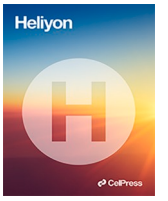
Heliyon 10 (2024) e34567

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Prevalence, antimicrobial susceptibility profiles and resistant gene identification of bovine subclinical mastitis pathogens in Bangladesh

Ahsan Al Emon [a,b], Hemayet Hossain [c], Md Shahidur Rahman Chowdhury [a], Md Anisur Rahman [a], Fatema Yeasmin Tanni [a], Mashuka Nahida Asha [a], Hafsa Akter [d], Md Mukter Hossain [a], Md Rafiqul Islam [a], Md Mahfujur Rahman [a,*]

[a] Department of Medicine, Faculty of Veterinary, Animal and Biomedical Sciences, Sylhet Agricultural University, Sylhet, Bangladesh
[b] Faculty of Agricultural and Nutritional Sciences, Christian Albrechts University of Kiel, Kiel, Germany
[c] Department of Anatomy and Histology, Faculty of Veterinary, Animal and Biomedical Sciences, Sylhet Agricultural University, Sylhet, Bangladesh
[d] Department of Biochemistry and Chemistry, Faculty of Biotechnology and Genetic Engineering, Sylhet Agricultural University, Sylhet, Bangladesh

ARTICLE INFO

Keywords:
SCM
Prevalence
Antibiotic resistance
S. aureus
E. coli
K. pneumoniae

ABSTRACT

Subclinical mastitis (SCM), a silent threat in the dairy sector of Bangladesh poses a significant economic impact and serves as a potential source of infection for healthy cows, hindering efforts to achieve milk self-sufficiency. Despite the importance of this issue, limited research has been conducted on mastitis in Sylhet region of Bangladesh. This study aimed to investigate the molecular prevalence, antimicrobial susceptibility profile and resistant genes detection on pathogens (*Staphylococcus aureus, Escherichia coli,* and *Klebsiella pneumoniae)* causing SCM. In a cross-sectional study utilizing convenience sampling, 325 milk samples were collected from apparently healthy dairy cows. Initially, SCM was detected using the modified Whiteside test (MWST) method. Suspected positive samples were then subjected to bacteriological culture and standard biochemical assays, followed by molecular identification through polymerase chain reaction (PCR). Finally, antimicrobial susceptibility testing was conducted on all PCR-positive samples using the Kirby-Bauer disk diffusion method on Mueller-Hinton agar. In the Sylhet district, the prevalence of SCM was 64.92 % (211 out of 325) at the individual animal level and 82.69 % (43 out of 52) at the herd level. Among the SCM-positive animals, *S. aureus* was found in 31.75 % (67 out of 211) of cases, *E. coli* in 81.99 % (173 out of 211), and *K. pneumoniae* in 66.82 % (141 out of 211). *K. pneumoniae* had the highest prevalence at 60 % in Zakiganj, *S. aureus* at 45 % in Zakiganj, and *E. coli* at 72 % in Bishwanath Upazila. Extended spectrum beta lactamase (ESBL) genes bla_{TEM}, bla_{OXA}, $bla_{CTX\text{-}M1}$, $bla_{CTX\text{-}M2}$, $MultiCase_{DHA}$ were identified. Additionally, antibiotic resistance genes *tet(A), AAC(3)-iv,* and *Sul1* were also detected. The pathogens exhibited resistance to Penicillins (ampicillin, amoxicillin), Cephems (cefuroxime, ceftazidime), and Tetracyclines (tetracycline). However, all three bacteria were highly sensitive to meropenem, amikacin, gentamicin, ciprofloxacin, and sulfamethoxazole-trimethoprim. These findings highlight the

* Corresponding author.
E-mail addresses: aaemon.bd@gmail.com (A.A. Emon), hemayet.vabs@student.sau.ac.bd (H. Hossain), shahidur.vetmed@sau.ac.bd (M.S.R. Chowdhury), anisrahman.sau2015@gmail.com (M.A. Rahman), fytannisau@gmail.com (F.Y. Tanni), nahidamashuka@gmail.com (M.N. Asha), hakter.sau@gmail.com (H. Akter), mukter.vetmed@sau.ac.bd (M.M. Hossain), rafiq.vetmed@sau.ac.bd (M.R. Islam), mahfuj.vetmed@sau.ac.bd (M.M. Rahman).

https://doi.org/10.1016/j.heliyon.2024.e34567
Received 4 March 2024; Received in revised form 11 July 2024; Accepted 11 July 2024
Available online 14 July 2024

critical need for targeted antimicrobial stewardship and effective control measures to mitigate the impact of SCM on dairy production and animal health in the Sylhet region of Bangladesh.

1. Introduction

There are around 6 million dairy cattle in Bangladesh, the majority of which are crossbred high-yielding cows, producing approximately 9.4 million metric tons of milk each year [1]. About 70 % of Bangladeshi farmers are smallholders with one to three cows per farm, generating 70–80 % of the country's milk requirements [2]. Every 305-day lactation period, domestic dairy cows produce approximately 200–250 L of milk [3]. Production diseases, such as mastitis, are the greatest hindrance to a dairy farm's ability to achieve maximum profit [4].

Mastitis is an inflammation of the mammary gland, which is considered the most prevalent and costly disease in the dairy industry [5]. It is characterized by changes in the physical and chemical characteristics, including the pathological or microbiological changes in the mammary gland or milk [6,7]. The economic impacts of mastitis include treatment-related expenses, decreased milk output, alterations in product quality, and culling [8]. Therefore, mastitis is quite costly to the dairy sector. The estimated annual loss due to mastitis in Bangladesh is Tk 122.6 million (US $2.11 million) [9]. Mastitis infections are also considered either clinical or subclinical [10].

Clinical mastitis has a detrimental influence on the economy of dairy farms because of abnormal milk, deterioration in milk quality [10], lower production (up to 70 %), milk discharge after treatment (9 %), treatment expenses (7 %), labor, premature culling (14 %), and death [9,11]. In contrast, subclinical mastitis (SCM) is defined by the absence of clinical symptoms as opposed to an increase in the somatic cell count of the milk [12]. Evidence demonstrates that the incidence of subclinical mastitis is 15–40 times higher than that of clinical mastitis [13].

Numerous previous studies have documented the state of SCM in Bangladesh and surrounding countries, including India, Sri Lanka, and Pakistan [9,14–16]. In Bangladesh, the majority of dairy cows are crossbred, and crossbred cows have a higher prevalence of SCM. The prevalence of SCM in crossbred dairy cows in Bangladesh has been reported as ranging between 28.5 % and 61.3 % [1].

Subclinical mastitis (SCM) is caused by several bacteria [10]. Inflammation in mammary gland tissues can be induced by both gram-positive and gram-negative bacteria in equal measure [17]. However, species of bacteria, such as *Staphylococcus* spp.*, Klebsiella* spp.*,* and *Escherichia coli*, play important roles in the development of these diseases [18–21].

Staphylococcus aureus (*S. aureus*)*, Escherichia coli* (*E. coli*)*,* and *Klebsiella pneumoniae* (*K. pneumoniae*) are known to produce several virulence factors that contribute to the pathogenesis of mastitis. *S. aureus* can produce a range of toxins and enzymes such as coagulase, hemolysins, and proteases [22], which help it evade the host immune response and damage host tissues [23]. *E. coli*, on the other hand, utilizes adhesins, endotoxins, and siderophores to establish infection and acquire essential nutrients in the host environment. *K. pneumoniae* is known for its thick polysaccharide capsule, which enhances its ability to resist phagocytosis and antimicrobial agents, making it a formidable pathogen in mastitis [24]. Understanding these virulence mechanisms is crucial for developing effective strategies to control and prevent mastitis in dairy herds. The presence of these ESBL and other antibiotic resistance genes is crucial, as they significantly complicate the treatment of subclinical mastitis by conferring resistance to a wide range of commonly used antibiotics. Furthermore, virulence factors associated with these pathogens enhance their ability to colonize and invade host tissues, exacerbating the severity of the disease and posing a considerable challenge for effective management and control in the dairy sector.

Staphylococcus spp. (coagulase-negative) are the most prevalent infectious bacteria that cause mastitis [25,26]. *S. aureus* infection of the mammary gland remains a significant concern in the global dairy sector [27]. *K. pneumoniae* has been shown to be one of the environmental causes [24] that lead to mastitis and there have been a significant number of studies conducted on the topic [28-31]. *Klebsiella* mastitis causes significantly greater harm to milk production and survival rates than *E. coli* mastitis [32,33]. In some cases, *E. coli* is the most influential bacterium responsible for clinical mastitis [29]. Mastitis is a highly costly disease that adversely affects milk productivity and quality [34]. It leads to a 30 % reduction in milk yield per affected quarter and an overall 15 % decrease per cow per lactation. The economic impact of mastitis includes decreased milk production, increased culling of cows, higher medication expenses, and costs related to cow death and replacement [35]. Coliform mastitis causes a greater incidence (30–40 %) of cow death and/or agalactia-related culling in dairy cows [36], as well as subclinical infections that persist for extended periods of time [37].

These pathogens are also involved in the transmission of significant zoonotic diseases to humans, including as tuberculosis, brucellosis, leptospirosis, and streptococcal sore throat [38,39]. Therefore, antibiotic therapy is a crucial method of infection control for both bovine mastitis and human illnesses [40]. According to the findings of earlier studies, the bacteria *S. aureus, E. coli,* and *K. pneumoniae* develop resistance to several classes of antimicrobial drugs because of the selective pressures exerted by antimicrobials [40]. Multidrug resistance has been increased all over the world that is considered a public health threat [41,42]. Several recent investigations reported the emergence of multidrug-resistant bacterial pathogens from different origins that increase the necessity of the proper use of antibiotics [43]. Besides, the routine application of the antimicrobial susceptibility testing to detect the antibiotic of choice as well as the screening of the emerging MDR strains [44–46]. The majority of pathogens causing subclinical mastitis exhibit resistance to multiple drugs [43] making the selection of appropriate antimicrobials for clinical therapeutic purposes a crucial and challenging task for veterinarians and farmers [17]. Therefore, the identification of suitable (sensitive) drugs for the treatment of subclinical mastitis is essential and holds significant benefits for both farmers and the affected animals. Regrettably, the essential approach is not adequately explored in the Sylhet region of Bangladesh. Consequently, farmers are consistently facing the silent threat of subclinical mastitis (SCM), given that cows affected by SCM may appear healthy. In addition, *E. coli* and *Klebsiella* are categorized as

"critical mastitis pathogens" [24] by the World Health Organization (WHO) in an order to tackle antibiotic resistance [47].

Subclinical mastitis not only caused massive economic harm to the dairy industry, but it also serves as a source of infection for healthy milk cows, which is one of the most significant impediments to Bangladesh reaching milk self-sufficiency. Since relatively little study has been conducted on mastitis in north western part of Bangladesh [48,49]. *S. aureus, K. pneumoniae,* and *E. coli* from bovine SCM in the Sylhet region have not yet been characterized at the molecular level. This study aimed to investigate the molecular prevalence and antimicrobial susceptibility profile and resistant genes detection of pathogens (*S. aureus, E. coli,* and *K. pneumoniae*) causing SCM.

2. Materials and methods

2.1. Study design, location and sampling strategy

A cross-sectional investigation was conducted in 12 Upazilas (sub-districts) situated in the Sylhet district of Bangladesh, encompassing Balaganj, Beanibazar, Biswanath, DakkhinSurma, Fenchuganj, Golapganj, Gowainghat, Jaintapur, Kanaighat, Osmaninagar, Sylhet Sadar, and Zakiganj. The geographical coordinates of these Upazilas ranged between approximately 24°36′ to 25°11′ North latitude and 91°38′ to 92°30′ East longitude, as depicted in Fig. 1. The minimum number of sample size was estimated according to the prevalence of SCM investigated by Kayesh et al. (2014) [50] in Bangladesh who found the prevalence 28.50 %.

$$\mathbf{n = \{Z^2 \times p \times (1\text{-}p)\} / d^2;}$$

Where, n = Desired sample size; Z = 1.96 for 95 % confidence interval; p = 0.285, Expected prevalence (28.5 %); d = 0.05, Desired absolute precision (5 %)

This thoughtful choice of prevalence percentage was intended to optimize the sample size. Following the calculation, a total of 313 milk samples were determined as necessary for estimating SCM. The study was then conducted using 325 milk samples (Pooled sample) collected from 325 cows across 52 herds, through convenience sampling strategy from November 2021 to May 2022.

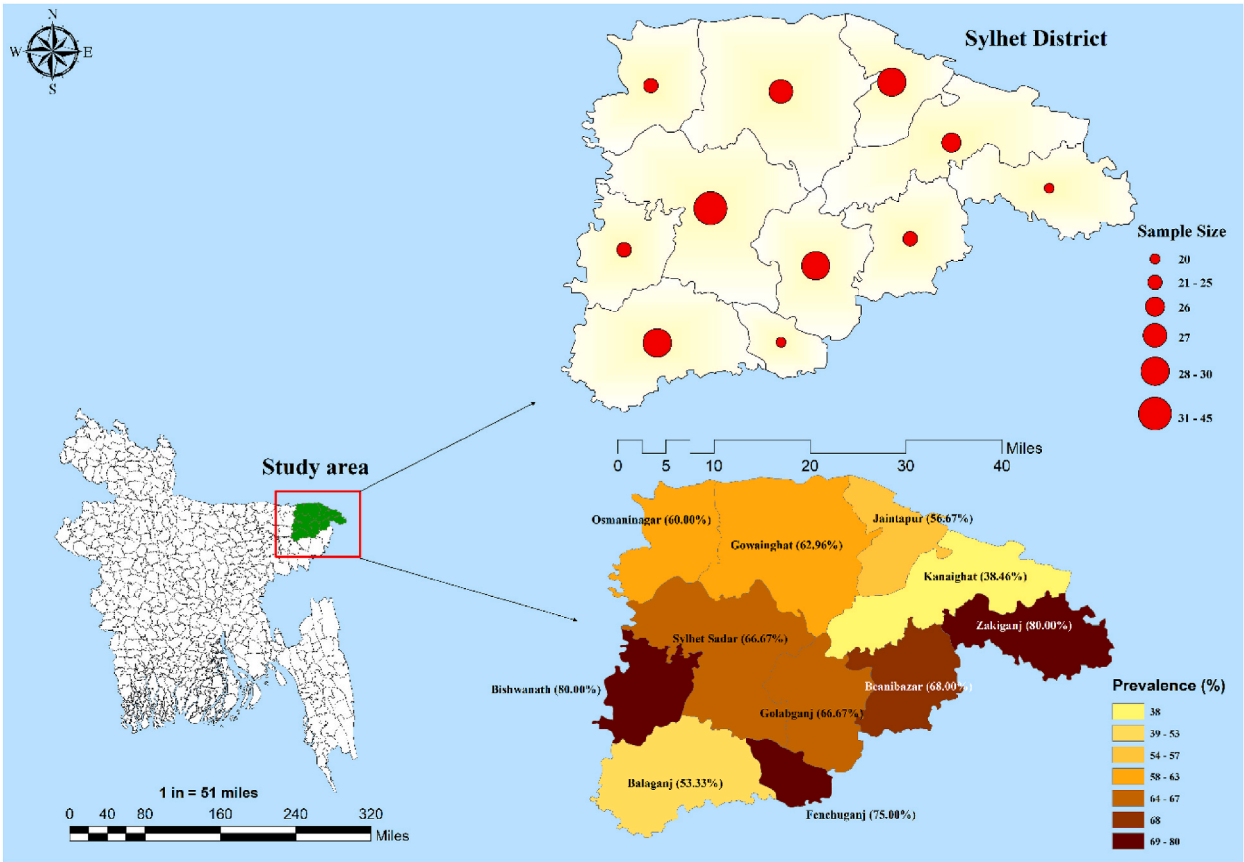


Fig. 1. Geographical mapping of study area showing sample size and prevalence of subclinical mastitis (SCM) in Sylhet district of Bangladesh. The map was created using ArcMap 10.7.

2.1.1. Collection of data on farm management

The data were collected through the direct methods with the face-to-face interview. A sequential series of pretested validated questionnaires were used for the systematic collection of data on various aspects viz., Husbandry practices, and individual animal level attributes. Our methods included the use of a pre-tested questionnaire including open and closed answers, administered by trained veterinarians (natives) in the native language. The different level predictor variables were collected in this study such as: Animal demographic variable (age, breed, lactation stage, parity, body condition score, milk yield); farm ownership information (age of the owner, sex, education level, establishment of farm); management type information (type of floor, rearing system, direct sunlight, shed cleaning, overall farm hygiene, grazing type, milking technique, bedding materials, stall partition, water supply).

2.2. Platform test of SCM

The modified Whiteside test (MWST), outlined by Dunn and Murphy [51], and served as the platform test to detect SCM [52] (Fig. 2A.). All the positive SCM milk samples underwent a pre-enrichment step by being cultured in Trypticase Soya Broth (HiMedia Laboratories Pvt. Ltd., Mumbai, India) at a 1:10 ratio. The cultures were then incubated at 37 °C for a duration of 24 ± 2 h. The positive response of the modified Whiteside test (MWST) for diagnosing subclinical mastitis is detailed in Table 2 and depicted in Fig. 1, featuring four grading reactions. Negative (−) cases were identified through the observation of an opaque and milky mixture. For traces of subclinical mastitis (SCM), the reaction displayed an opaque and milky appearance with fine coagulated particles. Grade (1+) SCM was identified by examining a less opaque but still fairly milky background, with larger coagulated particles present and densely dispersed. A Grade (2+) reaction exhibited a background with a slightly watery appearance and significant clumps of coagulated material. Finally, a Grade (3+) reaction produced a distinct watery background and a whey-like consistency with large masses of coagulated material forming strings and shreds.

2.3. Isolation and identification of bacteria by culture and biochemical methods

After the pre-enrichment process, each milk sample underwent the isolation of *S. aureus, E. coli,* and *K. pneumoniae* using established culture and biochemical methods as per previously outlined protocols [60–62]. *E. coli* was isolated using McConn's traditional culture methods [63]. The bacteria were then cultured on selective media, including Eosin Methylene Blue (EMB) and MacConkey agar (both from Oxoid, UK), where positive isolates exhibited a characteristic green metallic sheen. The purity of *E. coli* cultures was confirmed through Gram staining and various biochemical tests, such as sugar fermentation, MRVP, citrate utilization, motility, indole, and urease tests. For isolation of *K. pneumoniae*, the samples were cultured on EMB agar at 37 °C for 24 h. Presumptive colonies (circular, dome-shaped, mucoid pink) were subcultured on MacConkey (Oxoid, UK) and nutrient agar (HiMedia Laboratories Pvt. Ltd.,

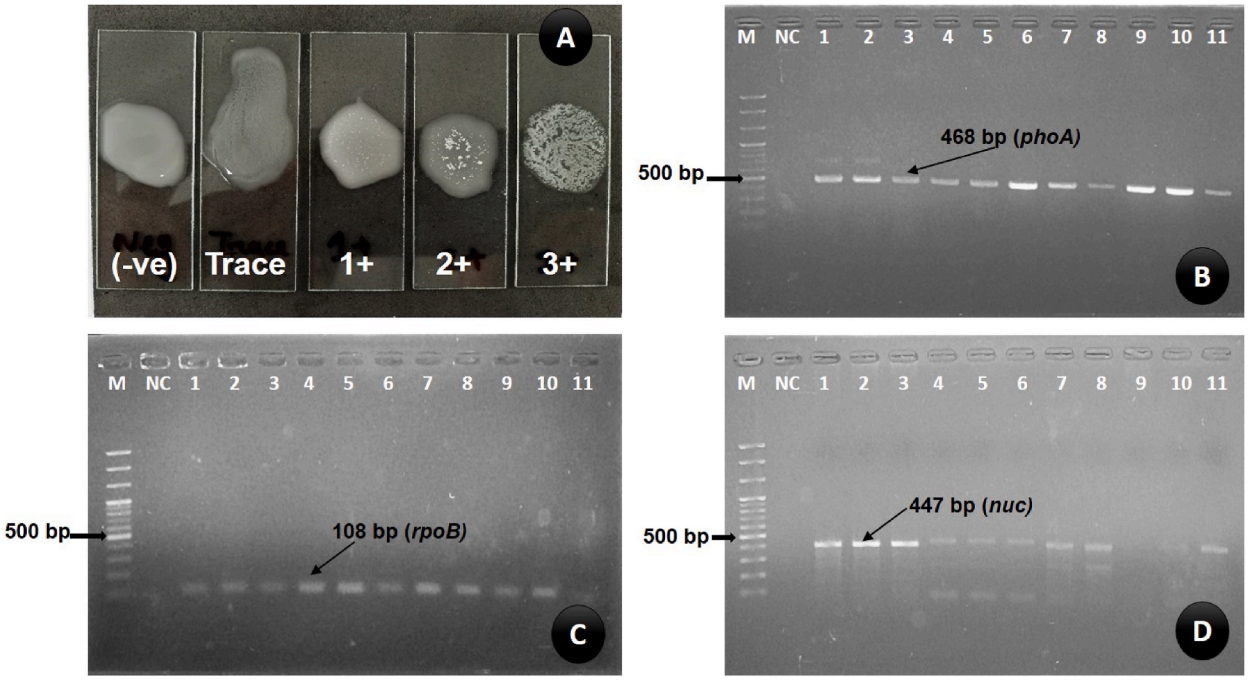


Fig. 2. Subclinical mastitis (SCM) detection according to MWST grading reaction (Fig. 2A); Amplified DNA of *E. coli* (Fig. 2B), *K. pneumoniae* (Fig. 2C), *S. aureus* (Fig. 2B) and their positive DNA bands were shown at 468 bp, 108 bp and 447 bp respectively. NC, M represent negative control and ladder DNA markers respectively.

Table 1
The primer sequences, amplicon sizes and target genes for Identification of specific organism and resistance genes by PCR.

Type of PCR	Primers (Gene)	Targeted Genes/Organism	Primer sequences	Amplicon size (bp)	Reference
UniPCR	*nuc*	*S. aureus*	F-GCGATTGATGGTGATACGGTT R-AGCCAAGCCTTGACGAACTAAAGC	447	[53]
	rpoB	*K. pneumoniae*	F-CAACGGTGTGGTTACTGACG R-TCTACGAAGTGGCCGTTTTC	108	[54]
	phoA	*E. Coli*	F-GGTAACGTTTCTACCGCAGAGTTG R-CAGGGTTGGTACACTGTCATTACG	468	[55]
mPCR-I	bla_{TEM}	*TEM-1*	F-CATTTCCGTGTCGCCCTTATTC R-CGTTCATCCATAGTTGCCTGAC	800	[56]
	bla_{SHV}	*SHV-1*	F-AGCCGCTTGAGCAAATTAAAC R-ATCCCGCAGATAAATCACCAC	713	
	bla_{OXA}	*OXA-1*	F-GGCACCAGATTCAACTTTCAAG R-GACCCCAAGTTTCCTGTAAGTG	564	
	$bla_{CTX\text{-}M1}$	*CTX-M-1*	F-TTAGGAAATGTGCCGCTGTA R-CGATATCGTTGGTGGTACCAT	688	
	$bla_{CTX\text{-}M2}$	*CTX-M-2*	F-CGTTAACGGCACGATGAC R-CGATATCGTTGGTGGTACCAT	404	
	$bla_{CTX\text{-}M9}$	*CTX-M-9*	F-TCAAGCCTGCCGATCTGGT R-TGATTCTCGCCGCTGAAG	561	
	MultiCase ACC	*ACC-1 & ACC-2*	F-CACCTCCAGCGACTTGTTAC R-GTTAGCCAGCATCACGATCC	346	
	MultiCase MOX	*MOX*	F-GCAACAACGACAATCCATCCT R-GGGATAGGCGTAACTCTCCCAA	895	
	MultiCase DHA	*DHA-1 & DHA-2*	F-TGATGGCACAGCAGGATATTC R-GCTTTGACTCTTTCGGTATTCG	997	
UniPCR	*tet(A)*	Tetracycline	F-GGCGGTCTTCTTCATCATGC R-CGGCAGGCAGAGCAAGTAGA	502	[57]
	AAC (3)-iv	Gentamycin	F-AGTTGACCCAGGGCTGTCGC R-GTG TGC TGC TGG TCC ACA GC	333	[58]
	Sul1	Sulfonamide	F-CGGCGTGGGCTACCTGAACG R-GCCGATCGCGTGAAGTTCCG	433	[59]

Table 2
Prevalence of bovine subclinical mastitis (SCM) in Sylhet district with their different predictors.

Predictors	Total No. tested	No. of positive	Prevalence % (95 % CI)	p-value
Animal and Herd level				0.011
Animal	325	211	64.92 (59.46–70.11)	
Herd	52	43	82.69 (69.67–91.77)	
Location				0.056
Sylhet Sadar	45	30	66.67 (51.05–80.00)	
Beanibazar	25	17	68.00 (46.50–85.05)	
Balaganj	30	16	53.33 (34.33–71.66)	
Bishwanath	25	20	80.00 (59.30–93.17)	
South Surma	22	18	81.82 (59.72–94.81)	
Fenchuganj	20	15	75.00 (50.00–91.34)	
Golapganj	30	20	66.67 (47.19–82.71)	
Gowainghat	27	17	62.96 (42.37–80.60)	
Jaintapur	30	17	56.67 (38.93–74.40)	
Kanaighat	26	10	38.46 (20.23–59.43)	
Osmaninagar	25	15	60.00 (38.67–78.87)	
Zakiganj	20	16	80.00 (56.34–94.27)	
Etiologic agents on SCM positive samples (n = 211)				<0.001
E. coli		173	81.99 (76.13–86.93)	
K. pneumoniae		141	66.82 (60.03–73.14)	
S. aureus		67	31.75 (25.53–38.50)	
MWST Grading on SCM positive milk (n = 211)				<0.001
Trace		36	17.06 (12.25–22.83)	
1+		43	20.38 (15.16–26.45)	
2+		56	26.54 (20.71–33.04)	
3+		76	36.02 (29.54–42.89)	

MWST: Modified Whiteside Test; CI: Confidence Interval.

Mumbai, India). Biochemical characteristics of *K. pneumoniae* were assessed using MIU agar for motility, indole, and urease tests, Simmons's Citrate Agar (HiMedia Laboratories Pvt. Ltd., Mumbai, India) for citrate utilization, and MRVP agar for Methyl Red/Voges-Proskauer tests (HiMedia Laboratories Pvt. Ltd., Mumbai, India). For isolation of *S. aureus*, samples were cultured on Mannitol Salt Agar (MSA; Oxoid, UK) plates at 37 °C for 24 h. Presumptive *S. aureus* colonies, indicated by yellow zones due to

mannitol fermentation, were subcultured on Nutrient agar (Oxoid, UK). The identity of *S. aureus* was confirmed by Gram staining (Gram stain kit, Remel, Kansas, USA), revealing grape-like clusters, and through biochemical tests such as coagulase, catalase, and DNase tests using standard protocols. Following the culture and biochemical testing, the positive samples were prepared for genomic DNA extraction [62] as well as polymerase chain reaction (PCR).

2.4. Bacterial genomic DNA extraction

The genomic DNA from isolated *S. aureus, K. pneumoniae*, and *E. coli* strains was extracted utilizing a DNA extraction kit (AddBio Inc. Ltd., Daejeon, Korea) in adherence to the manufacturer's instructions. Sets of reference primers specific to *S. aureus, E. coli,* and *K. pneumoniae* target genes were employed for amplification [64–67]. Detailed primer information, including sequences, is provided in Table 1. The requisite thermal cycling conditions, encompassing temperature and duration, are elucidated in Supplementary Tables 1–5.

2.5. Gel electrophoresis

A 100 mL solution of 1.5 % agarose was prepared for *S. aureus, K. pneumoniae,* and *E. coli,* incorporating Agarose LE from ADD BIO INC., Korea, and 1X TAE buffer. The solution was cooled to a temperature range of 50–55 °C, followed by the addition of 5 μL of a safe gel stain dye. Gel electrophoresis was conducted for 30 min at 100 V, and the outcomes were observed under UV *trans*-illumination using a gel documentation system [65,66] manufactured by Bio-Rad Laboratories Inc. in California, United States.

2.6. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was conducted on *S. aureus, E. coli,* and *K. pneumoniae* isolates using the Kirby-Bauer disk diffusion method on Mueller-Hinton agar (Hi Media Laboratories Pvt. Ltd., Mumbai, India) [68], and the diameter of the inhibition zone was assessed following the guidelines set forth by the Clinical and Laboratory Standards Institute (CLSI M100 2020). Employing the disk diffusion method, we evaluated the sensitivity and multi-drug resistance profile of *S. aureus, K. pneumoniae,* and *E. coli* against a panel of seventeen (17) routinely used antimicrobial disks (Oxoid, UK) including 11 different antimicrobial classes as Penicillin: ampicillin (AMP, 10 μg), amoxicillin (AMX, 10 μg); Tetracyclines: tetracycline (TE, 30 μg); Cephems: cefuroxime (CXM, 30 μg), ceftriaxone (CTR, 30 μg), ceftazidime (CAZ, 30 μg), cefotaxime (CTX, 30 μg); Quinolones: ciprofloxacin (CIP, 5 μg), nalidixic acid (NA, 30 μg); Macrolides: azithromycin (AZM, 15 μg); Folate pathway antagonists: trimethoprim-sulfamethoxazole (COT, 1.25/23.75 μg); Aminoglycosides: gentamicin (GEN, 10 μg), amikacin (AK, 30 μg); Monobactams: aztreonam (AT, 30 μg); Penems: meropenem (MEM, 10 μg), imipenem (IMP, 10 μg); Phenicols: chloramphenicol (CL, 30 μg); Lipopeptides: colistin (CL, μg). Isolates displaying resistance to at least three different antibiotic classes were classified as multidrug-resistant (MDR) [69]. Extensively Drug-Resistant (XDR) bacteria are non-susceptible to at least one agent in all but two or fewer antimicrobial categories [42] (i.e., bacterial isolates remain susceptible to only one or two categories). Pan Drug-Resistant (PDR) bacteria are non-susceptible to all agents in all antimicrobial categories, meaning the bacterial isolates are resistant to all available antimicrobial agents [70].

2.7. Multiple antibiotic resistance (MAR) index

The MAR index was computed and assessed based on the method outlined by Refs. [71,72] employing the formula: MAR= (The number of antibiotics to which an isolate was resistant)/(The total number of antibiotics tested).

MAR index values ranged from 0 to 1, with proximity to zero signifying high sensitivity and values nearing 1 indicating extreme resistance. A MAR index equal to or greater than 0.20 was considered indicative of a high-risk source for bacterial contamination or a state of significant "resistance" [73].

2.8. Statistical analysis

All data were collated, categorized, and structured within Excel spreadsheets. The prevalence of different diseases was computed using the following formula [74]:

$$\text{Prevalence} = \frac{\overset{\text{Number of current}}{\text{cases(new and preexisting)at a specified point in time}}}{\text{Population at the same specified point in time}} \text{X100}$$

A univariate analysis was performed employing the Chi-square test to evaluate associations among various explanatory variables. In instances where the expected count in a cell was below 5 and occurred in at least 20 % of the cells, Fisher's Exact Test was employed. Confidence intervals were determined using the Binomial exact test, with a significance level of less than 0.05 chosen to establish statistical significance. The data analysis was conducted using SPSS version 26 (SPSS, Chicago, IL). Additionally, we employed OriginPro (www.originlab.com) and utilized the Venn diagram file exchange format and this allowed creating informative Venn diagram offering a comprehensive view of the data [75,76]. The heatmap was created using OriginPro 2024 with "Heat map with Dendrogram" packages [76], and the correlation plot was created using the "metan" package on R and RStudio 4.3.2 version.

3. Results

Subclinical mastitis (SCM), a silent threat in the dairy sector of Bangladesh, poses a significant economic impact and serves as a potential source of infection for healthy cows, hindering efforts to achieve milk self-sufficiency. Despite the importance of this issue, limited research has been conducted on mastitis in the Sylhet region of Bangladesh. This study aimed to investigate the molecular prevalence, antimicrobial susceptibility profile, and resistant genes detection of pathogens (*S. aureus, E. coli*, and *K. pneumoniae*) causing subclinical mastitis. The identification of these mastitis-causing organisms was performed using both uniplex and multiplex PCR. Subsequently, antimicrobial susceptibility testing (AST) was conducted on the positive isolates to detect antibiotic resistance. Finally, ESBL and other commonly used antibiotic resistance genes were detected and evaluated, highlighting the critical need for targeted antimicrobial stewardship and effective control measures in the Sylhet region of Bangladesh.

3.1. Demography of farm owner and farm management

Most farm owners (61.5 %; 32/52) were middle-aged, between 30 and 50 years old. The majority of farm owners in the region were male (82.7 %; 43/52), with a smaller proportion being female (17.3 %; 9/52). Educational levels varied, with larger farm owners generally having higher education, while smaller farmers typically had education ranging from primary to higher secondary levels.

Most farms predominantly practiced a face-in rearing system (80.7 %) for the cows, with the majority featuring concrete flooring (82.7 %). A significant portion of the farms had facilities that allowed direct exposure to sunlight (65.4 %). Farm hygiene practices varied, with most farms (53.8 %) performing cleaning twice daily. Free grazing facilities were limited, with most farms prohibiting green grass grazing; only a small fraction of farms (15.4 %) allowed their animals to graze freely on recognized pasture land. Straw was commonly used as bedding material (44.2 %) for cows. Additionally, most farms (71.2 %) had stall partitions in their milking cow sheds. Hand milking was the predominant technique (90.4 %), with farmers typically employing a milking man for this purpose. The majority of farms (86.5 %) used deep ground water for drinking, ensuring safe water for the cows, while some relied on deep tube well water.

3.2. Detection of subclinical mastitis through Modified Whiteside Test approach

In Tables 2, it was observed that out of a total of 325 milk samples, the MWST indicated 211 samples as positive for SCM. Specifically, 36 milk samples were identified as trace SCM positive, 43 as Grade (1+) SCM positive, 56 as Grade (2+) SCM positive, and 76 as Grade (3+) SCM positive, with prevalence rates of 17.06 %, 20.38 %, 26.54 %, and 36.02 %, respectively.

Table 2 further provides information on the collection of milk samples from each of the 12 upazilas in the Sylhet district, detailing the number of SCM-positive cases identified and their prevalence rates. Notably, South Surma reported the highest prevalence of SCM at 81.82 %, followed by Zakiganj at 80 %, while Kanaighat exhibited the lowest incidence at 38.46 %.

3.2.1. Prevalence on cow-specific variable

The prevalence of subclinical mastitis (SCM) among cattle in Sylhet exhibited significant variations across different cow-specific

Table 3
Prevalence of SCM on cow-specific explanatory variable.

Variable	Category	x/N	Prevalence (%)	χ^2	p-value
Age (year)				15.8	<0.001
	3.5–5	51/103	49.5		
	5–8	149/207	72.0		
	8 above	11/15	73.3		
Breed				26.9	<0.001
	Indigenous	33/81	40.7		
	Cross (HF x local)	131/182	72.0		
	HF	46/62	74.2		
Lactation stage (days)				6.2	0.045
	Early (<90)	40/75	53.3		
	Middle (90–180)	93/137	67.9		
	Late (>180)	78/113	69.0		
Parity				6.9	0.008
	Primiparous	57/103	55.3		
	Multiparous	154/222	69.4		
Milk yield (litre)				21.2	<0.001
	Less than 5	13/40	32.5		
	5–15	150/218	68.8		
	15+	48/67	71.6		
Body weight (kg)				68.28	<0.001
	Less than 100	26/67	38.8		
	100–300	161/227	70.9		
	300+	24/31	77.4		

HF: Holstein Friesian, x = number of animals tested positive, N = Total number of animals tested.

explanatory variables (Table 3). Age-wise, the highest prevalence of SCM was observed in cows aged 8 years and above (73.3 %), while the lowest was in the 3.5–5 year age group (49.5 %) ($p < 0.001$). Breed-wise, Holstein Friesians (HF) had the highest prevalence (74.2 %), compared to indigenous breeds, which had the lowest (40.7 %) ($p < 0.001$). In terms of lactation stage, cows in the late lactation stage (>180 days) showed the highest prevalence (69.0 %), whereas those in the early lactation stage (<90 days) had the lowest (53.3 %) ($p = 0.045$). Multiparous cows exhibited a higher prevalence (69.4 %) compared to primiparous cows (55.3 %) ($p = 0.008$). Regarding milk yield, cows producing 15+ liters of milk per day had the highest prevalence (71.6 %), while those producing less than 5 L had the lowest (32.5 %) ($p < 0.001$). Finally, body weight was also a significant factor, with the highest prevalence found in cows weighing over 300 kg (77.4 %), and the lowest in those under 100 kg (38.8 %) ($\chi 2 = 68.28$, $p < 0.001$). These findings indicate strong associations between SCM prevalence and factors such as age, breed, lactation stage, parity, milk yield, and body weight (Table 3).

3.3. Detection of E coli, K. pneumoniae, S. aureus through PCR

Upon initial identification through cultural and biochemical examination, affirmative specimens underwent subsequent confirmation via PCR testing utilizing specific primer sets designed for detecting the *phoA* gene of *E. coli*, *rpoB* gene of *K. pneumoniae*, and *nuc* gene of *S. aureus* (Table 1). Subsequent analysis through agarose gel electrophoresis under a UV transilluminator authenticated all isolates, exhibiting discernible bands at 468bp for *E. coli*, 108bp for *K. pneumoniae*, and 447bp for *S. aureus* (Table 1, Fig. 2).

In the case of *E. coli*, following initial identification through culture and biochemical assays, all 173 positive samples were subsequently confirmed via PCR, displaying the expected 468bp band (Table 1, Fig. 2B.), resulting in an observed prevalence of *E. coli* at 81.99 %. For *K. pneumoniae*, subsequent to primary detection through culture and biochemical assessments, all positive samples underwent PCR analysis, with 141 samples confirming positivity as *K. pneumoniae*, as evidenced by the anticipated 108bp band (Table 1, Fig. 2C.). This yielded a calculated prevalence of *K. pneumoniae* at 66.82 %. Similarly, subsequent to concluding culture and biochemical examinations, all positive samples were definitively confirmed through PCR, with 67 samples affirming positivity for *S. aureus*, as indicated by the anticipated 447bp band (Table 1, Fig. 2D.). This resulted in a determined prevalence of *S. aureus* at 31.75 %.

All *E. coli* isolates from individual upazilas were validated through PCR, indicating that Bishwanath upazila demonstrated the highest prevalence rate at 72 %, while Kanaighat upazila reported the lowest prevalence rate at 26.92 % (Fig. 3). PCR confirmation for *K. pneumoniae* isolates in each upazila revealed Zakiganj upazila as having the highest prevalence rate at 60 %, whereas Jaintapur upazila exhibited the lowest prevalence rate at 23.33 % (Fig. 3). Additionally, PCR confirmation for *S. aureus* isolates from each upazila indicated Zakiganj upazila with the highest prevalence rate of 45.00 %, while Balaganj upazila displayed the lowest prevalence rate at 10.00 % (Fig. 3). Furthermore, the prevalence at the animal and herd levels was determined to be 82.69 % and 64.92 %, respectively (Fig. 4).

3.4. Mixed (multi-causal agents) infection

When examining SCM-positive isolates, those found to be positive for two or more causal agents were categorized as having a multi-causal agent infection. Out of 211 isolates that tested positive for SCM, 74 were identified as having a multi-causal agent infection, accounting for 35.07 % (95 % CI: 28.65–41.92). Fig. 4 depicts the correlation between causative agents and the occurrence of SCM. Interestingly, 14 samples tested positive for all three isolated organisms in this study. Among the cases of mixed infections, it was

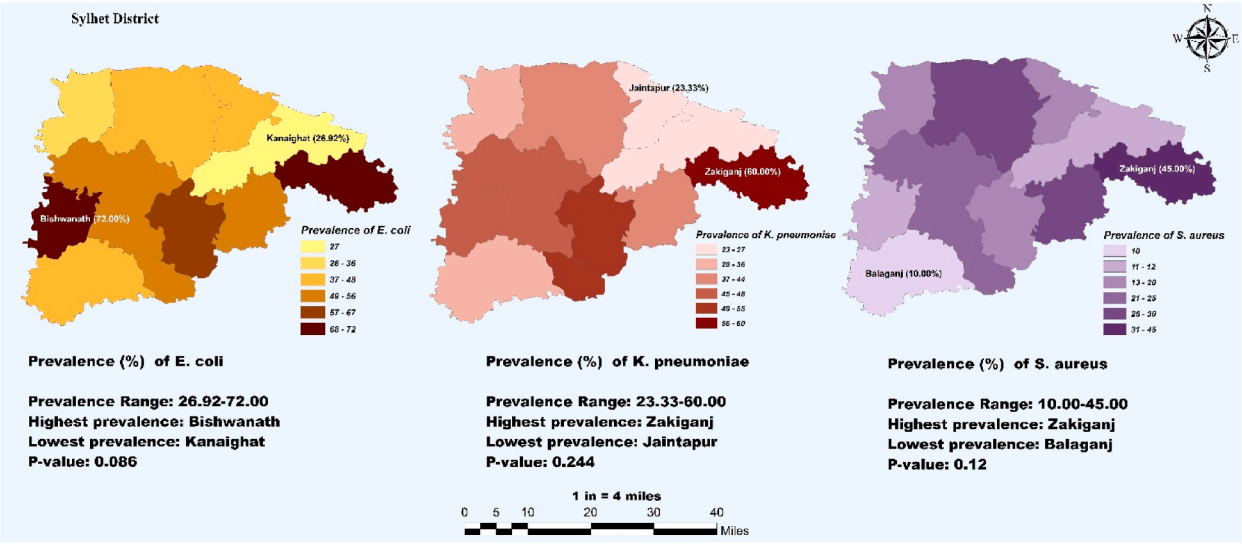


Fig. 3. Molecular prevalence of *E. coli*, *K. pneumoniae*, *S. aureus* isolated from milk samples in Sylhet district of Bangladesh.

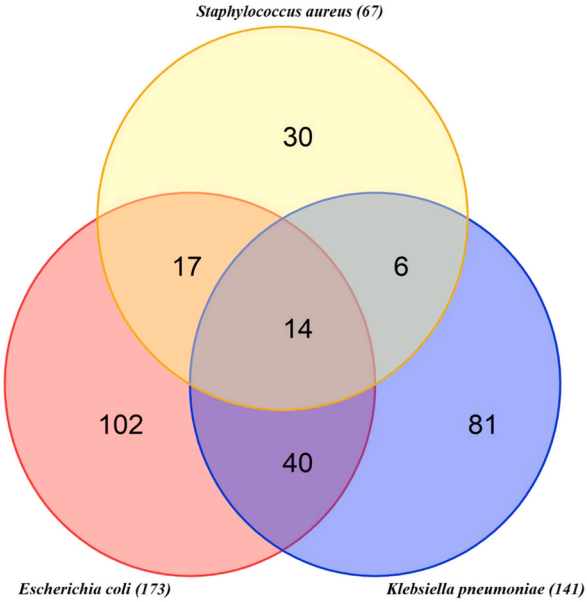


Fig. 4. Venn diagram showing the interaction among the pathogens that were responsible for causing SCM.

observed that *E. coli* and *K. pneumoniae* together were primarily responsible for the majority of SCM cases in this region.

3.5. Phenotypic characteristic of the recovered isolates and MDR, XDR, PDR and MAR index

Out of a total of 381 isolates from *E. coli, K. pneumoniae*, and *S. aureus*, 374 isolates (98.16 %) demonstrated MDR, surpassing a MAR index value of 0.20, as detailed in Table 4. Specifically, for *E. coli*, all identified multi-drug resistant isolates exhibited distinct MAR patterns ranging from 0.41 to 0.65 (Table 4, Fig. 5D.). Among these, the highest number of patterns was observed where the organism displayed resistance to 11 antibiotics across 7 classes (AMP-AMX-CXM-CTR-CTX-CAZ-TE-CL-AZM-C-NA). Similarly, for *K. pneumoniae*, all multi-drug resistant isolates showcased varied MAR patterns ranging from 0.24 to 0.76 (Table 3, Fig. 5D.). The most prevalent pattern involved resistance to 7 antibiotics from 5 classes (AMP-AMX-AK-CXM-CAZ-TE-CL). Regarding *S. aureus*, out of 67 isolates, 17 isolates with multi-drug resistance exhibited diverse MAR patterns ranging from 0.18 to 0.71 (Fig. 5D.). Among them, the predominant pattern featured resistance to 4 antibiotics across 2 classes (AMP-AMX-CXM-CAZ). No PDR (Pan Drug Resistant) isolates were detected among the mastitis-causing bacteria. For *E. coli*, no isolates were identified as either XDR (Extensively Drug Resistant) or PDR. However, 33 isolates of *K. pneumoniae* and 4 isolates of *S. aureus* were classified as XDR (Table 4).

3.6. Determination of antimicrobial susceptibility pattern

Table 5 presents the antibiotic sensitivity profile of 173 *E. coli* isolates. Notably, ciprofloxacin, amikacin, meropenem, and gentamicin exhibited the highest susceptibility among *E. coli* isolates. Specifically, 100 % of the isolates demonstrated susceptibility to ciprofloxacin, followed by 90.01 % to amikacin, 82.65 % to meropenem, and 79.77 % to gentamicin. Conversely, the utmost resistance was observed against ampicillin (100 %), amoxicillin (100 %), cefuroxime (100 %), and tetracycline (100 %) (Fig. 5A.).

The antibiotic sensitivity patterns of 141 *K. pneumoniae* isolates are delineated in Table 5 meropenem, sulfamethoxazole-trimethoprim, amikacin, gentamicin, and ciprofloxacin exhibited the highest susceptibility in *K. pneumoniae.* Meropenem demonstrated 100 % sensitivity, followed by 88.65 % for sulfamethoxazole-trimethoprim, 78.01 % for amikacin, 77.30 % for gentamicin, and 75.17 % for ciprofloxacin. Conversely, the highest resistance levels were noted against ceftazidime (100 %), tetracycline (100 %), amoxicillin (100 %), and ampicillin (88.5 %) (Fig. 5B.).

The antibiotic sensitivity profile of *S. aureus* is also depicted in Table 5. *S. aureus* exhibited heightened susceptibility to amikacin, meropenem, sulfamethoxazole-trimethoprim, ceftriaxone, and gentamicin. Specifically, 100 % sensitivity was observed for meropenem, amikacin, and sulfamethoxazole-trimethoprim, while ceftriaxone and gentamicin displayed 79.10 % and 71.64 % sensitivity, respectively. Conversely, cefuroxime (100 %), amoxicillin (100 %), and ampicillin (100 %) exhibited the highest resistance against *S. aureus* (Fig. 5C.).

3.7. Detection of resistant genes

Table 6 (six) illustrates the prevalence of various antibiotic-resistant genes in *E. coli, K. pneumoniae*, and *S. aureus* positive isolates, with corresponding *p*-values derived from the chi-square goodness of fit test. The bla_{TEM} gene is significantly more prevalent in *E. coli*

Table 4
Phenotypic resistant pattern, MDR, XDR, PDR and MAR index of isolated organisms.

Isolated organisms	Resistance phenotype	No. of resistant antibiotics (Class)	Total number pattern	Overall MDR isolates% (95 % CI)	MAR index	XDR	PDR
E. coli (N = 173)	AMP-AMX-CXM-CTR-CTX-CAZ-TE-CL-AZM-C-NA	11 (7)	52	100 % (97.89–100)	0.65	No	No
	AMP-AMX-CXM-CTX-CAZ-TE-CL-AZM-C-NA	10 (7)	17		0.59	No	No
	AMP-AMX-CXM-CTX-CAZ-TE-CL-AZM-NA	09 (6)	35		0.53	No	No
	AMP-AMX-CXM-CTX-CAZ-TE-AZM-NA	08 (5)	17		0.47	No	No
	AMP-AMX-CXM-CTX-CAZ-COT-TE-AZM-NA	09 (6)	17		0.53	No	No
	AMP-AMX-CXM-CTX-CAZ-COT-GEN-TE-AZM-NA	10 (7)	02		0.59	No	No
	AMP-AMX-CXM-CTX-CAZ-COT-GEN-TE-AZM	09 (6)	10		0.53	No	No
	AMP-AMX-CXM-CAZ-COT-GEN-TE-AZM	08 (6)	06		0.47	No	No
	AMP-AMX-CXM-CAZ-COT-GEN-TE	07 (5)	17		0.41	No	No
K. pneumoniae (N = 141)	AMP-AMX-AK-CXM-CTR-CTX-CAZ-IMP-TE-CL-AZM-C-COT	13 (09)	15	100 % (97.42–100)	0.76	Yes	No
	AMP-AMX-AK-CXM-CTR-CAZ-IMP-TE-CL-AZM-C-COT	12 (09)	01		0.71	Yes	No
	AMP-AMX-AK-CXM-CTR-CAZ-IMP-TE-CL-AZM-C	11 (08)	17		0.65	Yes	No
	AMP-AMX-AK-CXM-CTR-CAZ-IMP-TE-CL-AZM	10 (07)	14		0.59	No	No
	AMP-AMX-AK-CXM-CTR-CAZ-TE-CL-AZM	09 (06)	01		0.53	No	No
	AMP-AMX-AK-CXM-CAZ-TE-CL-AZM	08 (06)	17		0.47	No	No
	AMP-AMX-AK-CXM-CAZ-TE-CL	07 (05)	33		0.41	No	No
	AMP-AMX-AK-CXM-CAZ-TE	06 (04)	01		0.35	No	No
	AMP-AMX-AK-CAZ-TE	05 (04)	28		0.29	No	No
	AMX-AK-CAZ-TE	04 (04)	14		0.24	No	No
S. aureus (N = 67)	AMP-AMX-CXM-CTR-CTX-CAZ-IMP-TE-CL-AZM-C-NA	12 (08)	02	25.37 % (15.53–37.49)	0.71	Yes	No
	AMP-AMX-CXM-CTX-CAZ-IMP-TE-CL-AZM-C-NA	11 (08)	02		0.65	Yes	No
	AMP-AMX-CXM-CTX-CAZ-IMP-CL-AZM-C-NA	10 (07)	02		0.59	No	No
	AMP-AMX-CXM-CTX-CAZ-IMP-CL-AZM-C	09 (06)	05		0.53	No	No
	AMP-AMX-CXM-CTX-CAZ-IMP-CL-C	08 (05)	02		0.47	No	No
	AMP-AMX-CXM-CTX-CAZ-CL-C	07 (04)	03		0.41	No	No
	AMP-AMX-CXM-CTX-CAZ-CL	06 (03)	01		0.35	No	No
	AMP-AMX-CXM-CTX-CAZ	05 (02)	10		0.29	No	No
	AMP-AMX-CXM-CAZ	04 (02)	33		0.24	No	No
	AMP-AMX-CXM	03 (02)	07		0.18	No	No

Ampicillin (AMP), amoxicillin (AMX), gentamicin (GEN), amikacin (AK), cefuroxime (CXM), ceftriaxone (CTR), cefotaxime (CTX), ceftazidime (CAZ), imipenem (IMP), meropenem (MEM), tetracycline (TE), ciprofloxacin (CIP), colistin (CL), azithromycin (AZM), chloramphenicol (C), sulfamethoxazole-trimethoprim (COT), nalidixic Acid (NA); MAR: Multiple antibiotic resistant; MDR: Multi-drug resistant; XDR: Extensively drug resistant; PDR: Pan drug resistant.

(60.7 %) compared to *K. pneumoniae* (42.5 %) and *S. aureus* (16.4 %) ($p < 0.001$), indicating a higher resistance to certain beta-lactam antibiotics in *E. coli*. Conversely, the bla_{OXA} and $bla_{CTX\text{-}M1}$ genes do not show significant differences in prevalence among the three bacterial species ($p > 0.05$). Notably, the $MultiCase_{DHA}$ gene, associated with resistance to multiple antibiotics, is significantly more prevalent in *E. coli* (21.4 %) compared to *K. pneumoniae* (5.7 %) and *S. aureus* (7.5 %) ($p < 0.001$). There was no detection of $bla_{SHV,}$ $bla_{CTX\text{-}M9,}$ $MultiCase_{ACC}$, $MultiCase_{MOX}$. Furthermore, the *tet(A)* gene, which confers resistance to tetracycline, has the highest prevalence in *E. coli* (90.8 %), followed by *K. pneumoniae* (74.5 %), and is least prevalent in *S. aureus* (14.9 %) ($p < 0.001$). Similarly, the *AAC (3)-iv* gene, related to gentamicin resistance, is found at a significantly higher rate in *E. coli* (24.3 %) compared to *K. pneumoniae* (2.1 %) and *S. aureus* (10.4 %) ($p < 0.001$). Lastly, the *Sul1* gene, which provides resistance to trimethoprim-sulfamethoxazole, is most prevalent in *E. coli* (27.7 %).

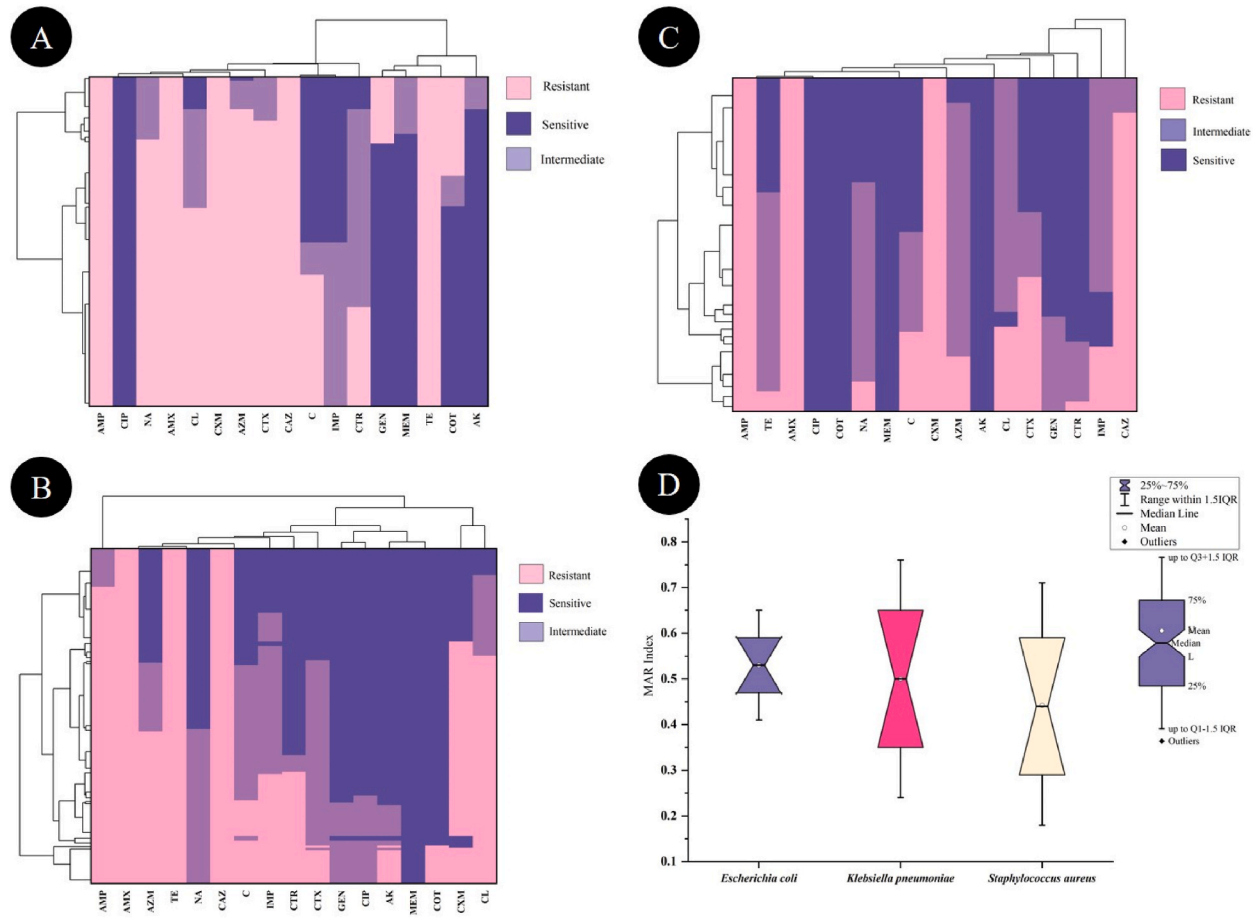


Fig. 5. Figure A–C showing the heat map of antimicrobial resistant pattern of *E. coli* (A), *K. pneumoniae* (B), *S. aureus* (C) isolated from milk samples and figure D showing the multiple antibiotic resistant (MAR) index of resistant patterns of isolated samples.

Table 5
Antibiogram profile of isolated bacteria from bovine subclinical mastitis (SCM) milk in Sylhet district.

Antibiotics	*E. coli* (*n* = 173)			*K. pneumoniae* (*n* = 141)			*S. aureus* (*n* = 67)		
	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)
AMP	0	0	100	11.34	0	88.65	0	0	100
AMX	0	0	100	0	0	100	0	0	100
GEN	79.77	0	20.23	77.30	22.70	0	71.64	28.35	0
AK	90.01	9.82	0	78.01	11.34	10.63	100	0	0
CXM	0	0	100	33.33	0	68.80	0	0	100
CTR	9.82	60.11	30.05	63.82	4.69	33.33	79.10	17.91	2.98
CTX	0	13.29	86.70	33.33	56.02	10.64	40.29	19.40	40.29
CAZ	0	0	100	0	0	100	0	10.44	89.55
IMP	50.29	49.71	0	20.56	46.80	32.62	16.41	64.17	19.40
MEM	82.65	17.36	0	100	0	0	100	0	0
TE	0	0	100	0	0	100	34.32	59.70	5.97
CIP	100	0	0	75.17	24.82	0	100	0	0
CL	9.82	30.05	60.11	7.80	24.11	68.08	4.47	70.14	25.37
AZM	1.15	8.68	90.01	34.04	20.56	45.39	7.46	76.11	16.41
C	50.28	9.82	39.89	34.75	41.84	23.40	46.26	29.85	23.88
COT	60.70	9.24	30.05	88.65	0	11.34	100	0	0
NA	0	19.07	80.92	53.90	46.09	0 %	31.34	59.70	8.95

Ampicillin (AMP), amoxicillin (AMX), gentamicin (GEN), amikacin (AK), cefuroxime (CXM), ceftriaxone (CTR), cefotaxime (CTX), ceftazidime (CAZ), imipenem (IMP), meropenem (MEM), tetracycline (TE), ciprofloxacin (CIP), colistin (CL), azithromycin (AZM), chloramphenicol (C), sulfamethoxazole-trimethoprim (COT), nalidixic Acid (NA); S = Sensitive, I = Intermediate, R = Resistan

Table 6
Antimicrobial resistant genes among the positive isolates of pathogens causing SCM.

Class	Resistant Genes	*E. coli* (N = 173) (%, x)	*K. pneumoniae* (N = 141) (%, x)	*S. aureus* (N = 67) (%, x)	*p*-value
ESBL	bla_{TEM}	60.7 (105)	42.5 (60)	16.4 (11)	<0.001
	bla_{OXA}	13.3 (23)	7.8 (11)	11.9 (8)	0.270
	$bla_{CTX\text{-}M1}$	17.9 (31)	14.9 (21)	25.4 (17)	0.134
	$bla_{CTX\text{-}M2}$	4.0 (7)	2.1 (3)	7.5 (5)	0.141
	$MultiCase_{DHA}$	21.4 (37)	5.7 (8)	7.5 (5)	<0.001
Tetracycline	*tet(A)*	90.8 (157)	74.5 (105)	14.9 (10)	<0.001
Gentamicin	*AAC (3)-iv*	24.3 (42)	2.1 (3)	10.4 (7)	<0.001
Trimethoprim- sulfamethoxazole	*Sul1*	27.7 (48)	12.1 (17)	0	<0.001

N: Number of samples tested, x: Number of positive samples; Chi-square goodness of fit test.

3.8. Resistant genotype and phenotype correlation

The correlation coefficient (r) indicates a moderately strong to strong positive relationship between the resistant genotype and phenotype (Fig. 6). A very strong and significantly positive correlation ($r = 0.91$, $p < 0.001$) was observed between the tetracycline phenotype (TE) and the *tetA* genotype. Similarly, a significantly strong correlation ($r = 0.87$, $p < 0.001$) was found between the gentamicin phenotype and genotype. Additionally, a moderately strong correlation ($r = 0.63$) was observed between the trimethoprim-sulfamethoxazole phenotype (COT) and its corresponding genotype (*AAC(3)-iv*).

4. Discussion

This investigation stands as one of the limited inquiries into bovine SCM in Bangladesh, providing an assessment of the overall prevalence of SCM and characterizing the three most common bacteria associated with SCM. The study, conducted in the Sylhet district of Bangladesh, disclosed an overall SCM prevalence of 64.92 %. Notably, this prevalence aligns closely with the findings (62.0 %) reported in a prior study [77] and falls within the spectrum of SCM prevalence (60–77 %) documented in recent research conducted in Bangladesh [78]. This result is much higher than the study findings where it is reported that SCM prevalence is 49.02 % through MWST only in Sylhet [79]. The prevalence of SCM varied widely between studies across the four countries, with rates ranging from approximately 20 %–80 %, though the overall average prevalence was notably high at 50 % [80]. The diverse occurrence of SCM in various countries could be linked to factors such as the hygiene and sanitation practices observed during the milking process, the specific stage of lactation, parity, the type of milking procedures employed, and potential genetic influences. This underscores the gravity of the situation within the continent's dairy industry, warranting careful consideration and attention.

Demographically, SCM was more prevalent among older cows, Holstein Friesian breeds, cows in late lactation stages, multiparous cows, high milk yielders, and heavier cows. These findings are consistent with other studies that have identified similar risk factors for SCM. The higher prevalence in older cows and high-yielding breeds like Holstein Friesians may be due to increased physiological stress and susceptibility to infections. Late lactation stages and multiparity are also associated with changes in immune function and udder health, which may predispose cows to SCM.

The prevalence of *E. coli* was 81.99 % a significantly elevated rate compared to the findings in Chitwan's dairy cattle in Nepal (16.98 %) and dairy cattle in Ethiopia (33.8 %) [81,82]. The variation in prevalence may be associated with distinct factors such as effective hygiene and sanitation practices, udder cleanliness, proper management of teat ends, and appropriate stall conditions [83].

This investigation also reveals that the prevalence of *K. pneumoniae* is recorded at 66.82 %, while *S. aureus* stands at 31.75 %. This observation aligns with the findings of XuehanLi [84]. However, it is noteworthy that the current study's prevalence of *K. pneumoniae* significantly exceeds the outcomes of two prior studies in China where prevalence rates were reported at 26.94 % and 23 %, respectively [85,86]. Additionally, the prevalence of *S. aureus* in the present study is somewhat lower (45.7 %) compared to a previous study conducted in Egypt [87].

The occurrence of SCM displayed variations across distinct locations within the Sylhet district. Notably, South Surma recorded the highest prevalence of SCM at 81.82 %, closely followed by Zakiganj at 80 %, whereas Kanaighat exhibited the lowest incidence at 38.46 %. The findings of the current study comparable with those of a prior investigation conducted in diverse regions of Bangladesh [1]. This variability in SCM prevalence could be attributed to factors such as geographical location, climatic conditions, housing types, milking methods, udder cleanliness during milking, hygienic practices, and the level of knowledge regarding farm bio-security among owners in different areas. The detection of extended-spectrum beta-lactamase (ESBL) genes (bla_{TEM}, bla_{OXA}, $bla_{CTX\text{-}M1}$, $bla_{CTX\text{-}M2}$, $MultiCase_{DHA}$) and other resistance genes (*tet(A), AAC(3)-iv, Sul1*) further confirms the presence of highly resistant bacterial strains. The prevalence of multidrug-resistant (MDR) isolates at 98.16 %, with MAR index values ranging from 0.18 to 0.76, underscores the urgent need for targeted antimicrobial stewardship programs. Notably, no pan-drug resistant (PDR) isolates were found, but the presence of extensively drug-resistant (XDR) isolates, particularly among *K. pneumoniae* and *S. aureus*, is alarming.

The results of the current study indicate that the Multiple Antibiotic Resistance (MAR) index for *E. coli* isolates falls within the range of 0.41–0.65. Notably, there were 52 distinct resistant patterns observed, involving 11 antibiotics across 7 classes. This contrasts with a study conducted in Ghana, where the MAR index ranged from 0.0 to 0.7, and 22 resistant patterns were identified [88]. Moreover, all *E. coli* isolates exhibited multidrug resistance, with a MAR index of 0.2 and above, significantly surpassing the results of a previous

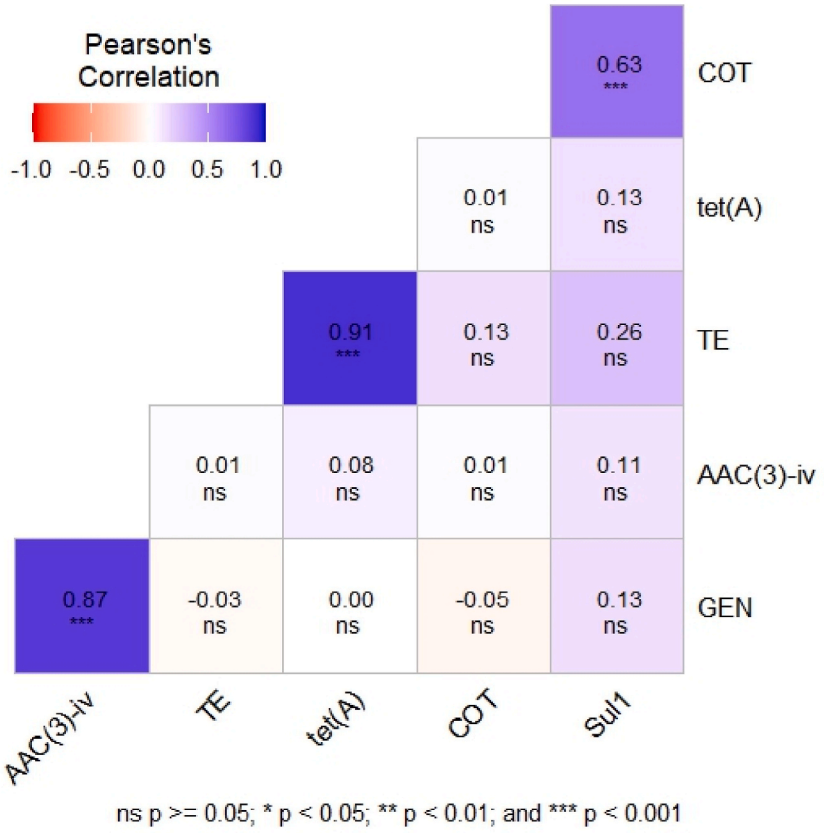


Fig. 6. Pearson's correlation coefficient among the resistant genotype and phenotype of commonly used antibiotics.

study where only 16 % of isolates were MDR, and 53 % had an MAR index of 0.2 and above [89]. For *K. pneumoniae*, all isolates in our study exhibited multidrug resistance, and the MAR index ranged from 0.24 to 0.76. This closely aligns with the results of a separate study in Pakistan, where 44.4 % of isolates were identified as MDR, and the MAR index varied from 0.21 to 0.92 [90]. Concerning *S. aureus,* around 25.37 % of isolates demonstrated MDR, showing MAR indices within the range of 0.18–0.71. This finding is comparable to another study conducted in Egypt, where only 16 % of isolates were identified as MDR, and the MAR index was reported as 0.5 [91].

The antibiotic susceptibility of PCR-positive bacterial isolates was evaluated against seventeen commonly used antibiotics. The antimicrobial susceptibility test (AST) revealed that all three tested microorganisms displayed multidrug resistance. Specifically, *E. coli* exhibited 100 % resistance to ampicillin, amoxicillin, tetracycline, ceftazidime, and cefuroxime, with the exception of ciprofloxacin, which showed 100 % sensitivity. This aligns closely with a prior study indicating the highest resistance against amoxicillin (94.5 %), ampicillin (89.5 %), and tetracycline (89.5 %) [92]. Notably, *E. coli* isolates exhibited a significantly higher level of MDR than previously reported [93]. Similarly, *S. aureus* exhibited MDR, with 100 % resistance to ampicillin, amoxicillin, and cefuroxime. The prevalence of penicillin resistance (100 %) surpassed rates reported in various regions, including Ethiopia (71.6 %) [94], Estonia (61.4 %) [95], Korea (52.9 %), Switzerland (31 %), Finland (32 %), and the USA (22.1 %) [96]. However, *S. aureus* displayed 100 % susceptibility to amikacin, meropenem, ciprofloxacin, and sulfamethoxazole-trimethoprim, with no resistance to gentamicin. These findings align with Gentilini [97] and Kaszanyitzky's [98] findings. Antibiotic sensitivity test results of *K. pneumoniae* showed that the bacteria are 100 % resistant to amoxicillin, ceftazidime and tetracycline followed by 88.65 % to ampicillin. However, *K. pneumoniae* showed 100 % sensitivity to meropenem as well as no resistance to gentamicin, ciprofloxacin and nalidixic acid. The present study findings are comparable to the findings of a study in Pakistan where it is stated that *K pneumoniae* showed high resistance to vancomycin, fusidic acid, amoxicillin, sulfamethazine, and chloramphenicol, while highly sensitive to ceftazidime, ciprofloxacin, levofloxacin, amikacin, gentamycin, tetracycline, and imipenem [99]. Additionally, our findings align with a Chinese study indicating low sensitivity of *K. pneumoniae* to penicillin (0 %) and amoxicillin (4 %), compared to higher sensitivity to gentamicin (92 %) [100]. Overall, the AST results revealed 100 % resistance to amoxicillin across all three bacteria and high resistance to ampicillin, cefuroxime, and tetracycline, except for *S. aureus*, which showed only 5.97 % resistance to tetracycline. In contrast, all three bacteria exhibited high sensitivity to meropenem, amikacin, gentamicin, ciprofloxacin, and sulfamethoxazole-trimethoprim. These findings underscore the concerning issue of multiple antibiotic resistances, emphasizing the role of antibiotic overuse and misuse in the development of bacteria resistant to multiple drug classes.

In conclusion, this research highlights the urgent need for comprehensive strategies to combat antimicrobial resistance in SCM pathogens. A collaborative approach involving veterinarians, farmers, and policymakers is crucial for sustainable dairy farming in Bangladesh. Future research should explore alternative therapies, including vaccine development and bacteriophage therapy, to manage and control SCM effectively.

5. Conclusion

This study elucidates the molecular prevalence, antimicrobial susceptibility profiles, and resistance gene identification of pathogens causing SCM in dairy cows in Sylhet, Bangladesh. The findings reveal a high prevalence of SCM at both individual animal (64.92 %) and herd levels (82.69 %). The predominant pathogens identified were *S. aureus* (31.75 %), *E. coli* (81.99 %), and *K. pneumoniae* (66.82 %), with a notable incidence of mixed infections, especially *E. coli* and *K. pneumoniae* , complicating SCM management. Antimicrobial susceptibility testing showed alarming resistance levels among the pathogens. *E. coli, K. pneumoniae*, and *S. aureus* exhibited significant resistance to multiple antibiotic classes, including penicillins (ampicillin, amoxicillin), cephems (cefuroxime, ceftazidime), and tetracyclines. However, these pathogens remained highly sensitive to meropenem, amikacin, gentamicin, ciprofloxacin, and sulfamethoxazole-trimethoprim. The presence of extended-spectrum beta-lactamase (ESBL) genes such as bla_{TEM}, bla_{OXA}, $bla_{\text{CTX-M1}}$, $bla_{\text{CTX-M2}}$, and other resistance genes like *tet(A), AAC(3)-iv*, and *Sul1* presents significant treatment challenges. These findings underscore the critical need for targeted antimicrobial stewardship programs and effective control measures to mitigate SCM's impact on dairy production and animal health. This includes regular monitoring of antimicrobial resistance, adopting best management practices, and prudent antibiotic use. Educating farmers on hygiene practices and proper milking techniques is also essential.

Funding

This study received financial support from the Ministry of Science and Technology (MoST), Government Peoples Republic of Bangladesh under special allocation for Science and Technology program with a project code: SRG-221364.

Ethical statement

This experiment holds an approved Animal Use Protocol #AUP2022037 from the Animal Experimentation and Ethics Committee (AEEC) at Sylhet Agricultural University, Bangladesh.

Data availability statement

Data are available on request from the corresponding author.

CRediT authorship contribution statement

Ahsan Al Emon: Writing – original draft, Visualization, Software, Resources, Methodology, Investigation, Formal analysis, Data curation. **Hemayet Hossain:** Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation. **Md Shahidur Rahman Chowdhury:** Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation. **Md Anisur Rahman:** Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation. **Fatema Yeasmin Tanni:** Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation. **Mashuka Nahida Asha:** Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation. **Hafsa Akter:** Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation. **Md Mukter Hossain:** Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation. **Md Rafiqul Islam:** Writing – original draft, Supervision, Software, Methodology, Investigation, Formal analysis, Data curation. **Md Mahfujur Rahman:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Md. Mahfujur Rahman reports financial support, equipment, drugs, or supplies, and travel were provided by Government of the People's Republic of Bangladesh Ministry of Science and Technology. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors would like to acknowledge the dairy farmers and their staffs who helped us to collect the samples.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e34567.

References

[1] M.A. Sayeed, M.A. Rahman, M.S. Bari, A. Islam, M.M. Rahman, M.A. Hoque, Prevalence of subclinical mastitis and associated risk factors at cow level in dairy farms in Jhenaidah, Bangladesh, Adv. Anim. Vet. Sci. 8 (2020) 112–121, https://doi.org/10.17582/JOURNAL.AAVS/2020/8.S2.112.121.

[2] M.M. Uddin, M.N. Sultana, B. Bruermer, K.J. Peters, Assessing the impact of dairy policies on farm-level profits in dairy farms in Bangladesh: benchmarking for rural livelihoods improvement policy, J. Rev. Global Econ. 1 (2012) 124–138. https://ideas.repec.org/a/lif/jrgelg/v1y2012p124-138.html. (Accessed 17 June 2024).

[3] L.N. Kong, J. Bin Li, R.L. Li, X.X. Zhao, Y. Bin Ma, S.H. Sun, J.M. Huang, Z.H. Ju, M.H. Hou, J.F. Zhong, Estimation of 305-day milk yield from test-day records of Chinese Holstein cattle, J. Appl. Anim. Res. 46 (2018) 791–797, https://doi.org/10.1080/09712119.2017.1403918.

[4] C. Odhong, R.G. Wahome, M. Vaarst, M. Kiggundu, S. Nalubwama, N. Halberg, S. Githigia, Challenges of conversion to organic dairy production and prospects of future development in integrated smallholder farms in Kenya. http://www.lrrd.org/lrrd26/7/odho26125.html, 2014. (Accessed 19 December 2021).

[5] N. Zaatout, D. Hezil, A meta-analysis of the global prevalence of methicillin-resistant Staphylococcus aureus (MRSA) isolated from clinical and subclinical bovine mastitis, J. Appl. Microbiol. 132 (2022) 140–154, https://doi.org/10.1111/JAM.15192.

[6] H.A. Hussein, K.A.E.H.A. El-Razik, A.M. Gomaa, M.K. Elbayoumy, K.A. Abdelrahman, H.I. Hosein, Milk amyloid A as a biomarker for diagnosis of subclinical mastitis in cattle, Vet. World 11 (2018) 34–41, https://doi.org/10.14202/VETWORLD.2018.34-41.

[7] P. Krishnamoorthy, K.P. Suresh, K.S. Jayamma, B.R. Shome, S.S. Patil, R.G. Amachawadi, An understanding of the global status of major bacterial pathogens of milk concerning bovine mastitis: a systematic review and meta-analysis (scientometrics), Pathogens 10 (2021), https://doi.org/10.3390/PATHOGENS10050545.

[8] T. Halasa, K. Huijps, O. Østerås, H. Hogeveen, Economic effects of bovine mastitis and mastitis management: a review, Vet. Q. 29 (2007) 18–31, https://doi.org/10.1080/01652176.2007.9695224.

[9] M.H. Kabir, M. Ershaduzzaman, K.H.M.N.H. Nazir, M.S. Islam, R. Khatun, M.S.A. Sarker, M.A. Yousuf, Y. Ali, N.R. Sarkar, M. Giasuddin, Development and validation of BLRI mastitis test kit at Bangladesh livestock research Institute regional station, sirajganj, J Adv Vet Anim Res 6 (2019) 425, https://doi.org/10.5455/JAVAR.2019.F363.

[10] K.A. Abd El-Razik, A.A. Arafa, E.A. Fouad, A.H. Soror, A.M. Abdalhamed, M. Elgioushy, Phenotypic and genotypic characterization of erythromycin-resistant Staphylococcus aureus isolated from bovine subclinical mastitis in Egypt, Vet. World 16 (2023) 1562–1571, https://doi.org/10.14202/vetworld.2023.1562-1571.

[11] W.N. Cheng, S.G. Han, Bovine mastitis: risk factors, therapeutic strategies, and alternative treatments — a review, Asian-Australas. J. Anim. Sci. 33 (2020) 1699, https://doi.org/10.5713/AJAS.20.0156.

[12] O. Radostits, C. Gay, C. Gay, K. Hinchcliff, P. Constable, A textbook of the diseases of cattle, horses, sheep, pigs and goats: veterinary medicine, Vet. Med. 10 (2007) 2045–2050.

[13] F.J. DeGraves, J. Fetrow, Economics of mastitis and mastitis control, Vet Clin North Am Food Anim Pract 9 (1993) 421–434, https://doi.org/10.1016/S0749-0720(15)30611-3.

[14] R.M.S.B.K. Ranasinghe, R.M.C. Deshapriya, D.I. Abeygunawardana, R. Rahularaj, C.M.B. Dematawewa, Subclinical mastitis in dairy cows in major milk-producing areas of Sri Lanka: prevalence, associated risk factors, and effects on reproduction, J. Dairy Sci. 104 (2021) 12900–12911, https://doi.org/10.3168/JDS.2021-20223.

[15] T. Ali, Kamran, A. Raziq, I. Wazir, R. Ullah, P. Shah, M.I. Ali, B. Han, G. Liu, Prevalence of mastitis pathogens and antimicrobial susceptibility of isolates from cattle and buffaloes in northwest of Pakistan, Front. Vet. Sci. 8 (2021) 746755, https://doi.org/10.3389/FVETS.2021.746755.

[16] D. Sharma, S. Kaniamuthan, A. Manimaran, A. Kumaresan, M. Sivaram, D. Rajendran, P.R. Wankhade, V. Sejian, S. Banu, Seasonal, physiological and bacteriological risk factors for subclinical mastitis in dairy cows maintained under different farming conditions, J. Dairy Res. 90 (2023) 164–172, https://doi.org/10.1017/S0022029923000389.

[17] A.L. Morales-Ubaldo, N. Rivero-Perez, B. Valladares-Carranza, V. Velázquez-Ordoñez, L. Delgadillo-Ruiz, A. Zaragoza-Bastida, Bovine mastitis, a worldwide impact disease: prevalence, antimicrobial resistance, and viable alternative approaches, Vet Anim Sci 21 (2023), https://doi.org/10.1016/j.vas.2023.100306.

[18] J.E. Breen, M.J. Green, A.J. Bradley, Quarter and cow risk factors associated with the occurrence of clinical mastitis in dairy cows in the United Kingdom, J. Dairy Sci. 92 (2009) 2551, https://doi.org/10.3168/JDS.2008-1369.

[19] J. Verbeke, S. Piepers, K. Supré, S. De Vliegher, Pathogen-specific incidence rate of clinical mastitis in Flemish dairy herds, severity, and association with herd hygiene, J. Dairy Sci. 97 (2014) 6926–6934, https://doi.org/10.3168/JDS.2014-8173.

[20] H.A. Naji, W.M.M. Saleh, M. Hanoon, I. Imad, Y. Salim, Serotyping, virulence gene expression and phenotypic characterization of *E. coli* O157:H7 in colibacillosis affecting buffalo calves in Basra governorate, Iraqi J. Vet. Sci. 33 (2019) 445–451, https://doi.org/10.33899/IJVS.2019.163198.

[21] H.W. Barkema, Y.H. Schukken, T.J.G.M. Lam, M.L. Beiboer, H. Wilmink, G. Benedictus, A. Brand, Incidence of clinical mastitis in dairy herds grouped in three categories by bulk milk somatic cell counts, J. Dairy Sci. 81 (1998) 411–419, https://doi.org/10.3168/JDS.S0022-0302(98)75591-2.

[22] B. Badawy, M. Elafify, A.M.M. Farag, S.M. Moustafa, M.Z. Sayed-Ahmed, A.A. Moawad, A.M. Algammal, H. Ramadan, M. Eltholth, Ecological distribution of virulent multidrug-resistant Staphylococcus aureus in livestock, environment, and dairy products, Antibiotics 11 (2022), https://doi.org/10.3390/antibiotics11111651.

[23] A.M. Algammal, M.E. Enany, R.M. El-Tarabili, M.O.I. Ghobashy, Y.A. Helmy, Prevalence, antimicrobial resistance profiles, virulence and enterotoxin-determinant genes of MRSA isolated from subclinical bovine mastitis samples in Egypt, Pathogens 9 (2020) 1–11, https://doi.org/10.3390/pathogens9050362.

[24] J. Yang, Y. Xiong, H.W. Barkema, X. Tong, Y. Lin, Z. Deng, J.P. Kastelic, D.B. Nobrega, Y. Wang, B. Han, J. Gao, Comparative genomic analyses of Klebsiella pneumoniae K57 capsule serotypes isolated from bovine mastitis in China, J. Dairy Sci. 107 (2024) 3114–3126, https://doi.org/10.3168/jds.2023-23721.

[25] C.M. Mbindyo, G.C. Gitao, C.M. Mulei, Prevalence, etiology, and risk factors of mastitis in dairy cattle in embu and kajiado counties, Kenya, Vet. Med. Int. 2020 (2020), https://doi.org/10.1155/2020/8831172.

[26] S. Dufour, J. Labrie, M. Jacques, The mastitis pathogens culture collection, Microbiol Resour Announc 8 (2019), https://doi.org/10.1128/MRA.00133-19.

[27] P. Rainard, G. Foucras, J.R. Fitzgerald, J.L. Watts, G. Koop, J.R. Middleton, Knowledge gaps and research priorities in Staphylococcus aureus mastitis control, Transbound Emerg Dis 65 (Suppl 1) (2018) 149–165, https://doi.org/10.1111/TBED.12698.

[28] R.N. Zadoks, J.R. Middleton, S. McDougall, J. Katholm, Y.H. Schukken, Molecular epidemiology of mastitis pathogens of dairy cattle and comparative relevance to humans, J. Mammary Gland Biol. Neoplasia 16 (2011) 357–372, https://doi.org/10.1007/S10911-011-9236-Y.

[29] R. Haftu, H. Taddele, G. Gugsa, S. Kalayou, Prevalence, bacterial causes, and antimicrobial susceptibility profile of mastitis isolates from cows in large-scale dairy farms of Northern Ethiopia, Trop. Anim. Health Prod. 44 (2012) 1765–1771, https://doi.org/10.1007/S11250-012-0135-Z.

[30] M.A. Munoz, F.L. Welcome, Y.H. Schukken, R.N. Zadoks, Molecular epidemiology of two Klebsiella pneumoniae mastitis outbreaks on a dairy farm in New York State, J. Clin. Microbiol. 45 (2007) 3964–3971, https://doi.org/10.1128/JCM.00795-07.

[31] M. Ohnishi, A.T. Okatani, K. Harada, T. Sawada, K. Marumo, M. Murakami, R. Sato, H. Esaki, K. Shimura, H. Kato, N. Uchida, T. Takahashi, Genetic characteristics of CTX-M-type extended-spectrum-β-lactamase (ESBL)-producing enterobacteriaceae involved in mastitis cases on Japanese dairy farms, 2007 to 2011, J. Clin. Microbiol. 51 (2013) 3117–3122, https://doi.org/10.1128/JCM.00920-13.

[32] Y.T. Gröhn, D.J. Wilson, R.N. González, J.A. Hertl, H. Schulte, G. Bennett, Y.H. Schukken, Effect of pathogen-specific clinical mastitis on milk yield in dairy cows, J. Dairy Sci. 87 (2004) 3358–3374, https://doi.org/10.3168/JDS.S0022-0302(04)73472-4.

[33] R.J. Erskine, P.C. Bartlett, J.L. VanLente, C.R. Phipps, Efficacy of systemic ceftiofur as a therapy for severe clinical mastitis in dairy cattle, J. Dairy Sci. 85 (2002) 2571–2575, https://doi.org/10.3168/JDS.S0022-0302(02)74340-3.

[34] K.A. Abd El-Razik, Y.A. Soliman, E.A. Fouad, A.A. Arafa, S.M. Syame, A.H. Soror, Macrolides-resistant Staphylococcus aureus associated with clinical mastitis in cattle and Buffalo in Egypt, J. Adv. Vet. Res. 13 (2023) 461–468.

[35] N. Ibrahim, Review on mastitis and its economic effect, Canadian Journal of Scientific Research 6 (2017) 13–22, https://doi.org/10.5829/idosi.cjsr.2017.13.22.

[36] G. Jones, G.M. Jones, T.L. Bailey, Understanding the basics of mastitis, Virginia Cooperative Extension 404 (233) (2009) 1–5, 2009.

[37] O.M. Radostits, S.H. Done, Veterinary medicine — a textbook of the diseases of cattle, horses, sheep, pigs and goats, in: The Canadian Veterinary Journal 51, tenth ed., 2010, p. 541.

[38] A. Khan, S. Firyal, I. Khan, A.R. Awan, M. Tayyab, M. Wasim, M.Z. Shabbir, M.M. Ali, A. Zohaib, Phenotypic and genotypic characterization of beta-lactams resistant staphylococcus aureus isolates from bovine mastitis and its zoonotic implications, Pak. Vet. J. 40 (2020) 523–526, https://doi.org/10.29261/PAKVETJ/2020.065.

[39] O.K. Dego, O.K. Dego, Control and prevention of mastitis: Part Two, animal reproduction in veterinary medicine. https://doi.org/10.5772/INTECHOPEN.93484, 2020.

[40] F. Gomes, M. Henriques, Control of bovine mastitis: old and recent therapeutic approaches, Curr. Microbiol. 72 (2016) 377–382, https://doi.org/10.1007/S00284-015-0958-8.

[41] A.M. Algammal, M.E.A. Hashem, K.J. Alfifi, A.S. Al-Otaibi, M. Alatawy, R.M. Eltarabili, W.A.A. El-Ghany, H.F. Hetta, A.M. Hamouda, A.A. Elewa, M.M. Azab, Sequence analysis, Antibiogram profile, virulence and antibiotic resistance genes of XDR and MDR gallibacterium anatis isolated from layer chickens in Egypt, Infect. Drug Resist. 15 (2022) 4321–4334, https://doi.org/10.2147/IDR.S377797.

[42] A.M. Algammal, R.A. Ibrahim, K.J. Alfifi, H. Ghabban, S. Alghamdi, A. Kabrah, A.R. Khafagy, G.M. Abou-Elela, N.M. Abu-Elala, M.G. Donadu, R.M. El-Tarabili, A first report of molecular typing, virulence traits, and phenotypic and genotypic resistance patterns of newly emerging XDR and MDR aeromonas veronii in Mugil seheli, Pathogens 11 (2022), https://doi.org/10.3390/pathogens11111262.

[43] M. Shafiq, M. Zeng, B. Permana, H. Bilal, J. Huang, F. Yao, A.M. Algammal, X. Li, Y. Yuan, X. Jiao, Coexistence of blaNDM–5 and tet(X4) in international high-risk Escherichia coli clone ST648 of human origin in China, Front. Microbiol. 13 (2022) 1–8, https://doi.org/10.3389/fmicb.2022.1031688.

[44] A.M. Algammal, K.J. Alfifi, M. Mabrok, M. Alatawy, D.A. Abdel-Moneam, S. Alghamdi, M.M. Azab, R.A. Ibrahim, H.F. Hetta, R.M. El-Tarabili, Newly Emerging MDR B. cereus in Mugil seheli as the First Report Commonly Harbor nhe, hbl, cytK, and pc-plc Virulence Genes and bla1, bla2, tetA, and ermA Resistance Genes, Infect. Drug Resist. 15 (2022) 2167–2185, https://doi.org/10.2147/IDR.S365254.

[45] G.E.S. Batiha, M. Alqarni, D.A.B. Awad, A.M. Algammal, R. Nyamota, M.I.I. Wahed, M.A. Shah, M.N. Amin, B.O. Adetuyi, H.F. Hetta, N. Cruz-Martins, N. Koirala, A. Ghosh, J. Echeverría, J.P. Pagnossa, J.M. Sabatier, Dairy-derived and egg white proteins in enhancing immune system against COVID-19, Front. Nutr. 8 (2021) 1–13, https://doi.org/10.3389/fnut.2021.629440.

[46] A. Elbehiry, E. Marzouk, M. Aldubaib, I. Moussa, A. Abalkhail, M. Ibrahem, M. Hamada, W. Sindi, F. Alzaben, A.M. Almuzaini, A.M. Algammal, M. Rawway, Pseudomonas species prevalence, protein analysis, and antibiotic resistance: an evolving public health challenge, Amb. Express 12 (2022) 1–14, https://doi.org/10.1186/s13568-022-01390-1.

[47] E. Leung, D.E. Weil, M. Raviglione, H. Nakatani, on behalf of the W.H.O.W.H.D.A.R.T.W. Group, The WHO policy package to combat antimicrobial resistance, Bull. World Health Organ. 89 (2011) 390, https://doi.org/10.2471/BLT.11.088435.

[48] S.C. Sarker, M.S. Parvin, A.K.M.A. Rahman, M.T. Islam, Prevalence and risk factors of subclinical mastitis in lactating dairy cows in north and south regions of Bangladesh, Trop. Anim. Health Prod. 45 (2013) 1171–1176, https://doi.org/10.1007/S11250-012-0342-7.

[49] S. Singha, G. Koop, Y. Persson, D. Hossain, L. Scanlon, M. Derks, M.A. Hoque, M.M. Rahman, Incidence, etiology, and risk factors of clinical mastitis in dairy cows under semi-tropical circumstances in chattogram, Bangladesh, Animals 11 (2021), https://doi.org/10.3390/ANI11082255/S1.

[50] M. Kayesh, M. Talukder, A. Anower, Prevalence of subclinical mastitis and its association with bacteria and risk factors in lactating cows of Barisal district in Bangladesh, Int. J. Biol. Res. 2 (2014), https://doi.org/10.14419/IJBR.V2I2.2835.

[51] H.O. Dunn, J.M. Murphy, O.F. Garrett, Nature of the material in milk responsible for the modified Whiteside test for mastitis, J. Dairy Sci. 26 (1943) 295–303, https://doi.org/10.3168/JDS.S0022-0302(43)92721-3.

[52] M.A., M. Rahman, M. Islam, M. Uddin, Prevalence of subclinical mastitis in dairy cows reared in Sylhet district of Bangladesh, Int. J. Biol. Res. 1 (2010) 23–28, et-al.

[53] O.G. Brakstad, K. Aasbakk, J.A. Maeland, Detection of Staphylococcus aureus by polymerase chain reaction amplification of the nuc gene, J. Clin. Microbiol. 30 (1992) 1654–1660, https://doi.org/10.1128/JCM.30.7.1654-1660.1992.

[54] M. Hayati, A. Indrawati, N.L.P.I. Mayasari, I. Istiyaningsih, N. Atikah, Molecular detection of extended-spectrum β-lactamase-producing Klebsiella pneumoniae isolates of chicken origin from East Java, Indonesia, Vet. World 12 (2019) 578, https://doi.org/10.14202/VETWORLD.2019.578-583.

[55] B. Shome, B.R. Shome, S. Das Mitra, M. Bhuvana, N. Krithiga, D. Velu, R. Shome, et al., Multiplex PCR assay for species identification of bovine mastitis pathogens, J. Appl. Microbiol. 111 (2011), https://doi.org/10.1111/j.1365-2672.2011.05169.x, 2011.

[56] C. Dallenne, A. da Costa, D. Decré, C. Favier, G. Arlet, Development of a set of multiplex PCR assays for the detection of genes encoding important β-lactamases in Enterobacteriaceae, J. Antimicrob. Chemother. 65 (2010) 490–495, https://doi.org/10.1093/jac/dkp498.

[57] N.A. Siddiky, S. Sarker, S.R. Khan, T. Rahman, A. Kafi, M.A. Samad, Virulence and antimicrobial resistance profile of non-typhoidal Salmonella enterica serovars recovered from poultry processing environments at wet markets in Dhaka, Bangladesh, PLoS One 17 (2022), https://doi.org/10.1371/JOURNAL.PONE.0254465.

[58] H. Heuer, E. Krögerrecklenfort, E.M.H. Wellington, S. Egan, J.D. Van Elsas, L. Van Overbeek, J.M. Collard, G. Guillaume, A.D. Karagouni, T.L. Nikolakopoulou, K. Smalla, Gentamicin resistance genes in environmental bacteria: prevalence and transfer, FEMS Microbiol. Ecol. 42 (2002) 289–302, https://doi.org/10.1016/S0168-6496(02)00342-2.

[59] G.K. Kozak, P. Boerlin, N. Janecko, R.J. Reid-Smith, C. Jardine, Antimicrobial resistance in escherichia coli isolates from Swine and wild small mammals in the proximity of swine farms and in natural environments in Ontario, Canada, Appl. Environ. Microbiol. 75 (2009) 559–566, https://doi.org/10.1128/AEM.01821-08.

[60] P.L. Preethirani, S. Isloor, S. Sundareshan, V. Nuthanalakshmi, K. Deepthikiran, A.Y. Sinha, D. Rathnamma, K.N. Prabhu, R. Sharada, T.K. Mukkur, N.R. Hegde, Isolation, biochemical and molecular identification, and in-vitro antimicrobial resistance patterns of bacteria isolated from bubaline subclinical mastitis in south India, PLoS One 10 (2015), https://doi.org/10.1371/JOURNAL.PONE.0142717.

[61] R. Hegde, S. Isloor, K.N. Prabhu, B.R. Shome, D. Rathnamma, V.V.S. Suryanarayana, S. Yatiraj, C.R. Prasad, N. Krishnaveni, S. Sundareshan, D.S. Akhila, A. R. Gomes, N.R. Hegde, Incidence of subclinical mastitis and prevalence of major mastitis pathogens in organized farms and unorganized sectors, Indian J. Microbiol. 53 (2013) 315, https://doi.org/10.1007/S12088-012-0336-1.

[62] M.F. Hoque, A.F. Mehedi, E.M.S. Chowdhury, H. Hossain, M. Imranuzzaman, M.N. Islam, E.A. Rana, A. Ahad, Prevalence and antibiotic susceptibility profile of Staphylococcus aureus in clinical and subclinical mastitis milk samples, Bangladesh, J. Vet. Med. 21 (2023), https://doi.org/10.33109/BJVMJJ2023FAM2.

[63] B.R. McConn, A.L. Kraft, L.M. Durso, A.M. Ibekwe, J.G. Frye, J.E. Wells, E.M. Tobey, S. Ritchie, C.F. Williams, K.L. Cook, M. Sharma, An analysis of culture-based methods used for the detection and isolation of Salmonella spp., Escherichia coli, and Enterococcus spp. from surface water: a systematic review, Sci. Total Environ. 927 (2024) 172190, https://doi.org/10.1016/j.scitotenv.2024.172190.

[64] S. Bobbadi, M.N. Bobby, B.K. Chinnam, P.N. Reddy, S. Kandhan, Phenotypic and genetic screening of Klebsiella pneumoniae isolates from human UTI patients for beta-lactamases and their genetic diversity analysis by ERIC and REP PCRs, Braz. J. Microbiol. 54 (2023) 1723, https://doi.org/10.1007/S42770-023-00984-6.

[65] B. Pinto, E. Chenoll, R. Aznar, Identification and typing of food-borne Staphylococcus aureus by PCR-based techniques, Syst. Appl. Microbiol. 28 (2005) 340–352, https://doi.org/10.1016/j.syapm.2005.01.002.

[66] B.R. Shome, S. Das Mitra, M. Bhuvana, N. Krithiga, D. Velu, R. Shome, S. Isloor, S.B. Barbuddhe, H. Rahman, Multiplex PCR assay for species identification of bovine mastitis pathogens, J. Appl. Microbiol. 111 (2011) 1349–1356, https://doi.org/10.1111/J.1365-2672.2011.05169.X.

[67] A.M. Algammal, A. Wahdan, M.M. Elhaig, Potential efficiency of conventional and advanced approaches used to detect Mycobacterium bovis in cattle, Microb. Pathog. 134 (2019), https://doi.org/10.1016/j.micpath.2019.103574.

[68] A.W. Bauer, W.M. Kirby, J.C. Sherris, M. Turck, Antibiotic susceptibility testing by a standardized single disk method, Tech. Bull. Registry Med. Tech. 36 (1966) 49–52.

[69] M.T. Sweeney, B.V. Lubbers, S. Schwarz, J.L. Watts, Applying definitions for multidrug resistance, extensive drug resistance and pandrug resistance to clinically significant livestock and companion animal bacterial pathogens, J. Antimicrob. Chemother. 73 (2018) 1460–1463, https://doi.org/10.1093/JAC/DKY043.

[70] A.P. Magiorakos, A. Srinivasan, R.B. Carey, Y. Carmeli, M.E. Falagas, C.G. Giske, S. Harbarth, J.F. Hindler, G. Kahlmeter, B. Olsson-Liljequist, D.L. Paterson, L. B. Rice, J. Stelling, M.J. Struelens, A. Vatopoulos, J.T. Weber, D.L. Monnet, Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance, Clin. Microbiol. Infection 18 (2012) 268–281, https://doi.org/10.1111/j.1469-0691.2011.03570.x.

[71] Y. Titilawo, T. Sibanda, L. Obi, A. Okoh, Multiple antibiotic resistance indexing of Escherichia coli to identify high-risk sources of faecal contamination of water, Environ. Sci. Pollut. Res. Int. 22 (2015) 10969–10980, https://doi.org/10.1007/S11356-014-3887-3.

[72] R. Mir, S. Salari, M. Najimi, A. Rashki, Determination of frequency, multiple antibiotic resistance index and resistotype of Salmonella spp. in chicken meat collected from southeast of Iran, Vet. Med. Sci. 8 (2022) 229–236, https://doi.org/10.1002/VMS3.647.

[73] P.H. Krumperman, Multiple antibiotic resistance indexing of Escherichia coli to identify high-risk sources of fecal contamination of foods, Appl. Environ. Microbiol. 46 (1983) 165–170, https://doi.org/10.1128/aem.46.1.165-170.1983.

[74] M.N. Asha, M.S.R. Chowdhury, H. Hossain, M.A. Rahman, A. Al Emon, F.Y. Tanni, M.R. Islam, M.M. Hossain, M.M. Rahman, Antibacterial potential of lactic acid bacteria isolated from raw cow milk in Sylhet district, Bangladesh: a molecular approach, Vet. Med. Sci. 10 (2024), https://doi.org/10.1002/VMS3.1463.

[75] M.S.K. Mahen, M.S.R. Chowdhury, H. Hossain, M.M. Hossain, M.R. Islam, M.M. Rahman, Investigating the infection dynamics and molecular detection of Cryptosporidium in Buffaloes in Sylhet, Bangladesh, Vet Parasitol Reg Stud Reports 52 (2024) 101043, https://doi.org/10.1016/j.vprsr.2024.101043.

[76] J. Al Naser, H. Hossain, M.S.R. Chowdhury, N.A. Liza, R.M. Lasker, A. Rahman, M.A. Haque, M.M. Hossain, M.M. Rahman, Exploring of spectrum beta lactamase producing multidrug-resistant Salmonella enterica serovars in goat meat markets of Bangladesh, Vet Anim Sci 25 (2024) 100367, https://doi.org/10.1016/j.vas.2024.100367.

[77] M. Islam, M. Islam, M. Islam, M. Rahman, M. Islam, Prevalence of subclinical mastitis in dairy COWS IN selected areas of Bangladesh, Bangladesh J. Vet. Med. 9 (2011) 73–78, https://doi.org/10.3329/BJVM.V9I1.11216.

[78] S. Singha, G. Koop, Y. Persson, D. Hossain, L. Scanlon, M. Derks, M.A. Hoque, M.M. Rahman, Incidence, etiology, and risk factors of clinical mastitis in dairy cows under semi-tropical circumstances in chattogram, Bangladesh, Animals 11 (2021) 2255, https://doi.org/10.3390/ANI11082255/S1.

[79] M. Das, A. Barman, S. Akter, S.R. Chowdhury, M. Rahman, Prevalence of subclinical mastitis and physicochemical properties of milk sample in dairy cows of Sylhet district, Int. J. Nat. Sci. 10 (2020) 30–36.

[80] M.S. Bari, M.M. Rahman, Y. Persson, M. Derks, M.A. Sayeed, D. Hossain, S. Singha, M.A. Hoque, S. Sivaraman, P. Fernando, I. Ahmad, A. Samad, G. Koop, Subclinical mastitis in dairy cows in south-Asian countries: a review of risk factors and etiology to prioritize control measures, Vet. Res. Commun. 46 (2022) 621–640, https://doi.org/10.1007/S11259-022-09948-X.

[81] S. Bhandari, D. Subedi, B.B. Tiwari, P. Shrestha, S. Shah, A.I. Al-Mustapha, Prevalence and risk factors for multidrug-resistant Escherichia coli isolated from subclinical mastitis in the western Chitwan region of Nepal, J. Dairy Sci. 104 (2021) 12765–12772, https://doi.org/10.3168/jds.2020-19480.

[82] E.J. Sarba, W. Wirtu, E.Z. Gebremedhin, B.M. Borena, L.M. Marami, Occurrence and antimicrobial susceptibility patterns of Escherichia coli and Escherichia coli O157 isolated from cow milk and milk products, Ethiopia, Sci. Rep. 13 (2023), https://doi.org/10.1038/s41598-023-43043-8.

[83] A.S. Neculai-Valeanu, A.M. Ariton, Udder health monitoring for prevention of bovine mastitis and improvement of milk quality, Bioengineering 9 (2022), https://doi.org/10.3390/BIOENGINEERING9110608.

[84] X. Li, F. Fang, J. Zhao, N. Lou, C. Li, T. Huang, Y. Li, Molecular characteristics and virulence gene profiles of Staphylococcus aureus causing bloodstream infection, Braz. J. Infect. Dis. 22 (2018) 487–494, https://doi.org/10.1016/J.BJID.2018.12.001.

[85] X. Wu, J. Liu, J. Feng, M.A.B. Shabbir, Y. Feng, R. Guo, M. Zhou, S. Hou, G. Wang, H. Hao, G. Cheng, Y. Wang, Epidemiology, environmental risks, virulence, and resistance determinants of Klebsiella pneumoniae from dairy cows in hubei, China, Front. Microbiol. 13 (2022) 1–14, https://doi.org/10.3389/fmicb.2022.858799.

[86] J. Cheng, M. Zhou, D.B. Nobrega, H.W. Barkema, S. Xu, M. Li, J.P. Kastelic, Y. Shi, B. Han, J. Gao, Genetic diversity and molecular epidemiology of outbreaks of Klebsiella pneumoniae mastitis on two large Chinese dairy farms, J. Dairy Sci. 104 (2021) 762–775, https://doi.org/10.3168/jds.2020-19325.

[87] A. Selim, K. Kelis, M.D.F. AlKahtani, F.M. Albohairy, K.A. Attia, Prevalence, antimicrobial susceptibilities and risk factors of Methicillin resistant Staphylococcus aureus (MRSA) in dairy bovines, BMC Vet. Res. 18 (2022), https://doi.org/10.1186/s12917-022-03389-z.

[88] F. Adzitey, N. Huda, A.H.M. Shariff, Phenotypic antimicrobial susceptibility of escherichia coli from raw meats, ready-to-eat meats, and their related samples in one health context, Microorganisms 9 (2021) 1–11, https://doi.org/10.3390/microorganisms9020326.

[89] O. Adenaike, O.S. Olonitola, Ameh, C.M.Z. Whong, Multidrug resistance and multiple antibiotic resistance index of Escherichia coli strains isolated from retailed smoked fish, J. Nat. Sci. Res. 6 (2016) 7–10.

[90] S. Saddam, M. Khan, M. Jamal, S.U. Rehman, P. Slama, P. Horky, Multidrug resistant Klebsiella Pneumoniae reservoir and their capsular resistance genes in cow farms of district Peshawar, Pakistan, PLoS One 18 (2023), https://doi.org/10.1371/journal.pone.0282245.

[91] H.M. Eid, H.S. El-Mahallawy, S.M. Roshdi, N.E.Y. Mohammed, N.H. Eidaroos, Multidrug-resistant and enterotoxigenic methicillin-resistant Staphylococcus aureus isolated from raw milk of cows at small-scale production units, J Adv Vet Anim Res 9 (2022) 113, https://doi.org/10.5455/JAVAR.2022.I575.

[92] M.A.S. Bag, M.S.R. Khan, M.D.H. Sami, F. Begum, M.S. Islam, M.M. Rahman, M.T. Rahman, J. Hassan, Virulence determinants and antimicrobial resistance of E. coli isolated from bovine clinical mastitis in some selected dairy farms of Bangladesh, Saudi J. Biol. Sci. 28 (2021) 6317–6323, https://doi.org/10.1016/j.sjbs.2021.06.099.

[93] L. Suojala, T. Pohjanvirta, H. Simojoki, A.L. Myllyniemi, A. Pitkälä, S. Pelkonen, S. Pyörälä, Phylogeny, virulence factors and antimicrobial susceptibility of Escherichia coli isolated in clinical bovine mastitis, Vet. Microbiol. 147 (2011) 383–388, https://doi.org/10.1016/J.VETMIC.2010.07.011.

[94] D. Kasew, B. Desalegn, M. Aynalem, S. Tila, D. Diriba, B. Afework, M. Getie, S. Biset, H.W. Baynes, Antimicrobial resistance trend of bacterial uropathogens at the university of Gondar comprehensive specialized hospital, northwest Ethiopia: a 10 years retrospective study, PLoS One 17 (2022), https://doi.org/10.1371/JOURNAL.PONE.0266878.

[95] P. Kalmus, B. Aasmäe, A. Kärssin, T. Orro, K. Kask, Udder pathogens and their resistance to antimicrobial agents in dairy cows in Estonia, Acta Vet. Scand. 53 (2011), https://doi.org/10.1186/1751-0147-53-4.

[96] P.J. Rajala-Schultz, A.H. Torres, F.J. DeGraves, W.A. Gebreyes, P. Patchanee, Antimicrobial resistance and genotypic characterization of coagulase-negative staphylococci over the dry period, Vet. Microbiol. 134 (2009) 55–64, https://doi.org/10.1016/J.VETMIC.2008.09.008.

[97] E. Gentilini, G. Denamiel, A. Betancor, M. Rebuelto, M. Rodriguez Fermepin, R.A. De Torres, Antimicrobial susceptibility of coagulase-negative staphylococci isolated from bovine mastitis in Argentina, J. Dairy Sci. 85 (2002) 1913–1917, https://doi.org/10.3168/JDS.S0022-0302(02)74267-7.

[98] É.J. Kaszanyitzky, S. Jánosi, Z. Egyed, G. Ágost, G. Semjén, Antibiotic resistance of staphylococci from humans, food and different animal species according to data of the Hungarian resistance monitoring system in 2001, Acta Vet. Hung. 51 (2003) 451–464, https://doi.org/10.1556/AVET.51.2003.4.3.
[99] S. Saddam, M. Khan, M. Jamal, S.U. Rehman, P. Slama, P. Horky, Multidrug resistant Klebsiella Pneumoniae reservoir and their capsular resistance genes in cow farms of district Peshawar, Pakistan, PLoS One 18 (2023), https://doi.org/10.1371/journal.pone.0282245.
[100] W. Cao, Y. Xu, Y. Huang, T. Xu, Isolation of pathogenic bacteria from dairy cow mastitis and correlation of biofilm formation and drug resistance of Klebsiella pneumoniae in jiangsu, China, Agriculture (Switzerland) 13 (2023), https://doi.org/10.3390/agriculture13101984.