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Molecular characteristics and antimicrobial susceptibility profiles of bovine mastitis agents in western Türkiye

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ABSTRACT

Importance: Identifying bovine mastitis agents using molecular methods to reveal their phylogenetic relationships and antimicrobial resistance profiles is essential for developing up-to-date databases in mastitis cases that cause severe economic losses.
Objective: This study examined bacterial mastitis agents in cows with clinical and subclinical mastitis observed in various dairy cattle farms to reveal their phylogenetic relationships and antibiotic resistance properties.
Methods: Sixty-two clinical and subclinical bovine mastitis milk samples were collected from 15 dairy farms. The polymerase chain reaction (PCR) was used to amplify the 16S rRNA gene regions of the bacteria. The 16S rRNA gene sequences obtained from sequencing include the V4–V6 regions. The strains were compared using a similarity analysis method that produced phylogenetic trees using the Molecular Evolutionary Genetics Analysis 11 program. Antibiotic susceptibilities were determined using the Kirby–Bauer disk diffusion method.
Results: Sixty-three bacteria were isolated and identified in this study. The most isolated bacteria from all mastitis cases were *Staphylococcus* spp. (30.2%), *Escherichia coli* (25.4%), *Streptococcus* spp. (14.3%), and *Aerococcus* spp. (7.9%), respectively. The phylogenetic trees were drawn from the 16S rRNA sequences. Some of these bacteria showed resistance to different types of antibiotics at varying rates.
Conclusions and Relevance: The bacteria isolated in this study originated from environmental sources. Regular cleaning of barns and proper hygiene practices are essential. Regular screenings for mastitis should be conducted in herds instead of the random or empirical use of antibiotics.

Keywords: Mastitis, bovine; dairy cow; phylogenetic analysis; subclinical infections

INTRODUCTION

Mastitis, which can be clinical or subclinical, is characterized by inflammation of the udder tissue. It is one of the most common and economically important diseases affecting the dairy industry [1]. More than 140 different pathogens have been reported in the etiology of mastitis

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Conflict of Interest
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[2]. The diversity of udder pathogens differs between countries and may vary in clinical and subclinical mastitis cases [3].

Many bacterial species, yeasts, or fungi have been isolated from mastitis cases. On the other hand, the most common etiological agents in bovine mastitis cases are *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus uberis*, *Escherichia coli*, coagulase-negative staphylococci (CNS), and other *Streptococcus* spp. [4]. These microorganisms can be classified as contagious or environmental pathogens. Contagious pathogenic microorganisms are agents adapted to survive in teat wounds and the mammary gland, while environmental pathogens are opportunistic agents in a contaminated environment [2,4]. While the transmission of contagious pathogens, such as *S. aureus* and *S. agalactiae*, from cow to cow occurs mainly during the milking process, opportunistic pathogens, such as *S. uberis* and *E. coli*, usually come from the contaminated environment and cause infection between milking or during the dry period [4].

In clinical mastitis (CM) cases, visible signs, such as redness, increased temperature, and swelling, can be detected through an inspection and palpation of the mammary glands. In subclinical mastitis (SCM) cases, however, there are no visible macroscopic signs of inflammation in the udder. It does not cause any changes in the appearance of the milk. Nevertheless, it is more common than clinical mastitis cases. SCM leads to milk loss and produces problems in the final products because it cannot be detected [2,5]. The methods used to diagnose mastitis cases in the field vary according to the course of the disease. CM can be diagnosed by observing physical changes in the udder and milk [1]. On the other hand, SCM is diagnosed using biochemical methods such as measuring somatic cell count, total dissolved solids, and electrical conductivity [6]. Nevertheless, microbiological diagnostic methods are considered the standard method in diagnosing the disease and determining the agent [7].

A polymerase chain reaction (PCR) and DNA sequencing are used widely in molecular biology and genetics. They detect microorganism diversity in bacterial flora that conventional methods cannot determine. Among various DNA regions, ribosomal RNA (rRNA) genes are used frequently in phylogenetic analyses because they are highly conserved among species [8]. Prokaryotic organisms have ribosomes of two subunits, 30S and 50S. 16S rRNA is present in the 30S size subunit. The 16S rRNA gene is found in all bacteria and is a universal region for bacterial identification [9]. Moreover, the function of 16S rRNA has remained the same over a long period [10]. The 16S rRNA gene is also large enough for bioinformatics studies (approximately 1,500 bp) [9]. The primary approach for treating and managing mastitis is antibiotics. On the other hand, the misuse of antibiotics has led to the emergence of multi-antibiotic-resistant bacteria, which is a growing threat to human and animal health globally [11].

Antimicrobial resistance has been an increasingly important problem in recent years among various bacterial species that cause infection in animals and humans [4,12]. The development of multiple resistance in some bacterial species means there are very few options among the preparations used in treatment. Initial treatment for bacterial infections of animals is usually based on field experience of the expected resistance of infectious agents. Studies indicate that regional differences may be observed in the antibiotic resistance and antibiotic resistance gene profiles of microorganisms that play a role in the etiology of mastitis [12]. This study examined the pathogenic bacteria that cause CM and SCM and revealed their phylogenetic relationships and antibiotic resistance properties.

METHODS

The Animal Experiments Local Ethic Committee of Mugla Sitki Kocman University approved this study under number E-40051172-100-359903.

Sampling

This study was conducted between April 2022 and November 2022, with samples taken from cows with mastitis during the lactation period on fifteen different farms. Milk samples from 62 cows with mastitis (CM, 30; SCM, 32) were collected. Scanning milk samples with California Mastitis Test (CMT; Kerbl, Germany) determined mammary quarters as CM and SCM. The milk samples that tested positive for the CMT were used [3]. The samples taken from animals with the symptoms of tenderness, increased temperature, and redness in the udder were classified as CM, while the samples taken from animals without any udder symptoms were classified as SCM samples. The udders of cows from which milk samples would be collected were cleaned with paper towels and disinfected using 70% ethyl alcohol. After the alcohol had evaporated, an average milk sample of 15–20 mL was milked into sterile sample containers. The samples were delivered to the laboratory under a cold chain on the same day and stored at 4°C until they were analyzed on the same day [5,13].

Bacterial isolations from milk samples

For bacterial isolation, milk samples were inoculated onto blood agar media containing 5%–7% sheep blood. The blood agars were incubated under aerobic conditions for 24–48 h. For identification purposes, pure cultures were obtained from the growth colonies and then transferred to blood agar for further analysis. The colony morphologies were assessed for pigment, color, size, and hemolysis properties, and their microscopic morphologies were determined by Gram staining. Catalase, oxidase, and other biochemical tests were applied to the strains. The pure cultures produced were stored at −20°C in sterile tubes containing glycerol (20%) tryptic soy broth (TSB) [3].

16S rRNA PCR analysis, gene sequencing studies, and phylogenetic analysis

DNA extraction for the 16S rRNA PCR analysis of isolated bacteria was performed with a commercial extraction kit (GeneJET Genomic DNA Purification Kit; Thermo Scientific, USA) according to the manufacturer's instructions. From the genomic DNA obtained from each isolate, the 16S rRNA gene regions were amplified by PCR using the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The amplification processes followed the kit protocol using a commercial master mix (2× Dream Taq PCR Master Mix; Thermo Scientific) kit. The reaction mixture was prepared in a 50 µL volume by adding 25 µL of 2× master mix, 2 µL of each of F and R primers (10 pmol each), 4 µL of template DNA, and 17 µL of DNAse-RNAse free water. The amplification process was conducted in 0.2 mL tubes with a conventional PCR device (Blue-Ray; Biotech, Taiwan). The reaction degrees and times were as follows. The first denaturation step was 2 min at 95°C (one cycle), followed by 35 cycles of 30 sec denaturation at 95°C, 30 sec annealing at 55°C, 1 min extension at 72°C, and finally 10 min extension step at 72°C (1 cycle). The amplicons were programmed to be kept at 4°C, and the desired region was amplified in the PCR thermal cycler. The amplicons were visualized using gel electrophoresis by preparing a 1% agarose gel with Tris-Borate-EDTA buffer (Sigma-Aldrich, USA) [13,14].

BM Labosis (Türkiye) purified and analyzed the obtained DNA sequences. The sequence analysis results were compared with the GenBank database at the National Center

Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) using the Basic Local Alignment Search Tool (BLAST) program. The accession numbers were assigned to the base sequences of the 16S rRNA gene region obtained by sequencing. In addition, 16S rRNA gene sequences of other bacterial species isolated from animal and human clinical samples in Turkey, which have an accession number in the GenBank (https://www.ncbi.nlm.nih.gov/nuccore/), were obtained, and a phylogenetic tree was drawn. These isolates were the subclinical bovine mastitis isolate *Staphylococcus haemolyticus* strain BM-TRKM1 (FJ654656.1) and *S. aureus* strain SIU1 (OK624658.1), *S. aureus* strain SIU3 (OK624659.1), *S. aureus* strain SIU4 (OK624660.1), *S. aureus* strain SIU5 (OK624661.1), *E. coli* strain EGE 3838360-21 (KY655051.1), *E. coli* strain EGE 3838360-3 (KY655034.1), *E. coli* strain EGE 3938785-9 (KY655039.1), *E. coli* strain EGE 3812419-12 (KY655042.1), and *E. coli* strain EGE 3975600-16 (KY655046.1) were isolated from human clinical samples. The maximum likelihood method and the Tamura–Nei model were used to infer the evolutionary history [15]. The initial trees for the heuristic search were obtained automatically by applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model. The analysis involved 74 nucleotide sequences, with 1,499 positions in the final dataset. Evolutionary analyses were performed using Molecular Evolutionary Genetics Analysis 11 (MEGA11) [16].

Determination of susceptibility of bacterial isolates to some antibiotics

The resistance of bacteria to antimicrobial agents was assessed using the agar disc diffusion technique following the Clinical and Laboratory Standards Institute (CLSI) guidelines [17,18]. Pure cultures obtained from the growth of isolates on TSB media were adjusted to 0.5 McFarland turbidity in sterile physiological saline. A 0.1 ml sample of each bacterial suspension was inoculated onto Mueller–Hinton agar or blood agar plates, and antibiotic discs (penicillin 10 U, cefoperazone 75 μg, enrofloxacin 5 μg, erythromycin 15 μg, gentamicin 10 μg, kanamycin 30 μg, neomycin 30 μg, tetracycline 30 μg, streptomycin 10 μg, ciprofloxacin 5 μg, trimethoprim/sulfamethoxazole 25 μg, ampicillin/sulbactam 20 μg; Bioanalyse, Türkiye) were placed at appropriate intervals. Petri dishes containing antibiotic disks were placed in an incubator at 37°C for 24 h. After incubation, the diameter of the inhibition zones was measured. The susceptibility and resistance statuses were determined according to the standards set by the CLSI and the European Antimicrobial Susceptibility Testing Committee (EUCAST) [17-19].

RESULTS

Bacterial isolations from milk samples

During the study, milk samples taken from 30 cows with CM and 32 cows with SCM from 15 different farms were examined bacteriologically. There was no growth in three of the SCM milk samples taken, and four of the CM milk samples were discarded due to contamination. In the samples taken from five of these farms, yeast was isolated from two milk samples with CM and three with SCM. Sixty-three bacterial strains obtained from 13 of these farms were identified as SCM and CM agents, and antibiogram analyses were performed. Genetic analysis was performed on 16S rRNA amplicons of bacterial isolates obtained from milk samples with CM and SCM. Twenty-six bacterial strains in the CM samples and 37 bacterial strains in the SCM samples were identified. The highest number of *Staphylococcus* spp. (n = 19) and *Escherichia* spp. (n = 16) species were detected in milk samples taken from all mastitis cases. Subsequently, *Streptococcus* spp. (n = 9), *Aerococcus* spp. (n = 5), *Acinetobacter* spp. (n = 3),

Klebsiella spp. (n = 3), *Corynebacterium* spp. (n = 2), and *Brevibacterium* spp. (n = 2), and other species (n = 4) were also detected. In this study, *Staphylococcus* spp. bacteria, the dominant genus, were isolated from 15 SCM samples and four from CM samples. In contrast, the second dominant genus, *Escherichia* spp., was identified as *E. coli*, 11 and five from CM and SCM samples, respectively. Of the nine *Streptococcus* spp. bacteria, which is the dominant third genus, five and four were isolated from the CM and SCM milk samples, respectively. When all samples were evaluated, two different bacterial agents were identified in two of the CM milk samples (K21 and K22) and in 10 of the SCM milk samples (SK5, SK10, SK11, SK12, SK13, SK14, SK18, SK19, SK20, and SK25) taken from the same farm. **Table 1** provides information on the identified bacteria, and **Fig. 1** presents their percentage distribution at the genus level.

16S rRNA gene region sequence analysis, identification, and phylogenetic analysis

The base sequence of the 16S rRNA gene region obtained as a result of the sequence was compared with the other sequences in the GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). Their similarity percentages were determined, and the bacteria were identified. **Table 1** lists the similarity percentages and accession numbers of the identified bacteria. **Fig. 1** shows the graphical distribution of the identified strains at the species and genus level.

The 16S rRNA gene has nine hypervariable regions represented in V1–V9 that can be used to distinguish different organisms [20]. The 16S rRNA gene sequences obtained from

Table 1. Similarity results of sequence analysis of the 16S rRNA gene region and distribution of all isolates by farm and their accession numbers in GenBank

Farm code	Floor substrate structure of farms	Mastitis case	Sample code	Isolate number	Identified isolate	Similarity rate (%)	AN of similar strains	GenBank AN of identified isolates in this study
DF1	Caoutchouc	CM	K19	K19A	*Aerococcus viridans*	99.82	MT502756.1	OR899228
	Caoutchouc	CM	K20	K20A	*Streptococcus uberis*	100	LC316933.1	OR898848
	Caoutchouc	CM	K21	K21A	*Staphylococcus simulans*	99.62	MT409926.1	OR898843
				K21B	*Staphylococcus chromogenes*	99.53	MT072170.1	OR898845
	Caoutchouc	CM	K22	K22A	*Corynebacterium lactis*	100	KF661298.1	OR898847
				K22B	*Staphylococcus chromogenes*	100	MT072191.1	OR898850
	Caoutchouc	SCM	SK1	SK1A	*Staphylococcus chromogenes*	99.91	MT072191.1	OR857400
	Caoutchouc	SCM	SK2	SK2A	*Escherichia coli*	100	KY367395.1	OR857415
	Caoutchouc	SCM	SK20	SK20A	*Staphylococcus xylosus*	99.23	ON678172.1	OR876248
				SK20B	*Acinetobacter johnsonii*	99.79	MN826577.1	OR876249
	Caoutchouc	SCM	SK21	SK21A	*Streptococcus uberis*	99.31	KX390233.1	OR876250
	Caoutchouc	SCM	SK22	SK22A	*Corynebacterium lactis*	100	KF661298.1	OR876251
	Caoutchouc	SCM	SK24	SK24B	*Aerococcus viridans*	100	MT502756.1	OR876262
	Caoutchouc	SCM	SK25	SK25A	*Staphylococcus epidermidis*	100	OR039103.1	OR876263
				SK25B	*Fundicoccus ignavus*	99.35	MN640404.1	OR915467
	Caoutchouc	SCM	SK26	SK26A	*Staphylococcus saprophyticus*	99.04	MH426600.1	OR876267
	Caoutchouc	SCM	SK27	SK27A	*Aerococcus urinaeequi*	99.86	MH329637.1	OR876271
	Caoutchouc	SCM	SK28	SK28A	*Streptococcus uberis*	100	LC317314.1	OR880221
	Caoutchouc	SCM	SK29	SK29A	*Aerococcus viridans*	99.79	MW175546.1	OR880222
	Caoutchouc	SCM	SK30	SK30A	*Streptococcus uberis*	100	LC317314.1	OR880223
				SK30B	*Staphylococcus epidermidis*	100	OR039103.1	OR880224
	Caoutchouc	SCM	SK32	SK32A	*Aerococcus viridans*	100	MT502756.1	OR880241
DF2	Caoutchouc	CM	K1	K1A	*Escherichia coli*	99.88	OR064346.1	OR889605
	Caoutchouc	CM	K2	K2A	*Escherichia coli*	100	OR064346.1	OR889624
	Caoutchouc	CM	K4	K4A	*Escherichia coli*	99.49	MN083301.1	OR889627
	Caoutchouc	CM	K5	K5A	*Escherichia coli*	99.60	MN083301.1	OR889631
	Caoutchouc	CM	K7	K7A	*Escherichia coli*	99.82	MN083301.1	OR889630

(continued to the next page)

Table 1. (Continued) Similarity results of sequence analysis of the 16S rRNA gene region and distribution of all isolates by farm and their accession numbers in GenBank

Farm code	Floor substrate structure of farms	Mastitis case	Sample code	Isolate number	Identified isolate	Similarity rate (%)	AN of similar strains	GenBank AN of identified isolates in this study
DF3	Sawdust on the concrete floor	CM	K3	K3A	*Streptococcus ruminantium*	100	LC316930.1	OR889625
	Sawdust on the concrete floor	SCM	SK4	SK4A	*Escherichia coli*	100	OR064346.1	OR857422
	Sawdust on the concrete floor	SCM	SK5	SK5A	*Staphylococcus chromogenes*	99.73	MT072170.1	OR858885
				SK5B	*Acinetobacter baumannii*	99.17	KJ806334.1	OR858891
	Sawdust on the concrete floor	SCM	SK6	SK6A	*Staphylococcus epidermidis*	99.90	LC499787.1	OR859457
	Sawdust on the concrete floor	SCM	SK7	SK7A	*Staphylococcus chromogenes*	99.90	MT072170.1	OR859534
	Sawdust on the concrete floor	SCM	SK8	SK8B	*Staphylococcus simulans*	99.44	MG798670.1	OR865979
	Sawdust on the concrete floor	SCM	SK9	SK9B	*Stenotrophomonas maltophilia*	99.46	MT533812.1	OR866003
	Sawdust on the concrete floor	SCM	SK10	SK10A	*Staphylococcus sp.*	98.89	MT321508.1	OR875844
				SK10B	*Escherichia coli*	99.65	KY367395.1	OR875856
	Sawdust on the concrete floor	SCM	SK11	SK11A	*Staphylococcus chromogenes*	99.43	MT072170.1	OR875870
				SK11B	*Escherichia coli*	99.88	MN083301.1	OR875873
	Sawdust on the concrete floor	SCM	SK12	SK12A	*Staphylococcus sp.*	98.88	MT261886.1	OR875935
				SK12B	*Staphylococcus aureus*	100	OQ954841.1	OR875936
	Sawdust on the concrete floor	SCM	SK13	SK13A	*Streptococcus uberis*	99.59	LC316933.1	OR875942
				SK13B	*Brevibacterium sp.*	99.34	MW931626.1	OR875981
DF4	Caoutchouc	CM	K17	K17A	*Streptococcus pasteurianus*	99.91	MT585424.1	OR898838
	Caoutchouc	SCM	SK14	SK14A	*Escherichia coli*	99.90	OR206334.1	OR875984
				SK14B	*Staphylococcus caprae*	99.72	MT225637.1	OR876247
DF5	Sawdust on the concrete floor	SCM	SK18	SK18A	*Staphylococcus devriesei*	100	FJ938168.1	OR880426
				SK18B	*Klebsiella pneumoniae*	99.35	OQ550178.1	OR880559
	Sawdust on the concrete floor	SCM	SK19	SK19A	*Glutamicibacter arilaitensis*	100	MH130322.1	OR915465
				SK19B	*Brevibacterium siliguriense*	99.42	MH489001.1	OR880560
DF6	Sawdust on the concrete floor	CM	K9	K9A	*Streptococcus uberis*	99.89	LC316933.1	OR889637
DF7	Sand base over concrete	CM	K10	K10A	*Escherichia coli*	100	OR064346.1	OR889642
	Sand base over concrete	CM	K11	K11A	*Escherichia coli*	99.45	OR064346.1	OR889640
DF8	Sawdust on the concrete floor	CM	K13	K13A	*Escherichia coli*	99.70	OR064346.1	OR889641
DF9	Sawdust on the concrete floor	CM	K8	K8A	*Klebsiella oxytoca*	99.63	MG557812.1	OR889635
	Sawdust on the concrete floor	CM	K12	K12A	*Escherichia coli*	100	OR064346.1	OR889643
	Sawdust on the concrete floor	CM	K14	K14A	*Klebsiella variicola* subsp. *variicola*	99.72	OQ406238.1	OR889644
DF12	Soil floor	CM	K24	K24A	*Escherichia coli*	99.81	OR064346.1	OR898851
DF13	Sawdust on the concrete floor	CM	K25	K25A	*Staphylococcus epidermidis*	100	MT613456.1	OR898853
	Sawdust on the concrete floor	CM	K29	K29A	*Streptococcus uberis*	99.33	LC316933.1	OR898865
	Sawdust on the concrete floor	CM	K30	K30A	*Acinetobacter seohaensis*	99.83	MT516326.1	OR898866
DF14	Soil floor	CM	K26	K26A	*Escherichia coli*	99.91	KX575726.1	OR898863
DF15	Soil floor	CM	K27	K27A	*Kocuria salsicia*	99.83	OP263486.1	OR915468

rRNA, ribosomal RNA; DF, dairy farm; AN, accession number; CM, clinical mastitis; SCM, subclinical mastitis.

sequencing in this study include the V4–V6 (V4: 576-682 and V6: 986-1043) regions. Based on the obtained sequences, the strains were compared using a similarity analysis method that produced phylogenetic trees. The phylogenetic trees were drawn from the 16S rRNA sequences of 74 bacterial species using the MEGA11 program (**Fig. 2**). The *S. aureus* strain SIU1 (OK624658.1), *S. aureus* strain SIU3 (OK624659.1), *S. aureus* strain SIU4 (OK624660.1), *S. aureus*

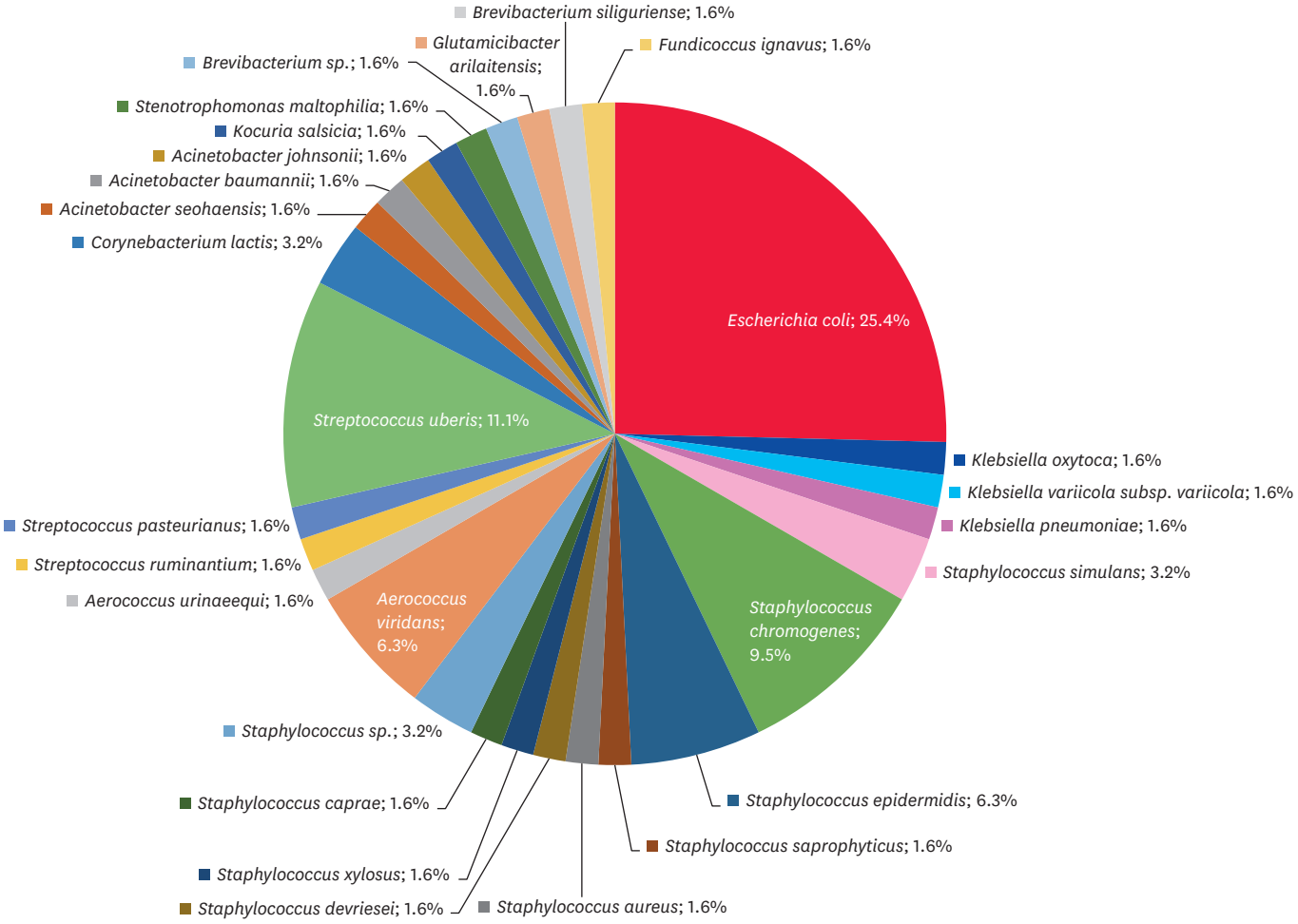

Fig. 1. Percentage distribution chart of identified strains according to the bacterial species.

strain SIU5 (OK624661.1) bacterial strains, whose sequences were obtained from GenBank for phylogenetic analysis, were located in a separate branch from other *Staphylococcus* spp. strains in the tree, as shown in **Fig. 2**. The nucleotide number of the 16S rRNA sequences of these bacterial strains was 469–477 bp; these sequences were located upstream of the V4 region. **Table 1** lists the genome sequences of all isolates deposited in the GenBank database with the accession numbers.

Antibiogram test results

Although all *Staphylococcus* spp. strains were sensitive to ciprofloxacin, 95% sensitivity to erythromycin, gentamicin, and sulfamethoxazole/trimethoprim was detected. Different rates of susceptibility to neomycin (89%), enrofloxacin (89%), penicillin (84%), and tetracycline (63%) were detected in the same strains. The highest resistance in these strains was against tetracycline (26%), followed by penicillin (16%) and neomycin (11%). At the same time, moderate sensitivity to tetracycline and enrofloxacin was detected at a rate of 11%. Multiple resistance was observed in the bacteria (*Staphylococcus epidermidis*) isolated from a CM milk sample (K25A) against sulfonamide, tetracycline, and aminoglycoside. In strains within the Enterobacteriaceae family, 100% sensitivity to neomycin and gentamicin and 74% sensitivity to kanamycin were observed. On the other hand, moderate sensitivity (63%) and resistance

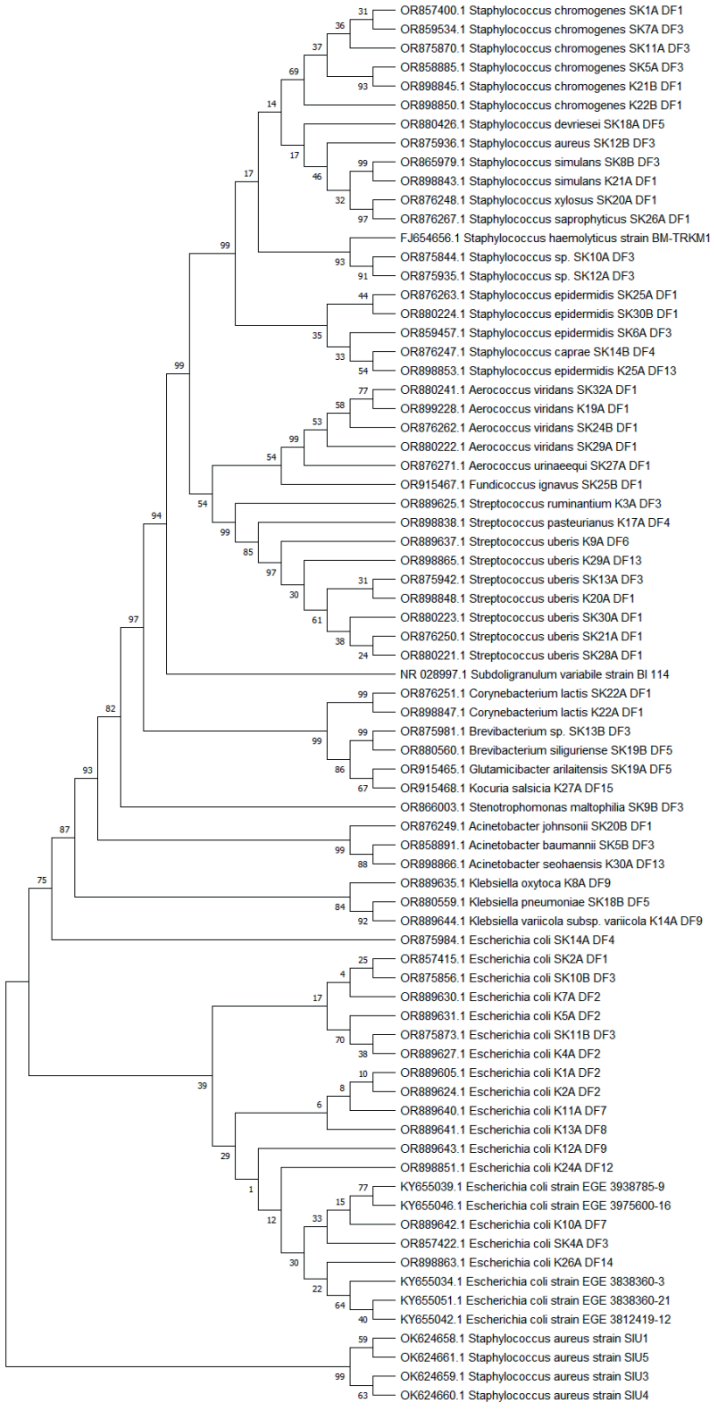

Fig. 2. Phylogenetic tree showing the taxonomic position of identified bacterial species and genus according to the 16S rRNA sequence. Outgroup NR 028997.1 *Subdoligranum variable* strain BI 114 is a partial sequence of 16S rRNA. The maximum likelihood method and the Tamura–Nei model were used to infer the evolutionary history. rRNA, ribosomal RNA; DF, dairy farm.

(16%) were found for streptomycin within the same group. The analysis showed that the group exhibited the highest resistance to erythromycin (84%), followed by penicillin (42%). Multiple antibiotic resistance from at least three different groups was detected in three *E. coli* strains (K13A, SK4A, and SK14A). All isolates of *Streptococcus* spp. showed sensitivity to

Table 2. Antibiotic resistance distributions of all identified strains

Bacteria	SAM	P	ENR	S	K	CFP	E	SXT	T	CN	N	CIP	MDR
Enterobacteriaceae	2 (11)	8 (42)	2 (11)	3 (16)	0 (0)	1 (5)	16 (84)	2 (11)	2 (11)	0 (0)	0 (0)	4 (21)	3 (16)
Staphylococcus spp.	-	3 (16)	0 (0)	-	-	-	1 (5)	1 (5)	5 (26)	1 (5)	2 (11)	0 (0)	1 (5)
Streptococcus spp.	2 (22)	2 (22)	0 (0)	-	-	0 (0)	2 (22)	1 (11)	6 (67)	-	-	-	1 (11)
Aerococcus spp.	1 (20)	0 (0)	0 (0)	4 (80)	2 (40)	0 (0)	1 (20)	3 (60)	1 (20)	1 (20)	0 (0)	0 (0)	2 (40)
Corynebacterium spp.	-	2 (100)	0 (0)	-	-	-	2 (100)	2 (100)	1 (50)	0 (0)	0 (0)	0 (0)	2 (100)
Acinetobacter spp.	0 (0)	-	-	-	-	-	-	0 (0)	1 (33)	0 (0)	-	0 (0)	0 (0)
Others	2 (33)	2 (33)	2 (33)	1 (17)	2 (33)	1 (17)	2 (33)	4 (67)	2 (33)	1 (17)	1 (17)	2 (33)	3 (50)

Data shown are number of resistant isolates (%).
SAM, ampicillin/sulbactam; P, penicillin; ENR, enrofloxacin; S, streptomycin; K, kanamycin; CFP, cefoperazone; E, erythromycin; SXT, sulfamethoxazole/trimethoprim; T, tetracycline; CN, gentamicin; N, neomycin; CIP, ciprofloxacin; MDR, multidrug resistance; -, no evaluation criteria.

cefoperazone. This was followed by enrofloxacin (89%) and penicillin, sulfamethoxazole trimethoprim, and erythromycin, all with the same sensitivity rate (78%). These isolates showed the highest resistance to tetracycline (67%). Multiple antibiotic resistance was observed in one strain (K9A). The highest resistance in the *Aerococcus* spp. strains was seen in streptomycin (80%) and sulfamethoxazole/trimethoprim (60%). Multiple antibiotic resistance was determined in two *Aerococcus viridans* strains (SK29A and SK32A) isolated from the same farm. **Table 2** lists the rates of phenotypic and multiple antibiotic resistance of the isolates to antibiotics from different groups.

DISCUSSION

Sixty-three bacterial strains in the CM and SCM milk samples collected in this study were identified genetically by 16S rRNA sequence analysis. Dairy cows with SCM and CM in acute or chronic form were included in this study. The 16S rRNA gene sequence, approximately 1,500 bp long, is used widely as a marker gene in determining bacterial species because of its highly variable and highly conserved regions among bacterial species [21]. Bacterial identification was achieved by 16S rRNA gene region sequence analysis. An analysis of just one of the hypervariable regions of the 16S rRNA gene, which contains different variable regions, is insufficient to distinguish all bacteria. Although regions V2, V3, and V6 are generally sufficient for discrimination at the genus level, they are also sometimes used for discrimination at the species level. According to the data obtained from the studies, V4–V6 regions are the most reliable regions that best represent the entire 16S rRNA gene sequence for bacterial phylogenetic analysis. A previous study also reported that V2 and V8 are the least reliable regions [22]. In this study, species were determined by considering the sequences of the V4–V6 regions.

When the bacterial strains in the results of this study were examined, the highest rates in the milk samples taken from all mastitis cases were bacteria from the Enterobacteriaceae family and *Staphylococcus* spp. species. These were followed by species belonging to the genera *Streptococcus* spp., *Aerococcus* spp., *Acinetobacter* spp., *Corynebacterium* spp., and *Brevibacterium* spp. On the other hand, similar or different findings have been reported regarding pathogens related to subclinical and clinical mastitis in different studies [3,23,24]. In this study, *E. coli* and *Streptococcus* spp. were responsible for most of the CM cases, and *Staphylococcus* spp. was responsible for most of the SCM cases. Except for one strain (K19A), all *Aerococcus* spp. isolates were isolated from the SCM samples.

Abdi et al. analyzed cows with CM and SCM. They reported the main causative bacteria isolated from bovine mastitis cases in the study area in decreasing order: *S. aureus* (34.2%), *S. uberis* (20.7%), *Streptococcus dysgalactiae* (18.7%), *E. coli* (17.6%), *Klebsiella pneumoniae* (6.7%), and *Klebsiella oxytoca* (2.1%) [25]. A recent bacteriological examination conducted by Babacan in the Balıkesir region of Türkiye collected milk samples from cows with CM and SCM [26]. The examination showed that *E. coli*, *K. oxytoca*, and *K. pneumoniae* were isolated in 6.6%, 1.41%, and 0.94% of the samples, respectively. In another study conducted in Austria [1], the most frequently identified pathogen group from clinical and SCM milk samples was staphylococci (50%), followed by streptococci (28%) and Enterobacteriaceae (14%). In the current study, similar to these studies, the most dominant bacterial species within Enterobacteriaceae, which is among the bacteria isolated at a high rate, was *E. coli* (25.4%). Of these, 11 and five were isolated from CM and SCM cases, respectively. Three different strains of the *Klebsiella* spp. species (4.8%) within the same group have been identified. One of these is responsible for SCM (*K. pneumoniae*) and two cases of CM (*Klebsiella oxytoca* and *Klebsiella variicola* subsp. *variicola*).

Antibiotic resistance is a top priority for health policymakers globally, with implications for human, animal, and environmental health [4]. Antibiotic resistance poses several challenges, including the difficulty of treating infections, the severity of the illnesses caused by resistant bacteria, and increased mortality rates. In addition, resistant bacteria can spread to humans through various channels, such as unpasteurized milk, wild animals, contaminated waterways, and the food chain [14]. The antibiotic susceptibilities of the mastitis agents identified in this study were determined using the Kirby–Bauer disk diffusion method. The Enterobacteriaceae isolates were most sensitive to gentamicin–neomycin and aminoglycosides, with 100% sensitivity rates for both. The isolates also showed high sensitivity rates for cefoperazone (89%) and sulfamethoxazole/trimethoprim (89%). On the other hand, the isolates were most resistant to erythromycin (84%), followed by penicillin (42%). In some cases, the *E. coli* strains were resistant to more than three groups of antibiotics. For example, one CM case (K13A) and two strains isolated from SCM milk (SK4A and SK14A) showed multiple resistance development. *S. aureus* is one of the leading bacterial causes of mastitis in dairy cows worldwide. Although there are different results in various regions of the world, CNS are isolated most frequently from cows with mastitis and are increasingly reported as mastitis pathogens [27]. The *Staphylococcus* species isolated in this study were determined to be CNS, except for one strain. Six of the nineteen *Staphylococcus* spp. isolates were identified as *Staphylococcus chromogenes* (two from CM and four from SCM). Four were identified as *S. epidermidis* (one from CM and three from SCM). Along with these species, different staphylococcal species have also been identified as predominant from SCM agents (24% from subclinical cases and 6% from clinical cases). Pascu et al. [2] reported that all staphylococcal strains isolated from mastitis cases were resistant to at least four antimicrobial agents, and multiple resistance (ampicillin, polymyxin B, tetracycline, tylosin, amoxicillin-clavulanic acid, oxacillin, erythromycin, methicillin, and novobiocin) were detected. The same study observed low resistance rates for kanamycin, gentamicin, amoxicillin, and cephalothin. Schabauer et al. [1] reported that *S. aureus* and non-*S. aureus* isolates identified from mastitis cases showed high sensitivity to different antimicrobials (78% for both). An examination of the antibiotic sensitivity status of all staphylococci isolated in this study showed that all of the strains were sensitive to ciprofloxacin. A high rate of sensitivity to gentamicin (95%), erythromycin (95%), and sulfamethoxazole/trimethoprim (95%) was observed, followed by enrofloxacin (89%), neomycin (89%), and penicillin (84%) with high sensitivity rates, respectively. The highest antibiotic resistance in all *Staphylococcus* spp. isolates were determined to be against tetracycline (26%). Similarly, higher resistance

rates to tetracycline have been reported in staphylococci isolated from mastitis cases compared to some antibiotics [1,2].

In this study, nine *Streptococcus* spp. bacteria were identified from different farms. Seven were identified as *S. uberis*, three of which were isolated from clinical and four from subclinical mastitis cases. *Streptococcus ruminantium* was identified from one clinical case, and *Streptococcus pasteurianus* was identified from another clinical mastitis case. The mastitis cases caused by microorganisms of the *Streptococcus* spp. genus have been reported in different countries. In some regions, such as Ireland, the *S. uberis* agent is commonly isolated among *Streptococcus* spp. [4]. A study in the Aydin province of Türkiye isolated *Streptococcus* spp. as the most responsible for mastitis after staphylococci and reported that the most common species among these isolates was *S. uberis*. In the same study, *S. agalactiae* was isolated at a high rate after *S. uberis* [28]. In the present study, *Streptococcus* spp. species were isolated as the most common mastitis causative agent after Enterobacteriaceae and *Staphylococcus* species. On the other hand, the most common species among streptococci was *S. uberis*. All strains exhibited sensitivity to cefoperazone when the antibiotic sensitivities of *Streptococcus* spp. isolates were examined. In addition, a high sensitivity rate was determined against enrofloxacin (89%), penicillin (78%), erythromycin (78%), and ampicillin/sulbactam (78%). The antibiotic group with the highest resistance was tetracycline (67%). The resistance levels vary among *Streptococcus* species in the literature. Cases of slowly but continuously increasing resistance to tetracyclines, especially in mastitis-related *S. uberis* agents, have been reported [4]. Moreover, all *S. uberis* agents except one strain from the isolates obtained in this study developed resistance to tetracyclines.

Among the bacteria isolated in this study, in addition to the bacterial genera mentioned above, *Aerococcus* spp. (7.9%) and *Acinetobacter* spp. (4.8%) were also isolated. Recently, mastitis cases caused by these bacteria were also reported [13,29]. In addition, different types of microorganisms were detected in varying proportions in the collected milk. Although there is insufficient information in the literature that these bacteria cause mastitis, *Corynebacterium lactis* isolated in the present study has been associated with infections in domestic animals. Although this bacterium has not been considered a pathogen, the determinants of the infections it causes remain unclear [30]. On the other hand, this bacterium is also isolated from raw milk collected from farms [31]. *Stenotrophomonas maltophilia*, which can cause mastitis as an opportunistic pathogen, has been isolated from various mastitis cases in recent years with different strains with high antibiotic resistance [32]. Previous studies have shown that entherogenic *S. maltophilia* migrates from the digestive system to the mammary gland, especially in animals fed highly concentrated rations, and causes mastitis [33,34]. In the current study, a *S. maltophilia* strain was isolated from a subclinical mastitis case, which was determined to have developed resistance to all antibiotics tested except the fluoroquinolone groups. In this study, *Kocuria salsicia* species were identified from a milk sample taken from a cow with clinical mastitis. Previous studies reported that this agent and some species of the related genus were isolated from clinical mastitis milk samples [35] and milking machines [36]. *Glutamicibacter arilaitensis*, *Brevibacterium sp.*, *Brevibacterium siliguriense*, and *Fundicoccus ignavus* bacteria identified in this study were isolated from milk samples taken from cows with subclinical mastitis. Although these bacterial species were isolated from milk samples from different sources [13,37-39], they have not been reported as etiological agents of cow mastitis.

Bovine mastitis is a complex disease influenced by various internal and external factors. Internal factors include age, health status, lactation period, and parity, while external factors encompass bedding material, udder hygiene, farm management, region, and climate [13]. Current studies have revealed increased causative agents of mastitis, environmental pathogens (e.g., CNSs), and other bacilli [2,14]. The bacteria isolated in this study originated from environmental sources, such as bedding, tools, and equipment used in care and feeding. The resistance to various antibiotics was detected in these bacteria at certain rates. The study showed that all milk samples had the highest rate of *E. coli*, followed by *S. chromogenes* and *S. epidermidis* from CNS, and *S. uberis*. In addition, the presence of *A. viridans* and other environmental bacteria was determined. Only a single SCM sample (SK12) contained *S. aureus*.

The optimal conditions for animal care and nutrition are essential to safeguard the welfare of the animals and prevent contamination. Regular barn cleaning and proper hygiene practices during milking are essential. It is essential to conduct routine mastitis screening in herds rather than resort to indiscriminate or empirical use of antibiotics. Resistance distributions of mastitis agents are generally herd-specific. The emergence of antimicrobial-resistant infections in the herd is a serious challenge for mastitis control. In this case, the treatment rates can be increased using more than one synergistically effective antimicrobial agent [2]. Milk samples should be taken from animals with clinical or subclinical mastitis, and causative agent and antibiogram analyses should be performed. This aids in the early detection and treatment of mastitis cases using the correct and appropriate antibiotic preparations, preventing udder and milk loss and serious economic losses. It is also important to determine the resistance profiles of local isolates to guide field-specific treatment regarding the antimicrobial resistance profiles of mastitis agents in bacterial mastitis cases. This helps prevent the development of new bacterial resistance cases in the future.

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