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Microbiological quality assessment of milk at different stages of the dairy value chain in a developing country setting

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ABSTRACT

The main objective of the study was to assess the microbiological quality of milk at different stages of the dairy value chain from farm to the factory in Bangladesh. A total of 438 raw milk samples (387 from primary producers, 32 from collectors, 15 from chilling plants, 4 from local restaurants) and 95 commercially processed milk samples were collected from northern part of Bangladesh. Almost 72% (n = 280) of samples at producer level and 100% from both collectors (n = 32) and chilling plants (n = 15) were contaminated with coliforms while 57% (n = 220) of samples from producers, 91% (n = 29) of samples from collectors and 100% (n = 15) from chilling plants were contaminated with fecal coliforms. Around 31% (n = 119) of samples from producers were positive for *E. coli* whereas > 60% (n = 20) and 100% (n = 15) samples from collectors and chilling plants, respectively were positive for *E. coli*. One quarter of samples from collectors were positive for *B. cereus* and coagulase positive staphylococci and 33% (n = 5) of samples from chilling plants were positive for both of these microorganisms. In case of commercially processed milk, 77% (n = 46) and 37% (n = 22) of pasteurized milk samples had a high aerobic plate count (APC) (10^4 CFU/ml) and coliform count (> 10 CFU/ml), respectively. None of the samples was positive for *Shigella* spp., *Salmonella* spp., and *Campylobacter* spp. Among 158 *E. coli* positive raw milk samples, 9% (n = 14) contained pathogenic *E. coli*, and enteroaggregative *E. coli* (EAEC) and Shiga-toxin producing *E. coli* (STEC) were found to be the predominant pathotypes. Of the 23 pathogenic *E. coli* identified from 14 samples based on their gene contents, > 95% (n = 22) were resistant to at least one antibiotic and 13% (n = 3) of isolates were resistant to ≥3 classes of antibiotics. Several factors including the time of milking, hygiene practices of the producers, cow breed and amount of milk produced by the cow were found to be significantly associated with high APC of milk samples. In conclusion, both raw and commercially pasteurized milk are highly contaminated with fecal organisms. For intervention, more emphasis should be given at producer's level as microorganisms introduced to milk at this stage get the longest time for survival and multiplication.

1. Introduction

Milk is a complex biological fluid which by nature serves as an excellent growth medium for many microorganisms (Godič Torkar and Golc Teger, 2008). It contains a wide range of nutrients including vitamins, proteins, fats and carbohydrates and therefore, nutritionally supports many different microorganisms under suitable growth conditions (Godefay and Molla, 2000; Parekh and Subhash, 2008). Milk contaminated with high level of spoilage bacteria often becomes unsuitable for further processing (Mhone et al., 2011). Microbial contamination can generally occur from various sources during the milking procedure: from within the udder, from the exterior of the udder, from the surface of milk handling and storage equipment and from milkmen if milking is done manually (Bramley and McKinnon, 1990; Oliver et al., 2005).

Microbiologically qualified milk means that the milk is free from

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pathogenic bacteria and harmful and toxic products of bacteria and that it is low in total bacterial counts. Total viable bacterial count is considered as one of the acceptance criteria for categorizing milk for human consumption and processing for dairy products (PMO, 2015). In most countries, the legal limits for total viable bacterial count in pasteurized milk range from 5×10^3 to 5×10^5 CFU/ml (Shojaei and Yadollahi, 2008). In Bangladesh, the acceptance limit for viable bacteria in pasteurized milk is $\leq 2 \times 10^4$ CFU/ml (BSTI, 2002). Among the coliforms, *Escherichia coli* is the most common contaminant of raw and processed milk and is a reliable indicator of fecal contamination (Kumar and Prasad, 2010). *E. coli* is a commensal microorganism inhabiting the intestine of animals and humans but its recovery from food may be of public health concern due to the possible presence of enteropathogenic and/or toxigenic bacteria, for instance, Shiga toxin-producing *E. coli*. *Staphylococcus aureus*, another important causative agent of food-borne diseases in humans is commonly associated with intoxications of food through its capacity to produce different kinds of potent enterotoxins (Balaban and Rasooly, 2000; Le Loir et al., 2003). Although heat may kill *S. aureus* cells, the enterotoxin may persist in food because it is more heat stable than the bacteria (Banwart, 1998). *Bacillus cereus* is a ubiquitous Gram-positive, spore-forming, motile rod, which is also responsible for spoilage of raw milk. It is also frequently found in pasteurized milk, causing deterioration of milk quality by producing lipases and proteases (Cromie et al., 1989; Meer et al., 1991).

In industrial countries, milk is produced at a commercial scale in dairy farms and collected through automated systems and further processing is done by maintaining good hygienic practices. However, in Bangladesh, milk is produced mostly in unorganized and informal ways. Even though very small proportion of milk is going to commercial processors, one of the biggest hurdles is to ensure safety and quality of milk all the way from the producers to the processing factory and to consumers. In this study, we aimed to assess the microbiological quality of raw milk through different stages of formal supply chain starting from the primary producer to the milk chilling stations of different commercial milk processing industries located in the northeast part of Bangladesh. Same assessment was done for commercially processed milk samples collected from retail outlets. In addition, factors associated with contamination of milk at the primary producer's level were investigated.

2. Materials and methods

2.1. Sampling site and sample collection

This study was conducted in 18 upazillas of 7 districts located in northern part of Bangladesh, including Bogra, Gaibandha, Nilphamari, Dinajpur, Joypurhat, Rangpur and Sirajganj. We collected a total of 438 milk samples of which 387 were from the primary producers (farmer), 32 from collectors (pooling points), 15 from chilling plants and 4 from local restaurants. The district and upazilla-wise distribution of milk samples are illustrated in Fig. 1. We determined the number of samples collected from each level of the value chain based on the study population enrolled in "Strengthening Dairy Value Chain (SDVC) project". The SDVC project was carried out by an international Non-Governmental Organization (NGO) called CARE Bangladesh (http://www.carebangladesh.org). During the time of our study, the total number of producers/farmers in the SDVC area was 15,309 and they were clustered in 519 small groups, distributed in nine districts.

For this study, we randomly selected a proportionate number of groups from each district with 6 producers per group. Of these 6 producers, we collected samples from 4 producers based on their availability and convenience. Accordingly, we selected 95 groups from which 570 (95 × 6) producers were initially included in the study, and finally samples were collected from 387 producers. We sampled one cow per producer and accordingly collected milk samples from 387 cows. We collected approximately 200 ml of milk in a sterile plastic bag (Fisher Scientific, China) by pouring milk out of a bucket immediately after milking the cow. We did not interrupt the normal timing and practice of milking which was in maximum cases the early morning and manual expression through hands.

In case of collection points, we followed the distribution chain from producers to collectors. We selected all collection points within the SDVC project area that collect milk from producers enrolled in our study (producers/collection point: minimum 4; maximum 29; median 13). Accordingly, we selected 32 collection points for sampling.

In case of chilling plants, we included all plants that collected milk samples from producers/collectors (producers/chilling plant: minimum 5; maximum 68; median 19 and collection points/chilling plant: minimum 1; maximum 4; median 1) enrolled in our study. A total of 15 chilling plants were included for sample collection.

As we selected primary producers within the SDVC project, majority of them deliver milk to collectors who supply the bulk to chilling plants belonged to different milk processing industries (formal chain). Only a few producers were found who sell milk to the local restaurants (informal chain). A total of 4 restaurants that used to receive milk from the producer groups enrolled in our study were identified, 3 of these were located in Bogra and one in Joypurhat district (Fig. 1). We collected milk samples from these restaurants at the point of reception.

We collected milk samples by maintaining an aseptic condition. Like at the primary producers, we collected at least 200 ml of milk from each of the collection points as well as the chilling plants in a sterile sampling bag (Fisher Scientific, China) and immediately placed them in a cool box having a temperature of +4 to +8 °C. Unlike primary producers, samples collected at collection points and chilling plants comprised milk originating from different producers. All samples were transported to the lab maintaining the cool chain for 6–8 h after collection and stored at +4 °C until analyzing of the samples. All samples were tested within 18–24 h of sample collection.

In addition to raw milk samples, we collected 95 commercially processed pasteurized milk (n = 60) and UHT milk (n = 35) from retail shops mostly located in Dhaka and Bogra. We collected products of those industries which have their chilling plants in the sampling area, including all medium and large size dairy industries in Bangladesh. For each product, we purchased 1–2 sealed packets of milk depending on the pack size so that we got at least 200 ml of product for analysis. We ensured that all products had different batch numbers and were within their shelf-life as labeled by the industry. All samples were placed in a cool box and transported to the laboratory by maintaining a cold chain for 6–8 h after collection.

2.2. Questionnaire-based survey

A questionnaire-based survey among all primary producers was carried out to determine factors associated with microbiological contamination of milk at primary producer level. Data were collected mainly related to the breeding type of cow, volume of milk produced by the cow, environmental condition surrounding the cattle house, construction materials of cattle house floor, type of food supplied to the cattle, source of drinking water for cattle, educational status of the farmer, time of milking, hygienic condition during milking and type of milk pot used for milking.

2.3. Microbiological analysis

All milk samples including both raw and commercially processed ones were tested for aerobic plate count (APC), lab pasteurized count (LPC), total coliform count (TC), thermotolerant coliform (fecal coliforms) count (FC), and enumeration of β-glucuronidase positive *E. coli*, *Bacillus cereus* and coagulase positive staphylococci by using standard methods described in the following. In addition, all samples were tested for foodborne pathogens including *Shigella* spp., *Salmonella* spp. and *Campylobacter* spp. using standard method as described in the

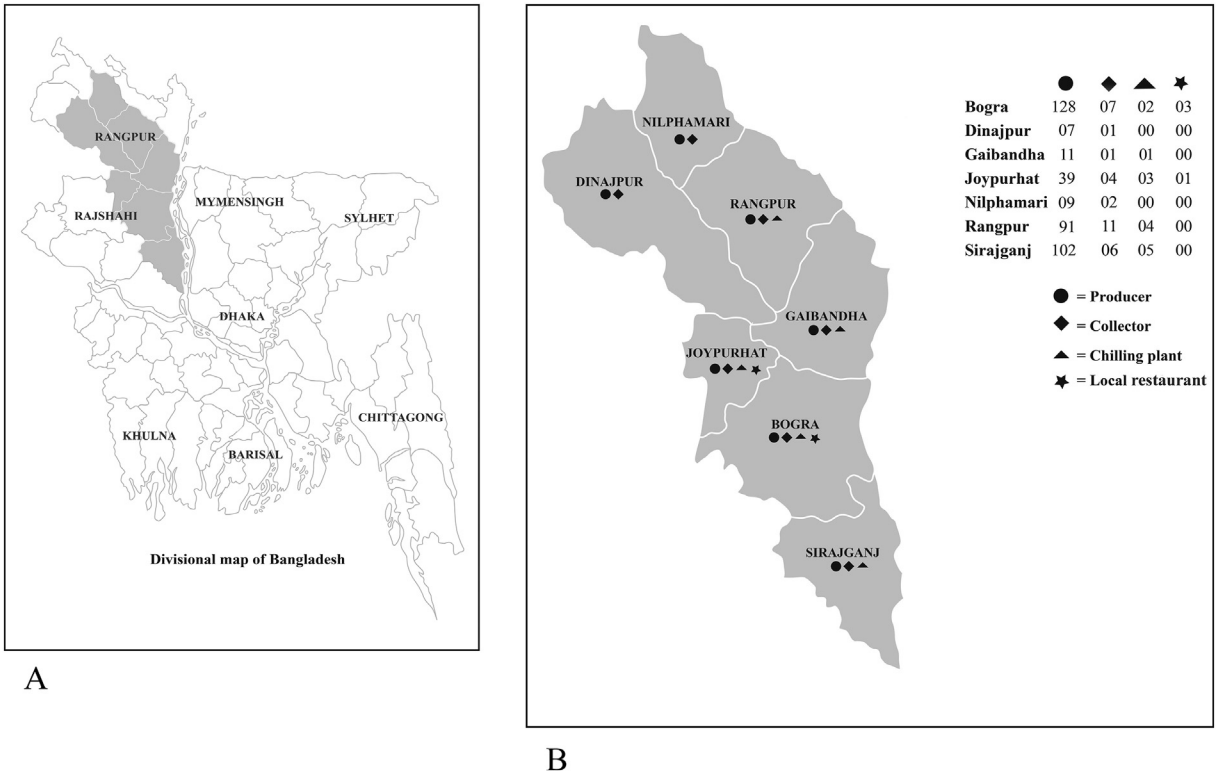

Fig. 1. A location-wise distribution of milk samples collected across the dairy value chain in Bangladesh. A: divisional map of Bangladesh highlighting the study sites; B: location of 7 districts along with the types and number of samples collected from each level of milk value chain.

following.

Ten milliliters from each milk sample were transferred into a sterile plastic bag. Then 90 ml of peptone salt solution were added and mixed well in a stomacher to get a 1:10 dilution. Subsequent dilutions of the sample were prepared by transferring 1 ml from the initial dilution to 9 ml of peptone salt solution (1:100 dilution) and so on up to 1:100,000 dilution.

2.3.1. Enumeration of indicator organisms

For APC, 1 ml of each dilution of a sample ranging from neat (undiluted) to 1:100,000 dilution was transferred to separate sterile Petri dishes and 15 ml of standard plate count agar (Oxoid, Basingstoke, UK) medium cooled to 45 ± 1 °C was added to each plate followed by immediate mixing of the sample dilutions and agar medium by pour plate technique (BAM, 2001).The plates were incubated at 35 ± 2 °C for 48 h ± 2 h promptly after solidification of molten agar. For enumeration of coliform, 1 ml of each dilution of a sample was mixed with crystal violet neutral red bile lactose agar (Oxoid, Basingstoke, UK) using pour plate technique. The plates were incubated at 37 °C according to the standard method (Anonymous, 2006a). Both typical and atypical colonies were counted and atypical colonies were confirmed by inoculating in brilliant green lactose bile broth (Oxoid, Basingstoke, UK). Colonies those produced gas in the Durham tubes were considered as coliform bacteria (Anonymous, 2006a). For fecal coliform, 100 µl of milk sample was inoculated directly onto membrane fecal coliform (m-FC) agar (Difco™, USA) supplemented with rosalic acid (600 µl/ 300 ml mixed in 1 N NaOH) using spread plate technique. The plates were incubated at 44° C for 18–24 h. For lab pasteurized count (LPC), milk samples were heated to 145 °F (62.8 °C) and held at that temperature for 30 min. Then the samples were inoculated in a similar way of aerobic plate count and the plates were incubated at 35 ± 2 °C for 48 h ± 2 h after solidification of molten agar. For enumeration of β-glucuronidase positive *E. coli*, 1 ml of diluted sample was inoculated on tryptone bile agar with x-glucuronide (TBX) (Oxoid, Basingstoke, UK) and incubated at 44 °C for 18–24 h according to standard method (Anonymous, 2001). Appearance of blue green colonies on TBX plate was indicative of the presence of *E. coli*. For enumeration of *Bacillus cereus* and coagulase positive *Staphylococcus aureus*, 100 µl aliquots of the same dilutions were inoculated onto mannitol egg yolk polymyxin agar (Oxoid, Basingstoke, UK) and Baird-Parker agar medium (Oxoid, Basingstoke, UK), respectively by spread plate technique and incubated at specific conditions as mentioned in Table 1 (Anonymous, 2004a; Anonymous, 1999). For all the tests, duplicate plates were used for each dilution. The colony counts were calculated according to the standard formula given in ISO 7218 and presented as CFU per ml of sample (Anonymous, 2007).

2.3.2. Detection and isolation of pathogenic bacteria

For isolation and identification of *Shigella* spp., *Salmonella* spp. and *Campylobacter* spp., the same amount of sample (25 ml) were taken for each of the organisms and mixed with selective enrichment media (225 ml) according to the standard method (Anonymous, 2002; Anonymous, 2004b; Anonymous, 2006b) (Table 1). Only for *Salmonella* spp., a pre-enrichment of the sample was carried out at 37 ± 1 °C for 18–24 h in buffered peptone water (Oxoid, Basingstoke, UK). The selective enrichment cultures were subsequently inoculated onto selective agar plates and incubated at specific conditions mentioned in Table 1. Typical colonies on culture plates were identified according to the standard procedures. All presumptive colonies of *Shigella* spp., and *Salmonella* spp., were identified by using API 20E biochemical test galleries (bioMerieux, Marcy l'Etoile, France) according to the manufacturer's instructions.

2.4. Detection of virulence genes in E. coli isolates by PCR

From each *E. coli* positive samples, 5 colonies with blue green appearance on TBX plate were randomly selected and sub-cultured on MacConkey agar. We then tested these 5 isolates by PCR for virulence

Table 1
Culture media and incubation conditions used in the study for isolating *B. cereus*, *S. aureus*, *Salmonella* spp., *Shigella* spp. and *Campylobacter* spp. from milk samples.

Organism	Enrichment media	Temperature and time	Selective agar media	Temperature and time
B. cereus	Not done		MYP agar	30 °C, 18–24 h
S. aureus	Not done		Baird-Parker agar	37 °C, 24–48 h
Salmonella spp.	RVS	41.5 ± 1 °C, 24 ± 3 h	XLD agar	37 °C, 24 ± 3 h
	MKTTn	37 ± 1 °C, 24 ± 3 h	BGA agar	
Shigella spp.	*Shigella* broth with 0.5 μg/ml novobiocin	41.5 ± 1 °C, 16–20 h (microaerophilic condition)	MacConkey agar Hectoen Enteric agar XLD agar	37 °C, 20–24 h
Campylobacter spp.	Bolton broth	41.5 °C, 44 ± 4 h (microaerophilic condition)	mCCDA Karmali agar	41.5 °C, 44 ± 4 h (micro-aerophilic)

Abbreviations: MYP, mannitol egg yolk polymyxin; XLD, xylose lysine deoxycholate; BGA, brilliant green agar; MKTTn, Muller-Kauffmann tetrathionate supplemented with novobiocin; RVS, Rappaport-Vassiliadis soya peptone; mCCDA, modified charcoal cefoperazone deoxycholate agar.

genes by using colony patch approach described in Islam et al., 2007. Briefly, single colonies were transferred from the MacConkey agar and plated onto trypticase soya agar (Difco) to create a grid pattern of 96 colonies (12 × 8). The plates were incubated overnight at 37 °C. The isolates were subjected to PCR for the detection of virulence genes as described below. DNA was extracted by boiling the suspensions of pooled colonies for 10 min (Islam et al., 2006) and 3 μl of the supernatant was used in the PCR. We used these DNA for detection of virulence genes in two multiplex setup, one for heat labile (*lt*), heat stable (*st*), attaching and effacing gene (*eae*), bundle forming pilus (*bfp*), antiaggregation protein transporter gene (*aat*) and genes for AggR-activated island (*aaiC*), invasion plasmid antigen H (*ipaH*) and invasion associated locus (*ial*) and the other one for Shiga toxin genes (stx_1 and stx_2) according to the procedures and programs described earlier (Talukdar et al., 2013; Islam et al., 2007). For the first multiplex PCR, 3 μl template DNA was suspended in 22 μl of reaction mix containing 2.5 μl of 10× PCR buffer with 1.5 mM $MgCl_2$ (New England Biolabs), 0.5 μl of 10 mM dNTPs (New England Biolabs), 0.4 μl each of *lt*, *st*, *bfp*, *aat*, *aaiC*, *ipaH*, *ial* primers, 0.44 μl of *eae* primers (Integrated DNA Technologies), together with 1 unit of Taq DNA polymerase (New England Biolabs) (5 U/μl). PCR cycling conditions consisted of initial denaturation at 96 °C for 4 min, followed by 34 cycles each of denaturation at 95 °C for 20s, annealing at 57 °C for 20s and extension at 72 °C for 1 min (Talukdar et al., 2013). PCR for stx_1 and stx_2 genes was carried out using the same volume of reaction mix (25 μl) comprising 2.5 μl of 10× PCR buffer with 1.5 mM $MgCl_2$, 0.5 μl of 10 mM dNTPs, 0.2 pmol of each primers, 1 unit of Taq DNA polymerase and 3 μl of template DNA. Primer sequences are listed in Table 2. Colonies that appeared positive for a particular gene in pooled PCR were further confirmed by individual colony PCR.

2.5. Antimicrobial susceptibility test

Antimicrobial susceptibility of all pathogenic *E. coli* isolates was determined by standard disk diffusion technique following the Clinical and Laboratory Standards Institute guidelines (CLSI, 2010). The antibiotics used in this study were ampicillin (10 μg), cefotaxime (30 μg), ciprofloxacin (5 μg), nalidixic acid (30 μg), trimethoprim/sulfamethoxazole (25 μg), aztreonam (30 μg), gentamicin (10 μg), kanamycin (30 μg), piperacillin-tazobactam (110 μg), chloramphenicol (30 μg), tetracycline (30 μg) and erythromycin (15 μg). The inhibition zones were measured and isolates were classified as resistant, intermediate or sensitive according to the interpretation guideline provided by the CLSI (CLSI, 2010).

2.6. Data analysis

Data collected from the field were coded, and visually checked for errors before entering into a database. Computer coding was pre-tested, and data entry and analyses were done using Statistical Package for Social Sciences (SPSS) Windows (Version 22; Chicago, IL, USA), Epi Info (Version 6.0, USD, Stone Mountain, GA, USA), and RStudio (Version 1.0.136.0). All data were validated by a series of logical and range checks, and statistical analyses included both descriptive and analytical methods. For categorical variables of interest, differences between categories were compared by χ^2 test and probability of < 0.05

Table 2
PCR primer sequences used in the study (Talukdar et al., 2013).

Target genes	Primer	Nucleotide sequences (5′–3′)	Annealing Temperature (°C)	Product size (bp)
elt B	LT-F	CACACGGAGCTCCTCAGTC	57	508
	LT-R	CCCCCAGCCTAGCTTAGTTT		
est A	ST-F	GCTAAACCAGTAGAGGTCTTCAAAA	57	147
	ST-R	CCCGGTACAGAGCAGGATTACAACA		
bfpA	bfpA-F	GGAAGTCAAATTCATGGGGG	57	300
	bfpA-R	GGAATCAGACGCAGACTGGT		
eae	eae-F	CCCGAATTCGGCACAAGCATAAGC	57	881
	eae-R	CCCGGATCCGTCTCGCCAGTATTCG		
aat	$_p$CVD432-F	CTGGCGAAAGACTGTATCAT	57	650
	$_p$CVD432-R	CAATGTATAGAAATCCGCTGTT		
aai C	aaiC-F	ATTGTCCTCACGCATTTCAC	57	215
	aaiC-R	ACGACACCCCTGATAAACAA		
stx_1	stx1-F	CACAATCAGGCGTCGCCAGCGCACTTGCT	58	606
	stx1-R	TGTTGCAGGGATCAGTGGTACGGGGATGC		
stx_2	stx2-F	CCACATCGGTGTCTGTTATTAACCACACC	58	372
	stx2-R	GCAGAACTGCTCTGGATGCATCTCTGGTC		
ial	ial-F	CTGGATGGTATGGTGAGG	57	320
	ial-R	GGAGGCCAACAATTATTTCC		
ipaH	ipaH-F	TGGAAAAACTCAGTGCCTCT	57	424
	ipaH-R	CCAGTCCGTAAATTCATTCT		

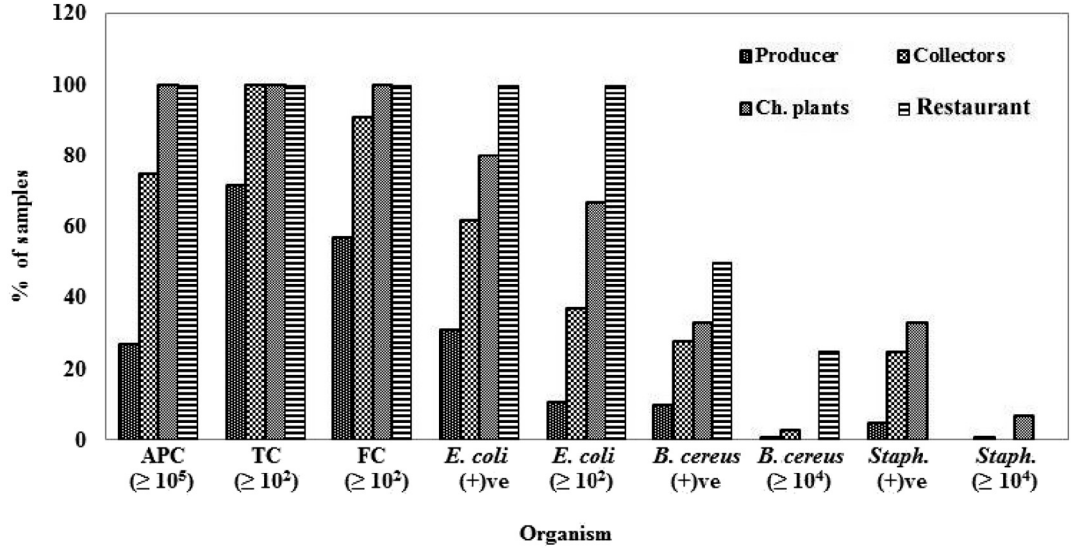

Fig. 2. Overall comparison of microbiological quality of milk samples collected at different points of dairy value chain.

was considered to be statistically significant. Finally, backward stepwise regression analyses were performed simultaneously adjusting for confounding variables for identifying factors that independently influenced the outcome.

3. Results

3.1. Raw milk samples collected from different points of milk value chain were highly contaminated

Almost 72% and 57% of raw milk samples, collected from primary producers were contaminated with coliform (≥100 CFU/ml) and fecal coliform (≥100 CFU/ml), respectively while 31% of samples were positive for *E. coli* and one in every ten samples (11%) was contaminated with high numbers of *E. coli* (≥100 CFU/ml). Around 10% and 5% of raw milk samples collected from producers were contaminated with *B. cereus* and *Staph. aureus*, respectively (Fig. 2).

All samples at collection points were contaminated with a high number of coliform bacteria (≥100 CFU/ml) and 91% of the samples contained fecal coliform (≥100 CFU/ml). More than 60% of samples were contaminated with *E. coli* and around 40% of samples had a high *E. coli* count (≥100 CFU/ml). Around 25% of samples were positive for *B. cereus* and coagulase positive staphylococci, however, in most of the samples, number of organisms was within the acceptable limit ($< 10^4$ CFU/ml) (de Oliveira et al., 2011; European Commission, 2001) (Fig. 2).

The level of microbial contamination in samples collected from chilling plants was even higher than that of collection points. Samples from all 15 chilling plants distributed in 5 districts were contaminated with high number of coliforms as well as fecal coliforms. *E. coli* was present in samples from all chilling plants (100%) and high count of *E. coli* was found in 67% of samples, mainly from chilling plants located in Bogra, Joypurhat and Sirajganj districts. Although *B. cereus* and coagulase positive staphylococci were detected in 33% of samples, the number of *B. cereus* in all of the positive samples and number of staphylococci in 27% of the samples was within the acceptable limits ($< 10^4$ CFU/ml). None of the raw milk samples collected from producers, collectors and chilling plants was positive for *Shigella* spp., *Salmonella* spp., and *Campylobacter* spp.

Bacterial counts increased gradually from producer to chilling plants/local restaurants (Fig. 3). All samples from chilling plants and local restaurants had a higher APC, TC and FC (Fig. 3). Similarly, the proportion of contaminated samples increased gradually from producers to the chilling plants/local restaurants (Fig. 2).

3.2. More than two-third of commercially processed pasteurized milk samples did not meet standard microbiological limits

Around 77% of pasteurized milk samples had a high APC ($> 10^4$ CFU/ml) while 37% and 15% of the samples were contaminated with coliforms and fecal coliforms, respectively. More than 31% and around 2% of pasteurized milk samples were found to be contaminated with coliforms and fecal coliforms, respectively at the level of > 10 CFU/ml. *E. coli* was isolated from 2 (3.3%) samples and *B. cereus* was present in 22% (n = 13) of the samples (geometric mean: 3.4×10^2 CFU/ml; range: 10–5.8×10^3 CFU/ml). Staphylococci, *Shigella* spp., *Salmonella* spp., *Campylobacter* spp. and pathogenic *E. coli* were not detected in pasteurized milk samples. Bacterial growth was not observed in any of the UHT milk samples.

3.3. A significant proportion of organisms in raw milk might be thermoduric

All milk samples from producers, collectors and chilling plants were tested for Laboratory Pasteurized Count (LPC) in order to enumerate the thermoduric bacteria. While none of the samples from producers had LPC in the range of 10^4 to 10^5 CFU/ml, around 10% of samples from collection points and 8% from chilling plants had LPC in this range (Table 3). Samples having high LPC also had high APC.

3.4. Raw milk contained pathogenic E. coli

Of the 158 *E. coli* positive raw milk samples, 14 samples (9%) were positive for pathogenic *E. coli*. For detection of pathogenic *E. coli*, we tested 5 *E. coli* colonies per sample for pathogenic genes. In some samples (n = 6), we found more than one colonies positive for pathogenic genes but of different types, hence we considered them as different *E. coli*. Accordingly, we identified a total of 23 pathogenic *E. coli* isolates from 14 samples. Of these 23 isolates, 9 isolates (39%) were positive for EAEC specific genes, *aaiC* and *aat*, same number of isolates (39%) were positive for STEC specific genes, stx_1 and stx_2, 3 isolates (13%) were positive for EIEC specific genes, *ipaH* and *ial*. One isolate was positive for EPEC specific gene (*eae*) and another one for ETEC specific gene (*st*).

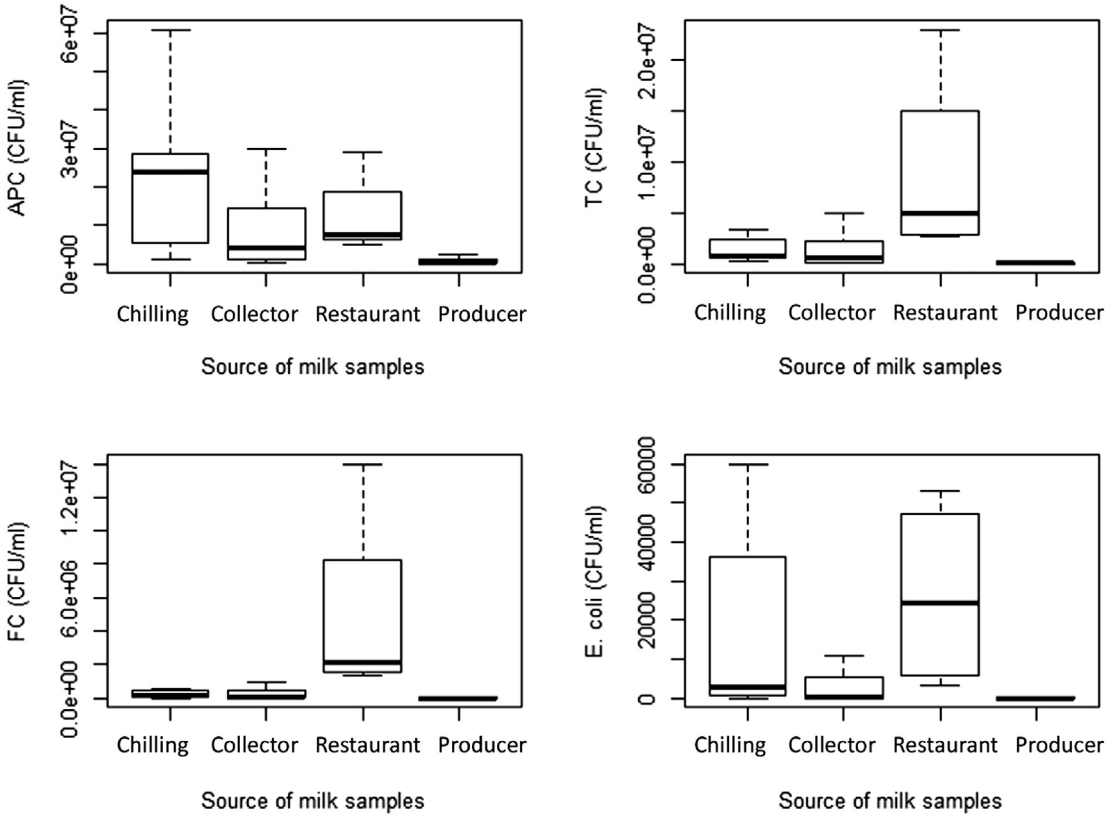

Fig. 3. Gradual change in bacterial counts in milk samples collected from different stages of dairy value chain.

3.5. Pathogenic E. coli isolated from raw milk samples were resistant to multiple antibiotics

Among the 23 pathogenic *E. coli* isolates, > 95% (n = 22) were resistant to erythromycin followed by 13% (n = 3) to ampicillin and 9% (n = 2) to tetracycline and nalidixic acid. > 95% (n = 22) isolates were resistant to at least one antibiotic and 13% (n = 3) of the isolates were resistant to three or more classes of antibiotics and thus defined as multi-drug resistant (MDR).

3.6. Multiple factors were involved in contamination of milk at primary producer level

A number of variables were found to be significantly associated with high APC of milk samples, including breed of the cow, volume of milk produced by the cow, the time of milking, and farmer's hand washing practices (Table 4). Significantly higher number of samples from commercial breed cow was contaminated than that of the local breed (P = 0.012). Higher number of samples from small-scale producers (who produce < 5 l of milk/day) were found to be contaminated than that of the large-scale producers (who produce > 5 l of milk/day)

Table 3
Association between high APC and LPC of milk samples collected from different stages of dairy value chain.

Producer level (APC versus LPC)	LPC/APC (CFU/ml)	No. (%) of sample positive for		P
		APC < 10^5 (N = 282)	APC > 10^5 (N = 105)	
No (%) of sample positive for	LPC < 10^3 (N = 332)	253 (89.7%)	79 (75.2%)	< 0.05
	LPC > 10^3 (N = 55)	29 (10%)	26 (24%)	
Collectors level (APC versus LPC)	**LPC/APC (CFU/ml)**	**No. (%) of sample positive for**		**P**
		APC < 10^5 (N = 8)	APC > 10^5 (N = 28)	
No (%) of sample positive for	LPC < 10^3 (N = 22)	6 (75%)	16 (57%)	0.36
	LPC > 10^3 (N = 14)	2 (25%)	12 (42%)	
Chilling plants level (APC versus LPC)	**LPC/APC (CFU/ml)**	**No. (%) of sample positive for**		**P**
		APC < 10^5 (N = 7)	APC > 10^5 (N = 8)	
No (%) of sample positive for	LPC < 10^3 (N = 3)	2 (28%)	1 (12%)	0.57[a]
	LPC > 10^3 (N = 12)	5 (71%)	7 (87%)	

[a] Fisher exact test was performed when expected frequency was < 5.

Table 4
Factors associated with high level of microbial contamination of milk at producer level.

Variables (N)	No. (%) of milk samples with an APC of		P
	$< 10^5$ CFU/ml	$\geq 10^5$ CFU/ml	
Breed of cow			
Local (198)	156 (78.8)	42 (21.2)	**0.012**
Commercial (189)	127 (67.2)	62 (32.8)	
Amount of milk produced by the cow			
Upto 5 l (306)	234 (76.5)	72 (23.5)	**0.003**
More than 5 l (81)	49 (60)	32 (39)	
Condition of surrounding environment of cattle house			
Clean (104)	74 (71.1)	30 (28.9)	0.595
Dirty (283)	209 (73.8)	74 (26.2)	
Construction materials used for cattle house floor			
Mud or mud with brick (358)	260 (72.6)	98 (27.4)	0.434
Concrete (brick with cement) (29)	23 (79)	6 (20)	
Type of food for cattle			
Grass and straw (347)	256 (73.8)	91 (26.2)	0.398
Straw and chaff (30)	20 (66)	10 (33)	
Source of drinking water for cattle			
Tubewell (378)	276 (73.0)	102 (27.0)	0.547
Well (9)	7 (77)	2 (22)	
Did cow urinate/defecate during milking?			
Yes (15)	12 (80)	3 (20)	0.392
No (372)	271 (72.8)	101 (27.2)	
Education of farmer			
Nil (46)	34 (73)	12 (26)	0.823
Primary (242)	175 (72.3)	67 (27.7)	
No of cow owned by the farmer			
One (210)	156 (74.3)	54 (25.7)	0.638
More than one (176)	127 (72.2)	49 (27.8)	
Who usually milks the cow			
Owner of the cow (129)	99 (76.7)	30 (23.3)	0.256
Others (258)	184 (71.3)	74 (28.7)	
Usual time of milking			
Morning (295)	206 (69.8)	89 (30.2)	**0.008**
Mid-day and evening (92)	77 (83)	15 (16)	
Does the same person milk every day?			
Yes (360)	263 (73.1)	97 (26.9)	0.908
No (27)	20 (74)	7 (25)	
How do the person wash hands before milking cow			
With water only (329)	234 (71.1)	95 (28.9)	**0.034**
With soap and water (58)	49 (84)	9 (15)	
Udder wipe before milking			
With cloth or tail of the cow (93)	69 (74)	24 (25)	0.790
Do not wipe (294)	214 (72.8)	80 (27.2)	
Type of milkpot used at the time of milking			
Plastic (62)	51 (82)	11 (17)	0.076
Metal (325)	232 (71.4)	93 (28.6)	

(P = 0.003). A significant association between the time of milking and microbial contamination was also found. Higher number of samples collected in the morning was contaminated than the number of samples collected at mid-day or in the evening (P = 0.008). Of the hygiene issues, hand washing practices among people who milk the cow was found to be significantly associated with microbial contamination. Higher number of samples from producers who washed their hands only with water before milking was found to be contaminated than that of the samples from producers who washed their hands with soap and water (P = 0.034). In multivariate analysis, high APC of milk sample was significantly associated with volume of milk produced by the cow, after controlling for other variables those appeared to be significant in bivariate analysis. The overall F-ratio was 3.33 (degrees of freedom = 2) which was statistically significant (P = 0.03). The adjusted R-square, a measure of goodness-of-fit was 0.0170, which suggested that either the model specification was not precise or the association of variables with APC were not always linear. Measurement errors also could not be ruled out.

4. Discussion

Food safety is paramount for healthcare management of highly congested and cognate settings such as Bangladesh. This becomes very critical when food supply sectors such as dairy chains and their operations remain unorganized and are not fully automated and regulated and largely depend on human handling.

The total bacterial count could often serve as a surrogate of the microbiological quality of milk, and reflects the standards of primary production operations, collection, transportation and storage (Hassainya et al., 2006). In our study, more than one quarter (27%) of milk samples at primary producer level had a high APC ($\geq 10^5$) whereas 100% of the milk samples from chilling plant had the same level of APC. Presence of coliform bacteria is generally considered as an indicator of poor hygienic practices in the handling of milk, while presence of *E. coli* reflects fecal contamination or the presence of other enteric bacteria in the samples (Labioui et al., 2009). In this study, we found that > 70% of the samples taken from primary producers were contaminated with high number of coliform bacteria ($\geq 10^2$) while 100% of samples taken from chilling plants were contaminated with the same number of coliforms. *E. coli* contamination increased from 31% to 100% from producer to chilling plant. *B. cereus* and *S. aureus* were found in 10% and 5% of samples obtained from primary producers, respectively, while both organisms were found in 33% of samples from the chilling plants. Presence of pathogenic *E. coli* in milk is more alarming as this might potentially lead to an outbreak of foodborne infections in the community, if milk is consumed in raw state or with inadequate treatment or sterilization (Langer et al., 2012; Scavia et al., 2009). In this study, 9% of *E. coli* positive samples were found to be positive for pathogenic *E. coli* and the predominant pathotypes were EAEC and STEC. In a previous study from Bangladesh, around 10% raw milk samples were found to be positive for STEC (Islam et al., 2010). A more recent study conducted in Brazil showed that among 101 raw milk samples collected from three different dairy farms, 31.1% were contaminated with STEC (Vendramin et al., 2014).

Antibiotic resistance is a global concern and inappropriate use of antibiotics in the food chain is one of the major drivers of antimicrobial resistance. In this study, we found that 95% of pathogenic *E. coli* isolates were resistant to erythromycin, followed by 13% being resistant to ampicillin, 9% to tetracycline as well as to nalidixic acid and only 4% isolates were resistant to chloramphenicol, cefotaxime, trimethoprim-sulfamethoxazole and aztreonam. Around 13% isolates were multi-drug resistant. A previous study reported that among 120 *E. coli* isolates from raw milk and unpasteurized cheese, 56% were resistant to nalidixic acid, 30% to gentamicin, 28% to trimetoprim-sulfamethoxazole and 23.4% to ampicillin (Bonyadian et al., 2014). Another study revealed all of the 19 *E. coli* O157 strains isolated from raw milk samples were resistant to penicillin and tetracycline, 94.7% to erythromycin, 84.2% to amoxicillin, oxacillin, sulphamethoxazole/trimethoprim, 68.4% to chloramphenicol and 42.1% to streptomycin (Reuben and Owuna, 2013). In contrast to these diverse studies, it appears that pathogenic *E. coli* isolates obtained from our study were less resistant to antibiotics. However, it might be interesting to analyse the non-pathogenic *E. coli* isolates for antibiotic resistance as they could also act as sources of many antibiotic resistance genes.

A significant association was found between the APC and LPC of milk samples collected from primary producers. Significantly higher number of samples with a high APC ($> 10^5$ CFU/ml) showed a higher LPC ($> 10^3$ CFU/ml) compared to samples having a low APC

($< 10^5$ CFU/ml) ($P < 0.05$) (Table 3). This result indicates that a major proportion of the total microbiota in milk could be thermoduric meaning that it could survive pasteurization. An increasing trend of samples with high LPC was observed from producers to chilling plants, while an opposite trend was observed entailing the samples with low LPC. All samples (100%) from chilling plants had a high APC ($> 10^5$ CFU/ml) and half of them (47%) had high LPC values suggestive of the possibility that thermoduric organisms thrived and dominated significantly. In order to find out if it concurs with the commercially processed milk in Bangladesh, we tested pasteurized milk samples of all available brands obtained from retail outlets. We found that around 80% of pasteurized milk samples had a high total APC ($> 10^4$ CFU/ml) and > 31% of samples were contaminated with coliform bacteria at a level of > 10 CFU/ml. According to the Bangladesh standard, accepted limit for viable bacteria in pasteurized milk is $\leq 2 \times 10^4$ CFU/ml (BSTI, 2002) indicating that a major proportion of commercially pasteurized milk at the point of sale is not meeting the safety criteria. Seamless surveillance and monitoring of the commercial milk value chain in the country should be implemented.

We interpret that the contamination of milk originates from the primary producer levels and amplifies at subsequent stages. At the primary producer level, contamination occurs due to soiled udders of the cows, unhygienic practices of the farmers, such as milking with dirty hands, use of unclean milk pots, wiping udder with cow tail, dirty cloths, urination or defecation by the animal during milking and an overall unclean environment. Soiled udders of cows are predominantly seen in cows during morning time than the mid-day/evening time, and this might be the reason that milk extracted during the morning hours was more contaminated than milk extracted at evening time (Table 4). Normally bathing/washing of cows is done at noon or in the afternoon and thus body of the cow is cleaner in the evening than in the morning. The unhygienic milking practices were observed to be more predominant among small scale farmers as majority of them are living in poverty, marginalized and illiterate (SDVC project). This was also evident from multivariate analysis of the factors associated with milk contamination which showed that significantly higher number of samples from small volume producers (< 5 l) were contaminated than medium or large volume producers (Table 4). We also found that a significantly higher number of milk samples from commercial breed cows were contaminated than that of the local breed cows. The reason behind this difference could not be explained by the available information and thus a further study focusing on this issue is warranted.

Microbial contamination increases at the collector level due to long holding time at ambient temperatures and pooling of milk from different producers in a single container. In addition, further contamination may be the outcome of poor hygienic conditions practiced by the collectors during handling of bulk milk. At the chilling plant, contamination of milk could potentially occur due to lack of temperature control which favors exponential growth of previously introduced microorganisms. Other factors may be due to extended time of transportation of the milk to the chilling plants and possibly also due to the dirty instruments employed for quality checks in chilling stations.

Finally, we believe that our observations clearly point at an unmet need for good hygienic practices required to be urgently introduced at each level of milk production in Bangladesh starting from producers to the processors. More emphasis should be given at producer's level because microorganisms introduced to milk at this stage get the longest time for survival and multiplication. Use of automated machines for milking could be an alternative for achieving safe handling of milk at producer level. Temperature controlled containers can be introduced at collectors' level to restrict the growth of microorganisms present in pooled milk. Transportation of milk from collectors to chilling stations should be done by maintaining cold chain. Small scale pasteurization plants can also be installed at collectors' levels so that processing of milk can be done at the field level. Further, the quality of this locally produced pasteurized milk must be monitored carefully on a regular basis. In addition, demand based marketing strategies need to be developed for proper distribution of processed milk to consumers.

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