Bacterial Analysis of Different Types of Milk (Pasteurized, Unpasteurized and Raw Milk) Consumed in Kathmandu Valley

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ABSTRACT

Objectives: The presence of pathogenic bacteria in milk is the major public health concern resulting in food borne illness. The aim of this study is to determine the microbial quality of three different types of milk consumed in Kathmandu Valley with respect to the acceptable standard guideline and measure the antibiotic susceptibility pattern of the *Escherichia coli* and *Staphylococcus aureus* isolates.

Methods: A total of 66 samples (16 pasteurized, 25 unpasteurized and 25 raw milk) were collected from various sites of Kathmandu Valley. Those samples were subjected for total plate count and total coliform count by pour plate method. Furthermore, identification was made for the presence of *E. coli* and *S. aureus* with biochemical tests.

Results: The mean total plate count (TPC) of pasteurized, unpasteurized and raw milk was $1.2X10^6$ cfu/ml, $2.3X10^7$cfu/ml and $2.0X10^7$cfu/ml respectively. And, the mean total coliform count (TCC) of pasteurized, unpasteurized and raw milk was 2.9 X 10^4cfu/ml, $6.3X10^5$cfu/ml and $1.6X10^5$cfu/ml respectively. Coliforms were detected in 50%, 84% and 56% of the pasteurized, unpasteurized and raw milk sample respectively. *E. coli* and *S. aureus* were isolated from 18.8% and 12.5% of pasteurized, 40% and 16% of unpasteurized and 20% and 24% of the raw milk samples respectively. Among total *E. coli* isolates *(n=18)*, 16.7% were susceptible to ampicillin whereas 100% isolates were susceptible to other tested antibiotics. Similarly, 33.3% and 66.7% of the isolated *S. aureus* were susceptible to penicillin and cefoxitin respectively, whereas all *S. aureus* isolates were sensitive to all other antibiotics.

Conclusion: The mean value of TPC and TCC of pasteurized and raw milk exceed the standard guideline by FDA. Higher total plate count and presence of coliforms (also *E. coli*) and *S. aureus* in this study necessitates the close monitoring of the pasteurization process and post pasteurization process (packaging, transportation, storage etc.).

Key words: milk, coliforms, pasteurization, hygienic

INTRODUCTION

Milk, a great source of nutrients including protein with all ten amino acids, essential fatty acids, immunoglobulin and other micronutrients has become important part of diet to all age group including expectant mothers (Wijesihha-Bettoni and Burligame 2013). Most of the people in the world consume pasteurized milk and few people prefer raw milk as they believe that raw milk is more beneficial, tastier and convenient than pasteurized one (Altalhi and Hassan 2009). There isalso abundancy in view that raw milk could reduce allergic reactions and cure other ailments.

Milk, from the synthesis in specialized cells of mammary gland to the secretion to the alveoli of udder, is virtually sterile (De Silva et al. 2016). The microbial contamination of milk then after occur within the udder, exterior of the udder or from the surface of milk handling and storage equipment (Bramley and McKinnon 1990). Milk can also be cross contaminated during food preparation and by infected workers

who don'tpractice good hygiene (Lore et al. 2006). Pathogens involved in causing food borne diseases due to the consumption of raw milk include *Escherichia coli, Listeria monocytogenes, Salmonella, Campylobacter, Brucella abortus, Staphylococcus aureus, Bacillus cereus, Mycobacterium* spp. *and Clostridium botulinum* (Chye et al. 2004). Post-treatment contamination of milk caused outbreaks of campylobacteriosis, salmonellosis, yersiniosis and staphylococcal enterotoxin "food poisoning" (Lecos 1985).

Presence of *E. coli* in milk and milk products reported to bring public health hazard. Enteropathogenic *E. coli*are potential to cause severe diarrhea and vomiting in infants and young children (Sousa 2005). *E. coli* in milk is also the indicator of fecal contamination of milk.Similarly, *S. aureus* is another important human pathogen that causes food borne infections including milk and milk products (Bergdoll et al. 1989). Although *S. aureus* is effectively killed by pasteurization, but the enterotoxins produced by the *S. aureus* retain their biological activity even after pasteurization, which is becoming a hazard for consumers (Asao et al. 2003). Therefore, this study aimed to determine the degree of bacterial load and occurrence of *S. aureus* and *E. coli* with their antimicrobial susceptibility pattern in three different types of milk (pasteurized, unpasteurized and raw) consumed in Kathmandu Valley.

MATERIALS AND METHODS

Study period and study site: This research was conducted from February 2016 to May 2016 at Microbiology Laboratory, Department of Microbiology, GoldenGate International College, Kathmandu, Nepal.

Sample and sampling method: Pasteurized packaged milk from local shops (n=16), unpasteurized milk from local dairy (n=25) and raw milk from cow farm (n=25) of Kathmandu valley were included in this study. All sixteen pasteurized milk samples were from different brands. For unpasteurized milk and raw milk, sample was placed separately in a sterile plastic bag and transported to the laboratory in an ice box within 2 hours of collection and promptly processed.

Sample preparation: During sample preparation, 10 ml of each sample was taken and added to 90 ml distilled water. Further, a serial 10-fold dilution was made until a dilution of 10^{-6} was obtained.

Enumeration of bacteria: After the sample was prepared, 1 ml of each dilution of every samples were transferred to the sterile petriplate and molten plate count agar (PCA) and violet red bile agar (VRBA) (at around 45°C) were poured into respective petriplates for the enumeration of total bacterial count and total coliform count respectively. The overlay plate method was used for the total coliform count. Then, the plates were incubated at 37°C for 24 hours for total plate (bacterial) count and total coliform count (Cheesebrough 2006). Since, one ml of sample was inoculated in each plate, the number of colonies in each plate indicated the cfu/ml of each dilution. However, different dilutions of each samples were inoculated, mean of all dilutions was calculated to get cfu/ml of the particular sample (Aneja 2003).

Isolation and identification of *S. aureus* and *E. coli*: One loopful each of the sample from 10^{-1} dilution was inoculated on to Mannitol Salt agar (MSA) and MacConkey Agar (MA). The plates were incubated at 37° C for 24 hours. *S. aureus* produce yellow colonies with yellow zone on MSA. The isolated colonies were taken and identified as Gram positive, catalase positive, oxidase positive, Coagulase positive and DNA-ase positive (Isenberg 2004). Lactose fermenting colonies on MacConkey agar were sub-cultured to obtain pure culture. Pure cultures were tested biochemically (catalase test, oxidase test, Indole test, Methyl Red test, Voges Proskauer test, Citrate utilization test, Triple sugar iron agar test, urease test, oxidative-fermentative test) for confirmation of *E. coli* (Isenberg 2004; Cheesbrough 2006).

Antimicrobial susceptibility testing (AST) of the identified microorganisms: In vitro antimicrobial susceptibility testing towards different antibiotics was performed by modified Kirby Bauer disc diffusion method on to Muller Hinton Agar (MHA) and zone size was interpreted by using CLSI guideline (2014).

Statistical analysis: All data obtained from the sample analysis were tabulated using SPSS v. 19 and Microsoft Excel.

RESULTS

Frequency Distribution of Total Plate Count of Different Milk Samples

Of 16 pasteurized milk samples, 7 (43.8%) were observed with total plate count in the range of 10^5 ($\times 10^5$) cfu/ml and other 7 (43.8%) samples with total plate count of $\times$ 10^6 cfu/ml respectively. Similarly, 13/25 (52%) unpasteurized milk samples were observed with

total plate count of × 10^7 cfu/ml and 9/25 with total plate count of × 10^6 cfu/ml respectively. And, 14/25 raw milk samples exhibited total plate count of × 10^7 cfu/ml and 5 each of 25 raw samples contained total plate count of × 10^5 cfu/ml and × 10^6 cfu/ml respectively.

Table 1: Frequency distribution of total plate count

Count/ml (in range)	Pasteurized n=16		Unpasteurized n=25		Raw n=25	
	No.	%	No.	%	No.	%
× 10^0	-	-	-	-	-	-
× 10^1	-	-	-	-	-	-
× 10^2	-	-	-	-	-	-
× 10^3	1	6.3	-	-	-	-
× 10^4	1	6.3	-	-	1	4
× 10^5	7	43.8	2	8	5	20
× 10^6	7	43.8	9	36	5	20
× 10^7	-	-	13	52	14	56
× 10^8	-	-	1	4	-	-
Total	**16**	**100**	**25**	**100**	**25**	**100**

Note: No. - number

Frequency Distribution of Total Coliform Count of Different Milk Samples

Of 16 pasteurized milk samples, 8 (50%) samples were obtained to contain less than 10 coliforms (i.e, in the range of × 10^0cfu/ml). Of remaining, 3 samples contained coliform in the range of × 10^3, and two samples each contained coliform in the range of × 10^4 and ×10^5 respectively. In unpasteurized samples (n=25), 9 samples exhibited coliform in the range of × 10^6, followed by 6 samples in the range of × 10^5 and 4 samples in the range of less than 10 coliforms (× 10^0cfu/ml). And, in raw samples, large proportion of the samples, i.e, 11/25 (44%) exhibited coliforms less than ten (× 10^0cfu/ml), followed by 5 samples in the range of × 10^5.

Table 2: Frequency distribution of total coliform count

Count/ml (in range)	Pasteurized n=16		Unpasteurized n=25		Raw n=25	
	No.	%	No.	%	No.	%
× 10^0	8	50	4	16	11	44
× 10^1	-	-	1	4	-	-
× 10^2	1	6.3	-	-	-	-
× 10^3	3	18.8	2	8	4	16
× 10^4	2	12.5	3	12	4	16
× 10^5	2	12.5	6	24	5	20
× 10^6	-	-	9	36	1	4
Total	16	100	25	100	25	100

Note: No. – number

Comparison of Mean Microbial Load among Three Different Types of Milk Samples

The mean total plate (bacterial) count was obtained higher in unpasteurized milk (2.3 × 10^7 ± 35.96) and raw milk (2.0 × 10^7 ± 19.18) in comparison to pasteurized milk (1.2 × 10^6 ± 1.17). Similarly, total coliform count was also higher in unpasteurized milk (6.3 × 10^5 ± 60.4) and raw milk (1.6 × 10^5 ± 36.44) in comparison to pasteurized milk (2.9 × 10^4 ± 5.51).

Table 3: Comparison of microbial quality of different type of milk samples

	Pasteurized cfu/ml	Unpasteurized cfu/ml	Raw cfu/ml
TPC	1.2 × 10^6 ± 1.17	2.3 × 10^7± 35.96	2.0. × 10^7±19.18
TCC	2.9 × 10^4 ± 5.51	6.3 × 10^5± 60.41	1.6 × 10^5±36.44

Note:TPC: Total Plate (Bacterial) Count; TCC: Total Coliform Count

Occurrence of *E. coli* and *S. aureus* in milk sample

Among all the milk samples, *E. coli* were isolated from 18 samples (3 from pasteurized, 10 from non-pasteurized and 5 from raw milk) and *S. aureus* were isolated from 12 samples (2 from pasteurized, 4 from non-pasteurized and 6 from raw milk).

Table 4: Occurrence of *E. coli* and *S. aureus* in different milk sample

Sample	*E. coli*	*S. aureus*
Pasteurized milk (n=16)	3	2
Non-pasteurized milk (n=25)	10	4
Raw cow milk (n=25)	5	6
Total number of samples (N=66)	**18**	**12**

Microbiological evaluation of three disfferent types of milk samples

Coliforms were found in 50% of the pasteurized milk, 84% of non-pasteurized milk and 52% of raw milk samples respectively. *E. coli* were isolated from 18.8% of pasteurized milk, 40% of non-pasteurized milk and 20% of raw milk samples respectively. *S. aureus* were isolated from 12.5% of pasteurized milk, 20% of unpasteurized milk and 24% of raw milk samples respectively.

Table 5: Microbial analysis of different type of milk samples

Sample	Pasteurized (%)	Non-pasteurized (%)	Raw(%)
Coliform	50	84	56
E. coli	18.8	40	20
S. aureus	12.5	16	24

Antimicrobial Susceptibility Testing of *E. coli* and *S. aureus*

All 18*E. coli* were susceptible to the tested antibiotics, namely amikacin, gentamycin, imipenem, chloramphenicol, cotrimoxazole, ceftazidime, piperacillin-tazobactam, cefotaxime and levofloxacin except ampicillins. However, only 16.7% of the *E. coli* isolates were susceptible to ampicillins.

Table 6: Antimicrobial susceptibility testing of *E. coli* (n=18)

Antibiotics	Susceptibility percentage
Ampicillin	16.7
Amikacin	100
Gentamicin	100
Imipenem	100
Chloramphenicol	100
Cotrimoxazole	100
Ceftazidime	100
Piperacillin-Tazobactam	100
Cefotaxime	100
Levofloxacin	100

All *S. aureus* (n=12) were susceptible to antibiotics, amikacin, gentamicin, erythromycin, levofloxacin, chloramphenicol, clindamycin and ofloxacin except penicillin and cefoxitin. In case of penicillin and cefoxitin 33.3% and 66.7% of the isolated *S. aureus* was susceptible respectively. Isolates resistant to cefoxitin are methicillin-resistant *S. aureus* (MRSA).

Table 7: Antimicrobial susceptibility pattern of *S. aureus* (n=12)

Antibiotics	Susceptibility percentage
Penicillin	33.3
Cefoxitin	66.7
Amikacin	100
Gentamicin	100
Erythromycin	100
Levofloxacin	100
Chloramphenicol	100
Clindamycin	100
Ofloxacin	100

DISCUSSION

In this study, the total plate (bacterial) count (TPC) results showed that none of the samples were free of bacterial contamination. The mean TPC of pasteurized, unpasteurized and raw cow milk was 1.2×10^6 cfu/ml, 2.3×10^7 cfu/ml and 2.0×10^7 cfu/ml respectively. The mean total plate count of pasteurized milk and raw milk obtained in this study exceeds the range as per FDA Pasteurized milk ordinance (FDA 2015). The mean TPC value was higher than the findings of Al-Mazeedi et al. (2013) where mean counts of the aerobic bacteria in the pasteurized milk from three different dairy companies were 3×10^4 cfu/ml, 9×10^1 cfu/ml and 5×10^3 cfu/ml respectively. High bacterial counts reflects poor production hygiene or ineffective pasteurization of milk. (Harding 1995). The mean TPC of raw milk obtained in this study is higher than the findings obtained by El-Diasty and El-Kaseh(2009), Tasci (2011) and Belbachir et al. (2015) who found mean Aerobic Plate Count of 6.1×10^5, 3.95×10^6 and 1.4×10^6 cfu/ml respectively but lower than those reported by Moustafa et al. (1988) and Mohamed and El Zubeir (2007) who found mean value of 1×10^9 and 5.63×10^9 cfu/ml respectively.

In this study, coliforms were present in 65.1% of total milk samples. The mean Total Coliform Count (TCC) of pasteurized, unpasteurized and raw milk was 2.9×10^4 cfu/ml, 6.3×10^5 cfu/ml and 1.6×10^5 cfu/ml respectively. The mean TCC of Pasteurized milk was found greater than the FDA Pasteurized Milk Ordinance (FDA 2015). The coliform was present in 50% of the pasteurized milk samples, which was in harmony with the research conducted by Silva et al. (2010) from Brazil. In contrary to this result, in similar research carried out in Kathmandu valley by Arjyal et al. (2004), out of 140 samples of 14 different brands, coliforms were detected in all sample except one. According to the annual report published by DFTQC (2011/2012) out of 65 milk and milk products analysed, 31 (47%) samples were found to be microbiologically unsafe.

The value of TCC of raw milk in this study (1.6×10^5) is less than the findings of Moustafa et al. (1988), Mohamed and El Zubeir (2007) and Hassan et al. (2015) where the mean TCC of raw milk sample was found to be 1×10^6, 3.3×10^6 and 1.8×10^6 cfu/ml respectively. But higher than the result reported by Belbachir et al. (2015) where mean TCC was 2.6×10^3 cfu/ml. The existence of coliform bacteria may not necessarily indicate a direct fecal contamination of milk but it is a precise indicator of poor hygiene and sanitary during milking and further handling processes (Hassan et al. 2015).

E. coli and *S. aureus* was isolated from 18.75% and 12.5% of the pasteurized milk sample respectively. In similar research carried out in Kathmandu valley by Arjyal et al. (2004); out of 140 pasteurized milk samples of 14 different brands, the presence of *S. aureus* (15%) was similar to this study. But the presence of *E. coli* was higher (i.e. 92%) than the current study. Out of 25 raw milk samples, *E. coli* and *S. aureus* was isolated from 20% and 24% of the samples respectively. Joshi et al. (2014) also reported the similar prevalence of *S. aureus*, i.e. 29.7%. Of 25 unpasteurized milk samples, *E. coli* and *S. aureus* were obtained from 40% and 16% samples respectively.

All the isolated *E. coli* were sensitive to all the administered antibiotics disc amikacin, gentamicin, imipenem, chloramphenicol, ctrimoxazole, ceftazidime, piperacillin-tazobactam, cefotaxime and levofloxacin except ampicillins. Among 18 *E. coli* isolates, 16.7%

were susceptible and remaining 83.3% were resistant to the antibiotic ampicillins. For *S. aureus*, all isolates were susceptible to the administered antibiotics amikacin, gentamicin, erythromycin, levofloxacin, chloramphenicol, clindamycin and ofloxacin except two antibiotics penicillin and cefoxitin. In case of penicillin only 33.3% isolates were susceptible and 66.7% isolates were resistant. And, 33.3% of S. *aureus* were resistant to cefoxitin, i.e. MRSA.

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