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Article

Microbial assessment of milk collected from different markets of Mymensingh, Gazipur and Sherpur districts of Bangladesh and determination of antimicrobial resistance patterns of the isolated bacteria

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Abstract: The study was conducted to determine the total viable count (TVC) and total coliform count (TCC) of unpasteurized, pasteurized and UHT milk samples to assess their microbiological quality. A total of 60 milk samples were collected from different markets of Mymensingh, Gazipur and Sherpur districts. The TVC of milk was performed to determine the bacterial load in supplied milk samples and TCC to determine the coliform bacterial load in collected milk samples. Milk samples were cultured onto various selective media for the isolation of bacteria. The isolated bacteria were identified by cultural properties on different selective media, biochemical tests and finally by PCR. Out of 60 samples 20 unpasteurized milk samples from different markets were found positive for *Staphylococcus aureus* and 19 unpasteurized milk samples were found positive for *Escherichia coli.* All the *S. aureus* were found positive for *S. aureus* specific 16S rRNA gene by PCR. Out of 19 isolates of *E. coli,* 15 were found positive for *E. coli* 16S rRNA gene by PCR. Results of antimicrobial susceptibility test showed that most of the isolates of *S. aureus* and *E .coli* were susceptible to azithromycin, streptomycin, gentamicin, norfloxacin, tetracycline and ciprofloxacin but resistant to amoxicillin and erythromycin. The findings of this study revealed the presence of multidrug resistant *S. aureus* and *E. coli* in unpasteurized milk samples that posseses a serious threat to public health.

Keywords: milk; *Staphylococcus aureus*; *Escherichia coli*; PCR; antimicrobial resistance

1. Introduction

Bangladesh, one of the developing countries, urban and peri-urban dairying constitutes an important sector of the agricultural production system. Trend of rapidly increasing human population together with growing urbanization creates increased demand for milk and milk products. Milk is a key contributor to improving nutrition and food security particularly in developing countries. Improvements in livestock and dairy technology offer significant promise in reducing poverty and malnutrition in the world (Hemme and Otte, 2010).

Being a nutritious food, milk serves as an ideal medium for the growth of various microorganisms (Chambers, 2002; Bonfoh *et al*., 2003). It is a highly perishable commodity and poor handling can exert both a public health and economic toll, thus requiring hygienic vigilance throughout the production to consumer chain (Richardson, 1985; Hayes *et al*., 2001). Estimation of bacterial content is a commonly used procedure to measure the hygiene and sanitary quality of milk.

Among microorganisms *Staphylococcus aureus* and *Escherichia coli* are most common contaminants and they are responsible for food-borne illness (Kumar and Prasad, 2010). Staphylococcal food poisoning (SFP) is one of the most common food-borne diseases (FBD) worldwide with high occurrence second to salmonellosis. Staphylococcal food poisoning is often associated with the ingestion of manually handled foods that contain one or more highly heat stable staphylococcal enterotoxins. The safety of milk with respect to FBD is of great

concern around the globe. This is especially important in developing countries where production of milk takes place under unsanitary conditions and the consumption of raw milk which is typically produced in small dairy farms under unsatisfactory hygienic conditions is a common practice (Lee *et al.,* 2003).

Among all micro-organism, *E.coli* is frequently contaminating organism and is reliable indicator of fecal pollution (Saxena *et al*., 2015). Most strains of *E. coli* are harmless. Some strains however, such as enterohaemorrhagic *E. coli* (EHEC), can cause severe food-borne disease. It is transmitted to humans primarily through consumption of contaminated foods, such as raw or undercooked ground meat products, raw milk and contaminated raw vegetables and sprouts. EHEC produces toxins, known as vero-toxins or Shiga-like toxins because of their similarity to the toxins produced by *Shigella dysenteriae.*

The indiscriminate use of antibiotics has led to the emergence of antimicrobial resistance in various isolates of bacteria. Consumable animal products have been suggested as a possible source of both resistant bacteria and resistant genes that can be transferred to humans directly (Pereira *et al*., 2009). The antibiotic-resistant strains of a number of pathogenic bacteria, including *S. aureus* and *E. coli* in foods which threaten public health have been the subject of many publications (Pereira *et al*., 2009; Thaker *et al*., 2012). So, keeping the above facts in mind, the present study was designed for the microbial assessment of milk samples and determination of antimicrobial resistance patterns of the isolated bacteria.

2. Materials and Methods

2.1. Collection and transportation of samples

A total of 60 samples consisting of 20 unpasteurized, 20 pasteurized and 20 UHT milk samples were collected from different markets of Mymensingh, Gazipur and Sherpur districts during the period of January 2015 to May 2015 and immediately brought to Bacteriology Laboratory of the Department of Microbiology and Hygiene, Bangladesh Agricultural University, Mymensingh through maintaining cool chain using ice box. After that samples were processed immediately for determination of TVC and TCC and isolation and identification of *S. aureus* and *E. coli*.

2.2. Enumeration of TVC and TCC

For the determination of TVC, 0.1 ml of each ten-fold dilution was transferred and spread on duplicate plate count agar (PCA) using a sterile micropipette for each dilution. The diluted samples were spread as quickly as possible on the surface of the plate with a sterile glass spreader. One sterile spreader was used for each plate. The plates were then kept in an incubator at 37^0 C for 24-48 hours. Following incubation, plates exhibiting 30-300 colonies were counted. The average number of colonies in a particular dilution was multiplied by the dilution factor to obtain the total viable count. The total bacterial count was calculated according to ISO (1995). The results of the total bacterial count were expressed as the number of organism or colony forming units per ml (CFU/ml) of milk sample. In case of TCC, the procedures of sampling, dilution and streaking were similar to those followed in TVC. Only in case of TCC, MacConkey agar was used. The calculation for TCC was similar to that of TVC.

2.3. Isolation of target bacteria

Isolation and identification of bacteria were performed according to the method described by Carter (1986). Initially samples were enriched in nutrient broth at 37º C for 24 hours. The overnight cultures were streaked on eosin methylene blue (EMB) agar for *E. coli* and mannitol salt (MS) agar for *Staphylococcus* spp. Inoculated plates were incubated at 37º C for 24 hours. Single well defined colony was further sub-cultured until pure culture obtained.

2.4. Identification of target bacteria

Cultural, morphological, and biochemical characteristics were studied to identify the bacterial flora. The cultural characteristics or colonial morphology of the bacteria grown on the EMB, MacConkey Agar (MCA) and MS agar were recorded. Gram's staining was performed to study the morphology and staining characteristics of the bacteria according to the method described by Cheesbrough (1985). Biochemical tests, such as sugar fermentation, Motility, catalase, coagulase, methyl red (MR), voges-proskauer (VP), and indole tests, were performed to identify the bacteria tentatively (Cheesbrough, 1985).

2.5. Molecular identification by polymerase chain reaction (PCR)

Bacterial DNA template was prepared by using boiling method (Englen and Kelley, 2000). All the samples were examined by two pairs of primers (Table 1) to detect 16S rRNA gene of *S. aureus* and *E. coli*. In case of *S.*

aureus, the PCR reactions were carried out using a thermocycler (ASTEC, Japan) with the following programme: initial denaturation with 1 cycle of 5 min at 95°C, 35 cycles each consisting of denaturation with 1 min at 94°C, annealing with 1 min at 53°C, extension with 1 min at 72 °C and a final extension step of 7 min at 72 °C. In case of *E. coli*, the PCR reactions were carried out using a thermocycler (ASTEC, Japan) with the following programme: initial denaturation with 1 cycle of 5 min at 95°C, 30 cycles each consisting of denaturation with 45 sec at 94°C, annealing with 45 sec at 52°C, extension with 1 min at 72 °C and a final extension step of 5 min at 72 °C. PCR products were separated on 2% agarose gel, stained with ethidium bromide and photographed using a gel documentation system (BioRad).

2.6. Antibiotic sensitivity test

All *S. aureus* and *E. coli* isolates were tested against eight commonly used antibiotics (HiMedia, India) by the method of disk diffusion as described by Bauer *et al.* (1966). For this purpose, eight different antibiotic discs were obtained from commercial sources (Himedia, India). The selected antibiotics used were ciprofloxacin (5 µg/disc), azithromycin (30 µg/disc), amoxicillin (30 µg/disc), gentamicin (10 µg/disc), norfloxacin (10 µg/disc), erythromycin (30 µg/disc), streptomycin (10 µg/disc), and tetracycline (30 µg/disc). The interpretation on susceptibility was done according to the guidelines of Clinical and Laboratory Standard Institute (CLSI, 2007) formerly known as NCCLS.

2.7. Statistical analysis

The data on total viable count (TVC) and total coliform count (TCC) obtained from the bacteriological examination were analysed in completely randomised design (CRD) using computer package subjected to Analysis of Variance using SPSS Software (Version 16, 2007) . The differences between means were evaluated by Duncan's Multiple Range Test. Correlation between TVC and TCC were also evaluated.

Table 1. Primers used in this study.

Primer name	Sequence (5'-3')	Target gene	Amplicon size (bp)	References
SauF 234	CGATTCCCTTAGTAGCGGCG	*S. aureus*	1267	Swaminathan and
SauR 1501	CCAATCGCACGCTTCGCC	16S rRNA gene		Feng, 1994
ECO-1	GACCTCGGTTTAGTTCACAGA	*E. coli*	585	Schippa *et al.*,
ECO-2	CACACGCTGACGCTGACCA	16SrRNA gene		2010

3. Results

3.1. Results of TVC and TCC

The mean and standard deviation of the total viable count (TVC) in unpasteurized milk of Mymensingh, Gazipur and Sherpur districts were log 7.59 ±0.567, log 7.47±0.547 and log 7.48±0.452CFU/ml, respectively (Table 2). The result of total viable count in three districts retail markets were not differed significantly ($P<0.05$). The maximum and minimum range of TVC in milk recorded at Mymensingh, Gazipur and Sherpur districts were log 8.17, log 8.12, log 8.15 and log 6.70, log 6.63, log 6.94, respectively (Table 3). However the average value of TVC at three districts were log 7.44, log 7.38 and log 7.55 as shown in Table 3.

The mean and standard deviation of the total coliform count (TCC) in unpasteurized milk of Mymensingh, Gazipur and Sherpur districts were log 3.52±0.038, log 3.62±0.174 and log 3.47±0.104 CFU/ml respectively (Table 2). The mean values of total coliform count (TCC) in milk of Mymensingh, Gazipur and Sherpur districts were not significant ($P>0.05$). Nevertheless no significant variation was demonstrated between the interaction of the three districts. The maximum and minimum range of TCC in milk recorded at Mymensingh, Gazipur and Sherpur districts were log 3.55, log 3.83, log 3.62 and log 3.48, log 3.42, log 3.38 respectively (Table 3). However, the average value of TCC at three districts were log 3.52, log 3.63 and log 3.50 as shown in Table 3.

Table 2. Determination of mean and standard deviation for microbiological quality of unpasteurized milk at different markets of Mymensingh, Gazipur and Sherpur districts.

Place of collection	TVC (Log CFU/ml)	TCC (Log CFU/ml)
Mymensingh	7.59 ±0.567[a]	3.52±0.038[a]
Gazipur	7.47±0.547[a]	3.62±0.174[a]
Sherpur	7.48±0.45[a]	3.47±0.104[a]
LSD	0.179	0.144
Level of sig.	NS	NS

Note: In a column figures with same letter do not differ significantly (p>0.05) whereas figures with dissimilar letter differ significantly (as per DMRT).
TVC= Total Viable Count; TCC= Total colifirm Count; LSD= Least Significant Difference; NS = Not significant
All counts are expressed in logarithms and CFU/ml

Table 3. Range of total viable count and total coliform count in unpasteurized milk obtained from different markets of Mymensingh, Gazipur and Sherpur districts.

Place of collection	TVC (Log CFU/ml)			TCC (Log CFU/ml)		
	Max	Min	Average	Max	Min	Average
Mymensingh	8.17	6.70	7.44	3.55	3.48	3.52
Gazipur	8.12	6.63	7.38	3.83	3.42	3.63
Sherpur	8.15	6.94	7.55	3.62	3.38	3.50

Note: All counts are expressed in logarithms and CFU/ml

3.2. Results of correlation between TVC and TCC at Mymensingh, Gazipur and Sherpur districts

The result presented in Figure 1 revealed negatively correlated between the total viable count and total coliform count in milk samples of Mymensingh district. Abruptly a weak relationship was observed between TVC and TCC. The value of correlation coefficient was R^2= 0.0467 and regression equation was, y= - 3.16784x+ 20.476. The result presented in Figure 2 revealed negatively correlated between the total viable count and total coliform count of milk samples of Gazipur district. Abruptly a weak relationship was observed between TVC and TCC. The value of correlation coefficient was R^2= 0.2989 and regression equation was, y= - 1.0246x+ 11.384. The result presented in Figure 3 revealed positively correlated between the total viable count and total coliform count of milk samples of Sherpur district. Abruptly a weak relationship was observed between TVC and TCC. The value of correlation coefficient was R^2= 0.3534 and regression equation was y= 1.6902x+ 1.4459.

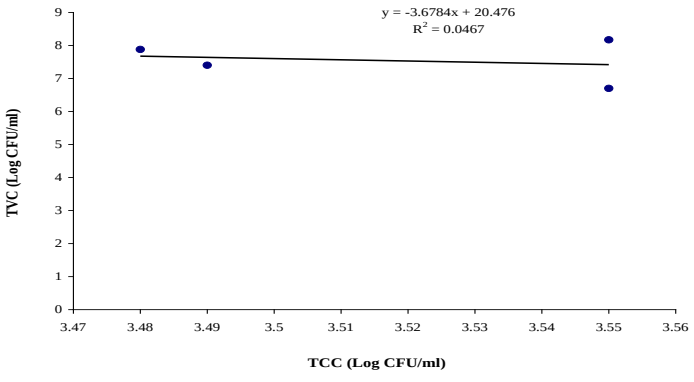

Figure 1. Correlation between total viable count (TVC) and total coliform count (TCC) in unpasteuzized milk at Mymensingh district.

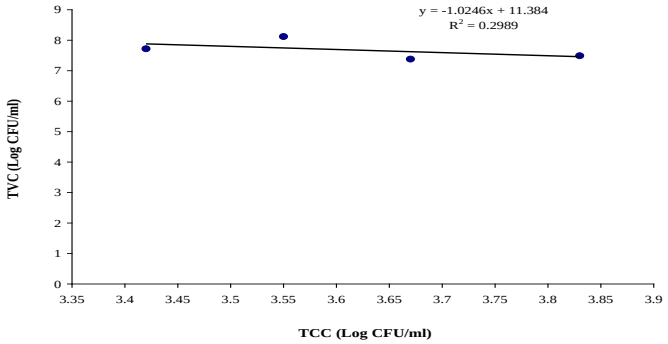

Figure 2. Correlation between total viable count (TVC) and total coliform count (TCC) in unpasteuzized milk at Gazipur district.

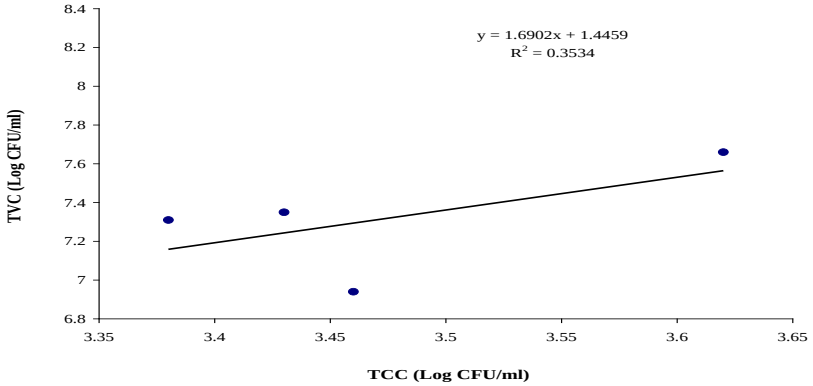

Figure 3. Correlation between total viable count (TVC) and total coliform count (TCC) in unpasteurized milk at Sherpur district.

3.3. Isolation of bacteria from milk sample

Two genera of bacteria were isolated from the collected milk samples. The isolates were identified as *S. aureus* and *E. coli* by observing their biochemical characteristics (Table 4).

Table 4. Enumeration of bacterial isolates in milk sample collected from different markets of Mymensingh, Gazipur and Sherpur districts.

Name of product (number of samples)	Bacterial genera	
	S. aureus	***E. coli***
Unpasteurized milk (20)	20	19
Pasteurized milk (20)	ND	ND
UHT milk (20)	ND	ND

ND= Not Detected

3.4. Molecular detection of bacteria

The optimized PCR assay was able to successfully amplify the target 16S rRNA gene (1267 bp fragment) from the DNA templates of all isolated *S. aureus.* Result of PCR for *S. aureus* is shown in Figure 4. PCR primers targeting 16S rRNA gene of *E. coli* amplified 585 bp fragments of DNA confirmed the identity of *E. coli*. Result of PCR for *E. coli* is shown in Figure 5.

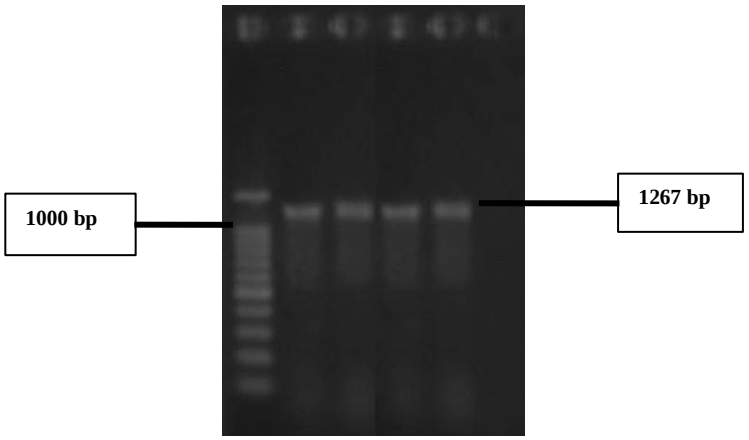

Figure 4. 16S rRNA gene based PCR of *S. aureus* Lane 1: 100 bp DNA ladder, Lane 2, 3, 4, 5: Tested samples were positive for 16S rRNA gene, Lane 6: Negative control.

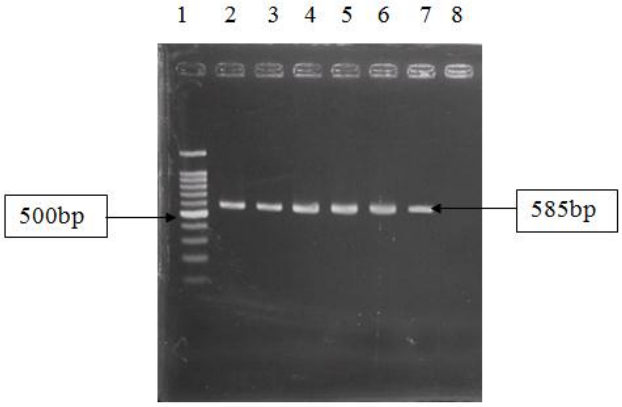

Figure 5. 16S rRNA gene based PCR for *E. coli* Lane 1: 100 bp DNA ladder, Lane 2, 3, 4, 5, 6: Tested samples were positive for 16S rRNA gene, Lane 7: Positive control, Lane 8: Negative control.

3.5. Results of antimicrobial susceptibility of isolated *S. aureus* and *E. coli*

The results of antimicrobial susceptibility test showed that most of the isolates of *S. aureus* and *E. coli* were susceptible to azithromycin, streptomycin, gentamicin, norfloxacin, tetracycline and ciprofloxacin but resistant to amoxicillin and erythromycin (Table 5).

Table 5. Results of antimicrobial susceptibility of the isolated *S. aureus* and *E. coli*.

Name of Bacteria (n)	Antimicrobial agents	Antimicrobial susceptibility profile		
		Sensitive (%)	Intermediate (%)	Resistant (%)
Staphylococcus aureus (20)	Amoxycillin	0(0)	2(10)	18(90)
	Azithromycin	20(100)	0(0)	0(0)
	Ciprofloxacin	18(90)	2(10)	0(0)
	Erythromycin	0(0)	2(10)	18(90)
	Gentamicin	18(90)	2(10)	0(0)
	Norfloxacin	17(85)	1(5)	2(10)
	Streptomycin	16(80)	2(10)	2(10)
	Tetracycline	4(20)	5(25)	11(55)
E. coli (19)	Amoxycillin	0(0)	3(16)	16(84)
	Azithromycin	16(84)	1(5)	2(11)
	Ciprofloxacin	17(89)	2(11)	0(0)
	Erythromycin	0(0)	0(0)	19(100)
	Gentamicin	19(100)	0(0)	0(0)
	Norfloxacin	19(100)	0(0)	0(0)
	Streptomycin	11(62)	5(26)	3(16)
	Tetracycline	4(21)	2(11)	13(70)

3.6. Results of antimicrobial resistance pattern of *S. aureus*

Out of 20 isolates of *S. aureus*, 9 (45%) isolates were resistant to 1 antimicrobial agent (AMX). Moreover, 3 (15%) and 2 (10%) isolates were resistant to 2 antimicrobial agents (AMX-E) and (AMX-TE) respectively. Furthermore 3 (15%) isolates were resistant to three antimicrobial agents (AMX-E-TE), 2 (10%) isolates were resistant to 4 antimicrobial agents (AMX-E-TE-NOR) and 1 (5%) isolates were resistant to 5 antimicrobial agents (AMX-E-TE-NOR-S). These results are shown in Table 6.

Table 6. Results of antimicrobial resistance pattern of *S. aureus.*

Isolates	Resistance profile	No. of isolates (%)
S. aureus (n=20)	No resistance demonstrated	-
	Resistance to 1 agent (AMX)	9 (45)
	Resistance to 2 agents (AMX-E)	3 (15)
	Resistance to 2 agents (AMX-TE)	2 (10)
	Resistance to 3 agents (AMX-E-TE)	3 (15)
	Resistance to 4 agents (AMX-E-TE-NOR)	2 (10)
	Resistance to 5 agents (AMX-E-TE-NOR-S)	1 (5)
	Total resistant isolates	20 (100)

3.7. Results of antimicrobial resistance pattern of *E. coli*

Out of 19 isolates of *E. coli*, 8 (42.10%) isolates were resistant to 1 antimicrobial agent (AMX). Moreover, 4 (21.05%) and 2 (10.52%) isolates were resistant to 2 antimicrobial agents (AMX-E) and (AMX-S) respectively. Furthermore, 2 (10.52%) isolates were resistant to three antimicrobial agents (AMX-E-TE), 2 (10.52%) isolates were resistant to 4 antimicrobial agents (AMX-E-TE-S) and 1 (5.26%) isolates were resistant to 5 antimicrobial agents (AMX-E-TE-S-AZM). These results are shown in Table 7.

Table 7. Results of antimicrobial resistance pattern of *E. coli.*

Isolates	Resistance profile	No. of isolates (%)
E. coli (n=19)	No resistance demonstrated	-
	Resistance to 1 agent (AMX)	8 (42.10)
	Resistance to 2 agents (AMX-E)	4 (21.05)
	Resistance to 2 agents (AMX-S)	2 (10.52)
	Resistance to 3 agents (AMX-E-TE)	2 (10.52)
	Resistance to 4 agents (AMX-E-TE-S)	2 (10.52)
	Resistance to 5 agents (AMX-E-TE-S-AZM)	1 (5.26)
	Total resistant isolates	19 (100)

4. Discussion

The present study was designed for the determination of TVC and TCC value in milk as well as for the isolation, identification and characterization of bacteria from unpasteurized, pasteurized, and UHT milk sample. Total 60 samples consisting of unpasteurized (20), pasteurized (20), and UHT milk (20) were aseptically collected from different markets of Mymensingh, Gazipur and Sherpur for bacteriological study. Only unpasteurized milk showed the positive result for TVC, TCC and *S. aureus*, and *E. coli*, were identified from it which were confirmed by cultural examination, morphological studies, staining characters and biochemical tests and finally PCR were performed for the amplification of specific 16S rRNA gene of isolated bacteria.

Milk is the best media for the growth of many bacteria in which some of them were pathogenic. As we know fresh milk is enriched with pathogenic and non-pathogenic *Staphylococcus* spp. which can be transmitted to human by milking and consumption of milk. *Staphylococcus* bacteria were found in the unpasteurized milk. This *S. aureus* might be hazardous if proper boiling of milk is not done during consumption. It also causes disease if proper hygienic procedure is not maintained during milking. In Bangladesh, Parveen (2000) characterized *S. aureus* isolated from human and animal samples and Das (2012) isolated and identified *S. aureus* from laboratory animals and human and also determined antibiogram profile. Jorgensen *et al*. (2005) stated that the presence of strains assigned to this *Staphylococcus* spp. in bulk milk or in raw milk products could reflect human contamination.

TVC of less than 20,000 CFU per ml which indicated the overall milk hygiene was satisfactory. Milk with a TVC consistently under 10,000 CFU per ml was achieved by many producers. TVC over 20,000 CFU per ml

indicated the hygiene needed improving and counts exceeding 100,000 CFU per ml showed a serious problem. Raw drinking milk must meet the criteria on Plate Count at 30°C (cfu per ml) ≤ 20,000, Coliforms (cfu per ml) < 100 (ACM, 2010). In this study, total viable count (TVC) in unpasteurized milk of Mymensingh, Gazipur and Sherpur districts were log 7.59±0.567, log 7.47±0.547 and log 7.48±0.452 CFU/ml which had the public health importance. The result of TVC in this study is supported by the result of Khan *et al.* (2008). The total coliform count (TCC) in unpasteurized milk of Mymensingh, Gazipur and Sherpur districts were log 3.52±0.038, log 3.62±0.174 and log 3.47±0.104 CFU/ml respectively. The result of TCC in this study is supported by result of Khan *et al.* (2008) and the result of TCC is higher than the result of Anderson *et al.* (2011).

In this study, it was showed that *S. aureus* produced smooth, convex, lustrous, circular colonies reaching a size of 0.5-1.5 μm in diameter and grown in an irregular three-dimensional bunch of grapes-like clusters of cells. In dependence on growth conditions, the colony pigmentation varies from grey, grey-white with yellowish to orange shades with typical β-haemolysis on the blood agar. Deresse *et al.* (2012), Sushma *et al*. (2012), Alzbeta *et al*. (2012) also recorded similar staining characteristics of *S. aureus.* The selected organism *S. aureus* gave positive result on catalase and coagulase test which were closely correlated with Sasidharan *et al*. (2011).

In this study, colony characteristics of *E. coli* observed in NA, EMB were similar to the findings of Ali *et al.* (1998). In Gram's staining, the morphology of the isolated bacteria exhibited Gram negative short rod arranged in single or paired and motile which was supported by several authors (Freeman, 1985; Buxton and Fraser, 1977).

The *E. coli* isolates revealed a complete fermentation of 5 basic sugars by producing both acid and gas which was supported by Russo (2005). The isolates also revealed positive reaction in MR test and Indole test but negative reaction in VP test (Honda *et al*., 1982; Buxton and Fraser, 1977).

PCR based molecular detection of these pathogens in the raw milk and milk products could be remarkably contribute to clarify the actual role in Staphylococcal food poisoning and other clinical symptoms associated with the consumption of milk and milk products. PCR could detect more specifically *S.aureus* than other method. The PCR based system for identification of *S. aureus* from milk has been used by many researchers (Annemuller *et al*., 1999; Brakstadt *et al*., 1992). In PCR among the isolated *E. coli* all are positive to 16s rRNA gene which were similar to the result of Hassan *et al.* (2014).

Results of antimicrobial susceptibility test showed that most of the isolates of *S. aureus* were sensitive to azithromycin, streptomycin, gentamicin, norfloxacin, tetracycline and ciprofloxacin but resistant to amoxicillin and erythromycin. These findings are slightly correlated to Guerin *et al*. (2003) who also observed similar type of findings. In this study, out of 20 *S. aureus* isolates 11 (55%) were detected as multidrug resistant. Resistant profiles of multidrug resistant *S. aureus* were similar to the results of Sharma *et al.* (2011). Results of antimicrobial susceptibility test showed that most of the isolates of *E. coli* were sensitive to azithromycin, streptomycin, gentamicin, norfloxacin and ciprofloxacin but resistant to amoxicillin and erythromycin, which was closely related with Memon *et al.* (2012). In this study, out of 19 *E. coli* isolates 11 (57.89%) were detected as multidrug resistant. This result was similar to the result of Sheikh *et al*. (2013).

5. Conclusions

The results of this study indicated that pasteurized and UHT milk is safe for human consumption but the unpasteurized milk from markets without any treatment has the public health importance. In addition, the findings of this study revealed the presence of multidrug resistant *S. aureus* and *E. coli* in unpasteurized milk samples that posseses a serious threat to public health.

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Conflict of interest

None to declare.

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