



# Microbiological Quality Assessment and Identification of Antibiotic Resistant Bacteria at Different Stages of the Milk Supply Chain in Dhaka City of Bangladesh

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## Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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## ABSTRACT

**Aims:** Milk works as an excellent medium for bacterial growth and can turn into a fatal source of food borne diseases when consumed without pasteurization. This study was carried out to examine the microbiological quality of milk from three different points of milk supply chain to investigate whether the dairy stakeholders are maintaining the consumer safety or not.

**Study Design:** A cross sectional study

**Place and Duration:** The study took place at the Food Microbiology lab of Institute of Nutrition and Food Science, University of Dhaka from November 2019 to February 2020.

**Methodology:** A total of 60 samples were studied including raw milk from collection centers, unpackaged pasteurized milk from processing plants and packaged pasteurized milks from retail shops. After carrying out the microbiological analysis the samples were examined for determining the total bacterial count (TBC) and total coliform count (TCC). Antibiotic susceptibility test was done using disk diffusion assay and detection of virulent gene in *Salmonella* spp. was done by Polymerase Chain Reaction (PCR) using specific *invA* primer.

**Results:** The results revealed that all raw milk samples were substandard in terms of TBC and TCC and pasteurized milks from processing plants maintained the standard quality. Importantly, packaged pasteurized milk samples from retail shops had high TBC ( $>4.0 \times 10^4$  CFU/mL) and TCC ( $1.2 \times 10^3$  CFU/mL) containing *Pseudomonas*, *Micrococcus*, *Streptococcus*, *Salmonella*, *Proteus*, *Staphylococcus*, *Bacillus* and *E. coli*. Bacteria like *Salmonella* (75%), *Proteus* (62.5%) and *Vibrio* (62.5%) possessed high Multiple Antibiotic Resistance (MAR) index and showed resistance towards antibiotics namely Ampicillin, Amoxicillin, Erythromycin and Colistin. Through further molecular analysis we detected *invA* virulent gene one of the *Salmonella* isolates which was collected from the pasteurized milk samples of the retail shops.

**Conclusion:** High bacterial load in raw milk and packaged pasteurized milk indicate that the milk we consume is substandard in microbiological quality. Precautionary measurements and careful processing of milk may reduce the prevalence of microbiological contamination in the milk supply chain.

**Keywords:** *Pasteurized milk; retail shops; multiple antibiotic resistance; invA gene; microbiological quality.*

## 1. INTRODUCTION

Milk is a nutrient-rich biological fluid that contains wide range of lipids, high-quality protein, vitamins, minerals, and other bioactive components [1]. This nutrient dense food not only helps children and adults to form healthy diets but also contributes to make our lives better by improving bone health, lowering blood pressure and reducing the risk for cardiovascular disease and type-2 diabetes [2]. Nevertheless, this beneficial compound can get contaminated at any stage of the milk supply chain resulting in endangered consumer health.

Milk supply chain includes stakeholders involved in the supply management ranging from farmers, manufacturers, retailers and consumers [2]. Milk is nearly a sterile fluid when secreted into alveoli of udder [3]. Therefore, the contamination in the supply chain occurs beyond this stage from the surface of milk handling and storage equipment, interior and exterior of the cow's udder as well as from feeding and housing practices [4]. Several studies isolated wide variety of bacteria from different stages of the dairy chain including *Staphylococcus aureus*, *Escherichia coli*, *Salmonella*, *Listeria monocytogenes* and *Campylobacter* [5,6]. Invention of pasteurization and improved dairy sanitation practices have dramatically decreased milk-borne diseases and other hazards related to milk but contamination can still occur due to process failure or post-pasteurization contamination. For example, a study conducted on five different brands of pasteurized milk found that all the milk samples were contaminated with high number of bacteria ( $3 \times 10^4$ - $7.2 \times 10^5$ ) [7]. Consumption of

inadequately pasteurized milk not just only causes foodborne diseases but at the same time increases our odds of consuming multi-drug resistant bacteria [8].

The dairy farmers of Bangladesh mostly handles milk in non-standardized way and supply to the consumers of both urban and rural areas [2]. Besides, in industries at commercial scale milk is produced mostly in unorganized and informal ways [9]. Ahmed et al. found presence of coliform bacteria in pasteurized milk samples of five different brands of Bangladesh. Several studies have been conducted on the microbiological quality of raw, pasteurized, and ultra-high-temperature (UHT) milk (Karmaker et al., 2020; Salauddin et al. in Bangladesh. However, there is only one study conducted by Islam et al. on the microbiological quality of the milk supply chain of only Rangpur division.

Dhaka being the capital and the most populated city of Bangladesh needs urgent evaluation of its milk supply chain. Our study is the first study to examine the microbiological quality of milk supply chain of Dhaka city. Therefore, the aim of our study is to assess the microbiological quality and to identify antibiotic resistant bacteria at three points of milk supply chain of Dhaka city. The study also aims to investigate how the microbiological quality differs between pasteurized milk of processing plants and pasteurized milk of retail shops. The study findings would help to identify at which stage of supply chain the contamination occurs and inform hazard managers to take interventions for ensuring the safety and quality of milk all the way from the producers to the processing factory and to consumers.

## 2. MATERIALS AND METHODS

### 2.1 Sampling Site and Sample Collection

The milk samples were procured from three leading milk processing companies of Bangladesh located at Manikganj, Tangail and Tejgaon industrial area. We collected a total of 60 (n=60) milk samples from three points of the supply chain which were collection centers, processing plants, and retail shops. Untreated raw milk (n=15) were collected from collection centers of the companies, whereas unpackaged pasteurized milk (n=15) samples were procured from processing plants of the companies. Moreover, 15 packaged pasteurized milk and 15 UHT milk samples of same three companies were randomly collected from retail shops of the local markets of Dhaka city. We carefully checked the expiry dates from the packet of the milk samples before buying them. The samples were collected with consent of the authorities of the companies and for privacy concern the original names of these organizations had not been disclosed. The companies were coded as company A, B and C respectively. The milk samples were collected in sterile conical flasks covered with aluminum foil paper. These were carried in medical ice box from the place of procurement to the laboratory and samples were stored below 4°C in the refrigerator.

### 2.2 Sample Processing

Samples were taken in amount of 10 mL from sterilized conical flasks into previously autoclaved cotton plugged test tubes. These test tubes were shaken for homogenization, and these whole milk samples were regarded as the initial dilution. After that 1 mL sample was pipetted and transferred into sterilized cotton plugged test tubes containing 9 mL of 0.1% peptone water. Then, they were mixed thoroughly by shaking 20 times and the solution was allowed to stand for 5–10 min. Thus, further serial dilutions were prepared up to  $10^{-5}$  according to American Public Health Association (APHA) [10] sample dilution guidelines.

### 2.3 Microbiological Analysis

For isolation of bacteria from the samples, the spread plate method [11] was followed in this experiment. Different types of non-selective and selective agar were used for the isolation procedure. These are Plate Count Agar (PCA),

MacConkey (MC), Salmonella-Shigella (SS), Eosine-Methylene Blue (EMB), Thiosulphate Citrate Bile Sucrose (TCBS) agar. For antibiotic susceptibility test, Muller-Hilton agar and Muller-Hilton broth were used.

From each serial diluted tube 50  $\mu$ L sample suspension was transferred to the petri-dishes which were pre-incubated with aforementioned agar mediums. After the suspension was evenly spread over the surface of the agar, they were incubated for 24-48 hours at 37°C. After 24-48 hours of incubation, bacterial colonies were visible on the agar surface.

The viable cells present in the samples were counted as shown below:

$$50 \mu\text{L} = X \text{ number of colonies}$$

Therefore, per mL of sample contained=

$$X \times \frac{1000}{50} \times \text{Dilution factor}$$

Bacterial colonies grown on different types of medium were collected and maintained in nutrient slant agar which were kept in refrigerator at 4°C as stock cultures for further analysis. Total Bacterial Count was calculated by using Plate Count Agar (PCA) media and total coliform count (TCC) was done using MacConkey agar medium. For determining the quality of pasteurized and UHT milk we used the acceptable limit of Bangladesh Standards and Testing Institutions (BSTI) which is  $<2 \times 10^4$  CFU/mL for pasteurized milk and  $<10^1$  CFU/mL for UHT milk.

For identification of the bacterial isolates, cultural, morphological, and biochemical characteristics were examined. Morphological characteristics were determined by Gram staining procedure and cultural characteristics were determined by observing color, shape, size, margin, elevation, consistency and opacity of the bacterial colonies. The biochemical tests which we carried out were Kligler's Iron Agar (KIA) test, Motility, Indole and Urea (MIU) test, Catalase, Oxidase and Methyl red-Voges-Proskauer (MR/VP) test.

### 2.4 Antibiotic Susceptibility Test

Antibiotic Susceptibility Test was done by standard disk diffusion technique by following Clinical and Laboratory Standards Institute guidelines [12]. We conducted the antibiotic susceptibility test against eight common

antibiotics which were Ampicillin (AMP, 25µg), Colistin (CT, 10µg), Ciprofloxacin (CIP, 5µg), Chloramphenicol (C, 30µg), Erythromycin (E, 15µg), Tetracycline (TE, 30µg), Azithromycin (AZM, 30µg) and Amoxicillin (AX, 10µg). The concentration of antibiotics was subjected to their availability in the markets. The inhibition zones were measured and isolates were classified as resistant, intermediate or sensitive according to the interpretation guideline provided by the Clinical and Laboratory Standard Institute (CLSI) [12]. Multiple Antibiotic Resistance (MAR) index was calculated as the ratio of number of antibiotics to which the isolate showed resistance to total number of antibiotics to which the isolate was exposed [13] and MAR index was expressed as percentage.

### 2.5 Detection of virulent gene by Polymerase Chain Reaction (PCR)

Isolated and presumed *Salmonella* colonies from nutrient agar plate were grown overnight in 5 ml nutrient broth in test tube at 37°C with aeration using shaking water bath set at 120 rpm. From nutrient broth bacterial culture, 1 mL culture was taken from each tube of pure culture broth and mixed culture broth in eppendorf tube and DNA was extracted by boiling method [14]. In this method, the tubes were centrifuged for 5 minutes at 10,000 rpm. Supernatant was removed and 200µl PCR water was added. The bacterial pellet was mixed by pipetting. After boiling, eppendorf tubes were placed in ice for 10 minutes. Then the tubes were centrifuged for 10 minutes at 10,000 rpm in centrifugation. About 150µL supernatant was collected from each tube and preserved in refrigerator at -20°C [15].

Amplification of the desired gene in the selected isolates was carried out by polymerase chain reaction (PCR) [16]. The PCR reaction was started from denaturation at 94 °C for 1 minute followed by annealing and extension at 72 °C for 30 seconds. These three steps were repeated sequentially for 35 cycles with a final extension for 7 minutes at 72 °C. The specific *invA* primer which targets the virulent *invA* gene was used for the detection of pathogenic gene in targeted isolate [17].

## 3. RESULTS

### 3.1 Total Bacteria Count (TBC) and Total Coliform Count (TCC) of the Samples

All raw milk samples had high TBC and TCC, and company “C” had the highest TBC ( $6.5 \times$

106 CFU/mL) and TCC ( $2.2 \times 10^3$  CFU/mL) among all the companies. According to the Bangladesh Standards and Testing Institutions (BSTI) the TBC of milk samples collected from processing plants before packaging was within acceptable limits ( $< 2 \times 10^4$  CFU/ml) as well as the Total Coliform Count was nil. However, the packaged pasteurized milk samples from retail shops contained TBC which was beyond the acceptable limits. Both TBC and TCC count of the packaged pasteurized milk samples of retail shops were higher than pasteurized milk samples of processing plants. There was no bacterial growth found in UHT milk samples of all three companies.

### 3.2 Identification of Bacterial Isolates Collected From Different Sampling Points

A total of sixty-six bacterial isolates were collected from all the samples. After conducting cultural, morphological and biochemical tests the isolates were identified.

Raw milk samples were highly contaminated with different kind of bacteria such as *Micrococcus*, *Vibrio*, *Salmonella*, *Shigella*, *Streptococcus*, *E. coli* and many more. About 66.6% *Micrococcus* isolates, 62.5% *Salmonella* isolates and 60% *Proteus* isolates were isolated from untreated raw milk. From pasteurized milk samples of processing plants, we isolated *Streptococcus* (14%), *Staphylococcus* (16.7%), *Micrococcus* (16.7%) and *Bacillus* (20%). *Vibrio*, *Enterobacter*, *Citrobacter* and *Shigella* were only present in raw milk samples of the collection centers. From Fig. 1, it is evident that highest percent of each bacterial isolate were isolated from collection centers. The unpackaged pasteurized milk samples of retail shops contained higher percent and number of bacteria than pasteurized milk of processing plants. (Fig. 1 Percent of bacterial isolates collected from three points of the supply chain)

### 3.3 Antibiotic Susceptibility Test and Multiple Antibiotic Resistance (MAR) Index

All Gram-negative isolates (n=29) were resistant to Ampicillin (100%). About 84.21%, 68.42% and 47.36% of the isolates were resistant to Amoxicillin, Erythromycin and Colistin respectively. Ciprofloxacin—a third generation drug—was resistant to none of the isolates and was intermediate to 5.26% of the isolates (Fig. 2).

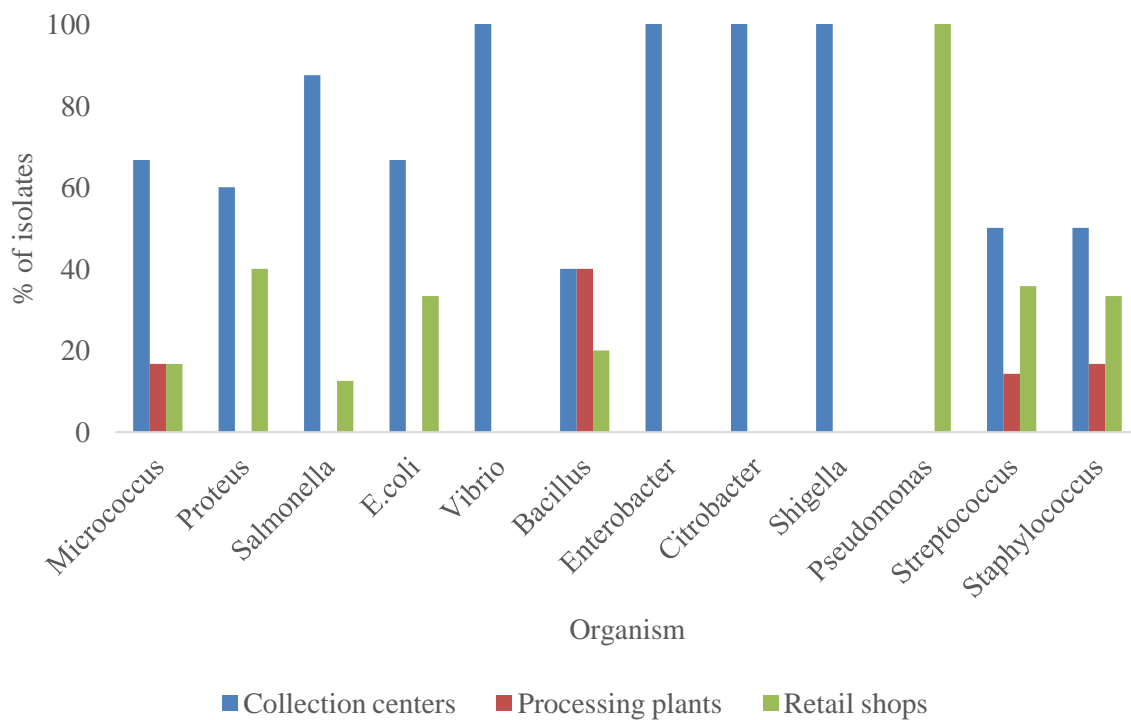
**Table 1. TBC and TCC of the milk samples from three points of the supply chain**

Company code	Total Bacteria Count (CFU/mL) <sup>a</sup>			Total Coliform Count (CFU/mL) <sup>a</sup>		
	Collection centers	Processing plants	Retail shops	Collection centers	Processing plants	Retail shops
A	$7.2 \times 10^5$	$1.6 \times 10^3$	$3.6 \times 10^4$	$2.4 \times 10^3$	0	$1.2 \times 10^3$
B	$6 \times 10^6$	$1.4 \times 10^3$	$2.6 \times 10^4$	$1.2 \times 10^4$	0	$1.3 \times 10^3$
C	$6.5 \times 10^6$	$5 \times 10^3$	$4.5 \times 10^4$	$3.4 \times 10^4$	0	$2.2 \times 10^3$

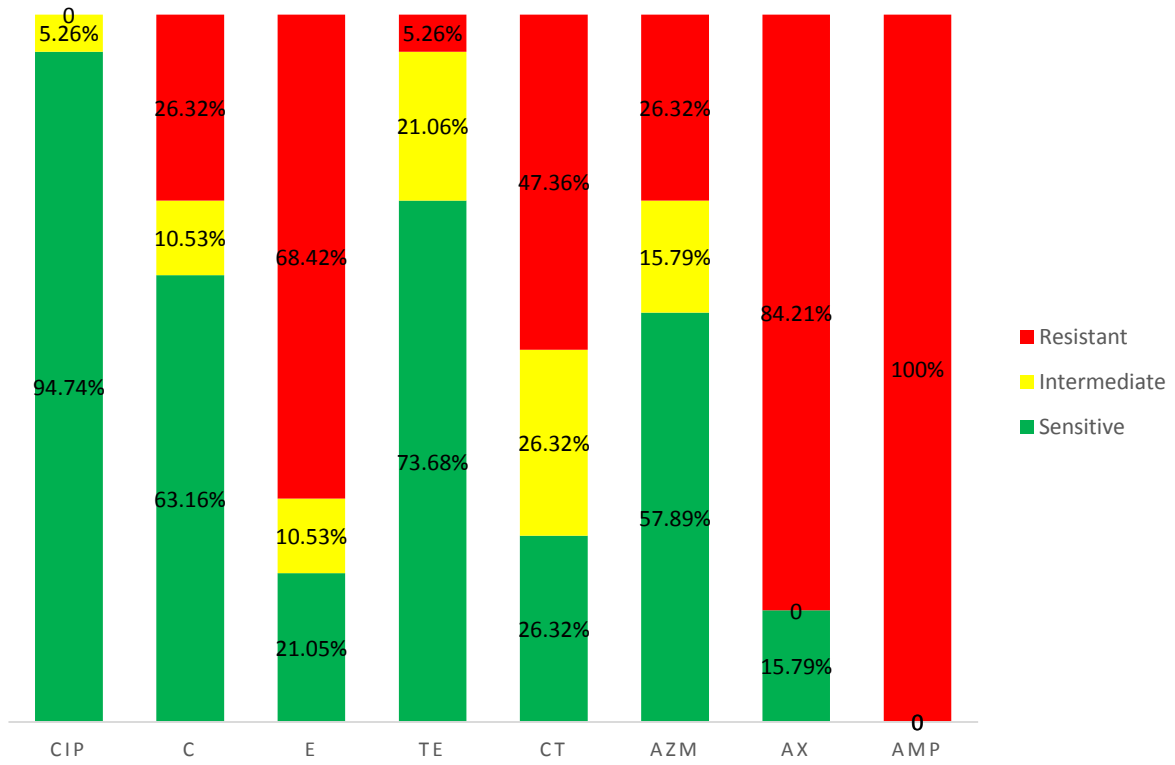
<sup>a</sup> Averages of samples collected from the sampling point of the companies

**Table 2. Multiple Antibiotic Resistance Index (MAR) of isolated bacteria**

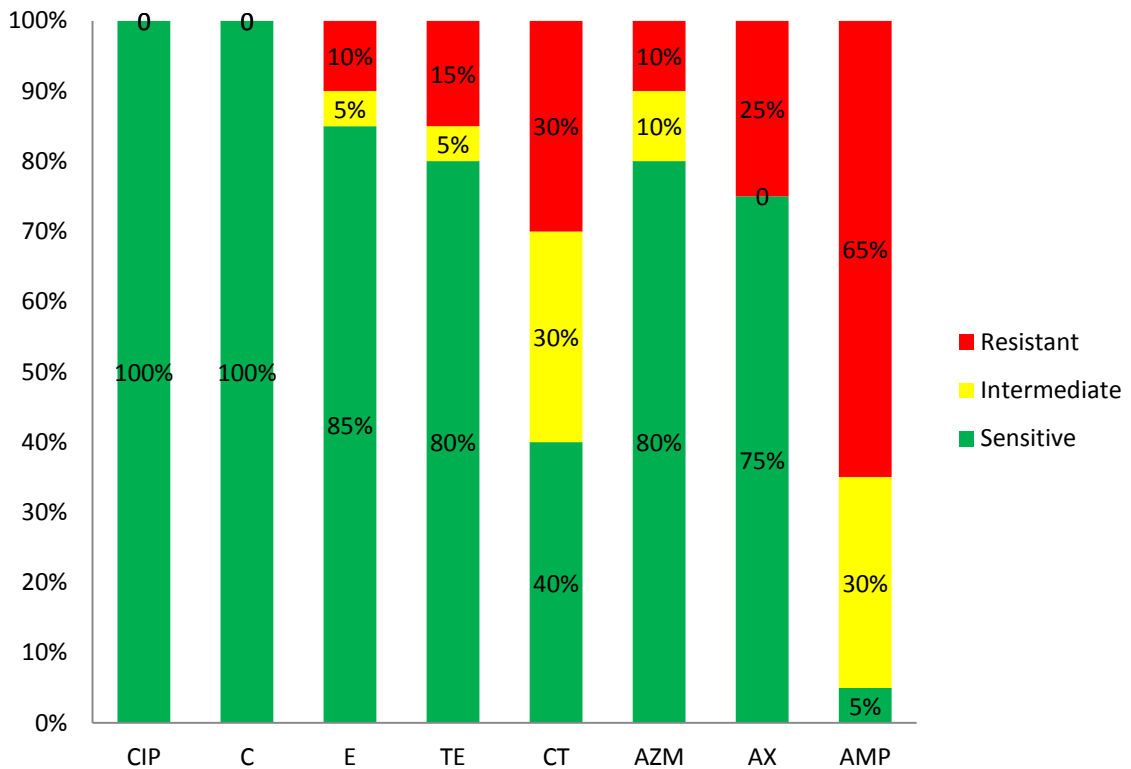
Bacterial spp.	MAR (%)
<i>Salmonella</i>	75
<i>Proteus</i>	62.5
<i>Vibrio</i>	62.5
<i>Pseudomonas</i>	50
<i>Shigella</i>	50
<i>E. coli</i>	37.5
<i>Bacillus</i>	37.5
<i>Staphylococcus</i>	37.5
<i>Streptococcus</i>	37.5
<i>Micrococcus</i>	37.5
<i>Enterobacter</i>	25
<i>Citrobacter</i>	25



**Fig. 1. Percent of bacterial isolates collected from three points of the supply chain**



**Fig. 2. Graphical representation of antibiotic resistance of Gram-negative isolates**



**Fig. 3. Graphical representation of antibiotic resistance of Gram-positive isolates**

All Gram-positive isolates (n=37) were sensitive to Ciprofloxacin and Chloramphenicol. Among all antibiotics, the highest percentage (65%) of bacteria were resistant to Ampicillin. Moreover, 30%, 10% and 25% isolates were resistant to Colistin, Azithromycin and Amoxicillin. (Fig. 3).

All bacterial isolates were not resistant to multiple antibiotics. *Salmonella* isolates showed the highest MAR index which was 75%. *Vibrio* and *Proteus* both showed 62.5% MAR index which is the second highest percentage. *Pseudomonas*, *Aeromonas* and *Shigella* had MAR index of 50% and *Streptococcus*, *Bacillus* and *E. coli* showed 37.5% MAR index.

### 3.4 Pasteurized Milk Samples Collected from Retail Shops Contained Pathogenic *Salmonella*

One *Salmonella* isolate collected from packaged pasteurized milk samples of retail shops, was tested for detection of pathogenic gene. For detecting the presence of virulent gene, *invA* primer was used which works as a good indicator of presence of *invA* virulent gene in *Salmonella* (Fig. 4: Agarose gel electrophoresis (on 1% agarose gel) showing polymerase chain reaction amplification products of *invA* gene (284 bp)).

## 4. DISCUSSION

Food safety and consumer safety are paramount for ensuring a healthy food system in an over populated and congested country like Bangladesh. Dairy sector which is one of the most emerging and a profitable area of our country needs much attention as it is not fully automated and largely depends on human handling.

Total bacterial count serves as an indicator of the microbiological quality of milk and also indicates the standards of dairy farmers, milk processing companies and retail shops. All raw milk samples of our study had high total bacterial count and total coliform count which implies that raw milks were highly contaminated with different types of bacteria. A study conducted with 22 raw milk collected from different dairy farms across Dhaka city showed similar findings, that the milk samples were substandard in terms of TBC and TCC [18]. Raw milks can be contaminated by various sources such as from the udder of the cow, handlers, milk collection vats, milking utensils, milk preservatives, disinfectants used

for cleaning the utensils as well as heavy metals, agrochemicals, hormones and which come from the veterinary applications and feed of the cows [19]. Moreover, herd management is also an important key for keeping the raw milk in aseptic condition as mastitis of cow is a common factor for contamination of milk [20]. In our study, the pasteurized milk samples collected before packaging from milk processing plants had TBC count within acceptable range and no coliform count. However, *Streptococcus*, *Staphylococcus* and *Micrococcus* were isolated from these samples which may be because of post processing contamination. As proper pasteurization kills pathogens, most milk-borne outbreaks of human illness have been associated with raw or inadequately pasteurized milk or with milk contaminated after pasteurization [8].

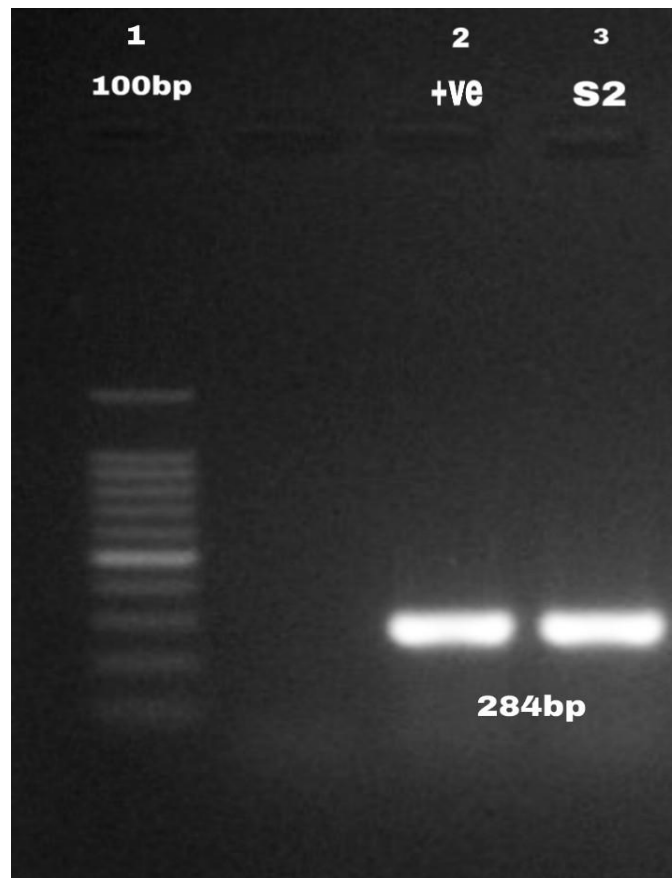
To find the microbiological quality of milks consumed by the people of Dhaka city we examined the packaged pasteurized milks from retail shops. We found that 100% of the milk samples had high TBC ( $>4.0 \times 10^4$  CFU/mL) and TCC ( $1.2 \times 10^3$  CFU/mL) though all of the samples were within expiry dates. This clearly indicates that the pasteurized milks of retail shops fail to maintain their quality at the point of sale. One possible explanation is that the contamination may be due to faulty holding temperatures of the retail shop refrigerators which should be below 4°C. Moreover, using of contaminated packaging materials or any type of leakage in the material may also lead to post processing contamination. UHT milk samples did not have any bacterial growth as it kills all the possible bacteria present in the milk with the high temperature short time technique [21]. A study conducted in Malaysia concluded that the reasons for the presence of bacteria in UHT milk may be due to milk quality, sanitation of process plant, status of packaging material and also the process of handling. We isolated a wide range of disease-causing bacteria from raw milks as well as pasteurized milks of retail shops such as *Salmonella*, *Streptococcus*, *Vibrio*, *Staphylococcus*, *Micrococcus* and *E. coli*. Presence of *Staphylococcus* which mainly comes from skin and nasal cavity; indicates the contamination of milk by the handler's sneezing or skin contact [22]. The high prevalence of *E. coli* in food of animal origin implies environmental and fecal contamination [23].

Antibiotic resistance has become one of the major global concerns of modern world as microorganisms have changed their pattern of

mechanism and become resistant to the common antibiotics. In our study, Gram negative isolates 100% showed resistance towards Ampicillin, about 84.21% resistance towards Amoxicillin and 68.42% towards Erythromycin. Improper hygienic standards and indiscriminate use of antimicrobials could be two of the main causes for the prevalence of these pathogenic resistant strains [24]. In our study, *Salmonella* showed MAR index of 75%, *Vibrio* and *Proteus* both showed MAR of 62.5% which implies that the bacteria are getting resistant towards several antibiotics. A previous study reported that approximately 60% of the antibiotic-resistant isolates were confirmed as multidrug resistant (MDR) [25]. In our study, we had isolated *Salmonella* from retail shop milk samples and after carrying out the PCR it was identified that the *Salmonella* contained virulent *invA* gene. Similar finding was observed in a study which concluded that, *invA* gene was found to be

present and functional in most *Salmonella* serotypes is responsible for the invasion of the cells of the intestinal epithelium allowing *Salmonella* pathogen to enter and survive inside the eukaryotic cells with subsequent diseases in variety of hosts [26].

Small sample size is a drawback of our study. At the same time, the strength of the study is that we collected milk samples directly from three leading dairy companies of Bangladesh. The study findings indicate that good hygienic practices and proper monitoring must be urgently introduced at each level of milk production in Bangladesh starting from producers to the processors. Finally, importance should be given at producer's level to hinder the survival and multiplication of microorganisms. Moreover, controlling temperature and proper maintenance of the cold chain from collection centers to processing plants should be practiced.



Lane 1, 100-bp DNA ladders; Lane 2, *invA* virulent gene (284 bp) used as positive control; Lane 3, PCR result of the S2 coded *Salmonella* isolate

**Fig. 4. Our presumptive strain (coded as S2) created the same product size (284 bp) and band intensity as the *invA* primer (Lane:2) which confirmed the presence of *invA* virulent gene in our tested S2 coded *Salmonella* isolate (Lane: 3)**



## 5. CONCLUSION

The result of the study focuses on the microbiological quality of the milk supply chain as well as on the antibiotic resistance of the common microorganisms. Raw milk collected from processing plants and packaged pasteurized milk from retail shops did not meet the microbiological standard. Different bacteria like *Salmonella*, *Proteus*, and *Vibrio* are getting resistant towards ampicillin, amoxicillin, erythromycin, and colistin. Moreover, detection of pathogenic *Salmonella* isolate from retail shops is a potential threat to consumer health and safety. Detailed attention and use of new technologies without involving human contact in the milking parlors may be a good way to reduce the contamination level. Moreover, milk processing companies should be cautious about their hygiene practices together with the proper maintenance of cold chain. The findings of this study would help to make the food safety authority mindful to monitor the milk processing companies as well as the retail shop sellers carefully on a regular basis.

## CONSENT

As per international standard or university standard, Participants' written consent has been collected and preserved by the author(s).

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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