Antibiogram analysis of Bovine milk at Khulna division in Bangladesh

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Abstract

As we enter the twenty-first century, antibiotic resistance is one of the most critical issues facing the world. Given this circumstance, we carried out a study to identify the isolates' patterns of antibiotic resistance and the existence of remnants of antibiotics in cow's milk in Bangladesh's southern regions especially in Khulna division. Aseptic collection and analysis of 210 cow milk samples-70 from Khulna, 70 from Satkhira, and 70 from Bagerhat-were conducted with the intent to detect antibiotic residues via a method called thin-layer chromatography. An antibiogram screening has been done on the isolates collected from these samples against eleven commonly utilized antibiotics in Bangladesh. *Staphylococcus* spp., *Escherichia coli*, and *Salmonella* spp. were the isolates identified in this study; their corresponding prevalence proportions were 61.43% (n=129), 29.05% (n=61), and 24.76% (n=52). The isolates in the current research displayed a variety of sensitivity to antibiotics. It was shown that erythromycin, ceftriaxone, penicillin, amoxicillin, and oxytetracycline were mostly ineffectual against the bacterial isolates. 7.62% of the milk samples (n=16) had antibiotic residues. The results of the investigation will undoubtedly help dairy farmers in adopting the most appropriate antibiotics with the goal of enhancing milk production and mitigating our population's daily milk intake deficit.

Keywords: Antibiotic; Antibiogram; Bovine milk; Prevalence; Antibiotic residues.

Introduction

Milk has been acknowledged as nature's finest food, and it is also among the most useful and widely consumed meals. Its excessive susceptibility to bacterial infection causes it to be highly perishable (Girma *et al*., 2014). Due to its special structure and traits, milk is a fantastic source of bacterial infection and a medium for microbial growth (Clayes *et al*., 2013, Singh *et al*., 2016). Milk might contain numerous microbes of significance to public health, including *Salmonella* spp., *Campylobacter jejuni*, *Listeria monocytogenes*, pathogenic strains of *Escherichia coli* (Chauhan *et al*., 2016), *Yersinia enterocolitica*, *Salmonella* spp., *Vibrio* spp., and enterotoxigenic strains of *Staphylococcus aureus* and *Klebsiella* spp. (Sharma and Malik, 2012). According to Rehman *et al*. (2014), milking operators and utensils could infect milk with different microbes throughout the lactating procedure. The teat canal is an additional pathway for pathogens to penetrate the udder, and these pathogens may migrate through milk. (Smith *et al*., 2007). The two major contaminants of milk are *Staphylococcus aureus* and *Escherichia coli*. According to Aycicek et al. (2005), the microbes in milk are primarily triggered by fecal contamination, although feed contamination is the main contributor of pathogen in feces. The occurrence of remnants of antibiotics in milk raises substantial concerns among dairy farmers, milk processing units, regulatory organizations, as well as consumers. Numerous antimicrobial medicines have recently been overused in poultry and farm animals for treatment, in addition to regulate the health of herds and flocks. Incorrect dosages, ignoring the withdrawal period, and reckless use of antibiotics is the main culprit for the rate of antimicrobial resistance, which has a detrimental effect on public health by lowering susceptibility to commonly used antibiotics through a variety of mechanisms, which includes mutations, conjugations, transformations, and more. (Zishiri *et al*., 2016). According to WHO (World Health Organization), intensive farming system has been developed in Southeast Asia where most of the farmers have limited technical knowledge, leading to the rising consumption of fertilizers, antibiotics, and pesticides. Antibiotic resistance becomes more prevalent by a range of variables, like inadequate level of biosecurity, hygiene, and sanitation, and a lack of awareness regarding regulations, appropriate policies, and the application of guidelines regarding antibiotic use in the livestock sector (Goutard *et al*., 2017). A lot of studies have been performed on antibiotic resistance pattern and determination of antimicrobial residue in the raw milk of commercial livestock at the several regions of Bangladesh. But there is no comprehensive study carried out in the southern regions particularly in Khulna division about these factors. Antibiotic traces in milk have not yet been monitored in this region. Consequently, no information is available on the presence of residues of antibiotics in milk that are produced and sold in this area. Considering the above facts, the present study was designed to assess the presence of antimicrobial drug residues in raw milk marketed at selected southern regions of Bangladesh and to build up awareness among livestock farmers, consumers, drug traders and practitioners about enormous use of antimicrobials in those areas.

Materials and methods

Sample Collection: From June 2021 to June 2022, this study was conducted in the southern districts of Bangladesh, including Khulna, Satkhira and Bagerhat. A total of 210 milk samples were aseptically collected from semi-intensive reared cattle of rural areas.

Transportation of Samples: After collection in sterile containers, the milk samples were transported to the laboratories maintaining the standard procedures described by (Jayarao *et al*., *2004*). One aliquot of each sample was shipped to the Bangladesh Livestock Research Institute (BLRI), Savar for isolation and characterization of the targeted bacteria like *Staphylococcus* sp., *Salmonella* sp., *Eschericia coli*. and another aliquot to the Department of Microbiology and Veterinary Public Health, Chittagong Veterinary and Animal Sciences University, Chittagong for thin layer chromatography (TLC).

Isolation and Identification of Bacteria

Cultural characterization: Cultural characteristics were isolated and then examined by following the methods of Poppelka *et al*., 2005.

Gram staining test: For identification of isolated bacteria, Gram's staining was performed according to the method described by Merchant and Packer(1967)

Biochemical characterization: Multiple biochemical examinations have been performed adopting the techniques indicated by Cheesbrough (1981), including the sugar fermentation test, MR-VP test, indole test, and catalase test. Pure colonies of the retrieved bacteria had been first enhanced into nutrient broth by incubating them for 24 hours at 37ºC to perform biochemical characterization.

Antibiogram Study: As per Bauer *et al*. (1966) the antibiogram study had been conducted. Table 1 provides those antibacterial agents' names, dosages per disc, and the measurement width of the zone of inhibition used for interpreting the outcomes.

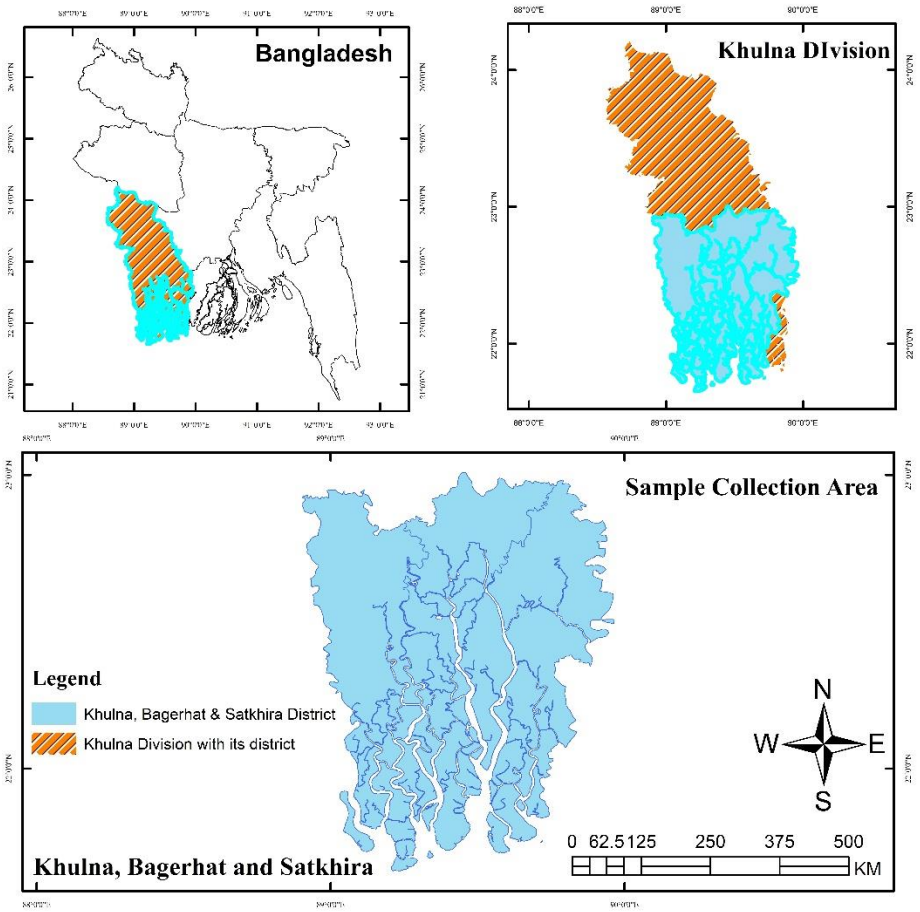

Figure 1: Sample Collection Area

Table 1: Antibacterials used to determine the pattern of antibiotic sensitivity.

Antibacterial agents	Concentration (µg/disc)	Interpretation of results (Zone diameter in mm)		
		Resistant	Intermediate	Sensitive
Amoxicillin	10 µg	≤ 11	14	≥ 15
Ceftriaxone	30 µg	≤ 12	13-15	≥16
Ciprofloxacin	5 µg	≤15	16-20	≥21
Gentamicin	10 µg	≤12	13-14	≥15
Oxytetracycline	25 µg	≤15	16-25	≥26
Penicillin	10 µg	≤11	14	≥15
Erythromycin	15 µg	≤13	14-15	≥16
Sulphonamide-trimethoprim	25 µg	≤14	15-16	≥17
Streptomycin	10 µg	≤11	12-14	≥15
Ampicillin	10 µg	≤11	14	≥15
Tetracycline	30 µg	≤11	12-14	≥15

*µg = microgram, mm=millimeter, R=resistant, I=intermediately sensitive, S=sensitive

For antibiotic sensitivity test, at first pure colonies of the isolated bacteria were enriched into nutrient broth by incubation at 37°C for 24 hours. Bacterial suspension was mixed with 0.1% peptone water, after overnight incubation. The tube containing bacterial suspension was observed on a sheet of white paper with black lines created on it to assess the bacterial suspension to 0.5 MacFarland's standards. The discs were positioned on the agar plate after the culture of bacterium had been distributed out on the Mueller-Hinton agar using a cotton bud.

Detection of Antibiotic Residues in Milk: Thin Layer Chromatography (TLC) test was performed to assess drug residues. This method was developed by Bele & Khale 2011. The procedures were as follows: At first 1 ml of milk was taken in a centrifuge tube. Then 1 ml of acetonitric- methanol-

deionized water was added at a ratio of 40:20:20. Proper mixing was done by shaking. After mixing, centrifugation of this mixture was done at 3000 rpm for about 10 minutes. Then supernatant was collected by eppendorf tube for TLC. Supernatant was incubated in TLC plate and was kept in 1:1 solution of methanol and acetone. To reach up to the mark, at least 20-25 minutes waiting was confirmed. Then dry the TLC plate at least for 2-5 minutes. Finally, it was observed under UV light to ensure the presence or absence of antibiotics in milk samples.

Data Analysis: Descriptive analysis was performed. To investigate resistance to antibiotics and the existence of remnants of antibiotics in cow's milk, data was collected and evaluated.

Results

Identification of Bacterial Agents

Out of 210 milk samples, 129 (61.43%) evaluated positive for *Staphylococcus* spp., 61 (29.05%) verified positive for *E. coli*, and 52 (24.76%) investigated positive for *Salmonella* spp. (Figure 2).

Cultural characterization

Staphylococcus spp. created smooth, convex, opaque, and golden, yellow-colored colonies greater than 1 mm on nutrient agar (NA), whereas *Salmonella* spp. generated circular, smooth, opaque, clear colonies, and *E. coli* formed smooth, circular, and colorless colonies. On EMB agar, *E. Coli* developed greenish-black colonies that exhibited a metallic shine. On blood agar, *Salmonella* spp. formed white, round, raised colonies without hemolysis, *Staphylococcus* spp. developed round, raised, opaque colonies with beta (β) hemolysis ranging from 1-2 mm in diameter, and *E. coli* produced colorless colonies without hemolysis. Colonies with blackish centers were formed on XLD agar by Salmonella species. On MS agar, Staphylococcus species produced yellow colonies with yellow zones.

Gram staining test

In Gram staining, *Salmonella* spp. was demonstrated as Gram negative, pink-colored, short, plump rod-shaped organisms which were organized in single or pairs forms, whereas *E. coli* has been demonstrated as Gram negative, pink-colored, and short, plump rod-shaped. Based on Gram staining, *Staphylococcus* spp. appeared spherical, violet-colored, and Gram-positive. They were grouped together resembling bunches of grapes.

Biochemical characterization

E. coli emitted gas and acid as it fermented five basic sugars, comprising mannitol, lactose, sucrose, maltose, and dextrose. Whereas *Salmonella* spp. showed fermentation with dextrose, maltose and mannitol and produced acid and gas. *Staphylococcus* spp. was revealed as fermenter of all the sugars only with the production of acid. *E. coli* was revealed as indole positive with red color in the reagent layer whereas *Salmonella* spp. and *Staphylococcus* spp. were detected as indole negative. All the isolates were detected as MR test positive with bright red color, whereas only *Staphylococcus* spp. was positive for VP test with pink color. In catalase test all the isolates were positive with the formation of bubble within few seconds.

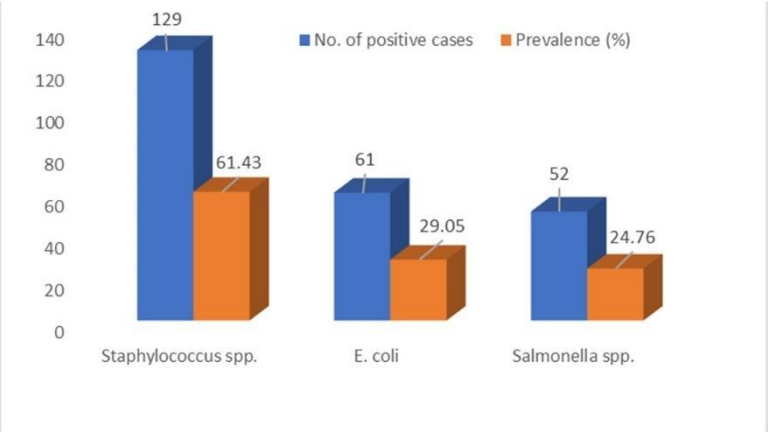

Figure 2: Prevalence of bacterial agents from milk samples

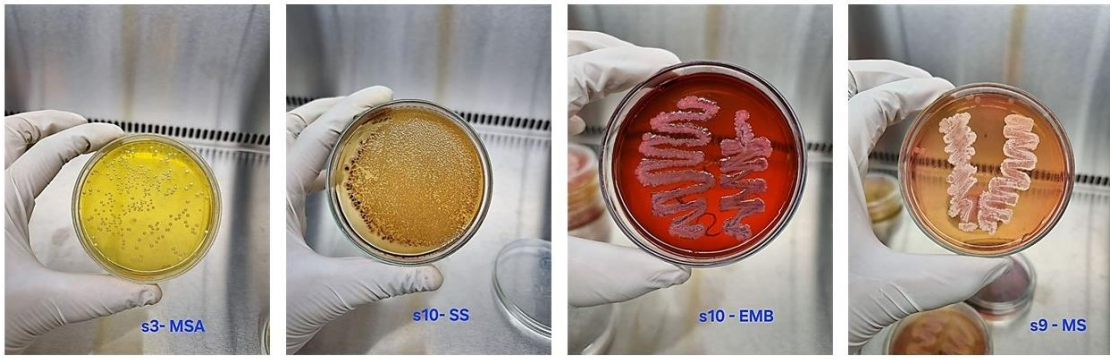

Figure 3: Different isolation growth on different agar media

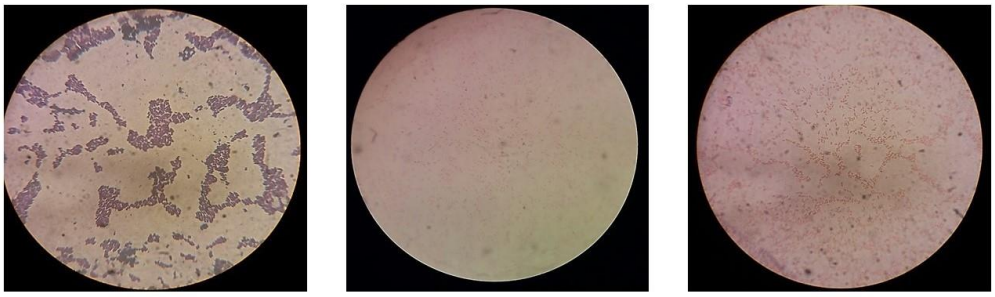

Figure 4: Different isolates after gram staining procedure

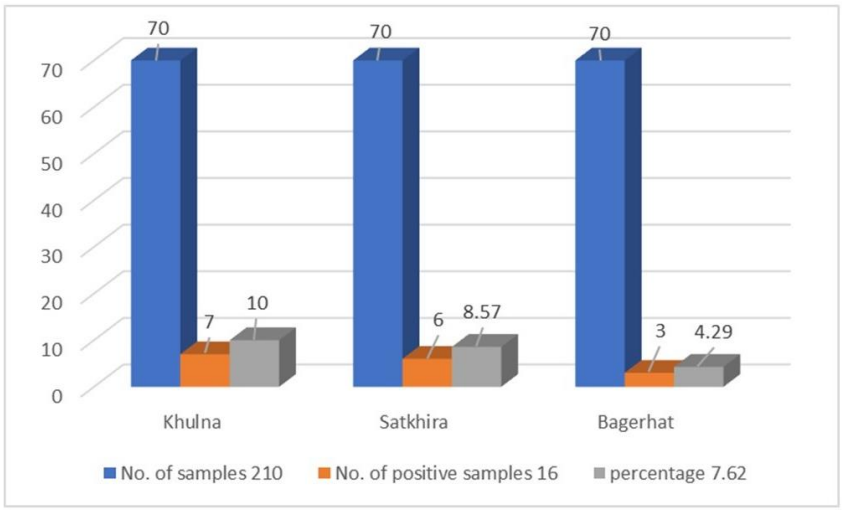

Figure 5: Antibiotic residues in milk samples

Antibiotic Resistance Pattern of the Bacterial Agents

The antibiogram study revealed that the isolated *Staphylococcus* spp. was highly resistant to ceftriaxone followed by amoxicillin, penicillin, ampicillin, erythromycin. It was observed that *Staphylococcus* spp. was highly sensitive to gentamicin, ciprofloxacin, streptomycin. Isolated *E. coli* showed varying degrees of sensitivity to antibiotics used in this study with highest sensitivity to ciprofloxacin followed by gentamicin, tobramycin, erythromycin but resistant to penicillin, tetracycline, amoxicillin and erythromycin. Isolated *Salmonella* spp. showed high resistance to

Table 2: Antibiotic resistance patterns of isolated bacteria

Antibiotics		***Staphylococcus* sp.** (n=93)	***E. coli*** (n=107)	***Salmonella* spp.** (n=83)
Sterptomycin	S	109 (84.50)	30 (49.67)	33 (63.46)
	I	4 (3.10)	9 (14.75)	7 (13.46)
	R	16 (12.40)	22 (36.06)	12 (23.07)
Erythromycin	S	33 (25.59)	6 (9.84)	27 (51.92)
	I	5 (3.88)	3 (4.92)	1 (1.92)
	R	91 (70.54)	52 (85.23)	24 (46.15)
Amoxicillin	S	0 (0.00)	10 (16.39)	0 (0.00)
	I	0 (0.00)	0 (0.00)	2 (3.85)
	R	129 (100.00)	51 (83.61)	50 (96.15)
Oxytetracycline	S	26 (20.16)	2 (3.28)	0 (0.00)
	I	9 (6.97)	0 (0.00)	0 (0.00)
	R	94 (72.87)	59 (96.72)	52 (100.00)
Ciprofloxacin	S	104(80.62)	41(67.21)	40 (76.92)
	I	7 (5.43)	4 (6.55)	4 (7.69)
	R	18 (13.95)	16 (26.22)	8 (15.38)
Penicillin	S	0 (0.00)	0 (0.00)	0 (0.00)
	I	0 (0.00)	0 (0.00)	0 (0.00)
	R	129 (100.00)	61 (100.00)	52 (100.00)
Ampicillin	S	10 (7.75)	19 (31.14)	6 (11.54)
	I	0 (0.00)	13 (21.31)	2 (3.85)
	R	119 (92.24)	29 (47.54)	44 (84.61)
Tetracycline	S	26 (20.16)	7 (11.48)	5 (9.61)
	I	5 (3.88)	1 (1.64)	0 (0.00)
	R	98 (75.97)	53 (86.88)	47 (90.38)
Gentamicin	S	129 (100.00)	33 (54.09)	49 (94.23)
	I	0 (0.00)	0 (0.00)	0 (0.00)
	R	0 (0.00)	22 (45.90)	3 (5.77)
Ceftriaxone	S	0 (0.00)	0 (0.00)	10 (19.23)
	I	0 (0.00)	4 (6.55)	5 (9.61)
	R	129 (100.00)	57 (93.44)	37 (71.15)
Sulphonamide-trimethoprim	S	67 (51.94)	11 (18.03)	28 (53.85)
	I	19 (14.73)	21(34.43)	7 (13.46)
	R	43 (33.33)	29 (47.54)	17 (32.69)

** S = Sensitive, I = Intermediate, R = Resistant

penicillin and oxytetracycline (**Table 2**). In this study, isolated *Salmonella* spp. was highly resistant to ceftriaxone followed by penicillin, amoxicillin, oxytetracycline and sensitive to gentamicin, ciprofloxacin, sulfamethoxazole, trimethoprim.

Antibiotic Residues in Milk

In accordance with the findings of this research, the overall incidence of antibiotic residues in milk in the Khulna division was 7.62% (n=16/210) **(Figure 5)**. Antibiotic residue frequency, in Khulna district was 10% (n=7/70); in Satkhira district was 8.57% (n=6/70) and in the Bagerhat district was 4.29% (n=3/70).

Discussion

Identification of Bacterial Agents: The results (Figure 2) found on this research have attracted a lot of attention and have been confirmed by several groups of scientists (Rahman *et al*., 2022;; Kou *et al*., 2021;).

Cultural characterization: In nutrient agar the results obtained for the *Staphylococcus* spp. were consistent with Murray *et al*. (2015); the findings for *E. coli* corresponded with the results reported by Madigan *et al*. (2018), whereas the outcomes for *Salmonella* spp. had been confirmed by Andrews *et al.* (2011). On the EMB agar the characterization of *E. coli* and findings of this research was similar to the findings of Madigan *et al*. (2018). The findings of *Salmonella* spp., *Staphylococcus* spp. and *E. coli* on blood agar were in the same line of findings with Murray *et al*. (2015) andWinn *et al*. (2006) respectively. The findings of Salmonella spp. were similar to the findings of Andrews *et al*. (2011). On MS agar the findings of *Staphylococcus* spp. were identical as the Forbes *et al*. (2007).

Gram staining test: The present research revealed that *Salmonella* spp. appeared pink-colored, short, compact rod-shaped, gram-negative bacteria that were grouped in single or pair formations employing Gram staining method. Muktaruzzaman *et al*. (2010) endorsed this conclusion. However, it has been

shown that *E. coli* is pink, short, plump, and rod-shaped; Prayekti & Sumarsono (2021) supported this assertion. *Staphylococcus* spp. were spherical, violet-colored, Gram-positive, and clustered like bunches of grapes, as confirmed by Fernandes Queiroga Moraes *et al*. (2021) and Tripathi & Sapra (2023).
Biochemical characterization: This study validates the results of Oktora *et al*. (2023), and Chitra *et al.* (2014) that E. coli ferments an array of sugars, producing gas and acid. The fermentation of dextrose, maltose, and mannitol by *Salmonella* spp. has been demonstrated by Karim *et al*. (2017) also developed gas and acid. Sugars were fermented by *Staphylococcus* spp., which simply generated acid (Chitra *et al*., 2014). *Salmonella* spp. and *Staphylococcus* spp. tested negative for indole synthesis, however *E. coli* tested positive (Karim *et al*., 2017; Chitra *et al*., 2014). The methyl red (MR) test yielded positive results for every isolate (Karim *et al*., 2017; Chitra *et al*., 2014). The Voges-Proskauer (VP) test indicated positive findings for *Staphylococcus* spp. (Chitra *et al*., 2014).
Antibiotic Resistance Pattern of the Bacterial Agents: The outcomes of the antibiogram investigation (Table 2) for *Staphylococcus* spp. have been supported by Sharma and Brinty (2014), and Salauddin *et al*. (2020). Results obtained for *E. coli* corresponded with those of Rehman *et al.* (2014). Conclusions of this investigation showed similarity to the findings of Hassani *et al*. (2022) and Rahman (2022) for *Salmonella* spp.
Antibiotic Residues in Milk: A previous study (Rahman *et al*., 2021) observed 7% overall prevalence of antibiotic residues in milk and another study (Brown *et al*., 2020) also found 10.50% overall prevalence of antibiotic residues in milk which supported our current findings. The outcomes of a previous study (Chowdhury *et al.*, 2015) line up with the 10% prevalence of antibiotic residues in Khulna district (n=7/70). The prevalence of antibiotic residues in Satkhira district was 8.57% (n=6/70), which was consistent with the conclusions of a different research (Islam *et al*., 2021). Antibiotic residue frequency in Bagerhat district was 4.29% (n=3/70), which was compatible with the results from a separate investigation (Kaya *et al*., 2010).

Conclusion

The isolates in the current study were significantly prone to ciprofloxacin and gentamicin and incredibly resistant to penicillin and amoxicillin. 7.62% of the milk samples exhibited antibiotic residues overall. The higher levels of antibiotic remnants found in the milk samples of Khulna district compared to other districts in Khulna division which indicates that antibiotics are used more often in milk yielding cattle of Khulna district. The results of this research will be helpful in enhancing the public's consciousness regarding not to use antibiotics for healthy milk production unless it is necessary. This research will also aid the researchers, veterinarians, Government of Bangladesh, different NGO's who are constantly working with the antimicrobial resistance.

Acknowledgment

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Conflict of interest

None of the authors have a conflict of interest to declare.

Informed consent

All necessary ethical protocols and informed consent procedures were diligently followed throughout the course of this study.

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