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Original article

High prevalence of multidrug-resistant extended spectrum βeta lactamase -producing *Escherichia coli* in raw milk in Bangladesh

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ABSTRACT

Background: Raw milk is essential in our daily diet for food safety but poses a risk of contamination with harmful bacteria like ESβL *Escherichia coli*. **Objectives:** The study was undertaken to characterize multidrug-resistant *E. coli* and determine the antibiogram profile of the isolates recovered from raw milk. **Methods:** A total of 80 raw milk samples were collected from 20 milk selling points in Sylhet and analyzed for physio-chemical parameters, microbial loads, ESβL *Escherichia coli*, and their virulent factors with the association of AMR. **Results:** Results demonstrated that the mean specific gravity, fat%, protein%, lactose%, acidity%, SNF%, pH, and added water% were 1.026±0.0003, 3.83±0.072, 2.83±0.046, 4.08±0.080, 0.20±0.003, 7.47±0.144, 6.38±0.035, 1.72±0.034, respectively. The mean aerobic mesophilic count and mean coliform count were $(2.72\times10)^7$ CFU/ml and $1.53\times(10)^6$ CFU/ml, respectively. We found the prevalence of *E. coli* was 60% (48/80) in raw milk, and PCR results revealed that all *E. coli* isolates were positive for the stx1 (6.25%), *eae*A (6.25%), *bla*$_{CITM}$ (8.33%), *bla*$_{SHV}$ (6.25%), *bla*$_{TEM}$ (14.58%), and *tet*A (50%) genes. The antibiogram results demonstrated that all the isolates were most resistant to ampicillin and tetracycline (100%), amoxicillin (79.17%), ceftriaxone and ceftazidime (62.5%), streptomycin (58.53%) and gentamycin (60%), whereas vancomycin (79.17%), ciprofloxacin (75%) and meropenem (54.17%) were the most sensitive. **Conclusion:** Our study underscores the urgent need for stringent hygiene measures in milk production and emphasizes judicious antibiotic use in safeguarding public health.

Introduction

Liquid milk is a critical component of our daily diet and plays a significant role in both ensuring food safety in developing countries like Bangladesh and meeting the rising nutritional demand. Milk is a must-eat food due to its numerous nutrients, including protein, carbohydrates, fat, vitamins, and minerals. Cow's milk is composed of 87.5% water, 3.8% fat, 3.1% protein, 4.6% lactose, 8.7% solid non-fat (SNF), and 12.5% total solid (TS). A significant component of them is milk fat, which can vary from 2.8% to 8.1% depending on the breed, lactation, diet, hygiene, and season [1].

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Milk quality depends on composition and hygiene during milking, storage, and transport. Poor practices can harm public health and the economy. Milk, due to its high nutrient content, is an ideal medium for microbial growth. Typically, fresh milk contains 2×10^5 cfu/ml microorganisms while pasteurized milk has 2×10^4 cfu/ml [2]. Udder and external sources like equipment, water, and workers can contaminate milk. High bacteria affect taste, spoilage, and safety, including zoonotic risks [3]. Shiga toxin-producing *Escherichia coli* (STEC), also known as Verotoxin-producing *E. coli* (VTEC), generates potent toxins, Stx1, Stx2 (or VT1, VT2), alongside other virulence factors like intimin, which fosters attachment to intestinal cells, leading to lesions. STEC triggers severe gastrointestinal issues, hemolytic uremic syndrome (HUS), and persistent diarrhea in both humans and animals. Pathogenic *E. coli*, with diverse virulence like *eae*A, can induce illness in both animals and humans [4]. There have been several recent reports of outbreaks linked to *E. coli* in milk and other food sources.

Extended-spectrum β-lactamase (ESβL) producing bacteria poses a significant public health concern in contemporary times. The existence of such bacteria frequently results from the improper utilization of antibiotics in managing infections and their irrational administration [5]. *Escherichia coli* (*E. coli*) stands as a prominent environmental contaminant, frequently linked to the presence of ESβL-encoding genes, specifically identified as $bla_{\text{CTX-M}}$ and bla_{TEM} [6]. ESβL-producing *E. coli* is highly prevalent in food products of animal origin [7]. Livestock and livestock products, including milk, can serve as potential sources for the transmission and dissemination of ESβL-producing bacteria. This presents a novel concern, given their direct integration into the human food chain. The global distribution pattern of ESβL enzymes in both human and animal populations holds research value in guiding preventive and therapeutic strategies against ESβL bacterial infections. Clinical trials for treating infections caused by ESβL -producing bacteria have exhibited limited success [8].

Antimicrobial resistance (AMR) is one of the biggest risks to global health in the twenty-first century. AMR is both a one-health concept and a one-world problem spreading internationally among humans and animals [9]. There is grave concern regarding the future incapacity to cure common bacterial diseases because of rising AMR rates in both humans and animals. *Escherichia coli* has been linked to numerous infections and there have been growing reports of its drug resistance [10]. We observed significant rates of resistance among this group of isolates since *E. coli* is one of the most frequent causes of sepsis and urinary tract infections (UTI). Many causes contribute to AMR, with increased or improper use of antibiotics for prevention, treatment, and promoting animal growth being the main culprits [11]. Poor environmental hygiene, poverty, inadequate healthcare systems, antibiotic-rich animal feeds, subpar or fake antimicrobials, and expensive second-line treatments could all potentially foster the emergence and growth of AMR pathogens in developing nations like Bangladesh where the burden of AMR is already reported to be high [12]. This issue, combined with the high prevalence of infectious diseases, the scarcity of available antibiotics, and inadequate laboratory diagnoses, creates the perfect storm for the creation and dissemination of resistant bacterial strains.

There have been a few studies on this topic in Bangladesh. However, this type of study on raw milk is rare in Sylhet. This is why the current study was conducted with the intention of isolation and molecular characterization of multidrug resistant ESBL producing *E. coli* recovered from raw milk marketed in Sylhet.

Material and methods

Ethical approval

The study did not require any ethical approval.

Collection of milk samples

We collected raw milk samples (n = 80) for this study from randomly selected 20 raw milk selling points in Sylhet in four consecutive weeks. All the samples were obtained in 500ml packages, transported in ice boxes to the Laboratory of Department of Dairy Science, Sylhet Agricultural University, Sylhet and analyzed immediately upon arrival.

Physico-chemical analysis

Specific gravity was determined with the use of lactometer following **Chaudhuri et al.** (1959). Several chemical analyses including acidity, protein, lactose, fat, Solids-Not-Fat (SNF), and added water were determined using Lactoscan SP Ultrasonic Milk Analyzer (Model: MT-25) which is manufactured by a Bulgarian company named Milkotronic Ltd. and pH was analyzed by Hanna

pH/ORP Meter (Model: HI 2211) manufactured by RP Scientific Store.

Microbiological analysis

In the present study, we performed total aerobic mesophilic count and coliform count to estimate colony forming unit for assessing the microbial load in the samples following **Tasci** [13].

Isolation and identification

Milk samples were directly streaked on Eosin Methylene Blue (EMB) agar and incubated at 37°C for 18-24 hours to isolate *E. coli*. Colonies with characteristic green metallic sheen were selected for biochemical tests and DNA extraction and confirmed to be *E. coli* by specie specific PCR. For future use, all samples were stored at -20°C in nutrient broth containing 15% glycerol [14, 15].

Detection of *pho*A gene in *E. coli* isolates

The detection of *pho*A gene in *E. coli* isolates was conducted following the methods described previously with slight modification [16]. Briefly, *E. coli* samples were sub-cultured on nutrient broth for 16-18 hours and the genomic DNA was extracted. For this, the supernatant was discarded after centrifugation of one mL of broth culture placed in a 1.5 mL tube, and the pellet was re-suspended in 100 µL sterile distilled water, boiled at 100°C for 15 minutes, and centrifuged at 13,500 rpm for 5 minutes. The extracted bacterial DNA was amplified with 12.5 µL of OneTaq Quick-Load 2x Master Mix with Standard Buffer (USA), 1 µL of each primer (20 picomole concentration), 6 µL of DNA template, and 4.5 µL of water to reach a final volume of 25 µL. DNA was amplified **(Supplementary table 1)** in a thermal cycler (BIO-RAD T100 Thermal Cycler). PCR products were isolated on 1.5% of agarose gel, and DNA amplicons were visualized with ethidium bromide.

Detection of virulence and antibiotic-resistant genes

Further, Uniplex PCR was performed for detecting each of the virulence genes (stx1 and *eae*A genes) and antibiotic-resistant genes (bla_{CITM}, bla_{SHV}, bla_{TEM}, and *tet*A genes) following **Grakh et al.** with slight modification [17] **(Supplementary table 1)**. The products of the Uniplex PCR were separated by electrophoresis on 1.5% of agarose gel (Invitrogen, USA) in 10X TAE buffer stained with ethidium bromide using a gradient of 5 V/cm by a gel documentation system (Cleaver Scientific, UK).

Antimicrobial susceptibility test

The antimicrobial susceptibility test was performed using the Kirby-Bauer disk diffusion method following [18, 19] and CLSI guidelines 2020 (**Supplementary table 2**). The test was performed on Mueller-Hinton agar (MHA) with an inoculum equivalent to 0.5 McFarland standards. The isolates were documented as susceptible, intermediate, and resistant according to the zone diameter interpretative standards recommended by CLSI guidelines.

Statistical analysis

Statistical analyses were performed using Microsoft Excel 365 and the graphical presentations were produced using GraphPad Prism version 9. We performed all the experiments in triplicate repeat and obtained descriptive statistics as mean ± standard error of mean (SEM), and minimum and maximum values. The standard errors of mean were calculated to control the precision of the examination and p-value less than 0.05 was considered statistically significant.

Results

Physico-chemical analysis

In this study, we analyzed all the milk samples for specific gravity, fat, acidity, SNF, lactose, pH and added water. Results demonstrated that the specific gravity showed slight variations among the samples that varied from 1.021 to 1.040 with the mean specific gravity of 1.026. The results of the Lactoscan milk analyzer revealed that the values of fat content varied from 3.15 - 5.65% with an average value of 3.83% which is higher than the BDS and FAO standard. However, the percentage of the mean value of protein, lactose, acidity, SNF, pH and added water were 2.83, 4.08, 0.20, 7.47, 6.38, and 1.72 respectively (**Figure 1, Supplementary table 3**), and compared to the BDS and FAO standards (**Figure 2, Supplementary table 4**).

Microbiological analysis

Samples were further subjected to microbiological analysis to observe microbial load in raw milk. Total aerobic mesophilic count and coliform count were performed and measured in Colony Forming Unit (CFU). The results demonstrated that the mean values of total aerobic mesophilic count and coliform count were $2.72 \times 10^7 \pm 0.34$ CFU/ml and $1.53 \times 10^6 \pm 0.20$ CFU/ml respectively and then they were compared with Bangladesh standard recommended

by Bangladesh Food Safety Authority (BFSA) (**Figure 3, Supplementary table 5**).

Detection of *pho*A, virulence and antibiotic resistant genes

We isolated 48 *E. coli* isolates out of 80 raw milk samples (60%) based on colony morphology (**Supplementary figure 1**) and biochemical tests. All the isolates were confirmed to be *E. coli* positive by PCR results targeting the *pho*A gene (**Figure 4**). The presence of various virulence (stx1 and *eae*A) and antibiotic-resistant genes (bla_{CITM}, bla_{SHV}, bla_{TEM} and *tet*A) in the isolates were then assessed. We found that *E. coli* isolates showed an accurate amplicon for stx1 and *eae*A (6.25%) genes. The presence of beta-lactamase producing genes were confirmed to be bla_{CITM} (8.33%), bla_{SHV} (6.25%), bla_{TEM} (14.58%) by PCR. In addition, *tet*A genes were detected in 50% of the isolates in this study (**Figure 5, Supplementary table 6, Supplementary figure 2-7**).

Antimicrobial susceptibility test

According to Clinical Laboratory Standards Institute (CLSI) guideline 2020, the antibiotic susceptibility test of the *E. coli* isolates was conducted. All the isolates (100%) exhibited no zone of inhibition against ampicillin and tetracycline. Most of the isolates were resistant to amoxicillin (79.17%) followed by ceftriaxone and ceftazidime (62.5%), cefoxitin (54.17%), imipenem (37.5%), azithromycin (41.67%) and meropenem (26%). In addition, 60% of the isolates were resistant to gentamycin and 58.33% to streptomycin. However, vancomycin (79.17%), ciprofloxacin (75%), meropenem (54.17%), sulfamethoxazole-trimethoprim (50%) were the most sensitive antibiotics. Most of the isolates were revealed to be resistant to the beta-lactam antibiotics used in the test. All the isolates showed resistance to more than one antibiotic class (MDR) in this study which makes them challenging to treat effectively with traditional antibiotics (**Figure 6**, **Supplementary figure 8, Supplementary table 7**).

Figure 1. Analysis of physico-chemical properties of raw milk samples. Analysis of physico-chemical properties of 80 market milk samples collected from 20 milk selling points in 4 consecutive weeks; Specific gravity (A); Fat percentage (B); Acidity percentage (C); Solids-Not-Fat percentage (D); Protein percentage (E); Lactose percentage (F); Added water percentage (G); pH (H). Mean value of selling points of physico-chemical properties and the milk selling points were placed in Y-axis and X-axis respectively; in X-axis 1-20 represents the number of milk selling points

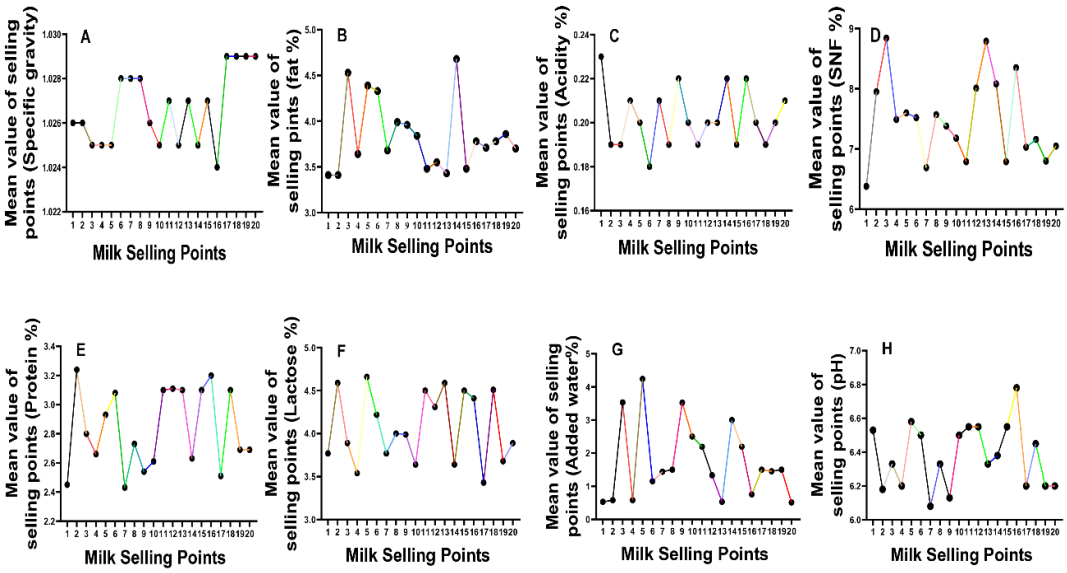


Figure 2. Comparison of chemical properties of raw milk samples with BDS and FAO standards. Comparison of chemical properties among Bangladesh Standard (BDS), Food and Agricultural Organization Standard (FAO), and mean value of 80 raw milk samples collected from 20 milk selling points in 4 consecutive weeks; the values of chemical properties were placed in Y-axis and the chemical properties in the X-axis

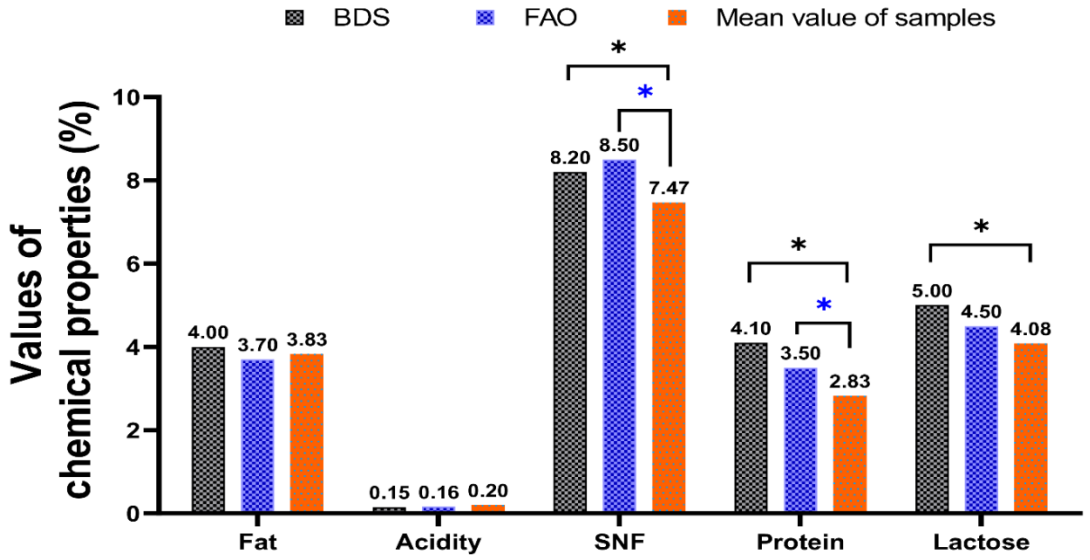


Figure 3. Microbiological analysis of raw milk samples and comparison with BFSA standard. Comparison of bacterial loads with Bangladesh standard recommended by Bangladesh Food Safety Authority (BFSA). All raw milk samples were assessed for aerobic mesophilic count and coliform count, and then compared with BFSA. The raw milk samples exposed with a significantly higher number of cfu/ml ($p < 0.001$) for aerobic mesophilic count (A) and coliform count (B) compared to those of BFSA. Data represent the average ± SEM of the levels derived from the collected samples (n=80 for A and B). *p<0.05, **p<0.01 and ***p<0.001 compared between the study findings and BFSA

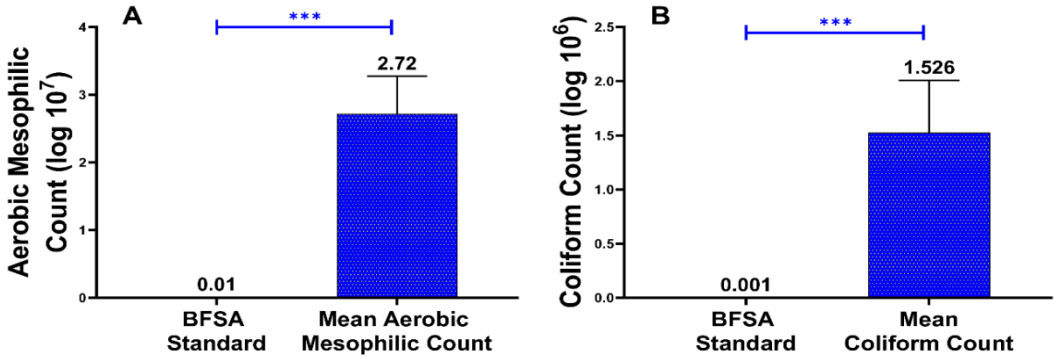


Figure 4. Gel image showing amplified DNA of genes of E. coli isolates. Amplified DNA of phoA gene of *E. coli* isolates. The positive isolates amplified at 468 bp. Lane M: 100bp DNA Ladder; Lane NC: (-ve) Control; Lane 1-5: *E. coli* positive isolates

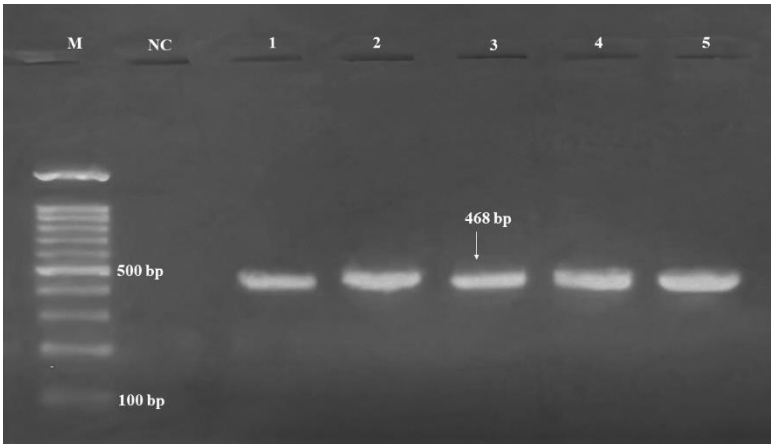


Figure 5. Gel image showing amplified DNA of genes of E. coli isolates. Amplified DNA of genes of *E. coli* isolates. Amplified DNA of stx1 gene at 302 bp. Amplified DNA of *eae*A gene at 454 bp. Amplified DNA of bla_{CITM} gene at 462 bp. Amplified DNA of bla_{SHV} gene at 768 bp. Amplified DNA of bla_{TEM} gene at 425 bp. Amplified DNA of *tet*A gene at 372 bp. Lane M: 100bp DNA Ladder; Lane NC: (-ve) control; ; Lane 1: positive isolate for stx1 gene; Lane 2: positive isolate for *eae*A gene; Lane 3: positive isolate for bla_{CITM} gene; Lane 4: positive isolate for bla_{SHV} gene; Lane 5: positive isolate for bla_{TEM} gene and Lane 6: positive isolate for *tet*A gene

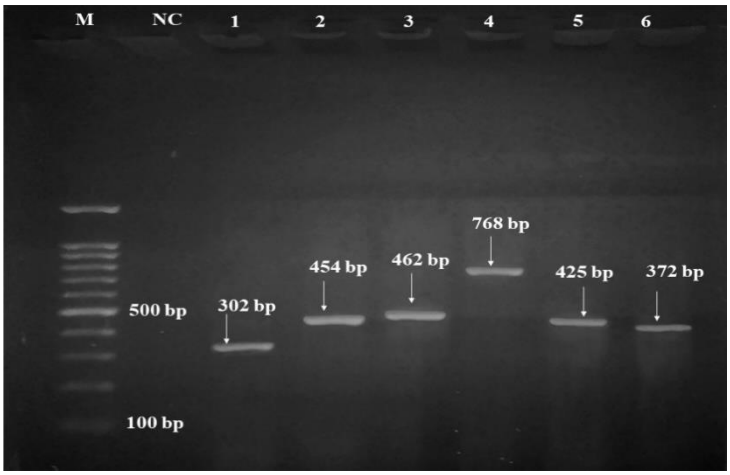
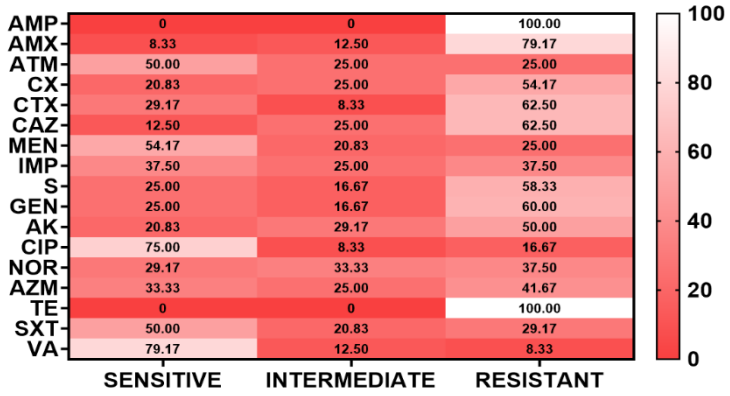


Figure 6. Antimicrobial resistance patterns of E. coli isolates. Antimicrobial susceptibility test by Kirby-Bauer disc diffusion method. Heat map showing antimicrobial resistance patterns of the isolates of *E.coli* recovered from raw milk; All the isolates of *E. coli* were assessed for their antimicrobial susceptibility against CLSI-2020 guided antimicrobial agents; The intensity of color from red to white in the vertical bar on the right side indicates the percentage of isolates from 0 to 100 respectively; The values inside each rectangle designate the percentage of resistance and sensitivity of isolates; AMP=Ampicillin, AMX=Amoxicillin, AT=Aztreonam, CX=Cefoxitin, CTX=Cefotaxime, CAZ= Ceftazidime, MEM=Meropenem, IMP= Imipenem, S=Streptomycin, GEN= Gentamycin, AK=Amikacin, CIP=Ciprofloxacin, NOR= Norfloxacin, AZM=Azithromycin, TE=Tetracycline, SXT=Sulfamethoxazole/Trimethoprim, VA=Vancomycin



Discussion

The demand for safe fresh milk is rising in the country with the increasing education and awareness. The physico-chemical properties of milk are largely related to the nutritional value and shelf life of milk. In our study, milk samples were evaluated for physico-chemical properties with the help of milk analyzer. The specific gravity showed slight variations among the samples. The highest value of specific gravity was recorded 1.040 and lowest value was 1.021 with the mean specific gravity of 1.026 which lies within the standard range recommended by FAO food databases. Related results of 1.029-1.032 were described by **Kader et al.** which is close to our findings [20].

Further, the chemical properties were assessed, presented in **Figure (1),** and compared to the Bangladesh standard and Food and Agriculture Organization standard presented in **Figure (2)**. In this study, fat % of the samples revolved around 3.83±0.072 % where the highest and lowest value were 5.65% and 3.15%, respectively. The mean value of fat% is higher than the FAO standards and previous findings [21] and slightly lower than the BDS. The acidity of milk is an important chemical parameter as it indicates the shelf life of milk and

milk products. Milk having higher acidity yields less amount of cream during cream separation which can be a challenge to the dairy industry. The mean acidity% was 0.20±0.003 which exceeds the BDS and FAO standards limit, and previous research findings [21, 22]. The mean values of SNF%, protein% and lactose% were 7.47%, 2.83% and 4.08%, respectively which are significantly lower (p<0.05) than the BDS and FAO standards. Further, the mean pH of the milk samples was 6.38 which is very close to the normal range (6.4-6.8) of raw milk. Added water % of 1.72 indicates the water adulteration in raw milk which can be a potential source of contamination.

To determine the microbial quality of milk, microbiological analysis of the samples was conducted. Mean values of total aerobic mesophilic count and coliform count were $2.72 \times 10^7 \pm 0.34$ CFU/ml and $1.53 \times 10^6 \pm 0.20$ CFU/ml, respectively. Total aerobic mesophilic count and coliform count of the raw milk samples revealed that all of them exceeded the standard limit of Bangladesh standard recommended by Bangladesh Food Safety Authority (BFSA). However, recent studies showed closely similar findings to this research findings. One recent study described by Alam et al. found that the mean values of total viable bacterial count and coliform count were 4.04×10^8 CFU/ml and 1.88×10^6CFU/ml in raw milk [23]. Another study showed that the mean viable bacterial count was from 5.8×10^8 to 1.3×10^7 CFU/ml which is close to our findings. And the mean coliform count was 4.2×10^4 to 1.0×10^4 CFU/ml which is lower than our findings [24]. The most frequent cause of high bacterial load is normally because of poor cleaning of the milking system. Bacterial count may be high due to milking dirty udders, maintaining an unclean milking, and housing environment and failing to rapidly cool milk to less than 4°C [25].

Our present study revealed that 60% of the isolates were positive for *E. coli* in raw milk. In Bangladesh, the occurrence rates of *E. coli* in raw milk were 82% [23] and 76% [26] which are higher than our findings. However, a much lower incidence of *E. coli (*26%) was reported by **Sultana et al.** than that of our findings [27]. In contrast of global prevalence, recent studies showed the prevalence rate of *E. coli* in raw milk were 34% and 45% in Northern China [28, 29], 82% in India [30], 74% in Pakistan [31] and 44% in Tanzania [32]. Overall, the findings showed that *E. coli* is a frequent strain in raw milk collected from dairy herds in Bangladesh. This high prevalence of *E. coli* in raw milk is concerning because it is associated with fecal contamination and the risk of enteric pathogenic microorganisms in food as a result.

The presence of virulence factors is a great concern of *E. coli* infections. Public consumers could potentially be harmed by *E. coli* that possesses certain virulent genes. In our study, we found 6.25% of the isolates harbored stx1 and *eae*A genes. Shiga Toxin can cause serious complications such as neurological disorder and hemolytic syndrome, or HUS [33]. Besides, enteropathogenic *E. coli* is one of the common causes of diarrhea in developed and developing countries like Bangladesh. In the study, we found 6.25% of isolates were *eae*A positive which can be classified as enteropathogenic *E. coli* (EPEC). Recent studies reported that the presence of eaeA gene in raw milk was 4.2% [34]. The incidence rates of stx1 and *eae*A genes in raw milk reported by **Elafify et al.** [35] were 28.8% and 11.2%, respectively. Moreover, our findings are comparable with the findings of [29] who reported 9% stx1 and 4.5% *eae*A genes in raw milk in Northern China.

Antibiotic sensitivity tests revealed significant resistance to most of the antibiotics used. Since these drugs are often employed in the treatment of human patients as well as in veterinary practice, the development of antimicrobial resistance by bacteria to these treatments poses a major threat to both human and animal medicine. It has been postulated that uncontrolled usage of antibiotics in animal treatment and their inclusion in animal feeds considerably contribute to the rise of antibiotic resistance across human and animal bacterial populations [36]. In the present study, we found the most frequent antibiotics resistant to ampicillin (100%) and tetracycline (100%). Among others, amoxicillin (79.17%), ceftriaxone and ceftazidime (62.5%) showed resistance to most of the isolates. However, vancomycin (79.17%) and ciprofloxacin (75%) were the most sensitive antibiotics to the isolates. Our findings were in agreement with several previous findings [23, 27, 31]. Similar findings were reported by **Alam et al.** (2017) aligning with our results for ampicillin, amoxicillin, and tetracycline and closely similar findings in case of ciprofloxacin.

The presence and proliferation of ESβL-producing genes in the environment may be closely linked to the dissemination of bacterial strains harboring these genes and the horizontal transfer

facilitated by transmissible plasmids [37, 38]. The primary mechanism of resistance to β-lactams in *E. coli* primarily involves the inactivation of β-lactam antibiotics through the hydrolysis of their β-lactam rings catalyzed by β-lactamase enzymes. *E. coli* strains carrying ESβL genes possess the ability to hydrolyze a wide range of cephalosporins and penicillin. ESβL enzymes, predominantly found in Enterobacteriaceae, often demonstrate resistance to multiple non-β-lactam antimicrobial agents [39]. In our study, we found three types of beta-lactamase encoding genes and one tetracycline resistant gene in *E. coli* isolates in raw milk. The incidence of these genes was bla_{CITM} (8.33%), bla_{SHV} (6.25%), bla_{TEM} (14.58%) and *tet*A (50%). So, these *E. coli* isolates can act as a reservoir of beta-lactamase producing genes leading to horizontal transmission of these genes which is a great public health hazard for the consumers. A current study conducted by **Liu et al.** [29] showed that the overall prevalence of bla_{SHV} and bla_{TEM} were 1.5% and 20.9%, respectively. Another study found that the occurrence of *tet*A gene in raw milk was 64.70% [40]. Thus, the findings of our study indicate that milk can be a good reservoir of bacteria harboring ESβL resistance genes, posing a potential risk to human health.

Conclusion

The study found high contamination of raw milk in the local market. The global concern of antibiotic-resistant bacteria is pronounced in developing countries where close human-animal interaction, raw milk consumption, and transmission of drug-resistant pathogens are prominent. To address this, monitoring antibiotic use in dairy animals and all milk processing stages is vital.

Conflict of interest

The authors of this study declare no knowledge of conflicting interests.

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None

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request. We kept the name of the raw milk selling points anonymous due to privacy or ethical restrictions.

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