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Raw Milk In Noakhali, Bangladesh: Quality Assessment and Antibiotic Resistance of Identified Microorganisms

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Abstract

Milk is an ideal food for all age groups. The current study was carried out to identify the microorganisms to assess the raw milk quality and the antibiotic resistance of those identified micro-organisms. Five raw milk samples along with two high treatment (UHT) milk samples from different locations of Noakhali district of Bangladesh were analysed. Bacterial isolation was performed by Nutrient Agar (NA) and MacConkey (MCA), Eiosin Methylene Blue (EMB) and Genital Menital Salt agar (GMSA). The isolates were then identified by Kliger's Iron Agar (KIA) test, Motility Indole Urease (MIU) test, Catalase and Oxidase tests. Antibiotics resistance tests were done for 13 different antibiotics. Among all these samples, Maijdee Bazar (S4) contained the highest load as 1.87×10^6 and the UHT samples contained no bacterial contamination. *E. coli* covered 47.05% whereas *Listeria*, *Bacillus* and *Yersinia* were in the same percentage as 5.88% among all isolates. *Salmonella* and *Staphylococcus* were 23.53% and 11.76%,respectively. *Listeria* and *Salmonella* were resistant to five different antibiotics by 46.15% and 38.46%of multiple antibiotic resistance index (MRI), correspondingly. However, *E. coli* and *Yersinia* were resistant to three antibiotics namely, Rifampcin (RIF), Cefotaxime (CTX), Amoxycillin (AMX) by about 23% as MRI percentage. *Bacillus* and *Staphylococcus* both were resistant to Cefepime (CPM) by 7.69% of MRI. Hence, it can be concluded that Rifampcin and Cefepime were most common antibiotics which were resisted by most of the isolates.Therefore, hygiene aspect of these milk sources needs to be taken into consideration with high priority. Also, the antibiotics which are resisted by different organisms will be detrimental for public health aspects.

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Introduction

Generally, people rarely consume raw milk.However, there are some peoplewho preferconsuming natural, unprocessed food. These people believe that raw unpasteurized milk, which has not been subject to any heating process,has particular healthy properties which reduces susceptibility to allergies, enhancesnutritional quality, and has a better taste.[1 2]

A sound sanitary manner is needed for the production and processing of wholesome and nutritious food according to consumer's preference. Milk is a nutrient densewhite fluid secreted by female mammary gland and widely consumed foodamong all age groups. Quality milk convey the meaning of normal chemical composition, being completely free of harmful bacteria and harmful toxic substances, free of sediment and extraneous substances, having lower degree of titratable acidity, having good flavour, being adequate in preserving quality, and having low bacterial counts. In Bangladesh, cattle rearing, milk production and distributionare mostly done by following traditional method. Modern technologies for cattle rearing, machine milking process are not common practices other than big farms.[3 4]

The factors associated with contamination of raw milk are the milking machine, milking area, faecal contamination, personal hygiene, poor storage condition.[15] Dairy farms mainly use antimicrobials because of the intra-mammary inflammation.[2] Uncontrolled usage of antimicrobial components may affect negatively on human health by residing the residues in cattle body. Whereas controlled usage of antimicrobials can help to rear healthy cattle which would be added value in dairy industry.[6]

Previously, Tekilegiorgis mentioned about a study in Ethiopia in 2018 where total bacterial count was 5×10^3 to 3.18×10^8 cfu/ml in raw milk and 4.4×10^1 to 4.43×10^5cfu/ml in pasteurized milk samples.[7] Regasa *et al*., (2019) reported about *Staphylococcus* aureus susceptibility was 16.6% and load count was 10^4-10^5cfu/ml[8] In Northern Italy (2016), the prevalence of *Listeria monocytogenes* was reported as 1.66%, more specifically 2.2% from bulk milk tank whereas 0.5% in vending machine of milk.[9] Ahmed *et al*., (2019) reported about five different pasteurized brands of milk from Bangladesh which ranged from 3.5×10^4 to 1.15×10^7cfu/ml. According to Bangladesh Standards and Testing Institution, the total bacterial count should not exceed 20,000 CFU/ml.[10] In 2015, raw, pasteurized milk and yogurt samples were collected from different zones of Dhaka city, Bangladesh. In that report, it has been mentioned that total viable count varied from 3.5×10^3cfu/ml to 4.2×10^6cfu/ml for raw milk samples. Along with this, all of them were contaminated with *E. coli* and Shigella-like species but *Listeria* was not present.[11] Previously, standard bacterial plate count for pasteurized milk was reported in Sylhet city, Bangladesh where the range was also higher than the recommended range (54200 to 68400 cfu/ml).[12]

In addition to this issue, some organisms are present as potential for food borne illnesses and some of them are comprised of genes which are antimicrobial resistant. They are also mentioned for the mycotoxin and presence of their metabolites in the milk and dairy products.[13] Higher level of yeastand lower level of mould compared to yeast had been reported in differentstudies on raw milk.[14 15]

As milk is an ideal media for growth of microorganisms, it is very crucial to investigate the microbial contamination load and associated microorganisms' presence in milk.[16] Although most of the cases milk is pasteurized before marketing, microorganisms can be a vital concern regarding health aspects, as there are several situations when pasteurized milk cannot be helpful.[15]

In several researches it has been mentioned that concern in the dairy industry has raised because of the disease outbreaks from the consumption of unpasteurized milk by farm employees, family members, associated neighbours and nearby local area population. Unpasteurized milk is also used in the cheese industry. Along with this, contamination in the milk processing industry also allow forming of biofilms and some improper pasteurization may not abolishfood borne microorganisms.[2] No previous data was found on the microbial quality assessment of milk in this area. As milk is widely consumed food among all age groups thus the present study aims to identify the presence of pathogenic microorganisms in raw milk and antibiotic resistance of those organisms in the mentioned region.

Materials and Methods

Sample Preparation

Five raw milk samples were collected from five different locations of Noakhali, Bangladesh.Two different brands of UHT milk samples were collected from the same region. Selection of collection zones and brandswasdone randomly. The samples were labelled as S1, S2, S3, S4, S5 and UHT andthose were collected from University student hall, Suborno-agro, Sonapur, Maijdee, VC- Bungalow and local shops, respectively. All of them were collected and transportedin ice boxtothe laboratory of Food Technology and Nutrition Science, Department of Noakhali Science and Technology University. Then samples werestored in the laboratoryat 4ºC. 1 ml of milk sample was mixed with 9ml of 0.9% sterile sodium chloridesolution in a sterilized cotton plugged test tube. Then it was mixed by stirring and shaking and this homogenized solution was then allowed for further serial dilution. The method was followed from Mokbul *et al*. (2016).[17]

Bacteriological Studies

For the isolation of bacteria, pour plate and streak plate techniques were followed. Nutrient Agar (NA) and MacConkey (MCA), Eiosin Methylene Blue (EMB) and Genital menital salt agar (GMSA) were used for isolation purpose. Nutrient agar was used for cultivating non-fastidious microorganisms. MacConkey agar was used for the isolation and differentiation of enteric bacteria. EMB and GMSA agar are highly selective media and they were used for isolating *E. coli* and *Staphylococcus*. All these media were prepared according to their manual. 10 folds dilution was done to reduce the density of the microorganisms.Pour plate technique was used and then it was incubated for 24 hours at 37^0C that were grown in NA, MCA, EMB and GMSA. Colonies were isolated and collected based on their color, shape, elevationand stored in the nutrient agar slant. Morphological and cultural tests were done immediately.

Isolate Identification

Biochemical characterization as Kliger's Iron Agar (KIA) test, Motility Indole Urease (MIU) test,Catalase and Oxidase tests were performed for bacterial identification. The procedureswere followed fromMokbul *et al*., (2016).[17]

Antibiogram Profiling

Isolated strains were inoculated, prepared in Mueller-Hington broth and adjusted to turbidity equal to 0.5 McFarland standards and were applied onto Mueller-Hinton agar using a wire loop. Sterilized swab was then used to spread the culture on the media. The inoculated plate was allowed to dry for a few minutes, after which sensitivity disks were applied to it using sterile forceps. Zones of inhibition around sensitivity disks were measured after 18-24hr of incubation at 37°C. The sensitivity of all isolates was tested against: Rifampcin (RIF) 5µg/disk, Cefotaxime (CTX) 30µg/disk, Amikacin (AK) 30µg/disk, Colistin (CL) 10µg/disk, Genetamicin (Gen) 10µg/disk, Chloramphenicol (c) 30µg/disk, Ciprofloxacin (CIP) 15µg/disk, Amoxycillin (AMX) 30µg/disk, Ceflriaxone (CTR) 30µg/disk, Kanamycin (K) 30µg/disk, Nitrofurantoin (NIT) 30µg/disk, Norfloxacin (NX) 10µg/disk, and Cefepime (CPM) 30 µg/disk according to the CLSI requirements using the disk diffusion method. The interpretation of zones of inhibition around the disks was done according to CLSI (2006) (American Public Health Association, 1913).

Statistical Analysis

SPSS software version 23.0 was used to perform Analysis of Variance (ANOVA) test in order to understand the significant difference between different samples. The level of significance was set at ≤0.05.

Result &Discussion

The study revealed thatall raw milk samples were contaminated and in certain cases pathogens were detected which is a public health concern. Bacterial load of all the samples were quite high (Table 1) and in commercial UHT milk, no microorganisms were found.The range of the bacterial load of raw milk samples were found in different agar as Nutrient agar 1.95×10^4 to 1.87×10^6, Macconkey agar 7.5×10^5 to 1.95×10^6, Eosin Methylene Blue agar 2.33×10^4 to 1.35×10^6, and Glucose minimal salt agar 1.0×10^2 to 1.70×10^6. Among all the samples, the sample from Maijdee bazar (S4) contained the highest bacterial load whereas sample from VC-bungalow (S5) contained lowest bacterial count. According to Bureau of Indian Standards (BIS) for raw milk plate count (SPC) (IS: 1479-1977, PART

111) if the bacterial count is (count/ml) greater than 200,000 then the milk is of better quality, if the count is from 2,000,01 to 1,000,000 then it is of good quality, 1,000,000 to 50, 00,000 is Fair, and if it is more than5,000,000 then it is called poor quality milk.[18] Hence comparing this study with standards, S5 quality was best among all, S3 was better quality and S1 and S4 showed fair quality of milk based on nutrient agar total viable count.According to statistical result, colony count varied significantly in NA for S1 compared to S3, S4 and S5. In MCA, S1, S2 and S3 varied significantly among each other.

Table 1: Bacterial Count in different raw milk samples (CFU/ml) collected from different locations

Sample	Number of tested samples	NA (CFU/ml)	MCA(CFU/ml)	EMB(CFU/ml)	GMSA(CFU/ml)
S1	3	$1.79\times10^{6}\pm 0.36\times10^{6a}$	$9.70\times10^{5}\pm 0.06\times10^{5a}$	$5.60\times10^{5}\pm 0.17\times10^{5acd}$	$7.50\times10^{4}\pm 0.11\times10^{4abce}$
S2	3	$4.50\times10^{5}\pm 0.18\times105^{ab}$	$1.31\times10^{5}\pm 0.31\times10^{5b}$	$4.50\times10^{5}\pm 0.18\times10^{5bcdd}$	$2.00\times10^{4}\pm 0.02\times10^{4ab}$
S3	3	$1.95\times10^{5}\pm 0.08\times10^{5bc}$	$9.50\times10^{5}\pm 0.08\times10^{5c}$	$1.35\times10^{6}\pm 0.56\times10^{6abcd}$	$1.00\times10^{4}\pm 0.05\times10^{4ac}$
S4	3	$1.87\times10^{6}\pm 0.32\times10^{6bd}$	$1.95\times10^{6}\pm0.08\times 10^{6abcde}$	$2.33\times10^{4}\pm 0.58\times10^{4abcd}$	$1.70\times10^{6}\pm 0.72\times10^{6abcde}$
S5	3	$1.95\times10^{4}\pm 0.08\times10^{4be}$	$7.50\times10^{5}\pm 0.44\times10^{5bde}$	$3.00\times10^{5}\pm 0.01\times10^{5cde}$	$1.00\times10^{2}\pm 0.05\times10^{2ade}$
UHT	3	No Detection	No Detection	No Detection	No Detection
F value		21.990	10.153	17.326	14.274
Level of significance p		0.000*	0.002*	0.000*	0.000*

All values are means of triplicate determinations ± standard deviation (SD). The value with different superscripts in a column differs significantly ($p\leq 0.05$)

Biochemical characterization tests were performed to identify the microorganisms in raw milk samples. After conducting the tests,*E.coli*, *Salmonella*, *Staphylococcus*, *Bacillus*, *Listeria monocytogens,* and *Yersenia* were identified in the samples (Table 2). About half (47%) of the identified microorganisms were *E. coli* and only 5.88% were *Bacillus*, *Listeria* and *Yersenia* (Table 3). Previously, microbial contamination assessment had been reported for raw, pasteurized and UHT milk. Amenu *et al*. (2019) reported 2.5% *E. coli* contamination in milk and milk products samples in Ethiopia.[19] *E. coli* contamination had been reported in Italy in 2009 in vending machine as well. They revealed 0.2% *E. coli*, 0.3% *Salmonella* spp., 1.5% *Campylobacter spp*., and 1.6% *Listeria monocytogens* contamination in all the samples.[20] In 2011 Hossain *et. al*., analysed samples of raw, pasteurized and UHT milk from twelve different local markets of different locations in Bangladesh. They concluded that most of the raw milk samples contained indicator and pathogenic organisms as coliform, Aeromonas, *Salmonella*, and *Staphylococcus*. Some raw and pasteurized milk also contained psychrophilic organisms.[4] In 2019, one study reported 10.8% S. aureus harbour in ready to consume raw milk and milk products in Ethopia,.[19] Huque *et al*., (2018) mentioned about total bacterial count in raw milk as 2.31×10^5 to 2.45×10^5 CFU/ml in Savar, Bangladesh.[21] In different zones of Dhaka, Bangladesh, total bacterial count varied between 4.2×106 to 3.5 × 103 CFU/ml.[11] In another review by Zastempowska *et. al*., (2016) it was mentioned that many societies consume raw milk and *Salmonella*, Shiga toxin producing *E. coli*. *Micobacteriumbravis, Campylobacter* were responsible for the disease outbreaks in many

cases.[13] Bianchi *et al*., (2009) mentioned in a similar statementthatunpasteurized milk can be a possible source of food-borne disease outbreak for many organisms.[20] All the organisms thatare reported in this study are in agreement with the previous reports from various researchers of the world.

Table 2: Biochemical tests&identification of microorganisms in raw milk samples

Isolates ID	KIA Test			MIU Test			H_2s production	Citrate test	Identification
	Slant	Lactose	Gas	Motility	Indole	Urease			
M/E/D	*K*	-	+	-	-	-	-	-	*Yersenia*
M/E/R	*K*	-	-	-	-	-	+	-	*Salmonella*
M/E/G	*A*	+	+	+	+	-	-	-	*E. coli*
M/M/P	*A*	+	+	+	+	-	+	-	*E. coli*
M/M/R	*A*	+	+	+	+	-	+	-	*E. coli*
M/E/P	*A*	+	+	+	+	-	+	-	*E.coli*
S/E/G	*A*	+	+	+	+	-	+	-	*E.coli*
V/E/P	*K*	-	-	-	-	-	+	-	*Salmonella*
V/M/R	*A*	+	+	+	+	-	+	-	*E.coli*
Su/E/ G	*A*	+	+	+	+	-	+	-	*E.coli*
Su/E/P	*K*	-	-	-	-	-	+	-	*Salmonella*
H/E/P	*K*	-	-	-	-	-	+	-	*Salmonella*
H/E/G	*A*	+	+	+	+	-	+	-	*E.coli*
H/N/P	*A*	+	-	+	-	-	-	+	*Listeria monocytogens*
V/N/P	*A*	+	-	+	-	-	-	+	*Bacillus*
M/N/P	*A*	+	-	-	-	+	-	+	*Staphylococcus*
S/N/P	*A*	+	-	-	-	+	-	+	*Staphylococcus*

Isolate ID: Sample/Media/Colour;(+) indicates positive; (-) indicates negative

Table 3: Percentage of isolates in the samples

Name of the microorganism	N(%)
E.coli 8	(47.05)
Salmonella	4(23.53)
Staphylococcus	2(11.76)
Bacillus	1(5.88)
Listeria monocytogens	1(5.88)
Yersenia	1(5.88)

After performing biochemical test, *E. coli*, *Salmonella*, *Staphylococcus*, *Bacillus*, *Listeria monocytogens* and *Yersinia* were detected. Similarly, Rahman *et al*., (2015) mentioned about *E. coli*, *Salmonella*, *Listeria* in raw milk samples in Dhaka city, Bangladesh.[11]

Figure 1 shows the antibiotic resistance study of the isolates. This examination has been done with six different isolates and they showed different resistance for different antibiotics.

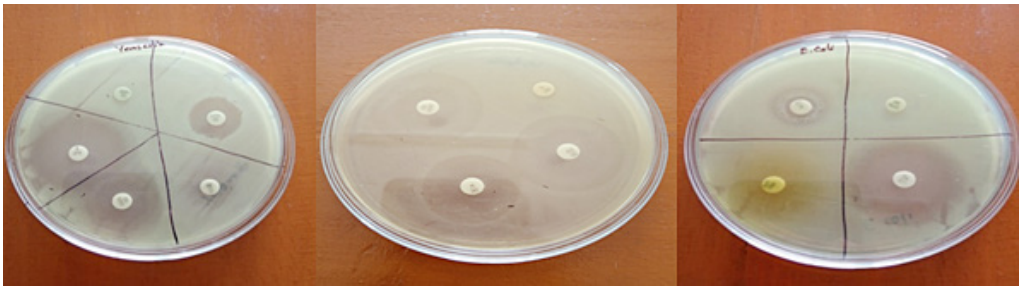

Fig. 1: Antibiotic resistance of the microorganisms

The antimicrobial resistance profiles of the bacterial isolates from raw cow milk are summarized in Table 4. All of the isolatesshowed antibiotic resistance though the MRI% varied organism to organism.

Table 4: Results of antibiotic profile with MRI percentage of isolates

Isolates	Antibiotics			MRI%
	Sensitive to	**Intermediate**	**Resistant to**	
E. coli	*Amikacin(AK), Colistin(CL), Genetamicin(Gen),Chloramp -henicol(c), Ciprofloxacin(CIP), Nitrofurantoin(NIT), Norfloxacin(NX)*	*Ceflriaxone (CTR), Kanamycin(K), Cefepime(CPM)*	*Rifampcin(RIF), Cefotaxime(CTX), Amoxycillin(AMX)*	23.0
Salmonella	*Amikacin(AK),Colistin(CL), Genetamicin(Gen),Chlora -mphenicol(c),Amoxycillin (AMX), Nitrofurantoin(NIT)*	*Ceflriaxone(CTR), Kanamycin(K)*	*Rifampcin(RIF), Cefotaxime(CTX), Ciprofloxacin(CIP), Norfloxacin(NX), Cefepime(CPM)*	38.46
Yersinia	*Amikacin(AK),Colistin(CL), Genetamicin(Gen),Chloramp -henicol(c),Kanamycin(K), Nitrofurantoin(NIT), Norfloxacin(NX)*	*Ciprofloxacin(CIP), Ceflriaxone(CTR), Cefepime(CPM)*	*Rifampcin(RIF) Cefotaxime(CTX) Amoxycillin(AMX)*	23.07
Listeria	*Amikacin(AK),Colistin(CL), Genetamicin(Gen),Ciprofl -oxacin(CIP),Kanamycin(K), Norfloxacin(NX)*	*Ceflriaxone(CTR)*	*Rifampcin(RIF), Cefotaxime(CTX), Chloramphenicol(c), Amoxycillin(AMX), Nitrofurantoin(NIT), Cefepime(CPM)*	46.15
Staphylococcus	*Rifampcin(RIF),Cefotaxime (CTX),Amikacin(AK),Colistin (CL),Genetamicin(Gen), Chloramphenicol(c),Ciproflo -xacin(CIP),Amoxycillin(AMX), Ceflriaxone(CTR),Kanamycin (K),Nitrofurantoin(NIT), Norfloxacin(NX)*		*Cefepime(CPM)*	7.69
Bacillus	*Rifampcin(RIF),Cefotaxime (CTX),Amikacin(AK),Colistin (CL),Genetamicin(Gen), Chloramphenicol(c),Ciprofloxacin (CIP),Amoxycillin(AMX),Ceflriaxone (CTR),Kanamycin(K),Nitrofu- rantoin(NIT),Norfloxacin(NX)*		*Cefepime(CPM)*	7.69

In this study, *Staphylococcus* was sensitive to RIF, CTX, AK, CL, Gen, C, CIP, AMX, K, NIT and NX. Similar finding was reported by Pol and Ruegg (2007) and Frey *et al*., (2013). They mentioned coagulase-negative Staphylococci from bovine milk was resistant to oxacillin, streptomycin, erythromycin, kanamycin, gentamycin.[22 23] *E. coli* showed intermediate resistance and complete resistant to CTR, CPM, K, RIF, CTX, AMX. Among these, Cefoxitin, Ceftriaxone, Kanamycin resistance were in agreement with previously reported research by.[24] The MRI percentage for *E. coli*, *Salmonella* and *Listeria* were23%, 38.46% and 46.15%, respectively. For these three organisms, multi-drug resistance was reported by Obaidat and Stringer (2019) in Jordan. They mentioned higher percentage of resistance as 93.8, 79.2, and 57.1for *L. monocytogenes*, *E. coli* and *S. enterica*, respectively. Both *Listeria* and *E. coli* were resistant to RIF CTX and AMX whereas *Salmonella* was resistant to RUF, CTX, CIP, NX and CPM. Kanamycin was in intermediate resistance level for both *E. coli* and *Salmonella*,whichwas also mentioned by Obaidat and Stringer (2019) in resistant category.[25] *Yersinia* was intermediate level resistant to CIP, CTR and CPM and resistant to RIF, CTX and AMX. These findings were similar to the study conducted by Bonardi *et al*., (2018). They also mentioned ciprofloxacin, nalidixic acid, ceftriaxone, tetracycline, ticarcillin as being sensitive to, and amoxicillin, cefoxitin, cephalexin as in resistant category for *Yersinia*.[26]

Conclusion

Considering bacterial load in raw milk, it was observed that VC-bungalow (S5) contained lowest bacterial count and Maijdee bazar (S4) contained the highest bacterial load which quality was labelled as fair comparing with the standard bacterial load. No bacterial presence was recorded in UHT milk. Along with *E. coli* other microorganisms as *Salmonella*, *Listeria*, *Bacillus*, *Yersinia* and *Staphylococcus* presence were observed. Other than *Staphylococcus* and *Bacillus*, all of them were resistant to three or more antibiotics which is alarming in global health aspect as well.However, at this age of globalization and commercialization, antibiotics resistance will affect country borders. So, this should be taken care of on a high priority basis. It is worth mentioning that an integrated monitoring and surveillance of the usage of different antibiotics for cattle is required. Proper education among farmers and throughout the community about the after-effect of antibiotic resistance is important as well to regulate the situation.

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Conflict of Interest

The authors have no conflict of interest.

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