk had very high coliform counts (table 1).

The cleaned teats had a mean of 3.03±2.13$\log_{10}$cfu/ml which was not significantly different (t=1.574, p=0.117) when compared to the count obtained from the teats that were uncleaned (3.12±1.97$\log_{10}$cfu/ml). However, total coliform counts of composite milk samples from animals with cleaned teats had a mean of 1.42±1.05$\log_{10}$cfu/ml was significantly lower (t=6.418, p-0.001) than the counts of milk obtained from cows with uncleaned teats (2.78±1.95$\log_{10}$cfu/ml) (table 2).

Table-1. Determination of Total Bacteria and Coliform level in Composite and Bulk raw milk samples collected from the selected farms

Bacteria count	Sample	Number (%) with bacterial count above acceptable levels	Number (%) with bacterial count within acceptable levels
Total plate count	Composite milk	190(95.48)	9(4.52)
	Bulk milk	13(100)	0(0)
Total coliform count	Composite milk	105(52.76)	94(47.24)
	Bulk milk	9(69.2)	4(30.8)

Table-2. Mean of Total Plate and Total Coliform Counts of Raw Composite Milk Samples in Relation to Teats Hygiene

Teats hygiene	No of samples	Mean±SEM	
		TPC($\log_{10}$cfu/ml)*	TCC($\log_{10}$cfu/ml)**
Cleaned Teats	57	3.03±2.13	1.42±1.05[b]
Uncleaned Teats	142	3.12±1.97	2.78±1.95[a]
Total	199	3.08±2.05	2.10±1.50
		t - 1.574	*t*-6.418
		p=0.117	p=0.001*

*=Significant difference exists at p≤0.05
SEM=Standard error mean
TPC: Total plate count
TCC: Total coliform count

Escherichia coli isolates obtained from this study were fifteen (15) representing 7.5%. The isolates obtained were distributed among Giwa, Sabon Gari and Soba Local governments as follows; n=67(5), n=76(5) and n=56(5) respectively (table 3). Out of the fifteen isolates characterized as *E. coli*, only 2 strains (14.29%) tested positive to the production of the toxin (table 4)

Table-3. Distribution of *E. coli* in raw milk obtained from different sampling area

Local Government Area	No of samples	No(%) of samples with *E. coli*
Giwa	67	5(7.4)
Sabon Gari	76	5(6.6)
Soba	56	5(8.9)
Total	199	15(22.9)

Table-4. Enterotoxin producing assay of *Escherichia coli* from raw milk samples

Isolates	IS_1	IS_2	IS_3	IS_4	IS_5	IS_6	IS_7	IS_8	IS_9	IS_{10}	IS_{11}	IS_{12}	IS_{13}	IS_{14}
Heat-Labile and Heat stable Toxin (RPLA)	++	+++	--	--	--	--	--	--	--	--	--	--	--	--

+++ = production of diarrhoeal enterotoxin detected by the Oxoid test;
----= absence of diarrhoeal enterotoxin;
IS=Isolate

All the isolates were found to be susceptible to Ceftriaxone and Ammox-Clav (n=15; %=100), one isolate was resistant to gentamycin, ciprofloxacin and chloramphenicol (n=15, 6.7%). Eight isolates were found resistant to nalixidic (n=15, 53.3%) and nine were found to be resistant to Sulphamethazole +Trimethoprim (n=15, 60%).

Table-5. Antibiotic Susceptibility of Isolated *E. coli* from Raw Milk Samples

Antibiotics /Conc in µg	No of ETEC resistant (n=2)	Number (%) Susceptible organism	Number (%) resistant organism	Number (%) Intermediate Organism
Nalixidic (30)	1	7(46.7)	8(53.3)	0
Ceftriaxone(10)	0	15(100)	0(0)	0
Amox-Clav(10)	0	15(100)	0(0)	0
Chloramphenicol(10)	1	14(94.3)	1(6.7)	0
Ciprofloxacin(5)	1	14(93.3)	1(6.7)	0
Gentamicin(10)	1	14(93.3)	1(6.7)	0
Sulphamethazole(25) +Trimethoprim	2	6(40)	9(60)	0

Reference strain *E. coli ATCC*25922

4. Discussion

Raw cow milk is considered as having unacceptable hygienic quality when the total aerobic mesophilic bacteria exceed 10^5 cfu/ml [14]. In this study it was observed that over 95% of the composite and bulk milk had bacterial counts above the acceptable limits recommended for milk products. Similar studies had established high counts for pooled raw milk [15, 16]. This is suggestive of unsanitary conditions and poor hygiene practice in the dairy farms. However, these high coliform counts might also have been contributed by faecal contamination during handling and could have been indicative of possible presence of other enteric pathogens which are of serious public health concern in consumption of such milk. The result observed in the TPC may be due to sub-clinical mastitis or the general condition of the milking environment which was a bit similar in the farms. There was a reduction in the total plate count and total coliform counts after disinfection of the teats. The result reported in this study is higher than the report of Mohamed and Fatima [17], lower than the reports of Sim, *et al.* [18] but agrees with Bramley and McKinnon [19] and Gran, *et al.* [20]. They reported a reduction of about 50% in total bacterial count when milk was drawn from washed udder.

The prevalence of *Escherichia coli* in this study is not significant. *E. coli* is a ubiquitous organism Hahn [21] and pathogenic strains if present could be harmful to consumers. Higher prevalence values of *E. coli* (60% and 70%) were reported by Ali and Abdelgadir [22] and Lingathurai and Vellathurai [23] respectively. Fulya [14] revealed a 10% prevalence of *E. coli* in the raw milk studied. Also, Crump, *et al.* [24] reported 13% prevalence of *E. coli* in raw milk. Hempen, *et al.* [25] also reported a prevalence of 23.5% of *E. coli* in raw milk. The heat-labile toxin and heat-stable toxins are the primary cause of diarrhoea in enterotoxigenic *E. coli*. In this present study, VET-RPLA kit was used to target the heat-labile toxin. *E. coli* could pass from handlers to the milk used in processing milk products. This represents a health hazard and should be considered important because of the recent considerations of animal as a source of *E. coli* that are pathogenic to man [26]. This observation was higher than that of Frank, *et al.* [26]. Recent discovery of several groups of toxigenic and virulent strains of *E. coli* makes this finding significant. The result from this study gives a higher prevalence than that reported by Frank, *et al.* [26] who reported the presence of 3.2% of ETEC strains in milk and milk products.

The susceptibility of *E. coli* in this study to Ceftiaxone explains that ceftriaxone, a third generation cephalosporin still retain considerable potency on *E. coli*. This agrees with the findings of Nazih [27], Mehdi, *et al.* [28]; Syed and Nousheen [29]. *E. coli* high degree of sensitivity to these antibiotics (Gentamicin, Ciprofloxacin and Chloraphenicol) suggests that they can be used for first-line treatment of *E. coli* infections. However, it should be noted that 6.7% of the *E. coli* strains were resistant to these drugs and irrational prescriptions may cause higher resistance in the future. The high level of resistances observed in some of the antibiotics used in this work may be a consequence of the abusive uses of antimicrobials in animal therapeutics as well as in food additives used to promote animal growth [30].

5. Conclusion

From this study, it can be concluded that the microbiological quality of most of the composite raw milk samples collected from the different local government areas of Kaduna state were not satisfactory as the presence of enterotoxigenic *E. coli* was detected and this suggests that consumption of raw milk has potential health risk to consumers. The level of total plate and coliform counts from the bulk samples were high. Some of the *E. coli* isolates showed resistance to some of the antibiotics used. The data suggest that selection pressure imposed by the use of these antibiotics whether therapeutically in veterinary medicine or as prophylaxis in the animal production, is a key driving force in the selection of antibacterial resistance in *E. coli*.

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