Polish Journal of Veterinary Sciences Vol. 28, No. 4 (2025), 639–650

DOI 10.24425/pjvs.2025.157278

Original article

Prevalence and antimicrobial resistance profiles of bacterial pathogens associated with subclinical mastitis and dairy farm environments

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Abstract

This study investigated the prevalence and antimicrobial resistance (AMR) profiles of bacterial pathogens linked to subclinical mastitis in dairy cows, as well as their occurrence in milk, faecal, and environmental samples collected in eastern Turkey between February and May 2024. A total of 2,400 milk samples were collected from 600 cows affected with subclinical mastitis, along with 292 rectal faecal samples, 150 raw milk samples consumed by the public, and hand and fecal samples obtained from animal breeders in 25 cattle enterprises. In addition, environmental samples such as water, soil, feed, and bedding (five samples per enterprise), were collected. Bacterial isolates cultured from all samples were identified using Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) mass spectrometry. Of the 600 cows examined, 292 (48.6%) were CMT-positive, and bacterial growth was detected in 261 (89.7%) of these samples. The predominant isolates were coagulase-negative staphylococci (CoNS, 30.8%), *Staphylococcus aureus* (19.5%), *Escherichia coli* (8.9%), and *Aerococcus viridans* (5.8%). Antimicrobial susceptibility of *S. aureus* and CoNS against 15 antibiotics and *E. coli* against 17 antibiotics was assessed using the disc diffusion method, and the *mec*A gene was screened by PCR. Among 121 *E. coli* isolates, no *mcr*, carbapenemase, or β-lactamase genes were detected by multiplex PCR. Tetracycline resistance was highest among *E. coli* isolates, particularly in milk samples from mastitic cows, rectal fecal samples, unpasteurised cow's milk, farmer's feces, soil, and feed, while resistance to amikacin, cefepime, cefoxitin, cephalexin, ertapenem, and norfloxacin remained lower. No resistance was observed against kanamycin. The *mec*A gene was identified in three *S. aureus* isolates (3/57, 5.3%): two from cows affected with subclinical mastitis and one from a farmer's hand. These findings highlighted the prevalence of major bacterial pathogens, potential therapeutic challenges and public health risks associated with the presence of AMR bacteria and raw milk consumption.

Keywords: one health, *Escherichia coli*, MALDI-TOF MS, mcr genes, mastitis, *Staphylococcus aureus*

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Introduction

The growing threat of antimicrobial resistance (AMR) among clinically significant bacteria presents a serious public health concern (El-Diasty et al. 2019). Residual antibiotics in milk and dairy products are particularly worrisome due to their harmful effects on human health (Sheikholeslami et al. 2022). Despite the escalating AMR crisis, progress in discovering and developing new antibiotics has stagnated, leaving a critical gap in treatment options (Sheikholeslami et al. 2022). The One Health paradigm which emphasize the link of animal, human, and environmental health is increasingly crucial (El-Diasty et al. 2019).

Mastitis, a leading cause of economic losses in the dairy industry, costs U.S. producers an estimated $2 billion annually (Hogan et al. 2011). *Staphylococcus aureus*, designated by the World Health Organization as a high-priority pathogen, is commonly linked to mastitis (World Health Organization 2017, Touaitia et al. 2025). This bacterium can be spread through direct physical contact or by consuming contaminated animal-derived products, and it is known for its environmental persistence, surviving in soil, water, and feces (Kadariya et al. 2014).

The efficacy of colistin, once considered a last-resort antibiotic for treating multidrug-resistant *E. coli*, has been undermined by the discovery of plasmid-borne resistance genes (mcr), first detected in 2015 (Liu et al. 2016). These genes, particularly variants *mcr*-1 to *mcr*-10, have been widely reported in animals, humans, food items, and environmental samples (Dadashi et al. 2022), including raw milk and cheese (Liu et al. 2016, Hammad et al. 2019). Studies also show evidence of direct and indirect transmission of resistant bacteria between animals and humans, underscoring the One Health concept (Anyanwu et al. 2020).

Although various resistance genes such as *mecA*, *mcr*, the major carbapenemases (*bla*OXA-48, *bla*NDM-1, *bla*IMP, *bla*VIM and *bla*KPC), β-lactamases (*bla*TEM-1, *bla*CTX-M and *bla*SHV-1) and OXA-48-like β-lactamases (*bla*OXA-162, *bla*OXA-163, *bla*OXA-181, *bla*OXA-204 and *bla*OXA-232) have been reported in Turkey across human, animal, and environmental sources, their transmission dynamics remain unclear. Notably, no comprehensive studies have investigated the full cycle of infection at the human-animal-environment interface. This study aims to fill this gap by providing comprehensive data to inform AMR control strategies, enhance national awareness and policy development.

Materials and Methods

Sample collection and ethics

This research was performed in compliance with the ethical guidelines sanctioned by the Local Ethics Committee for Animal Experimentation at Firat University, as outlined in protocol number FU-2024/04-04 and the Ethics Committee of Firat University of Medical Sciences (protocol number: FU-2024/04-42). The milk samples from 600 cows affected with subclinical mastitis collected from 25 different small family farms in Elazig province, eastern Turkey, between February and May 2024, were analysed. The cows were 3-9 years old, lactating (14-100 days post-calving), had given birth at least once, and had not received antibiotic treatment for at least 3 months. This study was conducted in compliance with the guidelines of the National Mastitis Council (Adkins et al. 2017).

Sampling procedure

A total of 2,400 milk samples were taken from the four quarters of the udders of 600 cows affected with subclinical mastitis. Each quarter was sampled individually, and no pooling of samples was performed. In addition, 292 rectal fecal samples from cows confirmed as California Mastitis Test (CMT) positive, 150 raw unpasteurized milk samples consumed by the public, and hand and fecal samples from livestock farmers in 25 dairy farms were collected. Environmental samples, including water, soil, feed, and bedding (five samples from each source per farm), were also obtained to assess bacterial contamination in the farm environment.

The teats of 600 animals were initially washed with hot water and thoroughly dried. They were then disinfected using 70% ethyl alcohol applied with cotton, after which the first stream of milk was discarded. Milk samples were subsequently collected and tested using the California Mastitis Test (CMT). For teats that yielded positive CMT results, bacteriological cultures were performed on the corresponding milk samples. Approximately 10 mL of milk was aseptically collected into sterile tubes containing Stuart transport medium, while an additional 5 mL was transferred into plastic tubes for somatic cell count analysis.

Classification of cows

Cows were classified as healthy and with subclinical mastitis by clinical observations, evaluation of mammary gland secretions, CMT score, somatic cell count (SCC) of milk and microbiological analysis of milk samples. The CMT test was performed as defined

by Jackson and Cockcroft (2002). SCCs were conducted using the DeLaval Cell Counter® (DeLaval International, Sweden) (Hisira et al. 2023). Animals with no visible milk abnormalities or obvious signs of local inflammation or systemic involvement, a negative CMT score and no bacterial growth on microbiological analysis of milk samples were classified as healthy, and animals with no clinical signs, a positive CMT score, an increase in SCC the milk and bacterial growth on microbiological analysis of milk samples were classified as subclinical mastitis (Zigo et al. 2022). All samples were sent to laboratories in Stuart transport medium under a cold chain for microbiological analysis.

Microbiological analysis

Milk samples (10 ml each) were vortexed and inoculated onto aesculin blood agar (modified), tryptic soy agar with 7% sheep blood, MacConkey agar, and MacConkey agar supplemented with 4 µg/ml colistin (Merck, Germany), then incubated aerobically at 37°C for 24-48 hours. Colony morphology and growth characteristics were assessed. Samples with ≥3 colony types were considered as contaminated and recollected; those with two were considered as mixed infections (Tartor et al. 2021).

Staphylococcus species were identified following Holko et al. (2019) via Gram staining, catalase (3% H_2O_2), coagulase, pigment production, and esculin hydrolysis. *S. aureus* was confirmed using latex agglutination. Gram-positive cocci observed in short or long chains under the microscope were considered presumptive streptococci. To verify their identification, the isolates were subjected to routine biochemical assays such as the catalase test, hippurate hydrolysis, and esculin hydrolysis (Abd El-Aziz et al. 2021).

Faecal samples (1 g, obtained directly from the rectum or after fresh defecation) were placed in a sterile stool collection container in a sterile Carry-Blair tube containing (Oxoid SR 0181E, UK) tryptic soy broth (enrichment broth) (Oxoid, Basingstoke, UK) supplemented with 9 mL of 20 mg/L novobiocin. The stool sample (approximately 1 g) was added to the medium and homogenised by vortexing for 1 minute. The prepared homogenate was incubated at 37°C for 18-24 hours under aerobic conditions for pre-enrichment. After pre-enrichment, a loop of broth was inoculated on MacConkey agar (Merck, Germany) and eosin methylene blue agar (Merck, Germany) and incubated under the same conditions.

The 25 g of soil, feed and bedding samples were pre-enriched in 225 ml buffered peptone water (BPW), then cultured on the same agar media as milk samples (Ngogang et al. 2021). Water samples (5 L) were filtered (0.45 µm, Millipore®) and filters were cultured on the same media, followed by incubation at 37°C for up to 48 hours (Hmede et al. 2019, Ngogang et al. 2021).

Bacterial identification was based on colony and cellular morphology and confirmed using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS). This was performed with the MALDI Biotyper Microflex LT system (Bruker Daltonics GmbH, Bremen, Germany) employing the MALDI Biotyper MSP identification method (Dubois et al. 2012). The confirmation of *S. aureus* isolates was carried out using MALDI-TOF and further verified through the deoxyribonuclease (DNase) test, following the procedure outlined by Noumi et al. (2020). The formation of biofilm (slime) by *S. aureus* was assessed using the Congo red agar technique as outlined by Zigo et al. (2022).

Detection of AMR genes by PCR and multiplex PCR

Genomic DNA was extracted from all *E. coli* and *S. aureus* isolates using the QIAsymphony automated system in conjunction with the QIAsymphony DSP Virus/Pathogen midi kit (Qiagen, Hilden, Germany), following the manufacturer's protocol. A total of 204 *S. aureus* isolates were analyzed for the *mec*A gene by PCR, while 121 *E. coli* isolates were examined via multiplex PCR targeting AMR-related genes. The multiplex PCR targeted mobilized colistin resistance genes (*mcr*-1 through *mcr*-9), carbapenemase genes (*bla*NDM-1, *bla*OXA-48, *bla*IMP, *bla*VIM and *bla*KPC), and β-lactamase genes (*bla*TEM-1, *bla*CTX-M, *bla*SHV-1) as previously described by Hasman et al. (2005), Ellington et al. (2007), Poirel et al. (2011), Rebelo et al. (2018) and Borowiak et al. (2020).

Each 25 µL PCR reaction consisted of 12.5 µL of TopTaq Master Mix (Qiagen), 5.5 µL of nuclease-free water, 0.5 µL of each primer (10 µM), and 2 µL of template DNA. Amplifications were carried out under the following cycling conditions: an initial denaturation at 94°C for 15 min; 25 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 90 s, and extension at 72°C for 60 s; followed by a final extension at 72°C for 10 min (Hasman et al. 2005, Ellington et al. 2007, Poirel et al. 2011, Rebelo et al. 2018, Borowiak et al. 2020).

PCR products were resolved on 1.5% ethidium bromide – stained agarose gels and visualized under UV illumination with a Gel Logic 2200 imaging system (Kodak Co., Rochester, NY, USA). Representative gel electrophoresis images of *mec*A gene detections are presented in Fig. 1.

Positive controls included *S. aureus* CCM 4750

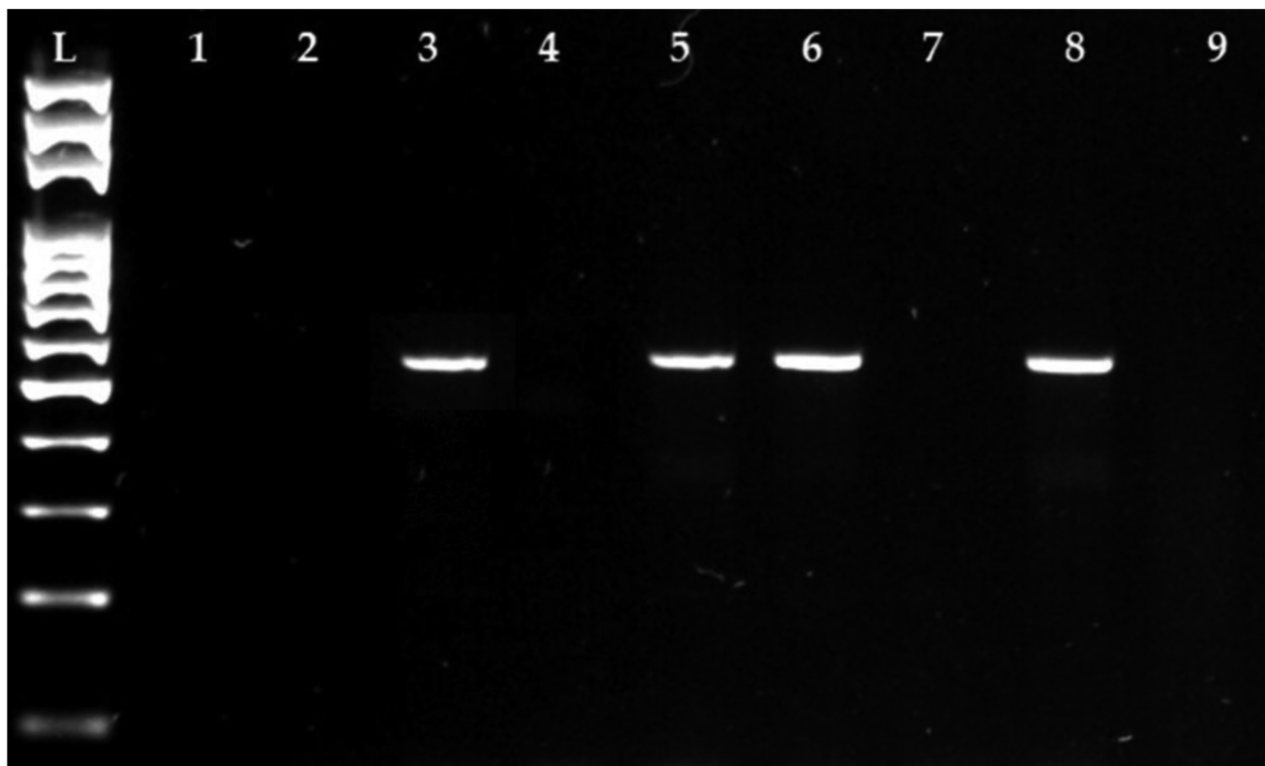

Fig. 1. Detection of the *mec*A gene in *Staphylococcus aureus (S. aureus)* isolates from cow's milk with subclinical mastitis and farmer's hand by PCR (527 bp). L: 100 bp ladder; Lines 1, 2, 4 and 7: isolate *S. aureus* isolates negative for the *mecA* gene; Line 3: isolate *S. aureus* from farmer's hand with *mec*A gene; Lines 5 and 6: isolate *S. aureus* from cow's milk with subclinical mastitis for *mec*A gene; Line 8: reference strain CCM 4750 *S. aureus* (positive control); Line 9: water (negative control).

(Czech Collection of Microorganisms, Brno, Czech Republic) for the *mec*A gene, and *E. coli* reference strains harboring specific AMR determinants: *E. coli* 2012-60-1176-27 (*mcr-1*), *E. coli* IncX4 plasmid (*mcr-2*), *E. coli* 2013-SQ352 (*mcr-3*), *E. coli* DH5α pCR2 (*mcr-4*), and *Salmonella paratyphi B* 13-SA01718 (*mcr-5*). These strains were generously provided by Assoc. Prof. Mehmet Cemal Adigüzel (Department of Microbiology, Faculty of Veterinary Medicine, Atatürk University, Turkey). Additional *E. coli* isolates containing representative carbapenemase and β-lactamase genes were kindly provided by Prof. Barış Otlu (Department of Medical Microbiology, Faculty of Medicine, Inonu University, Malatya, Turkey) and used as controls throughout the study.

Phenotypic AMR of *S. aureus* and *E. coli* isolates

The antimicrobial susceptibility of *S. aureus* isolates was assessed using the Kirby-Bauer disk diffusion technique, following the guidelines established by EUCAST (2024). Antimicrobial agents commonly used against *S. aureus* in animals and humans were selected for antimicrobial susceptibility testing. Approximately 15 antimicrobial agents commonly administered in both veterinary and human medicine was selected for testing. Antibiotic discs (Oxoid, UK), representing a range of antimicrobial classes, were used. Once pure *S. aureus* cultures were obtained, bacterial suspensions were prepared in sterile 0.85% saline solution and standardized to the turbidity of a 0.5 McFarland solution. The standardized suspensions were then evenly inoculated onto Mueller-Hinton agar plates. The following antibiotic discs were applied: clindamycin (CL; 10 µg), kanamycin (KAN; 30 µg), oxacillin (OXA; 1 µg), cefotaxime (CTX; 30 µg), sulphamethazole-trimethoprim (SXT; 25 µg), cefazolin (CZ; 30 µg), tetracycline (TET; 30 µg), erythromycin (E; 15 µg), ampicillin (AMP; 10 µg), penicillin (PEN; 10 µg), gentamicin (GEN; 10 µg), imipenem (IMP; 10 µg), amikacin (AMK; 30 µg), ciprofloxacin (CIP; 5 µg) and cefoxitin (FOX; 30 µg), following the methodology of Badawy et al. (2022a).

The tests were repeated with positive and negative controls to ensure data compatibility. *S. aureus* ATCC 25923 was utilised as the reference strain (positive control), while sterile distilled water was employed as the negative control. Isolates resistant to at least one antibiotic in three or more distinct antimicrobial classes were classified as MDR, according to the definition by Badawy et al. (2022b).

The antimicrobial susceptibility of *E. coli* isolates was evaluated using the Kirby-Bauer disk diffusion method, following the guidelines defined by EUCAST (2024). Bacterial suspensions were adjusted to the

0.5 McFarland standard and evenly distributed on Mueller-Hinton agar plates (Oxoid, UK). A variety of commercially available 17 antibiotic discs were applied, including: amikacin (AMK, 30 µg), ampicillin (AMP, 10 µg), streptomycin (STR, 10 µg), cefoxitin (FOX, 30 µg), cefixime (CFM, 5 µg), cephalexin (LEX, 30 µg), ceftazidime (CAZ, 30 µg), cefotaxime (CTX, 30 µg), ciprofloxacin (CIP, 5 µg), cefepime (FEP, 30 µg), ertapenem (ETP, 30 µg), gentamicin (GEN, 30 µg), kanamycin (KAN, 30 µg), tetracycline (TET, 30 µg), trimethoprim-sulfamethoxazole (SXT, 1.25/23.75 µg), norfloxacin (NOR, 10 µg), and amoxicillin/clavulanic acid (AMC, 20/10 µg), in line with the protocol described by Butaye (2013). The inoculated Mueller-Hinton agar plates were incubated at 37°C for 18 to 24 hours. Upon completion of the incubation period, the diameters of the zones of inhibition were measured and interpreted based on the EUCAST (2024) criteria.

Statistical analysis

Statistical evaluation was performed using SPSS version 26.0. The variations in antimicrobial resistance rates were analyzed using the chi-square test or Fisher's exact test, as appropriate for the data type. A p-value below 0.05 was regarded as statistically significant.

Results

The results of this current study indicate that key pathogens including *S. aureus*, *S. uberis*, and *S. agalactiae* along with CoNS, pose significant health risks to dairy cows. Out of 600 cows, 292 (48.6%) tested positive by the CMT, with bacterial growth in 89.7% (261/292) of samples. MALDI-TOF identified 51 bacterial species, with staphylococci most prevalent (50.3%), followed by *E. coli* (8.9%) and *S. chromogenes* (8.2%) (Table 1). Similar bacterial diversity was found in rectal feces, unpasteurized milk, farmer's hands and feces, water, soil, feed, and bedding samples.

Table 2 presents the evaluation of virulence factors in the tested staphylococci. *S. aureus* was the predominant pathogen identified, exhibiting multiple virulence factors such as hemolytic activity, gelatinase production, biofilm formation, and DNase activity. Except for *S. aureus*, statistical independence ($p<0.05$) of virulence factors was confirmed in *S. chromogenes* (Table 2). The *mecA* gene was identified in three *S. aureus* isolates (two from milk and one from a farmer's hand). The *S. aureus* isolates obtained from milk samples with mastitis exhibited the highest resistance to clindamycin (80.7%), followed by penicillin (70.2%) and ampicillin (63.2%) (Table 3).

In cow's faecal samples, 42 isolates (70%) of *S. aureus* were resistant to clindamycin, 38 (63.3%) to penicillin, 35 (58.3%) to ampicillin, 16 (26.7%) to tetracycline, 16 (26.7%) to erytromycin, 13 (21. 6%) to cefazolin; 12 (20%) to cefotaxime; 9 (15%) to kanamycin; 8 (13.3%) to gentamicin; 7 (11.7%) to imipenem; 5 (8.3%) to trimethoprim/sulphamethoxazole; 2 (3.3%) to cefoxitin; 2 (3.3%) to amikacin; and one (1.7%) to ciprofloxacin (Table 3).

S. aureus isolates recovered from unpasteurized cow's milk exhibited high resistance rates to clindamycin (76.2%), penicillin (71.4%), ampicillin (66.7%), and erytromycin (33.3%). Conversely, lower levels of resistance were noted against cefoxitin, imipenem, ciprofloxacin, gentamicin, amikacin, trimethoprim-sulfamethoxazole, and oxacillin (Table 3).

S. aureus, S. chromogenes, S. warneri, and *S. xylosus* isolates from various sources showed high resistance to penicillin and ampicillin, which was also confirmed by statistical analysis depending on the data distribution. In contrast, lower resistance to kanamycin, cefotaxime, cefoxitin, imipenem, and gentamicin was observed (Table 3).

Table 4 shows the antimicrobial resistance profile of 121 *E. coli* isolates from different sources. Based on statistical analysis accounting for data distribution, *E. coli* isolates exhibited the highest resistance to tetracycline across various sources, including milk samples from mastitic cows, rectal fecal samples, unpasteurised cow's milk, farmer's feces, soil, and feed. In contrast, low levels of resistance were observed for amikacin, cefepime, cefoxitin, cephalexin, ertapenem, and norfloxacin. No resistance were observed against kanamycin.

Discussion

Despite the many herd protection control programmes implemented in Turkey, rates of subclinical mastitis caused by *S. aureus* have been reported to range between 3.9-46.5% (Baştan et al. 2015, Sağlam et al. 2018). This study found a 19.5% prevalence lower than in Egypt (50-83%) (Gwida et al. 2021), Turkey (56%) (Gundogan and Avci 2014), China (46% and 43%) (Wang et al. 2018, Kou et al. 2021) but higher than in Ethiopia (15%) (Grima et al. 2021). These differences may be influenced by factors such as geographical region, climate, animal species, farm hygiene practices, animal health status, and the methods and hygiene standards used during milking (Badawy et al. 2022a).

Among CoNS, *S. chromogenes* was the predominant species, consistent with some international studies

Table 1. Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) identification results of bacterial isolates obtained from human, animal and environmental samples.

Bacteria	milk samples with mastitis % (n=292)	cow`s rectal fecal samples % (n=292)	unpasteurised cow's milk % (n=150)	farmers' hand % (n=25)	farmers' feces % (n=25)	water % (n=125)	soil % (n=125)	feed % (n=125)	bedding material % (n=125)
Acinetobacter iwoffii	-	-	1 (0.6)	-	-	-	-	-	-
Achromobacter xylosoxidans	-	-	-	-	-	2 (1.6)	-	-	-
Aerococcus viridans	17 (5.8)	-	-	-	-	-	-	-	-
Bacillus amyloliquefaciens	4 (1.4)	-	-	-	-	-	4 (3.2)	-	3 (2.4)
Bacillus cereus	3 (1)	2 (0.7)	5 (3.3)	2 (8)	1 (4)	7 (5.6)	4 (3.2)	8 (6.4)	10 (8)
Bacillus circulans	2 (0.7)	-	2 (1.3)	-	-	-	1 (0.8)	-	1 (0.8)
Bacillus licheniformis	3 (1)	5 (1.7)	4 (2.6)	-	-	-	7 (5.6)	10 (8)	5 (4)
Bacillus megaterium	1 (0.3)	-	2 (1.3)	-	-	-	4 (3.2)	3 (2.4)	4 (3.2)
Bacillus mycoides	-	-	-	-	-	-	3 (2.4)	-	-
Bacillus pumilis	3 (1)	2 (0.7)	-	-	-	2 (1.6)	6 (4.8)	-	-
Bacillus stearothermophilus	-	-	3 (2)	-	-	-	2 (1.6)	-	-
Bacillus subtilis	5 (1.7)	10 (3.4)	9 (6)	3 (12)	2 (8)	-	13 (10.4)	18 (14.4)	6 (4.8)
Bacillus thuringiensis	2 (0.7)	-	-	-	-	-	8 (6.4)	4 (3.2)	3 (2.4)
Brevibacillus choshinensis	1 (0.3)	-	-	-	-	-	-		-
Brevibacterium frigoritolerans	-	-	-	-	-	-	2 (1.6)		-
Brevibacterium luteolum	-	-	-	-	-	-	2 (1.6)		-
Burkholderia cepacia	1 (0.3)	1 (0.3)	-	-	-	-	2 (1.6)		-
Carnobacterium maltaromaticum	1 (0.3)	-	-	-	-	-	-		-
Citrobacter freundii		-	2 (1.3)	-	-	8 (6.4)	-		-
Corynebacterium bovis	9 (3)	10 (3.4)	4 (2.6)	-	-	-	-		-
Corynebacterium amycolatum	4 (1.4)	-	-	-	-	-	1 (0.8)		4 (3.2)
Corynebacterium xerosis	3 (1)	1 (0.3)	-	-	-	-	-		1 (0.8)
Corynebacterium freneyi	2 (0.7)	3 (1)	-	-	-	-	3 (2.4)		-
Corynebacterium confusum	1 (0.3)	-	-	-	-	-	-		-
Enterobacter aerogenes	-	-	2 (1.3)	-	-	-	-		-
Enterobacter cloacae	3 (1)	-	4 (2.6)	-	-	12 (9.6)	5 (4)	9 (7.2)	-
Enterococcus faecalis	7 (2.4)	9 (3)	6 (4)	-	4 (16)	-	7 (5.6)	17 (13.6)	6 (4.8)
Enterococcus faecum	3 (1)	6 (2)	-		-	-	2 (1.6)	-	-
E. coli	26 (8.9)	32 (10.9)	14 (9.3)	-	3 (12)	15 (12)	10 (8)	13 (10.4)	8 (6.4)
Klebsiella oxytoca	3 (1)	2 (0.7)	-	-	-	3 (2.4)	-	-	2 (1.6)
Klebsiella pneumoniae	2 (0.7)	2 (0.7)	-	-	-	2 (1.6)	-	-	-
Kluyvera ascorbata	-	-	-	1 (4)	-	-	-	-	-
Kocuria rhizophila	3 (1)	-	-	-	-	-	2 (1.6)	-	2 (1.6)
Lactobacillus acidophilus	2 (0.7)	-	2 (1.3)	-	-	-	-	-	-
Lactobacillus brevis	-	-	1 (0.6)	-	-	-	-	-	-
Lactobacillus casei	-	-	1 (0.6)	-		-	-	-	-
Lactobacillus plantarum	-	-	1 (0.6)	-	-	-	-	-	-
Lactococcus garviae	1 (0.3)	-	-	-	-	-	-	-	-
Morganella morganii	-	-	-	-	-	1 (0.8)	-	-	-
Micrococcus luteus	1 (0.3)	2 (0.7)	2 (1.3)	-	-	2 (1.6)	2 (1.6)	-	1 (0.8)
Paenibacillus pabuli	-	-	-	-	-	-	2 (1.6)	-	-
Pasteurella multocida	1 (0.3)	-	-	-	-	-	-	-	-
Proteus vulgaris	2 (0.7)	-	1 (0.6)	-	-	2 (1.6)	2 (1.6)	-	-
Pseudomonas aeroginosa	2 (0.7)	1 (0.3)	-	-	-	1 (0.8)	-	-	1 (0.8)
Pseudomonas fluorescens	2 (0.7)	-	3 (2)	-	-	-	-	-	-
Pseudomonas putida	1 (0.3)	1 (0.3)	-	-	-	3 (2.4)	3 (2.4)	-	-
Serratia liquefaciens	2 (0.7)	3 (1)	-	-	-	-	-	-	-
Serratia marcensens	1 (0.3)	-	-	1 (4)	-	-	-	-	1 (0.8)
Shewanella putrefaciens	-	-	-	-	-	2 (1.6)	-	-	-
Staphylococcus aureus	57 (19.5)	60 (20)	21 (14)	6 (24)	5 (20)	12 (9.6)	8 (6.4)	20 (16)	15 (12)
Staphylococcus auricularis	6 (2)	10 (3.4)	-	-	-	-	-	-	-
Staphylococcus capitis	3 (1)	-	2 (1.3)	-	-	1 (0.8)	-	-	-
Staphylococcus chromogenes	24 (8.2)	21 (7.2)	11 (7.3)	-	-	-	3 (2.4)	-	9 (7.2)
Staphylococcus cohnii	5 (1.7)	16 (6.8)	6 (4)	-	-	-	-	-	-
Staphylococcus epidermidis	7 (2.4)	15 (5.1)	5 (3.3)	9 (36)	6 (24)	14 (11.2)	12 (9.6)	15 (12)	16 (12.8)
Staphylococcus equorum	4 (1.4)	11 (3.8)	5 (3.3)	-	-	-	-	-	-
Staphylococcus haemolyticus	8 (2.7)	33 (11.3)	4 (2.6)	-	-	15 (12)	-	-	3 (2.4)
Staphylococcus hominis	2 (0.7)	7 (2.4)	-	-	-	-	-	-	-
Staphylococcus hyicus	4 (1.4)	-	3 (2)	-	-	-	-	-	-
Staphylococcus saprophyticus	1 (0.3)	-	-	-	-	2 (1.6)	-	-	-
Staphylococcus simulans	5 (1.7)	4 (1.4)	3 (2)	1 (4)	-	-	-	-	2 (1.6)
Staphylococcus xylosus	12 (4.1)	8 (2.7)	5 (3.3)	2 (8)	-	2 (1.6)	-	1 (0.8)	6 (4.8)

Staphylococcus warneri	8 (2.7)	2 (0.7)	3 (2)	-	-	1 (0.8)	-	1 (0.8)	2 (1.6)
Stenotrophomonas maltophilia	-	-	1 (0.6)	-	-	-	-	-	-
Streptococcus agalactiae	5 (1.7)	3 (1)	4 (2.6)	-	-	-	-	-	-
Streptococcus dysgalactiae	-	-	-	-	-	-	-	-	1 (0.8)
Streptococcus feacalis	-	-	-	-	-	10 (8)	-	-	-
Streptococcus pyogenes	-	-	3 (2)	-	-	-	-	-	-
Sreptococcus pluranimalium	-	-	-	-	-	-	-	-	1 (0.8)
Streptococcus uberis	8 (2.7)	3 (1)	-	-	-	-	-	-	3 (2.4)
Trueperella pyogenes	2 (0.7)	-	-	-	-	-	-	-	-
Vibrio parahaemolyticus	-	-	-	-	-	1 (0.8)	-	-	-
Mix infection	3 (1)	7 (2.4)	2 (1.3)	-	2 (8)	1 (0.8)	3 (2.4)	2 (1.6)	5 (4)
No growth	4 (1.4)	-	3 (2)	-	2 (8)	4 (3.2)	2 (1.6)	4 (3.2)	4 (3.2)

Table 2. The virulence factors of *Staphylococcus aureus (S. aureus)* and coagulase-negative staphylococci (CoNS) in milk samples with subclinical mastitis.

CoNS (147)	Hemolysins*	Gelatinase	DNAse**	Biofilm	*mecA*	The calculated test statistic
S. chromogenes (25)	3β/3δ	2	2	3	-	2.23*
S. xylosus (16)	5β/1δ	-	-	2	-	0.87
S. haemolyticus (13)	-	-	-	-	-	0.01
S. warneri (11)	4β/2δ	-	3	3	-	1.89
S. epidermidis (8)	2δ	-	-	2	-	0.74
S. simulans (6)	-	-	-	2	-	0.32
S. hyicus (5)	1δ	-	-	1	-	0.48
S. capitis (3)	-	-	-	-	-	0.01
S. hominis (2)	-	-	-	-	-	0.01
S. saprophyticus (1)	-	-	-	-	-	0.01
***S. aureus* (57)**	9α/4β	15	16	10	2	5.84*

Note: * Hemolysins; refers to the production of α-, β-, or δ-type hemolysins by staphylococci. **DNAse; indicates the ability to hydrolyze DNA of staphylococci; * The chi-squared analysis was conducted using a 0.05 significance level, corresponding to a critical value of $\chi^2 = 2.18$. The virulence factors in the isolated staphylococci were interpreted as statistically independent when the calculated test statistic (G) was greater than the critical value ($G > \chi^2$). Conversely, when the test statistic was lower than the critical value ($G < \chi^2$), the independence of the factors was not statistically supported.

(Jenkins et al. 2019, Andrade-Becerra et al. 2021). *S. aureus* contamination in milk poses a risk for dairy products, with prevalence rates varying across Turkey and other countries (Mus et al. 2019, Kou et al. 2021).

High somatic cell counts (SCC), a key indicator of infection and correlated with intramammary infection and udder pathogen presence, were found in 89.7% of culture-positive cases in this study. This concurrent with other studies (Tančin et al. 2013, Hisira et al. 2023) showing SCC as a marker of pathogen presence.

Antibiotic resistance in *S. aureus* was significant: 70.2% for penicillin, 31.5% for erythromycin, and 40.4% for tetracycline. Resistance to trimethoprim-sulfamethoxazole (10.5%) was also slightly higher than in previous reports (Güler et al. 2005, Ünal and İstanbulluoğlu 2009, Tel et al. 2009) from Turkey. In contrast, resistance rates (less than 50%) to penicillin and ampicillin in the majority of *Staphylococcus* species from cows with mastitis in Europe and the U.S. were generally lower (Rüegsegger et al. 2013, Leijon et al. 2021), while the resistance rates in China were notably high (more than 80% of *Staphylococcus* spp) (Zhang et al. 2016).

The *mecA* gene was identified in three *S. aureus* isolates, two from milk and one from a farmer's hand indicating possible transmission between humans and animals. This supports findings from similar studies in Turkey (Mus et al. 2019), Egypt (Sadat et al. 2022), China (Zhang et al. 2016, Kou et al. 2021) and Indonesia (Khairullah et al. 2024), but lower than some Egyptian reports (Algammal et al. 2020, Sadat et al. 2022). MRSA presence in milk and farmers' hands poses public health risks, highlighting prudent antibiotic use to prevent the spread of resistance.

CoNS also harbored *mec*A, though virulence genes were absent in MRSA isolates (Ünal and Çinar 2012). CoNS isolates showed notable resistance to penicillin (100%), tetracycline (44.9%), and clindamycin (29.6%) (Ünal and Çinar 2012), with low resistance (1.0%) to vancomycin and chloramphenicol (Benjelloun Touimi et al. 2020).

The global One Health paradigm to AMR emphasizes the need for coordinated surveillance across human, animal, and environmental sectors, particularly due to the role of mobile resistance genes such as *mcr-1* in accelerating the spread of AMR (Velazquez-Meza et al. 2022). Recent research has highlighted that the importation of food and animal feed can serve as a route

Table 3. Antimicrobial resistance of *Staphylococcus aureus (S. aureus)* (n=204) and coagulase-negative staphylococci (CoNS) isolates (n=386) from human, animal and environmental samples.

Bacteria	Sources	Penicillins			Cefalosporines			Carba-penems	Macrolides		Aminoglycosides			Quinolones	Tetracyc-lines	Sulfona-mides
		AMP	PEN	OXA	CTX	FOX	CZ	IMP	CL	E	AMK	GEN	KAN	CIP	TET	SXT
S. aureus	milk samples with mastitis (57)	36 (63.2)	40 (70.2)	4 (7)	15 (26.3)	3 (5.2)	16 (28)	10 (17.5)	46 (80.7)	18 (31.5)	3 (5.2)	7 (12.3)	13 (22.8)	3 (5.2)	23 (40.4)	6 (10.5)
	cow`s rectal fecal samples (60)	35 (58.3)	38 (63.3)	3 (5)	12 (20)	2 (3.3)	13 (21.6)	7 (11.7)	42 (70)	16 (26.7)	2 (3.3)	8 (13.3)	9 (15)	1 (1.7)	16 (26.7)	5 (8.3)
	unpasteurised cow's milk (21)	14 (66.7)	15 (71.4)	2 (9.5)	4 (19)	1 (4.8)	5 (23.8)	2 (9.5)	16 (76.2)	7 (33.3)	1 (4.8)	2 (9.5)	4 (19)	1 (4.8)	3 (14.2)	2 (9.5)
	farmer's hand (6)	3 (50)	4 (66.6)	1 (16.7)	0	1 (16.7)	0	1 (16.7)	4 (66.6)	2 (33.3)	0	1 (16.7)	0	0	4 (66.7)	0
	farmer's feces (5)	2 (40)	3 (60)	0	0	0	0	0	3 (60)	1 (20)	0	0	0	0	3 (60)	0
	water (12)	3 (25)	5 (41.7)	0	0	0	0	0	6 (50)	1 (8.3)	0	0	0	0	0	1 (8.3)
	soil (8)	3 (37.5)	4 (50)	0	1 (12.5)	0	2 (25)	1 (12.5)	5 (62.5)	1 (12.5)	0	0	1 (12.5)	0	1 (12.5)	0
	feed (20)	7 (35)	9 (45)	1 (5)	1 (5)	0	5 (25)	0	12 (60)	3 (15)	1 (5)	1 (5)	1 (5)	1 (5)	0	2 (10)
	bedding material (15)	9 (60)	10 (66.7)	1 (6.7)	6 (40)	1 (6.7)	4 (26.7)	1 (6.7)	11 (73.3)	2 (13.3)	1 (6.7)	1 (16.7)	2 (13.3)	0	1 (16.7)	0
	P value		< 0.05			0.28		0.35	< 0.05			0.32		0.63	0.078	0.37
S. chromogenes	milk samples with mastitis (24)	8 (33.3)	9 (37.5)	0	2 (8.3)	4 (16.7)	5 (20.8)	2 (8.3)	7 (29.2)	3 (12.5)	0	1 (4.2)	6 (25)	1 (4.2)	0	1 (4.2)
	cow`s rectal fecal samples (21)	5 (23.8)	7 (33.3)	0	2 (9.5)	3 (14.3)	3 (14.3)	1 (4.8)	5 (23.8)	2 (9.5)	0	3 (5)	5 (23.8)	1 (4.8)	0	1 (4.8)
	unpasteurised cow's milk (11)	3 (27.3)	4 (36.4)	0	2 (18.2)	1 (9.1)	2 (18.2)	1 (9.1)	3 (27.3)	1 (9.1)	0	1 (9.1)	3 (27.3)	1 (9.1)	0	0
	farmer's hand (0)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	farmer's feces (0)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	water (0)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	soil (3)	0	0	0	0	0	0	0	1 (33.3)	0	0	1 (33.3)	0	0	0	1 (33.3)
	feed (0)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	bedding material (9)	1 (11.1)	2 (22.2)	0	1 (11.1)	1 (11.1)	1 (11.1)	0	1 (11.1)	0	0	1 (11.1)	0	0	1 (11.1)	3 (33.3)
	P value		< 0.05			0.36		0.59	0.26			0.40		0.68	0.92	0.68
S. warneri	milk samples with mastitis (8)	3 (37.5)	4 (50)	0	1 (12.5)	1 (12.5)	1 (12.5)	0	2 (25)	1 (12.5)	0	1 (12.5)	1 (12.5)	2 (25)	0	1 (12.5)
	cow`s rectal fecal samples (2)	1 (50)	1 (50)	0	0	0	0	0	0	0	0	0	0	0	0	0
	unpasteurised cow's milk (3)	1 (33.3)	2 (66.7)	0	0	0	0	0	1 (33.3)	0	0	0	0	0	0	0
	farmer's hand (0)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	farmer's feces (0)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	water (1)	0	1 (100)	0	0	0	0	0	0	0	0	0	0	0	0	0
	soil (0)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	feed (1)	0	0	0	0	0	0	0	1 (100)	0	0	0	0	0	0	0
	bedding material (2)	1 (50)	1 (50)	0	0	0	0	0	0	0	0	0	0	0	0	0
	P value		< 0.05			0.87		1.0	0.25			0.73		0.78	1.0	1.0

S. xylosus	milk samples with mastitis (12)	2 (16.7)	3 (25)	0	0	0	0	0	2 (16.7)	1 (8.3)	0	0	0	1 (8.3)	0	1 (8.3)
	cow`s rectal fecal samples (8)	1 (12.5)	2 (25)	0	0	0	0	0	1 (12.5)	1 (12.5)	0	0	0	1 (12.5)	0	1 (12.5)
	unpasteurised cow's milk (5)	1 (20)	1 (20)	0	0	0	0	0	1 (20)	0	0	0	0	1 (20)	0	1 (20)
	farmer's hand (2)	1 (50)	1 (50)	0	0	0	0	0	0	0	0	0	0	0	0	0
	farmer's feces (0)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	water (2)	0	1 (50)	0	0	0	0	0	1 (50)	0	0	0	0	0	0	0
	soil (0)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	feed (1)	0	0	0	0	0	0	0	1 (50)	0	0	0	0	0	0	0
	bedding material (6)	1 (16.7)	2 (33.3)	0	0	0	0	0	1 (16.7)	1 (16.7)	0	0	0	0	1 (16.7)	0
	P value		< 0.05		1.0			1.0	0.22			1.0		0.56	0.86	0.55
Other CoNS*	milk samples with mastitis (45)	9 (20)	10 (22.2)	0	4 (8.8)	3 (6.6)	1 (2.2)	0	5 (11.1)	3 (6.6)	1 (2.2)	2 (4.4)	1 (2.2)	4 (8.8)	6 (13.3)	3 (6.6)
	cow`s rectal fecal samples (96)	17 (17.7)	20 (20.8)	0	9 (9.4)	3 (3.1)	1 (1)	0	8 (8.3)	4 (4.2)	0	2 (2.1)	1 (1)	4 (4.2)	5 (5.2)	0
	unpasteurised cow's milk (28)	6 (21.4)	7 (25)	0	3 (10.7)	2 (7.1)	1 (3.6)	0	2 (7.1)	3 (10.7)	1 (3.6)	1 (3.6)	0	2 (7.1)	2 (7.1)	3 (10.7)
	farmer's hand (10)	2 (20)	3 (30)	0	1 (10)	1 (10)	0	0	1 (10)	0	0	0	0	0	2 (20)	0
	farmer's feces (6)	1 (16.7)	2 (33.3)	0	0	0	0	0	1 (16.7)	0	0	0	0	0	0	0
	water (32)	7 (21.8)	8 (25)	0	0	0	0	0	16 (50)	0	0	0	0	0	1 (4.8)	0
	soil (12)	2 (16.7)	3 (25)	0	1 (8.3)	1 (8.3)	0	0	2 (16.7)	1 (8.3)	0	0	0	1 (8.3)	1 (8.3)	0
	feed (15)	1 (6.7)	2 (13.3)	0	0	0	0	0	1 (6.7)	0	0	0	0	0	0	0
	bedding material (21)	1 (4.8)	2 (9.5)	0	0	0	0	0	1 (4.8)	0	0	0	0	0	0	0
	P value		0.16		0.34			1.0	0.31			0.48		0.42	0.26	0.35

Table 4. Antimicrobial resistance of *Escherichia coli* (*E. coli)* (n =121) from different sources.

Sources	Penicillins		Cefalosporines						Carba-penems	Aminoglycosides				Fluoroquinolones		Tetra-cycli-nes	Sulfo-namides
	AMC	AMP	CTX	FEP	CFM	LEX	FOX	CAZ	ETP	AMK	GEN	KAN	STR	CIP	NOR	TET	SXT
milk samples with mastitis (26)	6 (23.1)	14 (53.8)	10 (38.5)	3 (11.5)	5 (19.2)	4 (15.4)	4 (15.4)	9 (34.6)	3 (11.5)	-	16 (61.5)	-	14 (53.8)	2 (7.7)	2 (7.7)	21 (80.8)	19 (73.1)
cow`s rectal fecal samples (32)	9 (28.1)	18 (56.3)	8 (25)	2 (6.3)	8 (25)	3 (9.4)	7 (21.9)	4 (12.5)	2 (6.3)	8 (25)	16 (50)	-	20 (62.5)	9 (28.1)	4 (12.5)	22 (71.8)	16 (50)
unpasteurised cow's milk (14)	6 (42.9)	11 (78.5)	4 (28.6)	1 (7.1)	3 (21.4)	1 (7.1)	2 (14.2)	3 (21.4)	-	2 (14.2)	5 (35.7)	-	1 (7.1)	8 (57.1)	1 (7.1)	11 (78.5)	9 (64.3)
farmer's feces (3)	1 (33.3)	2 (66.6)	1 (33.3)	1 (33.3)	-	-	-	1 (33.3)	-	-	1 (33.3)	-	-	1 (33.3)	-	2 (66.6)	2 (66.6)
water (15)	7 (46.7)	10 (66.7)	4 (26.6)	-	2 (13.3)	-	1 (6.7)	2 (13.3)	-	1 (6.7)	2 (13.3)	-	2 (13.3)	4 (26.6)	-	6 (40)	5 (33.3)
soil (10)	2 (20)	5 (50)	3 (30)	1 (10)	1 (10)	2 (20)	1 (10)	-	-	-	1 (10)	-	-	1 (10)	-	7 (70)	3 (30)
feed (13)	8 (61.5)	9 (69.2)	3 (23.1)	1 (7.7)	-	2 (15.4)	1 (7.7)	-	-	1 (7.7)	1 (7.7)	-	-	3 (23.1)	-	10 (76.9)	3 (23.1)
bedding material (8)	6 (75)	7 (87.5)	2 (25)	1 (12.5)	-	1 (12.5)	-	-	-	-	-	-	-	1 (12.5)	-	5 (62.5)	2 (25)
P value	< 0.05		0.21						0.46	0.30				0.26		< 0.05	0.17

for introducing *mcr*-1-carrying *E. coli* strains into countries where the gene was previously uncommon (Slettemeås et al. 2017).

This study reinforces the One Health perspective, emphasizing how AMR connects human, animal, and environmental health. The comparative data on *E. coli* from various environmental and animal sources revealed higher resistance in isolates from milk with mastitis, feces, and unpasteurized milk, suggesting cattle as AMR reservoirs and vectors of resistant strains.

Conclusion

S. aureus continues to be a leading pathogen responsible for bovine mastitis and a significant contributor to AMR, resulting in considerable public health concerns and economic losses. This study indicates the first regional investigation into the concurrent presence of multidrug-resistant *S. aureus* and *E. coli* across human, animal, and environmental sources. The findings highlighted the urgent need for robust infection prevention measures and judicious use of antimicrobials. These insights are vital for shaping future research directions, informing policy development, and strengthening investments in AMR monitoring and control strategies.

Acknowledgements

This research was funded by the Slovak Research and Development Agency under the Contract no. APVV-22-0457 and project VEGA no. 1-0162-23: *Reduction in antibiotic use in dairy mastitis control programs*. This article is also based upon work from COST Action EURESTOP, CA21145, supported by COST (European Cooperation in Science and Technology).

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