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ANTIBIOTICS SUSCEPTIBILITY PATTERNS OF BACTERIAL ISOLATES FROM UNPASTEURIZED MILK SOLD AT KADUNA STATE UNIVERSITY

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ABSTRACT

A total number of twenty four (24) random samples of unpasteurized milk were collected from three different locations in Kaduna State University, main campus to assess their microbiological quality and determine the antibiotics susceptibility patterns of the bacteria isolates. Total viable bacteria and coliform counts were carried out using pour plate method. Antibiotics susceptibility test was carried out using Kirby Bauer modified disc diffusion method, while multiple antibiotic resistance (MAR) index of isolates were also determined using recommended procedure. Results obtained revealed that the mean values of total viable bacterial counts ranged from 1.1 x 10^5 to 2.1 x 10^5 cfu/ml. Total coliform counts was from 4.6x10^4 to 7.8x10^5 cfu/ml. Six different bacteria were isolated from the samples, they were: *Bacillus* spp (18.8%), *E. coli* (18.8%), *Lactobacillus* spp (6.25%), *Proteus vulgaris* (18.8%), *Staphylococcus. aureus* (25.0%), and *Streptococcus* spp (12.5%). Antibiotic susceptibility test of the isolated organisms showed that Gram negative isolates were more resistant to antibiotic action than Gram positive isolates, 18.8% of Gram positive isolates and 75% of Gram negative isolates had MAR index greater than 0.2. The findings of this study showed that the unpasteurized milk samples were contaminated with bacteria including faecal contaminants and that these isolates may have originated from a high risk source of contamination where antibiotics are often used.

Keywords: antibiotics susceptibility, contaminants, unpasteurized milk,

INTRODUCTION

Milk is a white liquid nutrient rich food produced by the mammary glands of mammals. It is the primary source of nutrition for infant mammals before they are able to digest other types of food. It contains colostrum and many other nutrients including protein and lactose (Pehrsson *et al.,* 2000).

Milk is sterile when it is in the udder of a healthy animal but becomes contaminated with bacteria mainly during and/or after milking (Karimuribo *et al.,* 2005). These sources of contamination include disease causing organisms (pathogens) shedding in milk, infected udder, and animal skin, faecal soiling of the udder, contaminated milking and storage equipments and water used for cleanliness. Other bacterial sources are from air, milkers, handlers, drugs or chemicals used during treatment of animal and from water used for adulteration by unfaithful workers/sellers which may be contaminated and may cause additional health problems (Karimuribo *et al.,* 2005; Swai and Schooman, 2011).

Unpasteurized milk also known as raw milk is milk from cows, sheep or goats that has not been pasteurized to kill harmful bacteria. Such milk can carry dangerous bacteria such as *Salmonella*, *E. coli*, and *Listeria* spp. These are responsible for causing foodborne illnesses. Analysis by Centre for Disease Control and Prevention (CDC) between 1993 and 2006 as reported by Adam *et al*. 2012 showed that 1571 people in the United States became sick from drinking raw milk or eating cheese made from raw milk while 202 out of them were hospitalized. Seventy percent of deaths among children under five years are linked to biologically contaminated food and water (Unnevehr and Hirschhorn, 2000).

In Nigeria, sale and consumption of unpasteurized usually called 'nono' is very common both in the urban and rural settlements. The milking and processing is usually done by Fulani men while the sale of these unpasteurized milk is usually done by Fulani women. Studies had shown that these processes are usually carried out in unhygienic environments (Barrnabas *et al*. 2014, Nwankwo *et al*., 2015, Oladipo *et al*., 2016).

Bacteria drug resistance is a public health problem around the world especially in developing countries where misuse and abuse of antibiotics are often common. Antibiotics resistance usually cause treatment failure, increased cost and length of treatment (Noskin *et al.,* 2005). There can also be increased side effects due to the use of multiple and/or more powerful antibiotics.

The aim of this study was to assess the microbiological quality of unpasteurized milk sold at Kaduna State University, main campus and to determine the antibiotics susceptibility patterns of the bacteria isolates from the milk.

MATERIALS AND METHODS

Collection of Sample

A total of 24 unpasteurized milk samples were collected within the month of August, 2017 in sterile tubes from three different locations (Market, Female Hostel, and Gate) within Kaduna State University main campus.

Determination of Total Bacterial Count

Standard plate count method was used to determine the Total Colony Count of the bacteria in the "Unpasteurized Milk" samples (Sanders, 2012). A ten-fold serial dilution of the samples from each of the different locations was performed and plated out on Nutrient Agar (HKM, Guangdong Huankai Microbial Sci &Tech. Co. Ltd.) plates using the pour plate technique. The plates were incubated at 37˚C for 24 hrs. The total number of viable bacteria was counted and colony forming unit per ml (cfu/ml) was calculated. The bacteria isolates were refrigerated and kept on slants for further tests.

Determination of Total Coliform Count

Sterile pipette was used to transfer 0.1ml of the serially diluted sample into molten MacConkey agar medium (Oxoid Ltd, Basingstoke, Hampshire, England) and rocked gently to allow for uniform dispersion of the sample in the media, and then transferred into appropriately labelled plates. The plates were incubated at 37˚C for 24-48hrs. The numbers of coliforms bacteria isolated was counted and the colony forming unit per millimetre was calculated.

Growth on selective media

A 10-fold serial dilution of samples was done in normal saline and pour plated aseptically on de Mann Rogasa Sharpe (MRS) agar, mannitol salt agar, MacConkey agar media plates respectively. The plates were incubated at 37^0C for 24 – 48 hours.

Isolation and Identification of the Bacteria

Colonies that grew on nutrient agar plates were Gram stained in accordance with standard Gram staining procedure described by Totera *et al.,* 2003 and microscopic examination, catalase test, coagulase test, urease test, oxidase test, indole test, Triple sugar iron test, methy red and Voges proskauer tests were carried out (Cheesebrough, 2000).

Antibiotic Susceptibility Testing

Antibiotic susceptibility test of the isolated test bacteria against commonly prescribed antibiotics was determined using Kirby-Bauer modified disc diffusion method according to Clinical Laboratory Standard Institute (CLSI, 2012). The standard antibiotics discs used for Gram negative bacteria isolates include: Co-trimoxazole (30µg), Chloramphenicol (30 µg), Sparfloxacin (10 µg), Ciprofloxacin (10 µg), Amoxicillin (30 µg), Amoxicillin-Clavulanate (30 µg), Gentamicin (10 µg), Pefloxacin (30 µg), Ofloxacin (10 µg), Streptomycin (30 µg). Gram positive discs include: Co-trimoxazole (30 µg), Ciprofloxacin (10 µg), Amoxicillin (30 µg), Gentamicin (10 µg), Pefloxacin (10 µg), Streptomycin (30 µg), Ampiclox (30 µg), Cefuroxime, (20 µg), Ceftriaxone (25 µg), Erythromycin (10 µg)

Determination of Multiple Antibiotic Resistance Index

Multiple antibiotic resistance index (MAR) (number of antibiotics to which test isolate displayed resistance divided by total number of antibiotic to which the test organism has been evaluated for sensitivity) for each test isolate was calculated as recommended by Krumperman (Krumperman, 1983).

RESULTS

Total viable bacteria and coliform counts

Total viable bacteria and coliform counts (cfu/ml) from the three sample areas in Kaduna State University, main campus are presented in Table 1.

Table 1: Table showing mean of total viable bacteria and coliform counts (cfu/ml) from three sample areas

Locations	Mean Viable Bacteria Counts (cfu/ml)	Mean Coliform counts (cfu/ml)
Gate	$1.3x10^5$	$4.6x10^4$
Hostel	$2.1x10^5$	$1.9x10^5$
Market	$1.1x10^5$	$7.8x10^4$

Occurrence of bacteria isolated from each location

A total number of 24 isolates of different bacteria species were isolated from the unpasteurized milk samples from the various sites, these are presented in Table 2.

Table 2: Occurrence of bacteria isolated from each location

Organisms	Gate	Hostel	Market
Bacillus spp	1	3	1
E. coli	1	1	2
Lactobacillus spp	0	1	1
Proteus vulgaris	2	0	2
S. aureus	2	3	1
Streptococcus spp	2	0	1
TOTAL (%)	**8 (33.3)**	**8 (33.3)**	**8 (33.3)**

Antibiotic susceptibility profiles of Gram positive isolates

The percentage antibiotic susceptibility patterns of Gram positive isolates is presented in Table 3. The greatest activity was observed with gentamicin followed by pefloxacin while the isolates were highly resistant to ampiclox (ampicillin + cloxacillin) and amoxicillin.

Table 3: Percentage antibiotic susceptibility pattern of Gram positive isolates

Antibiotics	***S. aureus*** **No of isolates (%) n = 6**			***Streptococcus* spp No of isolates (%) n=3**			***Bacillus* spp No of isolates (%) n=5**			***Lactobacillus* spp No of isolates (%) n=2**		
	S	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**
Pefloxacin	6 (100)	0 (0)	0 (0)	3 (100)	0 (0)	0 (0)	5 (100)	0 (0)	0 (0)	1 (50)	1 (50)	0 (0)
Gentamicin	6 (100)	0 (0)	0 (0)	2 (66.7)	1 (33.3)	0 (0)	5 (100)	0 (0)	0 (0)	2 (100)	0 (0)	0 (0)
Ampiclox	1 (16.7)	0 (0)	5 (83.3)	1 (33.3)	0 (0)	2 (66.7)	2 (40)	0 (0)	3 (60)	1 (50)	0 (0)	1 (50)
Cefuroxime	4 (66.7)	1 (16.7)	1 (16.7)	3 (100)	0 (0)	0 (0)	4 (80)	1 (20)	0 (0)	2 (100)	0 (0)	0 (0)
Amoxicillin	0 (0)	0 (0)	6 (100)	2 (66.7)	1 (33.3)	0 (0)	1 (20)	0 (0)	4 (80)	2 (100)	0 (0)	0 (0)
Ceftriaxone	4 (66.7)	1 (16.7)	1 (16.7)	2 (66.7)	1 (33.3)	0 (0)	5 (100)	0 (0)	0 (0)	2 (100)	0 (0)	0 (0)
Ciprofloxacin	6 (100)	0 (0)	0 (0)	1 (33.3)	2 (66.7)	0 (0)	4 (80)	1 (20)	0 (0)	1 (50)	1 (50)	0 (0)
Streptomycin	6 (100)	0 (0)	0 (0)	1 (33.3)	2 (66.7)	0 (0)	5 (100)	0 (0)	0 (0)	2 (100)	0 (0)	0 (0)
Co-trimoxazole	6 (100)	0 (0)	0 (0)	3 (100)	0 (0)	0 (0)	3 (60)	2 (40)	0 (0)	0 (0)	2 (100)	0 (0)
Erythromycin	4 (66.7)	2 (33.3)	0 (0)	1 (33.3)	2 (66.7)	0 (0)	2 (40)	1 (20)	2 (40)	2 (100)	0 (0)	0 (0)

Key: S= Sensitive I = Intermediate R= Resistant

Antibiotic susceptibility profiles of Gram negative isolates

Chloramphenicol and amoxicillin-clavulanic acid showed the greatest activity against the Gram negative isolates while the least activity was observed with amoxicillin. The percentage susceptibility of the Gram negative isolates to antibiotics is presented in Table 4.

Table 4: Percentage antibiotic susceptibility pattern of Gram negative isolates

Antiobiotic	***E. Coli*** **(n=4) No of isolates (%)**			***P. vulgaris*** **(n=4) No of isolates (%)**		
	S	**I**	**R**	**S**	**I**	**R**
Co-trimoxazole	1 (25)	3 (75)	0 (0)	1 (25)	2 (50)	1 (25)
Chloramphenicol	3 (75)	1 (25)	0 (0)	3 (75)	1 (25)	0 (0)
Sparfloxacin	3 (75)	1 (25)	0 (0)	4 (100)	0 (0)	0 (0)
Amoxicillin-Clavulanate	3 (75)	0 (0)	1 (25)	3 (75)	1 (25)	0 (0)
Amoxicillin	1 (25)	0 (0)	3 (75)	1 (25)	3 (75)	0 (0)
Gentamicin	2 (50)	1 (25)	1 (25)	1 (25)	1 (25)	2 (50)
Ciprofloxacin	3 (75)	1 (25)	0 (0)	0 (0)	1 (25)	3 (75)
Streptomycin	0 (0)	1 (25)	3 (75)	3 (75)	1 (25)	0 (0)
Ofloxacin	4 (100)	0 (0)	0 (0)	0 (0)	2 (50)	2 (50)
Pefloxacin	0 (0)	1 (25)	3 (75)	2 (50)	2 (50)	0 (0)

Key: S= Sensitive I = Intermediate R= Resistant

Multiple Antibiotic Resistance (MAR) index value of different isolates

The result of MAR index showed that 3 (18.8%) of the Gram positive isolates and 6 (75%) of the Gram negative isolates had MAR index greater than 0.2.

DISCUSSION

The results obtained from the bacteriological assessment of unpasteurized milk sold at Kaduna State University, Main Campus showed that these products were contaminated with bacteria which are of public health concern. There was a considerable difference between the total bacterial viable counts and coliform count of unpasteurized milk samples from the various sites. The total bacterial count observed in this study was high, this is similar to the result observed in a previous study by Barnabas *et al*., 2015, though lower than those observed by Nwankwo *et al*., 2015 in a study on microbial evaluation of raw milk from a dairy farms in Udi Local Government Area, Enugu State, Nigeria. The total bacterial count observed in this study failed the microbial standard set by Food and Agricultural Organisation (FAO, 2006) which says bacterial count must not exceed 10^4cfu/ml from four or all samples examined.

Fresh milk had been reported to be contaminated by microorganisms, some of which are pathogenic. The contamination might be from external sources like air, soil and water, milking utensils and personnel as well as the disease pathogens of the animals (O'Connor and Tripachi; 2000, Livia, 2006).

Out of the bacteria isolated from the unpasteurized milk *Proteus vulgaris*, *S. aureus*, and *Streptococcus* spp were more prevalent at the university gate, *Bacillus* spp and *S. aureus* at the hostel, while *E. coli* and *Proteus vulgaris* were more prevalent at the market. The source of these organisms is likely the hands of the hawkers, udders of the cows, and/or containers (Calabash) used for the sales. Most of these organisms are known to cause several diseases in human, for instance, bacteria such as *Streptococcus* and *Bacillus spp*. are pathogenic and are of major health concern to man. *Streptococcus* spp are reported to be implicated in throat infections, blood infections, pneumonia and meningitis in new-borns (Akhigbemidu *et al*., 2015). *S. aureus* is part of the normal flora of the skin and since milk collection involves contact with the human skin, this could result in contamination of the unpasteurized milk collected, especially when good personal hygiene is not maintained. *S. aureus* might have also found their way into the unpasteurized milk through the carriers, since the organisms are found around the nose, throat, hands, and clothing of these carriers (Hague, 1997). Consumption of food contaminated by strains of *S. aureus* toxins may lead to Staphylococcal gastroenteritis (Akhigbemidu *et al.*, 2015). *S. aureus* is also known to cause infections such as skin lesions, wound infection and is a major cause of food poisoning. It is responsible for a long list of diseases such as folliculitis, furuncles, erysipelas, cellulites, scalded skin syndrome, impetigo, pneumonia, osteoporosis, toxic shock syndrome (Toxemia), and meningitis (Marjorie and Kattleen, 2006). Typically, the intensity of the disease symptoms produced may depend on the quantity of contaminated food ingested and susceptibility of the individuals to the toxin (Odu and Imaku, 2013). Isolation of *S. aureus* from milk products had been reported in previous studies ((Barrnabas *et al.*, 2014, Nwankwo *et al*., 2015, Oladipo *et al*., 2016).

E. coli was most prevalent in the samples collected from the market. Unpasteurized milk gets contaminated with *E. coli* mostly at the point of milking from the udder of the cow (Fredrick, 2016). The primary source of *E. coli* contamination in this study can be faecal contamination. The udder of lactating cows could have been contaminated by faeces as a result of the animal lying on dirty litters in the farms. Furthermore, milk samples collected from milking containers may have been contaminated by milk collected from the udder which was kept in them or from the unsterilized containers used for collecting the milk. Hands may have been contaminated by faeces on the udder during milking or from the hands of the milkers due to lack of proper personal hygiene. *E. coli* when found in water and food supplies, is indicative of a recent faecal contamination and is a threat to public health (Monica *et al*., 2000; Mora *et al*., 2005).

Generally, it was observed in this study that Gram positive isolates were more susceptible to antibiotics than the Gram negative isolates, the difference in their cell wall structure and composition might be responsible for this. A Gram positive organism lacks an outer membrane but has a thick layer of peptidoglycan and no lipopolysaccharide outer membrane (Salton and Milton, 1996). This facilitates access of cell-wall active antibiotics (eg. penicillin/betalactam or vancomycin-type antibiotics) to their site of action (the peptidoglycan). Gram negative bacteria (e.g. *E. coli*) has a thin single peptidoglycan layer and high lipopolysaccharide content due to the presence of an outer membrane. This makes them more resistant to antibiotic attack.

High percentage of Gram negative bacteria (75.0%) with MAR index greater than 0.2 observed in this study suggested that the isolates originated from a high risk source of contamination where antibiotics are often used **.** This is an indication of misuse or abuse of antibiotics in the university community where the samples were collected. Osundiya *et al.* (2013) in their work also observed that 66.7% of the Gram negative isolates had an MAR index greater than 0.2. Multiple antibiotic resistance index is useful in analysing health risk and also in checking the extent of antibiotics resistance (Saba *et al*., 2011)

CONCLUSION

The results obtained from this research showed that unpasteurized milk contains potential pathogenic bacteria which can constitute a lot of health hazard to consumers. The control of these microorganisms should be of great concern to dairy processing and the public as a whole to enable the prevention of their effects. To protect consumers and public health against milk-borne infections, proper hygienic milking and milk handling procedures are required.

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