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Antimicrobial Drug Resistance of *Escherichia coli* and *Staphylococcus aureus* Isolated from Milk and Milk Based Beverages of Dhaka City, Bangladesh

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Abstract

The increase of pathogens in milk is threatening for the human beings. This is an investigation on overall microbiological analysis of raw and pasteurized milk as well as the milk-based beverages and also determining the antibiotic resistance pattern of isolated *Escherichia coli* and *Staphylococcus aureus*. A total of 100 samples (raw milk, pasteurized milk, mattha, lassi and laban) were taken from various locations of the capital city of Bangladesh, Dhaka. Total Viable Count, Total Coliform Count and Yeast and Moulds Count were performed as the microbiological inspection of selected samples. *E.coli* and *Staphylococcus aureus* were identified by conducting morphological analysis, gram-staining and biochemical tests. Antibiotic resistance pattern of isolated *Escherichia Coli* and *Staphylococcus aureus* were also detected with 11 commonly used antibiotics by conducting disc-diffusion method, following the CLSI guideline. The TVC range was the highest in raw milk samples (3.8×10^4 - 4.1×10^8 cfu/ ml), and the lowest in pasteurized milk samples (1.2×10^2 - 5.4×10^3 cfu/ml), while 70% raw milk and 10% pasteurized milk samples strains were above the acceptable limit of Food and Drug Administration (FDA). Thirty-six *Escherichia coli* and thirty-two *Staphylococcus aureus* were isolated from all the 100 milk and milk-based beverage samples. The isolated *Escherichia coli* strains were most resistant to Penicillin G (81.58%), Erythromycin (78.94%) and Ampicillin (73.68%), and isolated *Staphylococcus aureus* strains were most resistant to Penicillin G (90.62%), Ampicillin (81.25%) and Methicillin (71.87%). respectively. Public awareness is needed to reduce the redundant use of antibiotics.

Keywords: Raw milk, Pasteurized milk, Disc-diffusion method, Antibiotic resistance pattern, Beverages

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INTRODUCTION

Milk is considered as vital food in terms of nutrition, especially for children and adults, and it is pondered as significant to compensate the protein deficiency in human and other mammals[1]. Milk can be contaminated by various sources during the milking procedure in dairy farms to the milk industry- consequently, microbiological quality assessment along with the entire production chain is crucial to take obligatory measure to indemnify human health[2]. Because of the short shelf life and higher moisture content milk is considered as an immense growing medium for pathogen such as, *Campylobacter jejuni, Staphylococcus aureus, Escherichia coli, Yersinia enterocolitica, Listeria monocytogenes*, which can cause different food-borne diseases[3]. Pasteurization is the method of heating milk for a certain time at a predetermined temperature to destroy pathogenic microbes and this process can uplift the shelf life of a food product but, it doesn't perish the slow growing and spore producing microorganisms[4]. Inappropriate handling after pasteurization can re-contaminate the pasteurized milk[5]. Pasteurization of milk and milk products is an emerging requirement of consumers found in developed countries. It is compulsory for all milk samples to be pasteurized in Canada, and no one can sell and distribute unpasteurized milk illegally under the regulation of Food and Drug[6]. FAO, FDA, EEC are the leading regulatory authorities for milk and milk products all over the world[7]. The acceptable limits for raw and pasteurized milk given by FDA are, TVC, $<1\times10^5$ (cfu/ml) and $<1\times10^4$ (cfu/ml) and coliform $<1\times10^3$ and <10 (cfu/ml) respectively but no *E.coli* is acceptable in 1ml of raw milk and pasteurized milk[8]. Enterohemorrhagic *Escherichia coli* O157:H7 is the most dominant food borne pathogen causing different diseases in human[9]. *Staphylococcus aureus* is commonly known as pathogenic bacteria, which is found in hospitals and in the community. There are several foodborne intoxications caused by *Staphylococcus aureus* which are connected to the contaminated milk consumption[10-12]. Fungi are conventional contaminants of milk and dairy products and they can generate damages in milk, such as bad odor and flavor, decolonization, disorganization

of structure and as well as economic losses[13-16]. Moulds that causes spoilage like *Aspergillus* and *Penicillium*, produce fungal toxins in milk and dairy products[17-20]. The prevalence of antibiotic resistance in *Escherichia coli* and *Staphylococcus aureus*, with high virulence potential is alarming in south Asia. Scarcity of appropriate data on milk-borne diseases and pathogens from countries with high infection record, such as Bangladesh, obstructs the controlling of disease and infections[11]. In order to have milk and milk products free from contamination of bacteria and other microbes, Hazard Analysis Critical Control Point (HACCP) must be obeyed, from milk collection through milk processing to milk storage[22].

In Bangladesh, although a lot of work has been conducted based on milk as well as milk based products for assuring quality and welfare, but these are not sufficient at all. In this regard, this study was planned after considering the public health aspect of Bangladesh to ensure the best quality of milk and dairy based products for consumption of mankind by inspection and investigation of the pathogenic microorganisms as well as find out the effective antibiotics against food-borne pathogens.

MATERIAL AND METHODS
Collection of Samples

An integrate set of 100 samples (n=100) were taken from various locations of the capital city of Bangladesh, Dhaka including, raw cow milk (n=20), pasteurized milk (n=20), mattha (n=20), lassi (n=20) along with laban (n=20). Among them, dairy farms were the collection source of raw cow milk samples whereas branded pasteurized milk samples were collected from grocery shops, also the mattha, lassi, labang (traditional milk based beverages of Dhaka city) samples were collected from local street vendors. Before sample collection the collecting tubes were cleaned aseptically[23]. The samples were analyzed immediately in the laboratory.

Sample Processing

Tenfold serial dilution of each sample was prepared in autoclaved saline water. Initially one milliliter of raw sample was mixed with nine

milliliter of saline in test tube in order to prepare 10^1 dilutions and this process was repeated until 10^7 dilutions for every samples[24-25].

Total Viable Count (TVC)

0.1 ml samples from 10^1–10^7 dilution were poured with fresh Plate Count Agar (PCA) plate. Incubation was done for 24 hours at 37°C. After that, colonies on plates were observed. Screening was done in order to identify isolated colonies and the real number of bacteria was counted in colony forming unit in (cfu/ml)[26-28].

Total Coliform Count (TCC)

Total Coliform Count was done by applying spread plate method on Mac Conkey agar medium. 0.1 ml of each samples from, 10^1–10^5 dilution were spread on Mac Conkey agar medium. Pink colonies were considered for the counting of Total Coliform Count (cfu/ml) after the period of incubation[26,27,29].

Yeast & Moulds Count (YMC)

For counting yeast and mold, 0.1 milliliter of samples from 10^1–10^5 dilution were spread properly on sterile Potato Dextrose Agar (PDA) plate. The PDA was mixed with 10% of lactic acid before it confers to the petri dish. The procedure was done by following ideal spread plate method. After incubation, the number of viable yeast & mould from plates had been counted in (cfu/ml)[30-32].

Isolation and identification of *Escherichia coli* and *Staphylococcus aureus*

Spread plate technique on EMB and BPA agar media had done for isolation of desired *E.coli* and *S. aureus* colonies respectively. *E.coli* and *S. aureus* were identified by conducting morphological, gram-staining and biochemical tests (Catalase, Oxidase, Mobility, Indole, MR and VP)[23].

Antibiogram of isolated *Escherichia coli* and *Staphylococcus aureus*

Kirby-Bauer procedure was applied to examine antimicrobial drug resistance pattern of pathogens[33]. The antibiotics tested for both *E.coli* and for *S. aureus* were - Methicillin (MET) 5µg, Penicillin G (P) 10µg, Ampicillin (AMP) 25µg, Amoxycillin (AML) 25µg, Gentamicin (GEN) 10µg, Streptomycin (S) 10µg, Azithromycin (AZM) 15µg, Erythromycin (E) 35µg, Ciprofloxacin (CIP) 5µg, Tetracycline (TIC) 30µg and Imipenem (IMP) 10µg.

The Mueller-Hinton agar plates (containing pure culture and antimicrobial discs) were examined after proper incubation and zone diameters of each antibiotic discs of complete inhibition were measured through the millimeter scale. The results were translated according to the interpretation table of the CLSI and EUCAST[34], European Committee on Antimicrobial Susceptibility Testing (2018). European Committee on Antimicrobial Susceptibility Testing (2019)[35,23,36,37]. The zone diameter results were compared with both CLSI and EUCAST standard zone diameter break points because, all the antibiotic resistance break points for each bacteria were not available in only one guideline. *Escherichia coli* ATCC@ 25922 and *Staphylococcus aureus* ATCC@ 29213 were used as control strains.

RESULTS

Microbiological assessment of raw milk and pasteurized milk

The TVC range of raw cow milk samples was the highest ($3.8×10^4$ - $4.1×10^8$ cfu/ml) whereas the lowest TVC range was observed in case of pasteurized milk samples ($1.2×10^2$ - $5.4×10^5$ cfu/ml). In terms of TVC, only 30% of raw cow milk had been found safe for customer consumption considering the limits given by FDA while, 90% of the pasteurized branded milk was of good quality (Fig. 1). 30% of raw milk was contaminated with coliform and six *E.coli* strain was detected (Table 1). The highest yeast and moulds were observed in raw milk ($6.1×10^2$ cfu/ml), while 40% found beyond the acceptable limit of FDA (Table 1).

Microbiological assessment of milk based beverages (mattha, lassi, laban)

Different non-branded milk based beverages were collected to analyse the quality based on different microbiological parameters. In terms of TVC different ranges of results were observed, such as- mattha ($7.8×10^3$– $1.6×10^6$ cfu/ml), lassi ($5.8×10^3$– $3.1×10^7$ cfu/ml) and laban ($1.9×10^4$– $2.3×10^6$ cfu/ml). In case of total coliform count, highest count was in mattha ($2.2×10^3$) and the lowest count was in lassi ($3.1×10^1$). Yeast and moulds were highest in mattha ($7.3×10^2$) and lowest in lassi ($7.2×10^1$). (Table 2).

Gram staining & Biochemical Identification

Gram-staining and specific biochemical

Table 1. Microbial count of raw cow milk and branded pasteurized milk in different microbiological parameters

Microbiological testing parameters	Microorganisms in raw milk (cfu/ml)	FDA standard for raw milk (cfu/ml)	Number and percentage of raw milk beyond the acceptable limit	Microorganisms in pasteurized milk (cfu/ml)	FDA standard for pasteurized milk (cfu/ml)	Number and percentage of pasteurized milk beyond the acceptable limit
TVC	3.8×10^4-4.1×10^8	$<1\times10^5$	14 (70%)	1.2×10^2 - 5.4×10^5	$<1\times10^4$	2 (10%)
TCC	7.0×10^1-2.2×10^3	$<1\times10^3$	6 (30%)	0 - 7.1×10^1	<10	2 (10%)
Yeast and Mould Count	1.8×10^1-6.1×10^2	$<1\times10^2$	10 (50%)	0 - 9.5×10^1	$<1\times10^2$	8 (40%)

Table 2. Microbial count of mattha, lassi, laban (milk based beverages) in different microbiological parameters

Microbiological testing parameters	Mattha (cfu/ml)	Lassi (cfu/ml)	Labang (cfu/ml)
TVC	7.8×10^3– 1.6×10^6	5.8×10^3– 3.1×10^7	1.9×10^4– 2.3×10^6
TCC	4.6×10^1-2.2×10^3	3.1×10^1– 8.4×10^2	7.0×10^1– 3.3×10^2
Yeast and Mould Count	1.1×10^2-7.3×10^2	7.2×10^1– 6.3×10^2	8.5×10^1– 3.6×10^2

(No national or international regulations based on microbiological analysis is available for mattha, lassi and laban.)

Table 3. Morphological, gram staining and biochemical tests of *Escherichia coli* and *Staphylococcus aureus*

Source and Colony	Gram Staining	Catalase	Oxidase	Mobility	Indole	MR	VP	Suspected microorganism
morphology EMB agar (round colony with green metallic sheen)	-	+	-	+	+	+	-	*Escherichia coli*
BPA (black colony with halo zone)	+	+	-	-	-	+	+	*Staphylococcus aureus*

tests were performed for the identification of *Escherichia coli* and *Staphylococcus aureus*. The suspected colonies from EMB and BPA medium which were observed as gram negative, rod and gram positive, cocci under the light microscope respectively, were chosen for the biochemical test. Catalase test and VP test were negative and other tests were positive for *E.coli*. On the other hand, catalase, MR and VP test were positive and other test results were negative for the isolated *Staphylococcus aureus*. (Table 3).

Number of pathogens according to their source

The highest number of *E. coli* was identified from mattha (10 out of 20) and laban (10 out of 20) sample and the lowest were identified from pasteurized milk (2 out of 20) samples. On the other hand, the highest (11 out of 20) and the lowest (3 out of 20) *Staphylococcus aureus* was present in raw and pasteurized milk respectively (Table 4).

Table 4. The number of *E.coli* and *S.aureus* in contaminated samples

Microorganisms	Raw milk	Pasteurized milk	Mattha	Lassi	Laban	Total
E.coli	6	2	10	8	10	36
Staphylococcus aureus	11	3	8	5	5	32

Table 5. Antibiotics used for determination of antibiotic resistance pattern

Names of antibiotics	Antibiotic conc.
Methicillin (MET)	5µg
Penicillin G (P)	10µg
Ampicillin (AMP)	25µg
Amoxycillin (AML)	25µg
Gentamicin (GEN)	10µg
Streptomycin (S)	10µg
Azithromycin (AZM)	15µg
Erythromycin (E)	35µg
Ciprofloxacin (CIP)	5µg
Tetracycline (TIC)	30µg
Imipenem (IMP)	10µg

Antibiogram

Disc-Diffusion Method was performed on Mueller-Hinton agar plate with 11 antibiotics commonly used in medical world for the treatment of disease associated with *E.coli* and *S.aureus*. Zone of inhibition were measured and compared with the zone diameter interpretive standard of CLSI & EUCAST. (Table 5).

Antibiotic resistance pattern of isolated *E.coli* and *Staphylococcus aureus*

There were 36 *E.coli* strains and 32 *S.aureus* strains isolated from all the 100 milk and milk based beverages samples. Antibiotic resistance patterns in those isolated pathogens were tested with 11 commercially available antibiotics. After performing the disc diffusion method, isolated *Escherichia coli* were found most resistant to Penicillin G (81.58%), Erythromycin (78.94%) and Ampicillin (73.68%), (Fig. 1). The *Staphylococcus aureus*, were most resistant to Penicillin G (90.62%), Ampicillin (81.25%) and Methicillin (71.87%) (Fig. 1). Penicillin G and Ampicillin both were tested against the Enterobacteriaceae (*Escherichia coli*) considering the antibiotics using frequency of the people of Bangladesh.

DISCUSSION

This study was performed for investigating the harmful microorganisms in raw milk, pasteurized milk and milk based
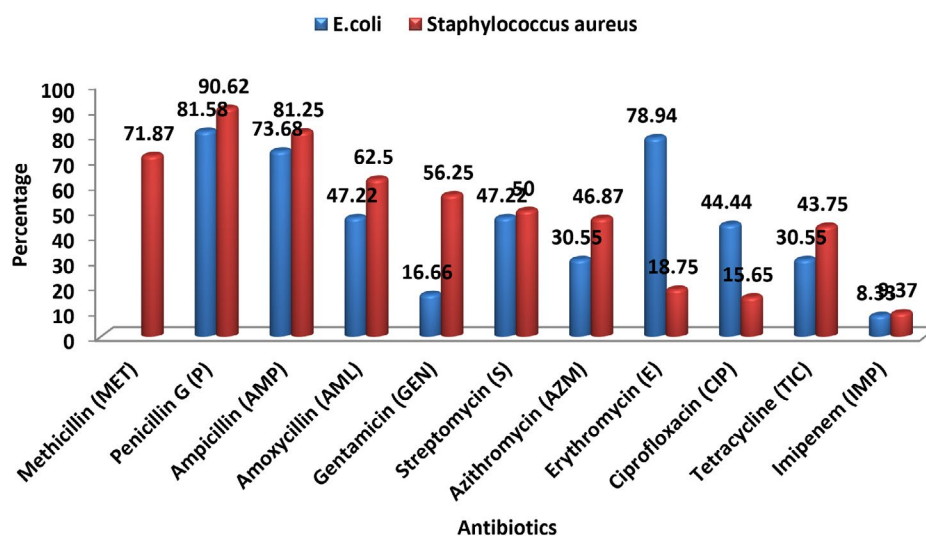

Fig. 1. Antimicrobial resistance patterns of isolated *E. coli* and *S.aureus* against commercial antibiotics

beverages available in Dhaka city and also for the determination of the antimicrobial resistance pattern of the strains- *Escherichia Coli* and *Staphylococcus aureus* taken from those samples with some commercial antibiotics. In this study, Total Viable Count for raw milk samples ranged from (3.8×10^4 - 4.1×10^8 cfu/ml). Unfortunately, 70% of raw cow milk and 10% of pasteurized branded milk samples were found beyond the acceptable limit of FDA. A study was done by Jubaida et al[38] on pasteurized, UHT and flavored milk and they found the TVC range in pasteurized milk between 2.3×10^2 (cfu/ml) to 4.69×10^3 (cfu/ml). In their study, only one pasteurized branded milk was found contaminated with coliform, but the number was under acceptable limit (<10/ml coliform in pasteurized milk according to BSTI). In our study we found almost similar result considering the study of Jubaida et al[38]. We found 2 pasteurized milk samples contaminated with coliform group among the 20 samples. Some evidence showed that dairy products such as lassi, mattha, and yogurt in Dhaka city yielded a total viable count of 10^2–10^4 cfu/ml. In our study we found slightly different results with mattha (7.8×10^3 – 1.6×10^6 cfu/ml), lassi (5.8×10^3 – 3.1×10^7 cfu/ml) and laban (1.9×10^4 – 2.3×10^6 cfu/ml). Among the 100 samples, 36% were contaminated with *Escherichia coli* and 32% with *Staphylococcus aureus*[39], conducted their study to examine the microbial contamination within common milk products of Dhaka, Bangladesh. Their samples included mattha, sweetened yogurt, lassi and many other dairy products. All samples were found contaminated with bacteria and fungus within 10^2-10^4 (cfu/ml) and 10^2-10^3 (cfu/ml). In our study we found, the highest population of yeast and moulds were in mattha (7.3×10^2 cfu/ml) and the lowest in lassi (7.2×10^1 cfu/ml). A study was performed by Nushrat et al[40], on microbial quality of dairy beverages commonly found in Dhaka. They had found (3.6×10^7 cfu/ml) and (1.7×10^3 cfu/ml) TVC in laban and strawberry milk shakes. They also found multi-drug resistant bacteria in some of their samples and all strains showed resistance against both Ampicillin and Colistin. Our study also identified multiple drug resistance pathogens in raw, pasteurized milk and milk based beverages of Dhaka city. In the present study, through disc-diffusion method, isolated *Escherichia coli* strains were most resistant to Penicillin G (81.58%), Erythromycin (78.94%) and Ampicillin (73.68%), and isolated *Staphylococcus aureus* strains were most resistance to Penicillin G (90.62%), Ampicillin (81.25%) and Methicillin (71.87%).

CONCLUSION

Multi drug resistant pathogens in food is alarming. Milk and milk based beverages are an essential part of diet in south Asia. Significance of maintaining hygiene during their preparation and storage is vital. The current study indicates poor hygiene and management of the food processing in some cases. Antibiotic resistance pattern is also important to identify the correct antibiotic immediately during the medical treatment. Public awareness is crucial to avoid unhygienic foods.

CONFLICT OF INTEREST

The authors declare that there are no conflict of interest.

AUTHORS' CONTRIBUTION

SP planned the study and supervised the research work. KWH performed the whole investigation. NT did the data analysis and methodology portions. KWH wrote down the original draft and all the authors reviewed it.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

Not applicable.

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