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Emergence of highly virulent multidrug and extensively drug resistant *Escherichia coli* and *Klebsiella pneumoniae* in buffalo subclinical mastitis cases

Md. Shahidur Rahman Chowdhury[1], Hemayet Hossain[2], Mohammad Nahian Rahman[1], Asikur Rahman[1], Piash Kumar Ghosh[1], Md Bashir Uddin[1], M. Nazmul Hoque[3], Md. Mukter Hossain[1] & Md. Mahfujur Rahman[1]✉

This study aimed to characterize virulence and antibiotic resistance genes in multidrug-resistant (MDR) and extensively drug-resistant (XDR) *Escherichia coli* and *Klebsiella pneumoniae* isolated from cases of subclinical mastitis (SCM) in buffaloes. A cross-sectional study was conducted on 1540 quarter milk samples collected from 385 buffaloes. Milk samples were screened using the California Mastitis Test and Modified Whiteside Test. Positive samples underwent bacterial culture, biochemical tests, biofilm detection and molecular analysis for pathogen identification and detection of virulence, resistance, and extended-spectrum beta-lactamase (ESBL) genes. The prevalence of SCM was 67.9% (1046/1540) at the quarter level and 80.8% (311/385) at the animal level. *E. coli* was identified in 9.5% (146/1540) of the samples, while *K. pneumoniae* was detected in 9.09% (140/1540). Virulence genes, such as *stx1* (27.4%), and resistance genes, including *aac(3)*-iv (77.4%) and *tetA* (76.7%), exhibited higher prevalence. Additionally, *β*-lactamase genes, notably bla_{TEM} (67.1%), and ESBL genes, such as $bla_{CTX\text{-}M1}$, were detected. Biofilm formation was detected in 83.6% (122/146) of *E. coli* isolates and 75.7% (106/140) of *K. pneumoniae* isolates. Antimicrobial susceptibility testing revealed significant resistance to ampicillin, amoxicillin-clavulanic acid, and aminoglycosides. MDR was observed in 31.5% of *E. coli* and 39.3% of *K. pneumoniae* isolates, with XDR rates of 8.9% and 12.9%, respectively. These findings underscore the alarming spread of resistant pathogens in SCM-affected buffaloes, emphasizing the urgent need for ongoing surveillance and targeted intervention strategies.

Milk and dairy products are crucial protein sources in lower middleincome ountries (LMICs). Buffaloes are the world's second-largest producer, accounting for 12% of global milk production, mainly from India (53%) and Pakistan (68%)[1,2]. In Bangladesh, the buffalo population stands at approximately 1.464 million, managed predominantly through household subsistence systems and extensive free-range (Bathan) grazing practices[3]. The nation's total buffalo milk production is estimated at 7.27 million metric tons per year[4]. However, buffalo milk is more expensive than cow milk because it contains more fat (6.0–8.5%) and nutrition[3,4]. Although cows produce over 90% of the milk in Bangladesh, persistent shortages have led to the promotion of buffaloes as an additional milk source[5,6]. However, subclinical mastitis (SCM) remains a major constraint, contributing to the decline in per capita milk production in the dairy sector[2]. Although SCM is 15–40 times more common than clinical mastitis (CM), it is more difficult to identify because of subtle changes in milk, which results in significant decreases in milk supply[7,8]. As determined by the CMT, the prevalence of SCM in Bangladesh ranges from 20 to 44% in cows and up to 81.6% in buffaloes[9,10]. It is highly prevalent in the dairy industry and causes substantial financial losses of $2.11 million USD yearly. It is caused mostly by contagious and environmental pathogens such as *Staphylococcus aureus, Streptococcus* spp., *Escherichia coli, and Klebsiella* spp.[9,11–13].

[1]Department of Medicine, Sylhet Agricultural University, Sylhet 3100, Bangladesh. [2]Department of Anatomy and Histology, Sylhet Agricultural University, Sylhet 3100, Bangladesh. [3]Department of Gynecology, Obstetrics and Reproductive Health, Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur 1706, Bangladesh. ✉email: mahfuj.vetmed@sau.ac.bd

In addition, rise of bacterial resistance poses a growing threat as resistance mechanism continue to spread globally[14]. The rise of multidrug-resistant (MDR) bacterial infections from various sources has been documented in a number of recent studies, which highlights the importance of using antibiotics appropriately. In addition to screening for emerging MDR strains, antimicrobial susceptibility testing should routinely use to identify the preferred antibiotics[15].

E. coli is the major cause of mastitis, leading to subclinical and clinical form of mastitis[16]. Shiga toxin (*stx1*, *stx2*) and MDR extended-spectrum *β*-lactamase (ESBL)-producing *E. coli* is among the most significant virulence factors identified in *E. coli* strains isolated from mastitic milk in buffaloes[17–19]. STEC virulence factors include Shiga toxins (*stx1* and *stx2*), which drive disease severity, intimin (*eae*), which aids host cell attachment, and hemolysin (*hlyA*), which promotes cell lysis and invasion[20]. Hemolytic uremia and hemorrhagic colitis are the two primary conditions linked to STEC infection in humans. Given that STEC has been implicated in a number of food-borne epidemics, it is of significant public health relevance[21]. Over the years, MDR *E. coli* strains have been consistently isolated from cases of SCM in dairy animals, underscoring a persistent issue. These MDR strains represent substantial public health hazards, particularly when raw milk is consumed without pasteurization[22].

Klebsiella spp. also play a significant role in mastitis, particularly *K. pneumoniae* and *K. oxytoca*, which are prominent species that cause mastitis in both environmental and host contexts, resulting in substantial global economic impacts[23,24]. Many strains of *K. pneumoniae* possess virulence factors that increase pathogenicity by promoting infection, increasing bacterial fitness, and facilitating the evasion of host immune responses. In addition, the presence of ESBL-producing MDR *Klebsiella* spp. in SCM is an emerging concern, and their presence in dairy herds can complicate treatment strategies and has significant implications for animal health, milk safety, and public health because of the potential for transmission of resistant bacteria[25]. Biofilms are structured communities of bacteria encased in a self-produced extracellular polymeric matrix composed of polysaccharides, proteins, and DNA[26]. Biofilm formation enables bacteria to withstand harsh conditions and resist antibacterial agents. Both *E. coli* and *K. pneumoniae* have the potential to produce biofilms, which enhance their protection against potent antibiotics and contribute to drug resistance. The key genes associated with biofilm formation include *lasR, lecA*, and *pelA* in *E. coli,* while *mrkA* and *mrkD* play a crucial role in *K. pneumoniae*[26].

Additionally, antibiotic resistance, often resulting from inconsistent antibiotic use, can reduce treatment efficacy[27]. Antibiotics are commonly used in dry cow therapy and to treat CM or SCM caused by bacterial pathogens. Given that culling animals to prevent disease spread is economically unfeasible, evaluating the antibiogram profile of bacterial strains is crucial for developing effective treatments[2]. For example, previous studies employing the disk diffusion method revealed that Shiga toxin-producing *E. coli* (STEC) serogroups isolated from mastitic milk presented the highest resistance to penicillin (100%), followed by tetracycline (57.44–92.2%), streptomycin (48.93–90.4%), nalidixic acid (88.3%), amikacin (86.5%), cephalothin (84.8%), ampicillin (46.80%), and sulfamethoxazole (40.42%). Additionally, 65.8% of the isolates were found to be MDR[28,29]. On the other hand, *Klebsiella* spp. presented the highest resistance to ampicillin (90–100%), followed by gentamicin (10–30%), tetracycline (60–80%), and trimethoprim-sulfamethoxazole (30–50%), and the lowest resistance to chloramphenicol (5–15%) and enrofloxacin (5–20%)[30–32]. Notably, most research has predominantly focused on cows and contagious pathogens, with limited studies on SCM in buffaloes caused by environmental pathogens like *E. coli* and *K. pneumoniae* which exhibit pathogenic effects similar to those of contagious mastitogens. Based on this background, the study aimed to assess the prevalence of *E. coli* and *K. pneumoniae* in milk samples from buffaloes with subclinical mastitis in Bangladesh, also focusing on their antimicrobial resistance patterns especially MDR/XDR pattern and virulence genes.

Methods

Ethical consideration

The study was approved by the Institutional Ethics Committee of Sylhet Agricultural University, Sylhet-3100, Bangladesh, under animal use protocol number #AUP2023001. All experimental procedures were conducted by trained professionals in strict compliance with the university's ethical guidelines and regulations. The welfare and well-being of all animals involved in the study were prioritized and carefully maintained throughout the research.

Study design, location and sampling strategy

A cross-sectional investigation was conducted in five predominantly buffalo populated Upazilas in Sylhet district of Bangladesh, namely, Jaintapur, Gowainghat, Kanaighat, Balaganj, and Fenchuganj. These regions are situated within geographic coordinates of approximately 24°36' to 25°11' North latitude and 91°38' to 92°30' East longitude, as depicted in Fig. 1. The study population required to estimate prevalence was calculated via a standard equation[33,34].

$$n = \frac{Z^2 \times Pexp \times (1 - Pexp)}{d^2}$$

where n = Desired sample size; Z = 1.96 for the 95% confidence interval; P_{exp} = 0.5, Expected prevalence (50%); d = 0.05, Desired absolute precision (5%).

On the basis of the calculations, to determine the prevalence of SCM, milk samples from 384 buffaloes (1536 quarter milk samples) were needed. With the goal of ascertaining the prevalence at the quarter and animal levels, 1540 quarter milk samples were collected from 385 swamp buffaloes. A random-cluster sampling technique was employed to accumulate the samples between February 2023 and June 2024.

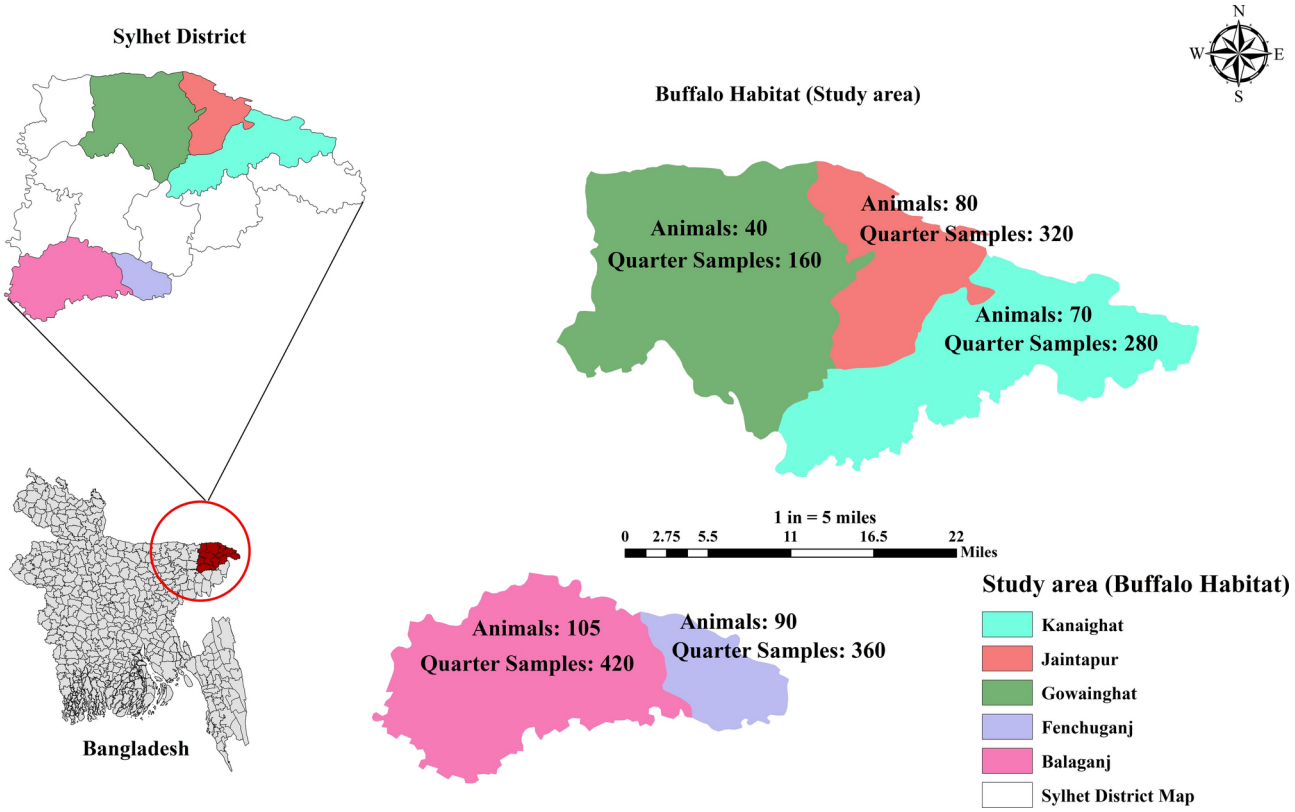

Fig. 1. Map of the study area in Sylhet district, Bangladesh, illustrating the locations of buffalo habitats, study sites, and the number of samples collected at both the animal and quarter levels. The map was generated using ArcMap 10.8 software.

Initial screening of the SCM

The milk samples were collected aseptically from each quarter of apparently healthy buffaloes, in accordance with the guidelines of National Mastitis Council (NMC)[35]. The modified Whiteside test (MWST), delineated by Emon et al.[36] and the California Mastitis Test (CMT)[37] were applied as preliminary screening methods for detecting SCM[38]. Following aseptic milk collection, the samples were mixed on a CMT paddle with reagent at an established ratio. The mixture was then gently swirled, and the reaction was monitored for changes in consistency. The milk was graded from strong (Grade 3+), which was defined by a fairly thick consistency and pronounced gel formation, to negative (Grade 0), where there was no change in viscosity and the milk remained liquid. Varying levels of thickening and gel formation/coagulation of SCM-positive milk were indicated by intermediate grades, which included a thick consistency with pronounced gel formation (grade 2+), noticeable thickening with slight coagulation (grade 1+), and slight thickening with no gel formation (graded as trace)[39]. Pre-enrichment of the predominantly positive milk samples was carried out in Trypticase Soy Broth (HiMedia Laboratories Pvt. Ltd., Mumbai, India) at a 1:10 dilution. The cultures were then incubated for approximately 24 h at 37 °C.

Isolation and identification of pathogens

The isolation and identification of *E. coli* was performed via the use of Eosin Methylene Blue (EMB) agar plates (HiMedia Laboratories Pvt. Ltd., Mumbai, India) following the guidelines and procedures of the NMC, USA. The agar plate was incubated at 37 °C for 18 to 24 h[40]. Further confirmation was achieved through biochemical assays[41], including Gram staining, catalase, coagulase, citrate, motility, indole, gas production, methyl red, urease, and triple sugar iron tests[42].

Klebsiella spp. *(K. pneumomiae, K. oxytoca)* were isolated and identified via McConkey agar medium (HiMedia Laboratories Pvt. Ltd., Mumbai, India). The plates were incubated at 37 °C for 24 to 48 h[26]. Confirmation was performed using biochemical tests, including Gram staining, catalase, coagulase, citrate, motility, indole, gas production, methyl red, urease, and triple sugar iron tests[43]. Following these analyses, positive samples were prepared for genomic DNA extraction and polymerase chain reaction (PCR).

Genomic DNA extraction

The well-established boiling method was utilized to extract genomic DNA from *E. coli* and several *Klebsiella* spp. as previously described by Aldous et al.[44]. The purity and concentration of the extracted DNA were evaluated using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). The monoplex PCR assays targeted the *alr, pehX, stx1*, and *stx2* genes for detection, along with *aac(3)-IV* and *sul1*, which confer

resistance to aminoglycosides and sulfonamides, respectively. The multiplex PCR assays focused on the *gyrA, rpoB,* $bla_{TEM,}$ $bla_{SHV,}$ $bla_{OXA,}$ $bla_{CTX\text{-}M\text{-}grp1,}$ $bla_{CTX\text{-}M\text{-}grp2,}$ $bla_{CTX\text{-}M\text{-}grp9,}$ $Multicase_{ACC,}$ $Multicase_{MOX,}$ $Multicase_{DHA}$, *tetA*, and *strA* genes.

Molecular detection of pathogens and resistance genes

The molecular detection of *E. coli, Klebsiella* spp. and antibiotic resistance genes (ARGs) was conducted using different PCR techniques. For the detection of *E. coli* and *K. oxytoca*, monoplex PCR was used to target the *alr* and *pehX* genes, respectively, utilizing reagents from Addbio Inc. (Daejeon, South Korea). Similarly, resistance to gentamicin and sulfonamides was assessed by monoplex PCR amplification of the *aac(3)-iv* and *sul1* genes, respectively. Additionally, multiplex PCR assays were executed to amplify specific genes for the presence of the *Klebsiella* genus (*gyrA*), *K. pneumoniae (rpoB)*, Shiga toxin-producing *E. coli* (*stx1* and *stx2*), and various ESBL genes, including $bla_{TEM,}$ $bla_{SHV,}$ $bla_{OXA,}$ $bla_{CTX\text{-}M\text{-}grp1,}$ $bla_{CTX\text{-}M\text{-}grp2,}$ $bla_{CTX\text{-}M\text{-}grp9,}$ $MultiCase_{ACC,}$ $MultiCase_{MOX,}$ and $MultiCase_{DHA}$, along with the *tetA* and *strA* genes for tetracycline and streptomycin resistance, respectively. The composition of the PCR mixture and amplification conditions for these monoplex and multiplex assays are detailed in Supplementary Table S1. All amplified products were verified through gel electrophoresis on a 1.8% or 1.5% low-melting agarose gel, and a 100 bp plus ladder was used for size verification. The primer sequences for all the targeted genes are outlined in Supplementary Table S2.

Qualitative detection of biofilm producer

Congo red agar (CRA) method

Following the procedure outlined by Freeman et al.[45], the Congo Red Agar (CRA) method was used to evaluate the bacterial isolates' capacity to form biofilms. Brain Heart Infusion (BHI) agar was supplemented with 0.8 g/L Congo Red and 36 g/L sucrose to create the CRA media (HiMedia Laboratories Pvt. Ltd., Mumbai, India). After being streaked onto the CRA plates, the bacterial isolates were cultured for 24 to 48 h at 37 °C in an aerobic environment. Colony morphology was used to determine biofilm production: colonies that were crystalline, dry, and black were categorized as strong biofilm producers, whereas those that were smooth or red were categorized as weak or non-biofilm producers.

Crystal violet microtiter plate (CVMP) assay

The biofilm-forming ability of bacterial isolates was assessed using the Crystal Violet Microtiter Plate (CVMP) assay following the protocol of Kouidhi et al.[46]. Overnight bacterial cultures were adjusted to 0.5 McFarland standard and diluted 1:100 in Tryptic Soy Broth (TSB) supplemented with 1% glucose to promote biofilm formation. A 96-well flat-bottom microtiter plate was used, where 200 µL of the diluted bacterial suspension was inoculated into each test well. Negative control wells contained only sterile TSB with 1% glucose. The plate was incubated at 37 °C for 24 h under static conditions. Following incubation, non-adherent cells were removed by gently washing three times with phosphate-buffered saline (PBS, pH 7.2). The adhered biofilm was then stained with 0.1% crystal violet for 15 min at room temperature. Excess stain was removed by washing the wells three times with sterile distilled water, and the bound dye was solubilized using 95% ethanol. Finally, the absorbance was measured at 570 nm (OD570) using a microplate reader, and biofilm production was classified based on OD570 values, with non-biofilm ($OD \leq 0.2$), weak ($0.2 < OD \leq 0.4$), moderate ($0.4 < OD \leq 0.6$), and strong ($OD > 0.6$) biofilm producers determined accordingly.

Antimicrobial susceptibility testing

The Kirby–Bauer disk diffusion method was used to perform antimicrobial susceptibility testing (AST) on Mueller–Hinton agar (HiMedia Laboratories Pvt. Ltd., Mumbai, India) plates in accordance with the guidelines established by the Clinical and Laboratory Standards Institute (CLSI)[47]. Thirteen antibiotics, belonging to eight different antimicrobial classes, included the following: penicillin (ampicillin 10 µg, amoxicillin-clavulanic acid 20/10 µg), tetracycline (tetracycline 30 µg), macrolides (azithromycin 15 µg), quinolones (ciprofloxacin 5 µg, nalidixic acid 30 µg), folate pathway antagonists (trimethoprim-sulfamethoxazole 1.25/23.75 µg), phenicol (chloramphenicol 30 µg), cephalosporins (cefoxitin 30 µg, ceftriaxone 30 µg), and aminoglycosides (gentamicin 10 µg, amikacin 30 µg, streptomycin 10 µg). A bacterial suspension was prepared by selecting 3–5 colonies from a fresh overnight culture and suspending them in normal saline to match the 0.5 McFarland turbidity standard ($\sim 1.5 \times 10^8$ CFU/mL). The 0.5 McFarland turbidity standard was prepared using the protocol followed by[48]. The suspension was then evenly streaked across the entire surface of a Mueller–Hinton agar (MHA) plate using a sterile cotton swab. After allowing the plate to dry for 3–5 min, antibiotic discs were placed, maintaining a minimum center-to-center distance of 24 mm. The plate was then incubated at 37 °C for 16–18 h. Finally, the diameter of the inhibition zone was measured in millimeters using a ruler compared to the CLSI breakpoints. Each assay was conducted in triplicate to ensure accuracy and reproducibility.

Evaluation of MAR index, MDR, and XDR patterns in bacterial isolates

The multiple antibiotic resistance (MAR) index was obtained using the following formula outlined by Naser et al.[33]. MAR = (number of antibiotics exhibiting resistance by an isolate)/ (total number of antibiotics subjected to testing). The MAR index values ranged from 0 to 1, with values closer to 0 denoting increased sensitivity and those close to 1 denoting strong resistance. A high-risk reservoir of bacterial contamination or a significant degree of resistance was indicated by a MAR value of 0.20 or above. Furthermore, MDR is defined as nonsusceptibility to at least one agent in three antimicrobial categories or three classes of antibiotic whereas nonsusceptibility to at least one agent in all but 2 or fewer antimicrobial categories is termed as XDR[49].

Statistical analysis

Excel spreadsheets were used for meticulous compilation, organization, and structuring of the accumulated data. A chi-square test was conducted to investigate associations among diverse explanatory variables through univariate analysis. Using the binomial exact test, confidence intervals were computed, with a significance threshold set at $p < 0.05$. SPSS version 26 (SPSS, Chicago, IL) was used to perform all the statistical analyses.

Geospatial mapping and plotting

ArcMap 10.8 (ArcMap 10.8, Esri, USA), with a shapefile sourced from www.diva-gis.org, was used to map the study area. The creation of a dot map effectively visualizes the sample cluster of buffalo habitat in Sylhet, Bangladesh. We generated the plot using GraphPad Prism 8.4.

Results

Phenotypic characteristics of the recovered pathogens

The recovered *E. coli* isolates exhibited circular, opaque colonies with a characteristic green metallic sheen on EMB agar. In Gram staining, they appeared as Gram-negative, rod-shaped (bacilli) bacteria, displaying a pink coloration. Biochemically, *E. coli* was catalase-positive, coagulase-negative, citrate-negative, indole-positive, methyl red-positive, urease-negative, and demonstrated positive fermentation for both glucose and lactose in the TSI test.

For *Klebsiella* spp., the colonies appeared as mucoid, sticky, pink colonies on MacConkey agar. Biochemically, they were Gram-negative, catalase-positive, coagulase-negative, citrate-positive, indole-negative, gas production-positive, methyl red-negative, and urease-positive.

Animal and quarter-level prevalence of SCM

At the animal and quarter levels, there was significant geographical variation in the occurrence of SCM (Fig. 2). The high pathogen burden and regional variation within the study area were highlighted by the quarter-level prevalence, which was 67.9% (95% CI 65.5–70.3%), and the overall animal-level prevalence across all areas, which was 80.8% (95% CI 76.5–84.6%).

Prevalence patterns of *E. coli* and *K. pneumoniae*

A bacteriological examination of 1,540 quarter milk samples collected from buffaloes revealed an overall *E. coli* prevalence of 9.5% (n = 146) and *Klebsiella* spp. of 21.5% (n = 332) based on molecular tests (Table 1). The prevalence of *E. coli* and *Klebsiella* spp. was assessed using culture-biochemical assays and PCR across

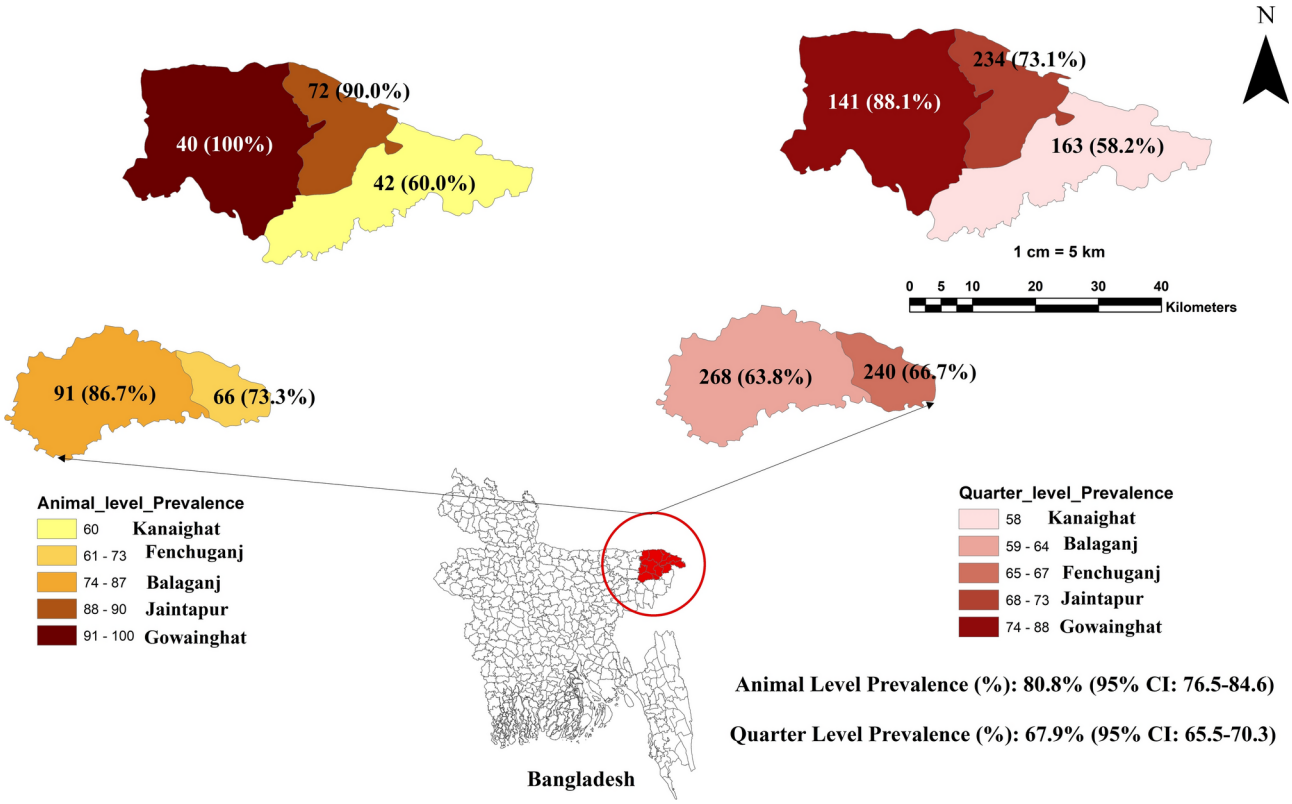

Fig. 2. Choropleth map depicting the prevalence of subclinical mastitis of buffalo at both the animal and quarter levels across various locations in Sylhet district, Bangladesh. The map was created using ArcMap 10.8 software.

Quarter	Screening Test (n, %)				PCR			
	MWST	CMT	C & B for *E. coli*	C & B for *Klebsiella* spp.	*E. coli*	*Klebsiella* spp.	*Klebsiella pneumoniae*	Mixed infection
LF	272 (70.6)	270 (70.1)	40 (10.4)	131 (34.0)	32 (8.3)	96 (24.9)	58 (60.4)	12 (9.4)
LR	260 (67.5)	260 (67.5)	43 (11.2)	139 (36.1)	39 (10.1)	105 (27.2)	24 (22.9)	9 (6.3)
RF	254 (66.0)	254 (66.0)	54 (14.0)	96 (24.9)	54 (14.0)	71 (18.4)	38 (53.5)	10 (8.0)
RR	262 (68.1)	262 (68.1)	25 (6.5)	143(37.1)	21 (5.5)	60 (15.6)	20 (33.3)	7 (8.6)
Total	1048 (68.1)	1046 (67.9)	162 (10.5)	509 (33.1)	146 (9.5)	332 (21.5)	140 (42.2)	38 (7.9)

Table 1. Percentages of *E. coli* and *K. pneumoniae* isolates identified through different methods and from various quarters of buffalo in Sylhet district, Bangladesh. The table included data on the outcomes of the Modified White Side Test (MWST) and California Mastitis Test (CMT) as screening methods, cultural and biochemical (C & B) identification for *E. coli* and *Klebsiella* spp., and PCR-based confirmation for *E. coli*, *Klebsiella* spp., *Klebsiella pneumoniae*, and mixed infections across left fore (LF), left rear (LR), right fore (RF), and right rear (RR) quarters of buffalo. Total percentages were provided for each category.

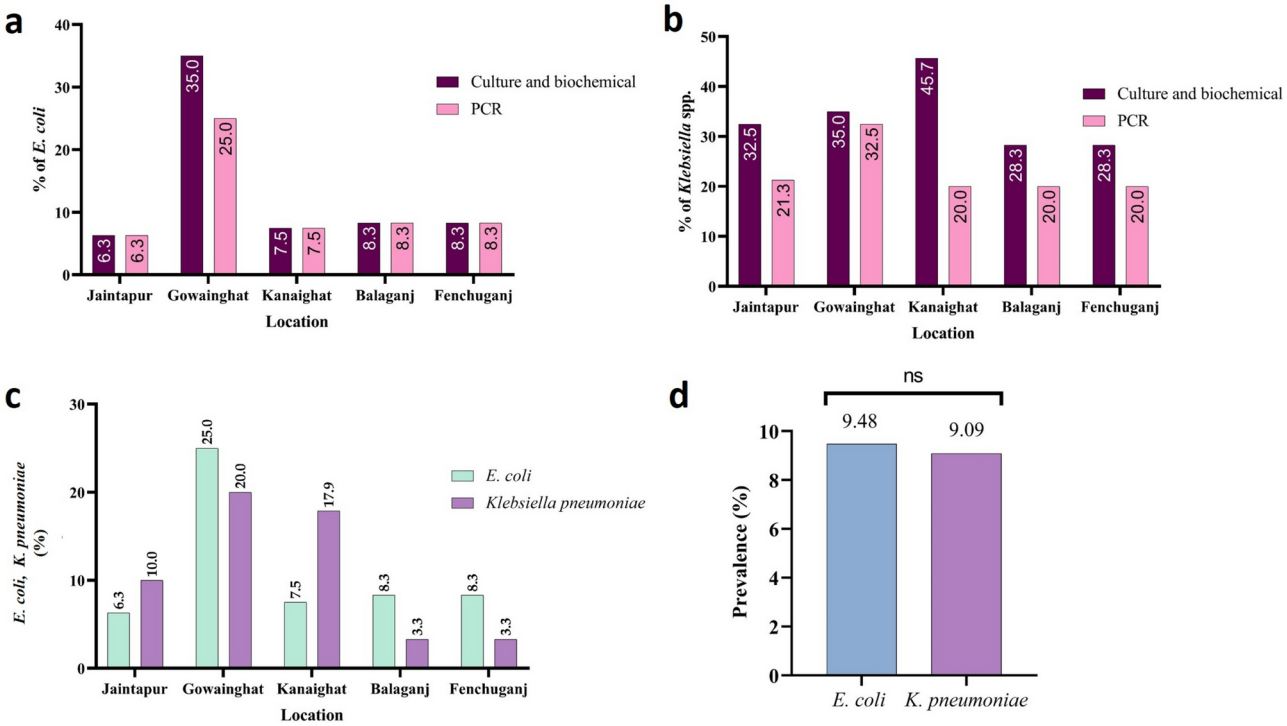

Fig. 3. The prevalence of mastitogens causing subclinical mastitis in buffalo across various geographical locations in Sylhet district, Bangladesh. (**a**) The prevalence (%) of *E. coli* in different locations, (**b**) The prevalence (%) of *Klebsiella* spp. in these areas; (**c**) Comparison of the prevalence between *E. coli* and *K. pneumoniae* across the locations; (**d**) The overall prevalence (%) of *E. coli* and *K. pneumoniae* combined.

five distinct habitats in Sylhet (Fig. 3a, b). Gowainghat had the highest prevalence of *E. coli* (35% by culture/biochemical methods), indicating a significant bacterial presence in this area, while other regions displayed relatively lower prevalence levels (6.3%–8.3%). Conversely, the prevalence of *Klebsiella* spp. was highest in Kanaighat (45.7% culture), followed by Gowainghat (35%) and Jaintapur (32.5%), suggesting favorable local conditions. Gowainghat was detected as a pathogen hotspot, with a molecular prevalence of 25% for *E. coli* and a 20% for *K. pneumoniae* (Fig. 3c). Kanaighat presented a significant *K. pneumoniae* prevalence (17.9%), whereas Balaganj and Fenchuganj presented minimal pathogen presence (3.3% each), suggesting lower bacterial pressure. An aggregate view of both pathogens was shown, with *E. coli* being slightly more prevalent (9.48%) than *K. pneumoniae* (9.09%), as illustrated in Fig. 3d. This study did not find any *K. oxytoca* in SCM-positive milk samples.

Prevalence based on tests and individual quarters

The results of initial screening and PCR testing across four quarters (*e.g.*, left front; LF, left rear; LR, right front; RF, and right rear; RR) for the prevalence of *E. coli* and *Klebsiella* spp. are detailed in Table 1. The average positive rates for the MWST and CMT tests were 68.1% and 67.9%, respectively. Both the primary isolation (culture/

biochemical) and PCR assays revealed that *Klebsiella* spp. were more common than *E. coli*, accounting for 33.1% of the total samples. *K. pneumoniae* was identified in 42.2% of the *Klebsiella* spp. positive samples, with the LF quarter showing the highest percentage at 60.4%. *E. coli* detection was lower at 9.5%, with the RF quarter having the highest PCR positivity at 14.0%. A total of 7.9% of the samples had mixed infections, with LF having the highest rate at 9.4%.

Prevalence of virulence, antimicrobial resistance and ESBL-encoding genes

The prevalence of virulence and AMR genes was notably high in *E. coli* and *Klebsiella* spp. isolates obtained from various geographic locations, including Jaintapur, Gowainghat, Kanaighat, Balaganj, and Fenchuganj (Fig. 4). The *stx1* gene was detected in 27.4% of the *E. coli* isolates (Fig. 4a). Surprisingly, *stx2* was not identified, indicating that *stx1*-mediated virulence dominated in these isolates. Among the AMR genes, the aminoglycoside resistance gene *aac(3)-iv* was the most prevalent, found in 77.4% of the isolates. This was closely followed by the tetracycline resistance gene *tetA*, which occurred in 76.7% of the isolates. The sulfonamide resistance gene *sul1* was present in 57.5% of the isolates, while the streptomycin resistance gene *strA* was detected in 20.5%. Molecular screening of β-lactamase genes showed a high prevalence of bla_{TEM} (67.1%), but bla_{OXA} and bla_{SHV} were not detected. The ESBL genes, including $bla_{\text{CTX-M-grp1,}}$ $bla_{\text{CTX-M-grp2,}}$ and $bla_{\text{CTX-M-grp9}}$ showed moderate detection rates, ranging from 8.2 to 52.7%. A similar concerning pattern was observed in *Klebsiella* spp. isolates (Fig. 4b). Specifically, the AMR genes *aac(3)-iv* and *tetA* were identified in 50.0% and 48.6% of the isolates, respectively, while *sul1* and *strA* had detection rates of 37.9% and 25.0%, respectively. The *β*-lactamase gene bla_{TEM} was the most common, found in 75.7% of the isolates, while bla_{OXA} and bla_{SHV} were not detected. The ESBL genes were also widespread, with $bla_{\text{CTX-M-grp1}}$ in 39.3%, $bla_{\text{CTX-M-grp2}}$ in 47.1%, and $bla_{\text{CTX-M-grp9}}$ in 22.9%. Additionally, the *AmpC β*-lactamase gene $Multicase_{\text{DHA}}$ was found in 55.5% of *E. coli* and 51.4% of *Klebsiella* isolates, indicating its important role in resistance to cephalosporins.

The phenotype-genotype correlation was varied in both pathogens. In most cases these antibiotics showed moderate to strong co-resistance patterns (Fig. 6a, b). The correlation analysis of *E. coli* isolates revealed that certain antibiotics, such as tetracycline, azithromycin, and nalidixic acid, exhibit moderate to strong positive correlations with other antibiotics, including chloramphenicol and trimethoprim-sulfamethoxazole (Fig. 6a). Similarly, in *K. pneumoniae*, a moderate to strong significant phenotypic correlation was observed between streptomycin and tetracycline, chloramphenicol, and nalidixic acid. In most cases, significant genotypic correlations were also evident (Fig. 6b). Additionally, antimicrobial resistance genes, particularly $bla_{\text{CTX-M-grp1}}$, $bla_{\text{CTX-M-grp2}}$, bla_{TEM}, and *sul1*, show a significant association with resistance phenotypes in *E. coli*. Some of these correlations are statistically significant ($p < 0.05$ to $p < 0.001$).

Antimicrobial susceptibility profiling

The AST profiles of 140 K. *pneumoniae* isolates and 146 *E. coli* isolates are presented in Fig. 5a-d. *E. coli* showed the highest susceptibility to cefoxitin (79.45%), streptomycin (78.77%), ciprofloxacin (78.0%), tetracycline (75.34%), chloramphenicol (71.23%), and trimethoprim-sulfamethoxazole (67.81%). In contrast, significant resistance was observed against ampicillin (82.88%), amoxicillin-clavulanic acid (70.55%), gentamicin (56.85%), and amikacin (56.16%) (Fig. 5a, c). An identical situation was observed with *K. pneumoniae*, which showed strong sensitivity to cefoxitin (80.0%), ciprofloxacin (77.14%), chloramphenicol (69.29%), tetracycline (68.57%),

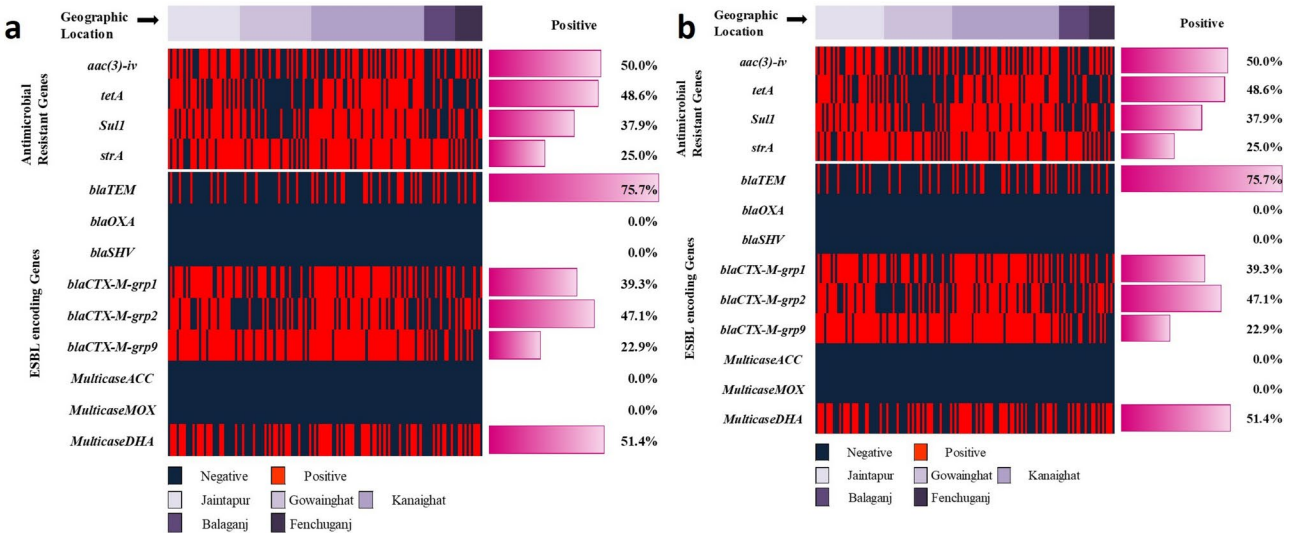

Fig. 4. The percent of different genes in between *E. coli* and *K. pneumoniae* positive isolates. (**a**) Combined heatmap and bar diagram illustrating the percentages of virulence, antibiotic resistance, and ESBL-encoding genes in *E. coli* positive isolates; (**b**) The percentages of antibiotic resistance and ESBL-encoding genes in *K. pneumoniae* positive isolates.

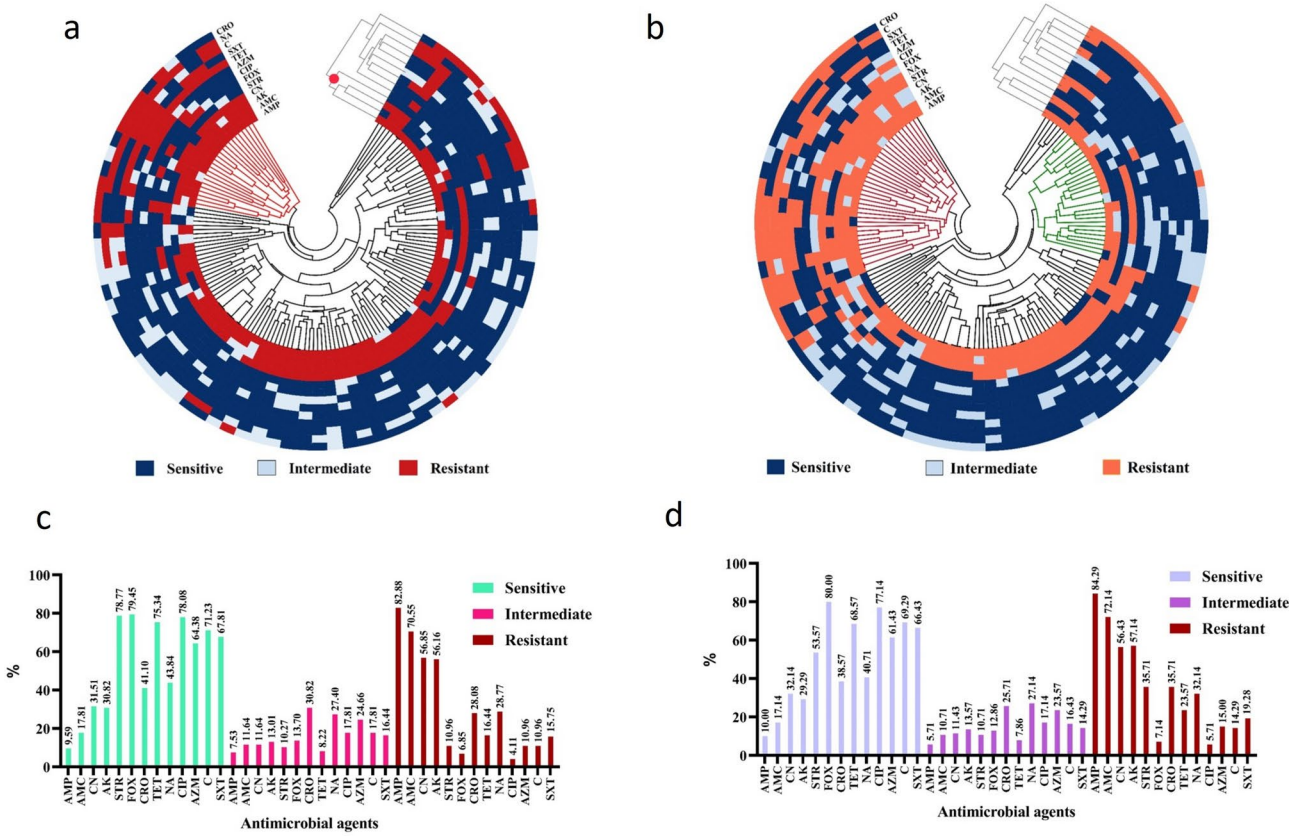

Fig. 5. Polar heatmap and bar diagram representing the antibiogram profiles of *E. coli* and *K. pneumoniae* isolates from subclinical mastitis-positive milk samples of buffalo. (**a**) The sensitive, intermediate, and resistant patterns of *E. coli* isolates; (**b**) The sensitive, intermediate, and resistant patterns of *K. pneumoniae* isolates; (**c**) The bar diagram showing the percentages (%) of sensitivity, resistance, and intermediate responses to different antimicrobials for *E. coli* isolates; (**d**) The bar diagram showing the percentages (%) of sensitivity, resistance, and intermediate responses to different antimicrobials for *K. pneumoniae* isolates.

and trimethoprim-sulfamethoxazole (66.43%). However, significant resistance was noted to ampicillin (84.29%), amoxicillin-clavulanic acid (72.14%), gentamicin (56.43%), and amikacin (57.14%) (Fig. 5b, d).

Biofilm detection

Biofilm formation was detected using the CRA method and the CVMP assay. The presence of biofilm contributes to antibiotic resistance in both pathogens, potentially leading to the development of MDR, XDR, or PDR strains.

In *E. coli*-positive isolates, biofilm formation was detected in 69.9% (102/146; 95% CI 61.7–77.2) using the CRA plate method, whereas the CVMP assay identified biofilm formation in 83.6% (122/146; 95% CI 76.5–89.2). Among biofilm-producing isolates detected by the CVMP method, 37.67% were strong producers, followed by 30.14% weak and 15.78% moderate producers. In *K. pneumoniae*-positive isolates, biofilm formation was detected in 62.1% (87/140; 95% CI 53.6–70.2) using the CRA plate method, whereas the CVMP assay detected biofilm in 75.7% (106/140; 95% CI 67.8–82.6). Among biofilm-producing isolates identified via CVMP, 42.14% were strong producers, followed by 27.86% moderate and 5.71% weak producers (Fig. 6c).

MAR index, MDR and XDR patterns of the screened bacterial isolates

A total of 140 *K. pneumoniae* isolates and 146 *E. coli* isolates were analyzed for MDR and multiple antimicrobial resistance index (MARI) (Table 2). Among *E. coli* isolates, 31.5% (46/146) were classified as MDR, with 8.9% (13/146) potentially showing XDR characteristics, and the average MARI was 0.62. In *Klebsiella* spp., 39.3% (55/140) of the strains were MDR, with 12.86% (18/140) possibly exhibiting XDR, and the average MARI was 0.66. The highest MARI for *Klebsiella* spp. was 0.92, indicating resistance to 12 of the 13 antibiotics tested, while *E. coli* showed a MARI of 0.85. The dominant resistance genes in both species included *aac(3)-iv*, *tetA*, and *sul1* (Table 2).

Discussion

Subclinical mastitis (SCM) in swamp buffaloes is a significant concern in dairy farming, as it reduces milk production and quality without visible symptoms, leading to economic losses. It also serves as a reservoir for antimicrobial-resistant pathogens, such as *E. coli* and *Klebsiella* spp., complicating treatment. Effective detection

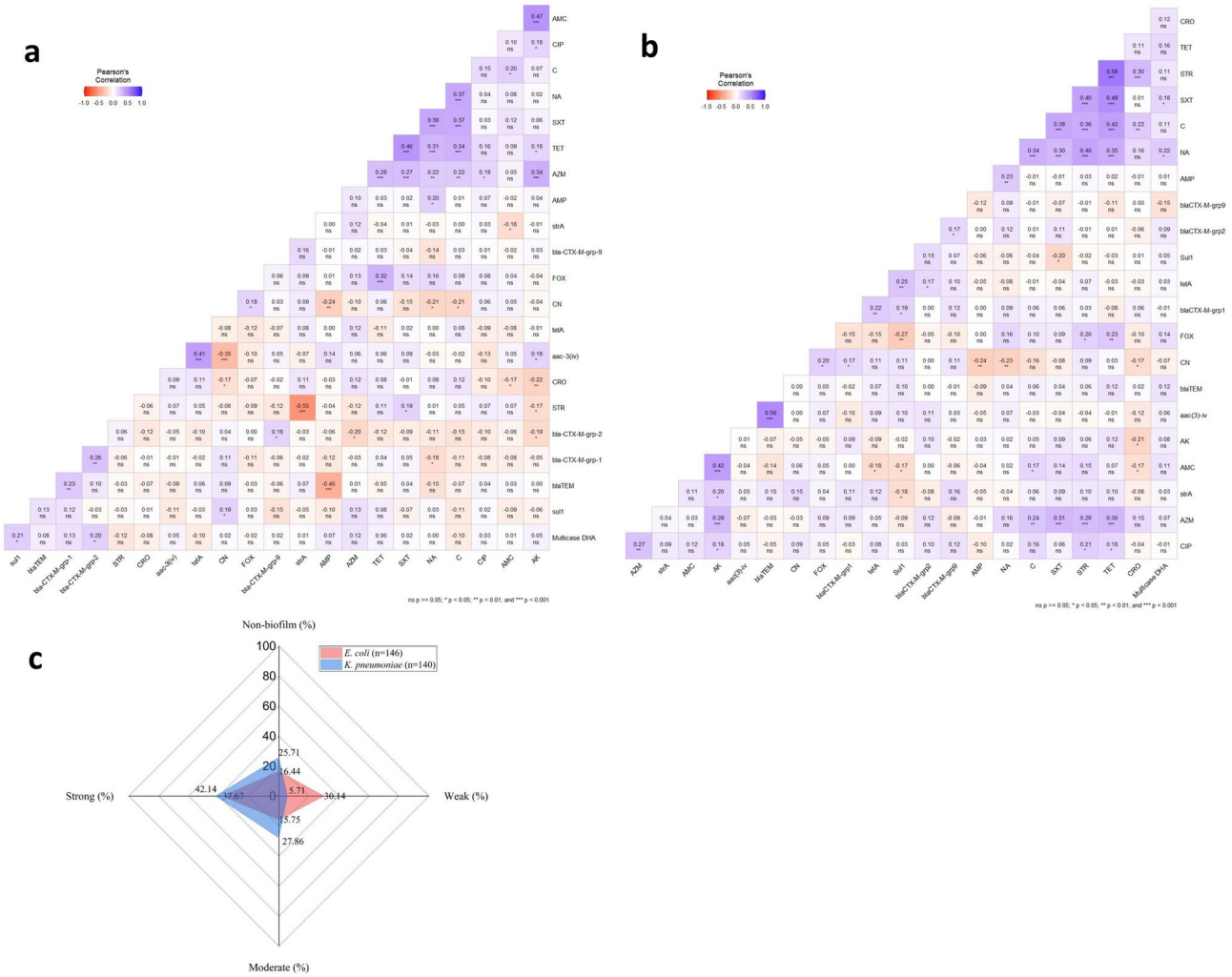

Fig. 6. The overall phenotype-genotype correlation of *E. coli* (**a**) and *K. pneumoniae* (**b**) positive isolates. Biofilm production was represented as a percentage (%) using a radar map (**c**). The figures (**a**, **b**) were created using RStudio (R 4.3.3) with the Metan package, while the radar map was generated using Origin 2024b.

and management are essential for improving dairy productivity and ensuring food safety. This study therefore thoroughly examined SCM in buffaloes in the Sylhet district of Bangladesh, concentrating primarily on the isolation and identification of bacterial pathogens, namely, *E. coli* and *Klebsiella* spp., and their prevalence. The study also revealed that the prevalence of SCM was significantly higher at the animal than quarter level. The findings of this research on the prevalence of SCM at the animal level (80.8%) closely correspond with those of earlier investigations in Bangladesh and Pakistan (81.6% and 62.2% respectively)[10,50]. However, these rates are significantly higher than those reported in other studies from Bangladesh (51.5%), India (26.20%) and the Philippines (42.76%)[13,51,52]. The findings of this research on the prevalence of SCM at the animal level (80.8%) closely correspond with those of earlier investigations in Bangladesh and Pakistan (81.6% and 62.2% respectively). Additionally, they exceed the 64.92% prevalence reported in another investigation of bovine SCM in the Sylhet district of Bangladesh[36]. The quarter-level prevalence of SCM in this investigation closely aligns with the findings reported in studies from India (45.8–61.6%) and Pakistan (68.63%)[52,53]. The prevalence is markedly higher than study noted (19.3%) in Chitwan, Nepal, underscoring the regional variability in SCM prevalence[51,54]. The higher animal-level prevalence of SCM compared to quarter-level prevalence is due to a single SCM-positive quarter designating the entire buffalo as SCM positive[51]. SCM incidence varies by country and is influenced by factors such as hygiene practices, milking techniques, lactation stage, and genetics[55,56].

Among the different upazilas in Sylhet district, the highest prevalence of *E. coli* and *Klebsiella* was detected in Gowainghat. The prevalence of *Klebsiella* spp. was the highest (80%) in another study in this region[57] which indicates the overall burden of *Klebsiella* in this region comparatively higher than other region of Sylhet. This may be attributed to environmental factors such as frequent flooding, wetland conditions, and high moisture content, which can facilitate bacterial persistence and transmission. *K. pneumoniae* thrives in moist environments, and the waterlogged soil, stagnant water, and frequent human-animal interactions in these areas may create conditions favorable for its survival and spread[57,58].

Organisms	No. of isolates	%	Resistance type	Phenotypic pattern	Antibiotics (class)	ARGs	MARI
E. coli	4	2.74	P. XDR	AMP-AMC-CN-AK-FOX-CRO-TET-NA-AZM-C- SXT	11 (8)	*aac(3)-iv, tetA, sul1*	0.85
	3	2.05	P. XDR	AMP-AMC-CN-AK-CRO-TET-AZM-C-SXT	9 (7)	*aac(3)-iv, tetA, sul1*	0.69
	4	2.74	P. XDR	AMP-AMC-CN-AK-CRO-TET-NA-AZM-C-SXT	10 (8)	*aac-3 (iv), tetA, sul1*	0.77
	2	1.37	P. XDR	AMP-AMC-CN-AK-STR-CRO-TET-NA-AZM-SXT	10(7)	*aac-3 (iv), tetA, sul1, strA*	0.77
	5	3.42	MDR	AMP-AK-CRO-NA-AZM	5(5)	*aac-3 (iv)*	0.38
	6	4.11	MDR	AMP-AMC-CN-AK-FOX-TET-NA-CIP	8 (5)	*aac-3 (iv), tetA*	0.62
	3	2.05	MDR	AMP-AMC-CN-AK-STR-FOX-CRO-TET-SXT	9 (5)	*aac-3 (iv), tetA, sul1, strA*	0.69
	2	1.37	MDR	AMP-AMC-CN-AK-STR-FOX-CRO-NA-CIP	9 (4)	*aac-3 (iv), strA*	0.69
	3	2.05	MDR	AMP-AMC-AK-STR-NA	5 (3)	*aac-3 (iv), strA*	0.38
	2	1.37	MDR	CN-AK-STR-FOX-CRO-TET-C-SXT	8 (5)	*aac-3 (iv), tetA, sul1, strA*	0.62
	3	2.05	MDR	AMP-CN-C-SXT	4 (3)	*aac-3 (iv), tetA*	0.31
	2	1.37	MDR	AMP-AMC-CN-AK-STR-TET-SXT	7(4)	*aac-3 (iv), tetA, sul1, strA*	0.54
	4	2.74	MDR	AMP-AMC-CN-AK-STR-FOX-CRO-TET-NA-CIP-SXT	11(6)	*aac-3 (iv), tetA, sul1, strA*	0.85
	3	2.05	MDR	AMP-AK-STR-CRO-TET-NA-SXT	7(6)	*aac-3 (iv), tetA, sul1, strA*	0.54
Total	46	31.5					
K. pneumoniae	2	1.42	P. XDR	AMP-AMC-CN-STR-FOX-CRO-TET-NA-CIP-AZM-C-SXT	12(8)	*aac-3 (iv), tetA, sul1, strA*	0.92
	4	2.86	P. XDR	AMP-AMC-CN-STR-CRO-TET-AZM-C-SXT	9(7)	*aac-3 (iv), tetA, sul1, strA*	0.69
	5	3.57	P. XDR	AMP-AMC-CN-CRO-NA-CIP-AZM-C-SXT	9(7)	*aac-3 (iv), sul1*	0.69
	3	2.14	P. XDR	AMP-AMC-CN-STR-CRO-TET-NA-AZM-C-SXT	10(8)	*aac-3 (iv), tetA, sul1, strA*	0.77
	4	2.86	P. XDR	AMP-CN-CRO-TET-NA-AZM-SXT	7 (7)	*aac-3 (iv), tetA, sul1*	0.54
	6	4.29	MDR	AMP-CN-STR-CRO-NA-CIP-AZM-SXT	8 (6)	*aac-3 (iv), strA, sul1*	0.61
	5	3.57	MDR	AMP-AMC-CN-AK-STR-FOX-CRO-TET-NA-CIP-SXT	11 (6)	*aac-3 (iv), tetA, sul1, strA*	0.85
	4	2.86	MDR	AMP-AMC-CN-STR-CRO-TET-NA-AZM	8(6)	*aac-3 (iv), tetA, strA*	0.61
	3	2.14	MDR	AMP-CN-AK–NA-AZM-C-SXT	7(6)	*aac-3 (iv), sul1*	0.54
	7	5.0	MDR	AMP-AMC-CN-AK-STR-FOX-CRO-TET-SXT	9 (5)	*aac-3 (iv), sul1*	0.69
	6	4.29	MDR	AMC-AK-STR-CRO-TET-NA-CIP-C	8(6)	*tetA, strA*	0.61
	2	1.42	MDR	AMP-CN-CRO-TET-NA-C	6(6)	*aac-3 (iv), tetA,*	0.46
	4	2.86	MDR	AMP-CN-AK-CRO-NA-CIP-AZM-C	8 (6)	*aac-3 (iv), tetA*	0.61
Total	55	39.3					

Table 2. Antibiotic resistance profiles, resistance types, and antibiotic resistance genes (*ARGs*) of *E. coli* and *K. pneumoniae* isolates from subclinical mastitis-positive milk samples of buffalo in Sylhet district, Bangladesh. The table included data on phenotypic resistance patterns, antibiotic classes, resistance types (MDR: multidrug-resistant; P. XDR: possible extensively drug-resistant), resistance percentages, antibiotic resistance genes (ARGs), and multiple antibiotic resistance index (MARI). Antibiotics are abbreviated as per CLSI 2023 guidelines. AMP: Ampicillin, AMC: Amoxicillin-clavulanate, CN: Gentamicin, AK: Amikacin, FOX: Cefoxitin, CRO: Ceftriaxone, TET: Tetracycline, NA: Nalidixic acid, CIP: Ciprofloxacin, AZM: Azithromycin, C: Chloramphenicol, SXT: Trimethoprim-sulfamethoxazole, STR: Streptomycin.

Conversely, the low prevalence of *K. pneumoniae* in Balaganj and Fenchuganj suggests a lower bacterial load or reduced environmental reservoirs for its transmission. Tanni et al.[57] also found comparatively lower prevalence of Klebsiella on this region. These regions may have better drainage systems, lower humidity, or reduced contamination sources, which limit bacterial survival and dissemination.

The prevalence of *E. coli* and *K. pneumoniae* varied across seasons by the pathogen fluctuations over different quarters likely influenced by climatic changes, rainfall patterns, and human activities. Due to increased waterborne transmission, monsoon seasons may contribute to higher bacterial spread, while drier months limit bacterial persistence in the environment. Hence, environmental dynamics play a key role in bacterial prevalence across different timeframes.

The prevalence of *E. coli* was noticeably greater than that reported in previous study in Nepal[59]. The variability in SCM prevalence may result from differences in geography, climate, housing systems, milking practices, udder cleanliness, hygiene protocols, and biosecurity awareness among livestock owners[60]. In this study, the prevalence of *E. coli* was slightly greater than that of *Klebsiella* spp., which is consistent with the findings of another study from a health perspective[61].

The prevalence of *E. coli* observed aligns closely with previous findings from studies in western Chitwan and Egypt, where 14% and 15.4% of buffaloes were reported to be affected by *E. coli*[59,62]. However, these results contrast significantly with studies from Nineveh Governorate, Iraq (42%) and coastal regions of Bangladesh (25%)[17,18]. For *Klebsiella* spp., the prevalence in the current study notably higher than those reported in an Indian study, which reported a prevalence of 9.1%[63]. These discrepancies highlight the variability in bacterial prevalence across different geographical regions. In addition, the LF quarter presented the highest prevalence of SCM caused by *K. pneumoniae*, which contradicts the findings of other studies in Nepal, where the LF quarter

presented the highest prevalence of SCM caused by *E. coli*. However, the findings of these studies are consistent with the findings of our study in the case of mixed infections (*E. coli* and *Klebsiella* spp.) [59,64]. The high prevalence in the forequarters (*e.g.*, LF and RF) could be due to the spread of infection via the milker's hand. Usually, milking is performed by hand, and since the LF quarter is milked first in most instances, that forequarter has greater chances of becoming infected. This might have led to the higher prevalence of CMT in the left forequarter[59]. Another study conducted in the Doaba region of Punjab, India, found that the LH quarters were more affected, which contrasts with the findings of our study[64].

Moreover, AMR in *K. pneumoniae* and *E. coli* from mastitic milk is mediated by the production of β-lactamases (ESBLs and carbapenemases), efflux pumps, and target site changes, leading to MDR and XDR strains[65,66]. Plasmid-mediated genes (*tetA, aac(3)-iv, bla*$_{\text{CTX-M}}$, *mcr-1*) facilitate horizontal gene transfer, imparting resistance to tetracyclines, aminoglycosides, β-lactams, and colistin[67,68]. Geographical variations in resistance are due to genetic mobility, antibiotic selective pressure, and environmental determinants, highlighting the global spread of AMR[69]. However, *stx1* was detected in 27.4% of the *E. coli* isolates, whereas *stx2* was noticeably absent. This finding is consistent with the results of another study in Iraq, where only one isolate of *E. coli* possessed the *stx1* gene (4.8%), and none of the isolates had the *stx2* gene[17]. However, the findings of the present study are much greater than those of another study in the coastal area of Bangladesh, where the *stx1* and *stx2* genes were 2.6% and 1.3%, respectively[18]. The results of this study sharply contrast with those of a previous investigation conducted in migratory and captive wild birds in Bangladesh, where *stx2* was found in 12% of *E. coli* isolates, while *stx1* was not detected at all[70]. The differences in the prevalence of *stx1* and *stx2* across regions may be attributed to variations in their ecological niches, genetic diversity, and the mobility of *stx*-encoding prophages. Additionally, factors like host reservoirs and environmental conditions could help explain these discrepancies. For instance, while *stx1* is more commonly linked to specific animal reservoirs, *stx2* is often associated with more severe human disease due to its higher toxicity and broader host range, influencing its distribution patterns in different regions[71,72]. The most prevalent AMR genes, *tetA* and *aac(3)-iv*, were identified in several other studies, supporting the findings of the present study. In this study, *tetA* and *aac(3)-iv* were the most frequently detected AMR genes in *E. coli* (76.7% and 77.4%, respectively) and *Klebsiella* spp. (48.6% and 50%, respectively)[73–76]. The genes *tetA* and *aac(3)-iv* are most frequently identified in *E. coli* and *Klebsiella* spp. because of their strong association with mobile genetic elements such as plasmids, facilitating their horizontal transfer, and their role in resistance to commonly used antibiotics such as tetracyclines and aminoglycosides[76–78]. The genes *sul1* and *strA* are less commonly detected than *tetA* and *aac(3)-iv are,* likely due to their association with resistance to sulfonamides and streptomycin, antibiotics with reduced usage in contemporary clinical and agricultural settings, leading to decreased selective pressure and lower prevalence. In contrast, *β*-lactamase genes, particularly *bla*$_{\text{TEM}}$, presented high prevalence rates in this study, with 67.1% in *E. coli* and 75.7% in *Klebsiella* spp., which aligns partially with findings from Barcelona (71.93%) and Mexico (96%) in *Klebsiella* spp. and from Bangladesh (60.7%)[36], Iraq (81%) and Indonesia (77.78%) in *E. coli*, reflecting regional and antibiotic usage variations[42,76,78,79]. The prevalence of *Klebsiella* spp. in this study is slightly higher than that reported in another study conducted in Bangladesh, which found a prevalence of 42.5%[36]. Furthermore, the prevalence of *bla*$_{\text{CTX-M-grp2}}$ was noticeably high in both *E. coli* (52.7%) and *Klebsiella* spp. (47.1%) among the *bla*$_{\text{CTX-M}}$ gene variants (*bla*$_{\text{CTX-M-grp1}}$, *bla*$_{\text{CTX-M-grp2}}$, and *bla*$_{\text{CTX-M-grp9}}$) examined in this study. This contrasts sharply with findings from Pakistan, where *bla*$_{\text{CTX-M-grp1}}$ was the most prevalent gene, detected in 56.1% of *E. coli* and 41.2% of *Klebsiella* spp., whereas *bla*$_{\text{CTX-M-grp2}}$ and *bla*$_{\text{CTX-M-grp9}}$ showed minimal occurrence (0–2.9%)[80]. However, the detection of *bla*$_{\text{CTX-M-grp2}}$ in 65.22% of *E. coli* isolates in the Philippines and 44.8% of *Klebsiella* spp. isolates in Brazil aligns closely with the results of this study[81,82]. In addition, the prevalence of *bla*$_{\text{CTX-M-grp1}}$, and *bla*$_{\text{CTX-M-grp2}}$ in another SCM study in Bangladesh is somewhat lower than the present study findings[36]. The variation in the distribution of *bla*$_{\text{CTX-M-grp1}}$, *bla*$_{\text{CTX-M-grp2}}$, and *bla*$_{\text{CTX-M-grp9}}$ across geographic regions may stem from differences in genetic mobility, plasmid associations, host and geographic dissemination, selective pressures exerted by antibiotic use, and regional variations in CTX-M variants [80,81]. Additionally, the AmpC β-lactamase gene *Multicase*$_{\text{DHA}}$ was detected in both *E. coli* (55.5%) and *Klebsiella* spp. (51.4%), whereas *Multicase*$_{\text{ACC}}$ and *Multicase*$_{\text{MOX}}$ variants were entirely absent, a finding that is consistent with previous research on bovine subclinical mastitis (SCM) in Banglades[36]. These observations underscore the complex interplay of genetic and environmental factors shaping the distribution of resistance genes in diverse settings.

Moreover, cefoxitin exhibited the highest sensitivity to the isolates of both *E. coli* and *Klebsiella* spp. in this study, a finding that sharply contrasts with previous reports from Canada and Bangladesh, where cefoxitin demonstrated 100% resistance to *E. coli* and 68.6% resistance to *K. pneumoniae*[83]. Ciprofloxacin was highly effective, with susceptibility rates of 78% in *E. coli* and 77.14% in *Klebsiella* spp., which aligns with some SCM-related studies in Bangladesh[36] and Nepal[59]. Similarly, tetracycline also demonstrated noticeable sensitivity against *E. coli* (75.34%) and *Klebsiella* spp. (68.57%), which is consistent with findings from Nepal[84], where tetracycline was 100% effective against *E. coli* and exhibited minimal resistance (19.9%) to *K. pneumoniae* in the United States[85]. Tetracycline also found completely (100%) resistant to *E. coli* and *Klebsiella* spp. in Bangladesh which is fully contradictory to the present study findings[36]. Streptomycin resulted in 78.77% susceptibility in *E. coli*, which is close to the 85% susceptibility reported in Ethiopia[86]. Sulfamethoxazole-trimethoprim exhibited moderate effectiveness against *E. coli* (67.81%) and *Klebsiella* spp. (66.43%), closely aligning with a study in Bangladesh reporting 87.5% susceptibility[70]. Furthermore, chloramphenicol displayed high sensitivity to *E. coli* (83%) and *Klebsiella* spp. (51%) in Jaipur, India, which is consistent with the results of this study[87]. In contrast, *E. coli* and *Klebsiella* spp. presented high resistance rates to ampicillin and amoxicillin-clavulanic acid and moderate resistance rates to gentamicin and amikacin in this study. These results are in agreement with studies conducted in Egypt, where *E. coli* presented 80%, 60%, and 50% resistance rates to amikacin, ampicillin, and amoxicillin-clavulanic acid, respectively [88]. Similarly, *Klebsiella* spp. isolates were found to be completely resistant to ampicillin and amoxicillin-clavulanic acid (100%), whereas gentamicin resistance was observed in

50% of the isolates[89]. These findings revealed an alarming trend of resistance to commonly used antibiotics, with increasing challenges for infection therapy involving these pathogens.

The MDR 46 (31.5%), and possibly XDR 13 (8.9%) of the *E. coli* isolates detected in this study are closely aligned to a previous study reporting 79 (30.3%) MDR and 22 (8.4%) XDR *E. coli* isolates[90], but much lower than another study in Bangladesh that found 98% MDR and 16% XDR[91]. Among 140 *K. pneumoniae* isolates, 55 (39.3%) were MDR, and 18 (12.86%) were possibly XDR, aligning with another study reporting 75 (37.5%) MDR and 25 (12.5%) XDR isolates[90]. However, this study's results were notably lower than other studies in Bangladesh that reported 87% MDR and 22.73% XDR[92,93]. The MDR prevalence in *E. coli* here contrasts with a study in Nepal, where 78% were MDR, but the XDR prevalence of 7% is similar to our findings[94]. Likewise, the MDR prevalence in *K. pneumoniae* differed from a study conducted in Iran, although the XDR prevalence of 13% was similar[95]. The MAR index (MARI) for *E. coli* (0.62) aligns with findings from Bangladesh, where 96% of isolates had a MAR index > 0.3, and 64% exceeded 0.5[91]. The MARI for *K. pneumoniae* (0.66) is also consistent with a Nigerian study reporting a MARI value of 0.79[96].

Most biofilm-producing isolates exhibited a high likelihood of developing MDR or XDR. In both pathogens, strong to moderate biofilm producers were predominantly associated with MDR and XDR profiles. Furthermore, Biofilm in *E. coli* is controlled by *lasR, lecA*, and *pelA*, affecting quorum sensing, lectin adhesion, and extracellular matrix production, respectively, for the promotion of bacterial persistence and antibiotic resistance[97]. In *K. pneumoniae, mrkA* and *mrkD* are essential for type 3 fimbriae-mediated adhesion and biofilm maturation, where they promote the colonization of host surfaces and medical devices by bacteria[98]. These biofilm-related genes are responsible for immune evasion and enhanced antimicrobial tolerance, thereby making infections more difficult to eradicate.

These results highlight regional differences in resistance patterns while reflecting global trends. The variation in MDR and XDR prevalence among different organisms may result from factors such as intrinsic resistance mechanisms, acquired resistance, antibiotic exposure, gene mobility, environmental influences, virulence, biofilm formation, and potential diagnostic or surveillance biases[99–101]. Limitations of this study include the reliance on conventional techniques like PCR, rather than more advanced methods such as whole-genome sequencing.

Conclusion

The study highlights a high prevalence of SCM, with significant proportions of *E. coli* and *K. pneumoniae* exhibiting MDR and XDR, along with an alarming levels of virulence genes (*stx1*) and AMR genes (*aac(3)-iv*, *tetA*, and β-lactamase genes such as bla_{TEM} and $bla_{\text{CTX-M-grp1}}$). The detection of resistance to critical antimicrobials, including aminoglycosides and β-lactams, and the prevalence of MDR/XDR strains draw attention to the growing threat posed by AMR in dairy production systems. Therefore, improved surveillance, sensible antibiotic use, and robust biosecurity measures should be considered to mitigate the spread of resistant pathogens and safeguard animal and public health.

Data availability

The data supporting the findings of this study can be obtained from the corresponding authors upon reasonable request.

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Author contributions

M.S.R.C.: Conceptualization, Data curation, Methodology, Investigation, Writing-original draft, Writing- reviewing & editing; H.H.: Methodology, Investigation, Formal analysis, Visualization, Software, Writing-original draft, Writing- reviewing & editing; M.N.R., A.R. & P.K.G.: Methodology, Investigation, Writing-original draft; M.B.U.: Validation, Visualization, Writing- reviewing & editing. M.N.H. & M.M.H.: Supervision, Validation, Visualization, Writing- reviewing & editing; M.M.R.: Conceptualization, Methodology, Investigation, Funding acquisition, Project administration, Resources, Formal analysis, Software, Supervision, Validation, Visualization, Writing-original draft, Writing- reviewing & editing.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to M.M.R.