The Relationship between Biofilm Formation and Multiple Antibiotic Resistance of *Streptococcus* Isolates from Bovine Milk with Mastitis

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ABSTRACT

Due to the increase in multi-antibiotic resistance (MDR) among streptococci, which have the ability to colonize different surfaces and form biofilms, treatment options have begun to be limited in diseases caused by these bacteria. The success of antibacterial therapy in mastitis may be related not only to the antibiotic susceptibility of the etiological factors, but also to the biofilm formation capacity, which is one of the most important virulence factors in bacteria. The aim of this study was to investigate the relationship between biofilm production and multi-antibiotic resistance of streptococcal isolates obtained from cow's milk with mastitis. For this purpose, 71 streptococcal isolates obtained from 282 subclinical mastitis milk from 27 farms were used. After the isolates were obtained by conventional methods, polymerase chain reaction (PCR) identifications were carried out. While the biofilm forming capacities of the isolates were determined phenotypically by the microplate method (MP); resistance to nine antibiotics belonging to nine antimicrobial families was evaluated by disc diffusion tests. Isolates resistant to at least three or more antimicrobial drug classes were considered multi-antibiotic resistant (MDR). Pearson Chi-Square ($\chi 2$) test was used to examine the relationship between biofilm forming capacity of isolates and multiple antibiotic resistance. Of the 71 streptococci obtained, 42 (59.1%) were identified as *S. uberis*, 13 (18.3%) as *S. agalactiae*, 8 (11.3%) as *S. parauberis* and 8 (11.3%) as *S. dysgalactiae*. Fifty one (71.8%) of the isolates were biofilm producers. Biofilm production was found in 84.7% (11/13), 75.0% (6/8), 69.1% (29/42), and 62.5% (5/8) in *S. agalactiae, S. parauberis, S. uberis* and *S. dysgalactiae* isolates, respectively. The most common resistance was to tetracycline (70.4%), followed by clindamycin (39.4%), ampicillin and vancomycin (35.2%), erythromycin (33.8%), and cefotaxime (32.4%). While 67.6% of the isolates were susceptible to chloramphenicol, 91.5% to linezolid and levofloxacin, 43.7% were MDR. The rate of multidrug resistance was higher in *S. agalactiae* (84.6%) than in *S. parauberis* (50%), *S. dysgalactiae* (50%) and *S. uberis* (28.6%). A significant correlation was found between the biofilm forming capacity of the isolates and multiple antibiotic resistance (P=0.016). It was concluded that biofilm-forming isolates showed higher resistance to antibiotics. As a result of this study, it was determined that mastitic streptococci isolates have the ability to produce biofilms that can significantly affect the course of the disease, and significantly affect multi-antibiotic resistance. The high biofilm forming capacity of MDR streptococcal isolates (87.0%, 27/31) might designate their high pathogenic potential that may pose a threat to human health.

Key words: *Streptococcus* spp.; Mastitis; Biofilm; Multiple Antibiotic Resistance.

INTRODUCTION

One of the most common etiological factors of mastitis, which cause economic losses in dairy cattle all over the world, are streptococci, which can be classified as both a contagious and an environmental pathogen (1). Not all bacteria belonging to the streptococcal genus, many of which are facultative anaerobes, are pathogenic (2). Some streptococci (*S. agalactiae, S. dysgalactiae, S. uberis, S. parauberis*) can cause severe problems in cattle such as mastitis, arthritis, meningitis, death and they can be transmitted zoonotically. Some streptococci (*S. agalactiae, S. dysgalactiae*) can cause important health problems such as sepsis, meningitis, pneumonia, endocarditis, cellulitis, joint and urinary system infections in humans, (1, 2).

S. agalactiae, a contagious pathogen, can colonize the gastrointestinal tract of dairy cows (1). *S. agalactiae* can survive for prolonged periods by forming biofilms in bovine mammary glands which may lead to subclinical mastitis (1). These streptococci are generally β-hemolytic and classified in Lancefield group B (2) and the Christine–Atkinson–Munch–Peterson (CAMP) test is positive (2). Environmental or contagious classification is unclear for *S. dysgalactiae* (2). Due to its ability to survive in the host and in the environment, it is defined as an intermediate pathogen (3). *S. dysgalactiae* are generally γ-hemolytic and CAMP and esculin tests are negative. Lancefield falls into the group C (3). Mostly α-hemolytic, *S. uberis* is primarily environmental, but cases of transmission have been observed. In addition to a variable CAMP phenotype capable of partial hemolysis, aesculin and sodium hippurate tests are positive (4). Lancefield classification is quite difficult of *S. uberis*. *S. uberis* and *S. parauberis*, which cannot be distinguished using phenotypic methods, and the only difference is D-glucuronidase production by *S. uberis* (5).

The "gold standard" method for identifying mammary gland pathogens is *in vitro* culture (2). However, it is difficult and time-consuming to identify streptococcal species with classical phenotypic microbiological tests (2,6). In addition, some bacteria such as *S. uberis* and *S. parauberis* cannot be distinguished by biochemical analyses (2). It is recognised that early detection procedures, when combined with appropriate antimicrobial therapy, increased recovery rates and reduced the time required to return to normal milk (6). Accurate and prompt identification of the pathogen is important not only for antimicrobial therapy, but also for monitoring and controlling the infection rate at the farm level. It has been shown that streptococcal identification with polymerase chain reaction (PCR) can provide better results when compared with phenotypic tests (6). By amplifying species-specific genes (16S rRNA, 23S rRNA), streptococci can be detected directly and rapidly. In addition, phylogenetically closely related organisms such as *S. parauberis* and *S. uberis* can also be identified (7).

A biofilm is defined as "a cluster of bacterial cells embedded in a biopolymer matrix that exhibits increased tolerance to antimicrobials compared to planktonic cells and resists the antimicrobial properties of host defenses" that can adhere to both biological and non-biological surfaces. It has been suggested that biofilm is the default growth mode for bacteria (7). Biofilm formation is an important factor in the pathogenesis of many diseases (8). Biofilm-forming bacteria are better protected against the influence of the host immune system *in vivo*, become less susceptible to the activity of antibiotics, and thus have a higher likelihood of survival (9). Therefore, investigating biofilm production, which is one of the important virulence mechanisms in bacteria, is extremely important for the development of effective prevention programs. The *in vitro* biofilm forming abilities of bovine mastitis pathogens have been investigated by many traditional biofilm methods (10,11). In a study conducted in Poland, it was shown that 83.7% of streptococcal isolates obtained from cow milk with mastitis had the ability to form biofilms *in vitro* by MP method (11).

Mastitis treatment and prophylaxis are the most common goals for antibiotic treatment in dairy cows (12). However, in some cases, the infection can become chronic even when treated with antibiotics. In this case, there is a risk of selection developed in favor of microorganisms resistant to antibiotics (1, 2). Continuing antibiotic treatment in cases where antibiotics cannot destroy microbial agents increases the risk of "development of antibiotic resistance", which is an important threat to human and animal health (13). The widespread use of antibiotics in dairy farming increases the risk of the emergence of microorganisms resistant to antimicrobials, which can later enter the food chain and affect human health (1, 12, 13). The standard approach in the treatment of streptococcal mastitis is the administration of ß-lactam and macrolide antibiotics (1,2). Increased antimicrobial resistance rates against tetracycline, erythromycin and enrofloxacin have been detected in streptococci isolated from mastitis (14).

In various studies, antibiotic resistance (15, 16, 17) and biofilm formation (10, 11, 17) were investigated in streptococcal isolates obtained from bovine milk with mastitis. However, to the best of the knowledge of the authors, there is no study in Türkiye examining the relationship between biofilm forming capacity of important streptococcal strains isolated from mastitis cases and multi-antibiotic resistance. Therefore, in this study, it was aimed to investigate the effect of biofilm formation abilities of streptococcal isolates, which is one of the major pathogens causing intramammary infections, with multi-antibiotic resistance.

MATERIAL AND METHODS

Bacterial Isolates

California Mastitis Test (CMT, Bavivet CMT Liquid, Kruuse®, Langeskov, Denmark) was applied after udder cleaning in dairy farms visited for mastitis screening. The procedures and interpretations were performed previously (2). Approximately 5-10 ml milk sample taken from a single mammary lobe with the highest CMT score was sent to the laboratory under aseptic cold conditions. Bovines that were not treated with antibiotics for at least three weeks were utilized in this study. CMT positive milk samples were collected from 282 dairy cattle in 27 farms. Milking machines were used at all enterprises. The cows' ages varied between three and eleven years and the numbers of cows were between 9 and 35, on each farm.

Isolation and Identification

Milk samples were centrifuged at 3500 rpm for 5 minutes and the supernatant was discarded. The sediment was vortexed and a loopful was inoculated onto Blood agar (Merck, 110886) and Edwards medium (Oxoid, CM0027B, Hampshire, England), both supplemented with 7% defibrinated sheep blood. The plates were incubated at 37°C for 48±2 hours under microaerofil conditions. The grown cultures were examined microscopically after Gram staining, and the phenotypic traits were analysed (i.e., type of catalase production, haemolysis, esculin hydrolysis). For further analysis, only Gram positive, catalase negative cocci were selected (2). All bacterial isolates were stored at -20°C in brain heart infusion broth (Merck 1.10493, Germany) supplemented with 20% glycerol. The final identification was performed using PCR.

Biofilm Assay

A modification of the method previously reported by Toledo-Arana *et al.* (2001) was used for the quantitative determination of biofilm formation in this study (18). Briefly, *Streptococcus* isolates were grown overnight in tryptic soy broth (TSB, Oxoid CM0129B, Hampshire, England) at 37°C. The culture was diluted 1:40 in TSB, and 200 µl of this cell suspension was used to inoculate sterile 96-well polystyrene flat bottom microtiter plates (Sarstedt, Nümbrecht, Germany). After 48 hours at 37°C under microaerofil condtions, wells were gently washed three times with 200µl of phosphate-buffered saline (PBS), dried in an inverted position, and stained with 1% crystal violet for 15 minutes. The wells were rinsed once again, and crystal violet was solubilized in 200µl of ethanol-acetone (80:20, vol/vol). The optical density at 595ηm (OD595) was determined using a microplate reader (BioTek ELx808 ELISA Reader, Vermont, USA). Each assay was performed in triplicate. Interpretation of biofilm production was according to the criteria described by Stepanović *et al.* (19). The optical density cut-off value (ODc) was the sum of the average OD of the negative control and three times the standard deviation of the negative control. Classification of strains was performed according to the following criteria: No biofilm producer (NB) (OD≤ODc), weak biofilm producer (WB) (ODc<OD≤2xODc), moderate biofilm producer (MB) (2xODc<OD≤4xODc) and strong biofilm producer (SB) (4xODc<OD).

Staphylococcus aureus 25923 was used as positive control and the negative control was sterile TSB media.

DNA Extraction

DNA extraction from *Streptococcus* spp. was performed with using a commercial genomic DNA extraction kit (Fermentas, Vilnius, Lithuania) according to the recommendations of the manufacturer. DNA purity and quantity controls were also performed. The OD260/OD280 ratio indicated that the DNA purity ratio was 1.6-2.0 (20). Then, DNA was electrophoresed on 1% agarose gel and the presence of DNA bands in the UV transilluminator were investigated.

Genotypic Identification

Firstly, the bacterial presence and DNA extraction was confirmed by amplification of the 16S rRNA gene. For the PCR performed using 16S rRNA universal primers, *S. uberis* ATCC 700407 strain was used as positive control, and mas-

Table 1. All primers used in this study.

Primers	Sequence (5'-3')	T_m	Amplicon (bp)	Reference
Universal	AGAGTTTGATCCTGGCTCAG	58.4	1371	Edwars *et al*., 1989, Zheng *et al*., 1996
	GACGGGCGGTGTGTACAA	58.4		
StrF	AGAGTTTGATCCTGGCTCAG	58.4	500	Conrads ve ark 1997
StrR	GTACCGTCACAGTATGAACTTTCC	63.5		
Sdy 105	AAAGGTGCAACTGCATCACTA	57.4	281	Riffon ve ark. 2001
Sdy 386	GTCACATGGTGGATTTTCCA	56.4		
Sag 40	CGCTGAGGTTTGGTGTTTACA	59.4	405	Riffon ve ark. 2001
Sag 445	CACTCCTACCAACGTTCTTC	58.4		
Sub 1546	TGATGGGGAGCGAAAATAAG	56.4	624	Riffon ve ark. 2001
Sub 2170	CCCAACAACGCCTCAAACGA	60.5		
Spa 2152	TTTCGTCTGAGGCAATGTTG	56.4	718	Riffon ve ark. 2001
Spa 2870	GCTTCATATATCGCTATACT	52.3		

T_m: Melting Temparature

termix without DNA was used as negative control. Sequence analysis of amplicons was performed to identify the bacteria.

Subsequently, genus and species-level identifications of isolates phenotypically determined to be *Streptococcus* species were confirmed by PCR using 16SrRNA gene as species-specific primers (6). The sequenced field strains of streptococci was used as a positive control; Enterococcus *faecalis* ATCC 29212 strain and mastermix without DNA was used as a negative control in PCR. The oligonucleotide primers and expected band sizes and references used for sequence analysis, genus and species level identifications in *Streptococccus* isolates are presented in Table 1.

Primers were utilized in a 25µl reaction containing 12.5µl of 5x FIREPol® Master Mix Ready to Load (Tartu, Estonia), 1µl of each primer of 0.1 mM concentrations, 8.5µl of water, and 3µl (20 ηg) of DNA template. The reaction was performed uaing a Boeco (Hamburg, Germany) thermal cycler.

The DNA was amplified using the following protocol: initial denaturation at 95˚C for 5 minutes followed by 30 cycles of denaturation (95˚C for 30 seconds), annealing for 30 seconds [56˚C (universal 16S rRNA, Str, Sdy, Sag, Sub, Spa)] and extension (72˚C for 1 minute), with a single final extension for 7 minutes at 72˚C. On electrophoresis, a 2% agarose gel stained with Safe View (100 ml/6µl) (ABM, Richmond, Canada) was used and the gel was exposed to 100 volts for 45 minutes. After electrophoresis, the gel was placed in the chamber of the transilluminator device, which was connected to the computer and photographed under UV light (Vilbert Lourmat, Collegien, France). When the amplified product formed a band of the expected size (Table 1), it was assumed to carry the gene examined.

Sequence Analysis

The DNA fragments were visualized by UV after electrophoresis. Samples with the expected size (1371 bp) of the amplified DNA were purified using the GeneJet Gel Purification Kit (ThermoScientific, Waltham, United States) according to the manufacturer's instructions. After the purification process, the amplicons were sent to Macrogen Europe (Amsterdam, The Netherlands). Sequence analysis was carried out using ABI Primse sequencing system. Sequences were compared using the Nucleotide-nucleotide BLAST (blastn) program available at the gene bank (**www.ncbi.nlm.nih.gov**).

Antimicrobial Susceptibility Test

For each *Streptococcus* spp. isolate confirmed by the PCR, the antimicrobial resistance against nine antibiotics (tetracycline, clindamycin, erythromycin, vancomycin, ampicillin, cefotaxime, linezolid, chloramphenicol, levofloxacin) (Oxoid, Hampshire, United Kingdom) belonging to nine different antibiotic families was tested by the disk diffusion method (Table 2). A bacterial suspension of 0.5 McFarland standard turbidity was first prepared using a 24 h culture (21). A sterile

Table 2. Antimicrobial resistance rates of isolates.

Antimicrobial Classes (Antibiotic), (Abbreviation)	Disk Content	Zone Diameter (mm)		*Streptoccus* spp.	*S. uberis* (n=42)	*S. parauberis*	*S. agalactiae*	*S. dysgalactiae*
	(µg)	S≤	R≤	(n=71) (%)	(%)	(n=8) (%)	(n=13) (%)	(n=13) (%)
Tetracyclines (Tetracycline, TET)	30	23	18	50 (70.4)	27 (64.3)	4 (50.0)	12 (92.3)	7 (87.5)
Lincosamides (Clindamycin, CD)	2	19	15	28 (39.4)	13 (30.9)	4 (50.0)	11 (84.6)	0 (0.0)
Macrolide (Erythromycin, E)	15	21	15	25 (35.2)	16 (38.1)	4 (50.0)	4 (3.0)	1 (12.5)
Glycopeptid (Vancomycin, VA)	30	17	–	25 (35.2)	10 (23.8)	5 (62.5)	6 (46.1)	4 (50.0)
Ampicillin (AMP)	10	24	–	24 (33.8)	16 (38.1)	0 (0.0)	8 (61.5)	0 (0.0)
Cephems (Cefotaxime, CTX)	30	24	–	23 (32.4)	9 (21.4)	4 (50.0)	6 (46.1)	4 (50.0)
Oxazolidinones (Linezolid, LZD)	30	21	–	6 (8.4)	2 (4.8)	0 (0.0)	0 (0.0)	4 (50.0)
Phenicol (Chloramphenicol, C)	30	21	17	2 (2.8)	2 (4.8)	0 (0.0)	0 (0.0)	0 (0.0)
Fluoroqinolons (Levofloxacin, LEV)	5	17	13	1 (1.4)	12.4	0 (0.0)	0 (0.0)	0 (0.0)

cotton swab was dipped into the bacterial suspension, and the swab was pressed and twisted against the inner surface of the test tube to remove excess fluid. The swab was streaked across a Mueller-Hinton agar (MHA) (Oxoid, Hampshire, United Kingdom) with 5% sheep blood surface in a zigzag manner. The MHA plate was turned 45° clockwise and streaked again using the same swab, and this step was repeated one more so that the swab had been streaked across the agar a total of three times. The antibiotic discs were placed onto the agar using a pair of sterile forceps. Antibiotics disks were placed onto MHA plate which were were incubated at 35±2°C for 20–24 h under microaerofil conditions. Zone diameters of susceptibility test results were categorized as sensitive (S), intermediate (I), or resistant (R) and evaluated as previously reported for *Streptococcus* spp. (21). The specific breakpoint values are presented in Table 2.

Staphylococcus aureus ATCC 25923 (Oxoid, Hampshire United Kingdom) and *E. coli* ATCC 25922 (Oxoid, Hampshire United Kingdom) was used as the quality control microorganism.

Multiple Antibiotic Resistance (MDR) and Multiple Antibiotic Resistance Index (MAR)

Multiple drug resistance was defined as resistance to three or more antimicrobial classes (22). The MAR for each isolate was determined by dividing the number of antibiotics to which the isolate was resistant by the total number of antibiotics tested (23).

Statistical Analysis

SPSS (Statistical Package for Social Sciences) version 23.0 (SPSS Inc., Chicago, IL, USA) package program was used for statistical analysis of the data obtained. Pearson Chi-square ($\chi 2$) test was used to compare frequency data. A P-value of <0.05 was considered statistically significant at the 95% confidence interval. The $\chi 2$ test was used to reveal statistical difference between biofilm formation capacity and MDR of isolates.

RESULTS

Biochemical Tests and Phenotypic Identification

Isolated from 282 subclinical mastitic bovine milk 71 (25.2%) were catalase negative. Gram-positive cocci were identified as *Streptococcus* spp. Esculin negative, hemolyzed (α, β or γ). Twenty-one (29.6%) isolates were evaluated as *S. agalactiae* or *S. dysgalactiae*. Esculin positive, hemolyzed (α or γ) 50 (70.4%) isolates were evaluated as *S. uberis* or *S. parauberis*.

Further identification processes were performed using molecular methods.

PCR

Sequence Analysis

Firstly, bacterial presence and DNA extraction were confirmed with amplification of the 16S rRNA gene. Samples with expected size (1371 bp) amplicons were further analyzed by sequencing. As a result of the sequence analysis, 50.7% (36/71) of the isolates were *S. uberis*, 18.3% (13/71) *S. agalactiae*, 11.2% (8/71) *S. dysgalactiae*, 11.2% (8/71) *S. parauberis* and 8.4% (6/71) was identified as S. *hongkongensis*.

Genus and Species Specific PCR

All 71 isolates phenotypically identified as *Streptococcus* spp. were identified as *Streptococcus* spp. (500 bp) by *Streptococcus*

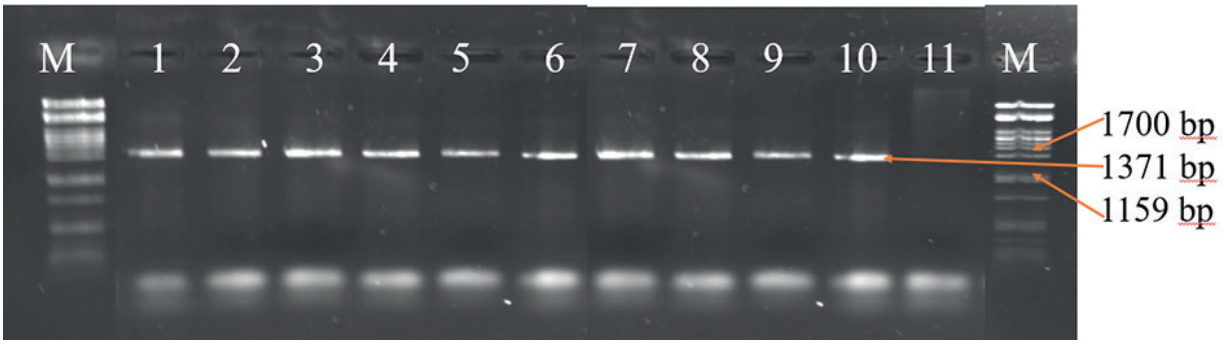

Figure 1. PCR performed by using 16S rRNA universal primers. M: Marker (Lambda phage DNA restricted with PstI enzyme) 1-9: PCR performed by using isolated microorganism's DNA. 10: Positive Control (*S. uberis* ATCC 700407) NC: Negative Control (Mastermix without DNA).

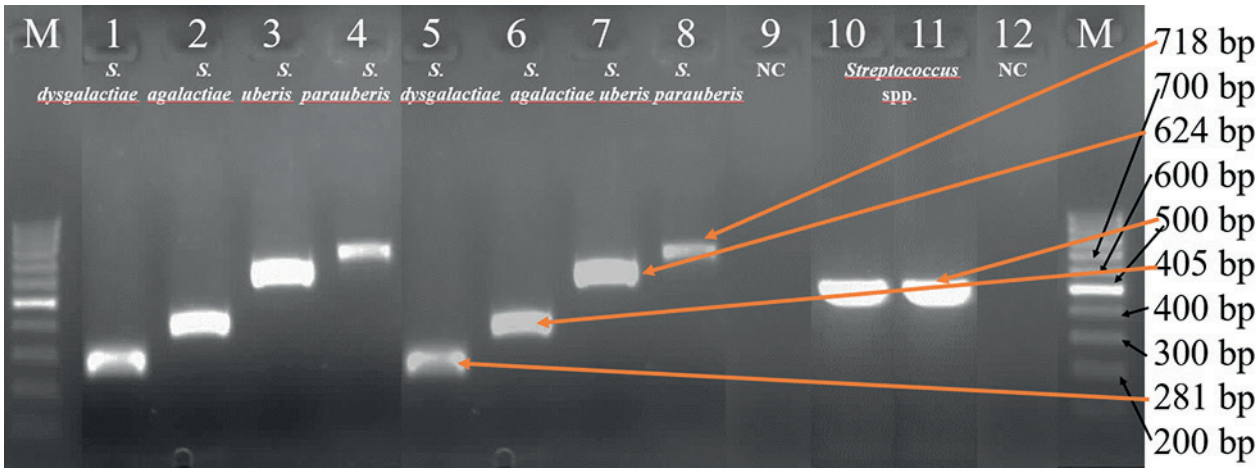

Figure 2. Gel electrophoresis image of *Streptococcus* spp. isolates 1,5: *S. dysgalactiae* (281 bp), 2,6: *S. agalactiae* (405 bp), 3,7: *S. uberis*, 4,8: *S. parauberis* (718 bp), 10: *Streptococcus* spp. sequenced field strain (500 bp) 11: Positive Control: *S. uberis* ATCC 700407 9: NC: *E. faecalis* ATCC 29212 12: NC: Mastermix without DNA M: 100 bp DNA Ladder, (Fermentas, USA).

genus specific PCR. After species-specific PCR, eight isolates were identified as *S. dysgalactiae* (281 bp), thirteen isolates as *S. agalactiae* (405 bp), fourty two isolates as *S. uberis* (624 bp), eight isolates as *S. parauberis* (724 bp) (Figure 2, Figure 3).

Biofilm Formation

Among the isolates examined, while 20 (28.2%) isolates did not form biofilms; 51 (71.8%) were able to produce biofilm: Six (8.4%) were SB producers, whereas 20 (28.2%) and 25 (35.2%) were MB and WB producers, respectively. Biofilm assay for *S. agalactiae* isolates using the MP method is presented in Figure 4. While 11 (84.7%) of *S. agalactiae* isolates were positive for biofilm formation, biofilm production was found to be 75.0%, 69.1%, and 62.5% in *S. parauberis, S. uberis* and *S. dysgalactiae* strains, respectively (Figure 4, Table 3).

Antimicrobial Resistance

The rates of resistance to antimicrobial drugs were tetracycline 70.4%, clindamycin 39.4%, erythromycin 35.2%, vancomycin (35.2%) ampicillin (33.8%), cefotaxime (32.4%), linezolid (8.4%), chloramphenicol (2.8%) and levofoxacin (1.4%). The most effective antibiotics against isolates were levofloxacin and linezolid (91.5% susceptibility rate) (Figure 5.).

MAR index and MDR

MAR index of 62.0% (44/71) *Streptococcus* isolates were found above 0.2 (Table 4.). Thirty one (43.7%) of the isolates were found to be resistant to multiple antibiotics. Nine *S. uberis* isolates (12.7%, 9/71) were sensitive to all antibiotics examined, while 18 (25.3%) isolates were resistant to one and 13 (18.3) to two antibiotics. The MAR index and MDR

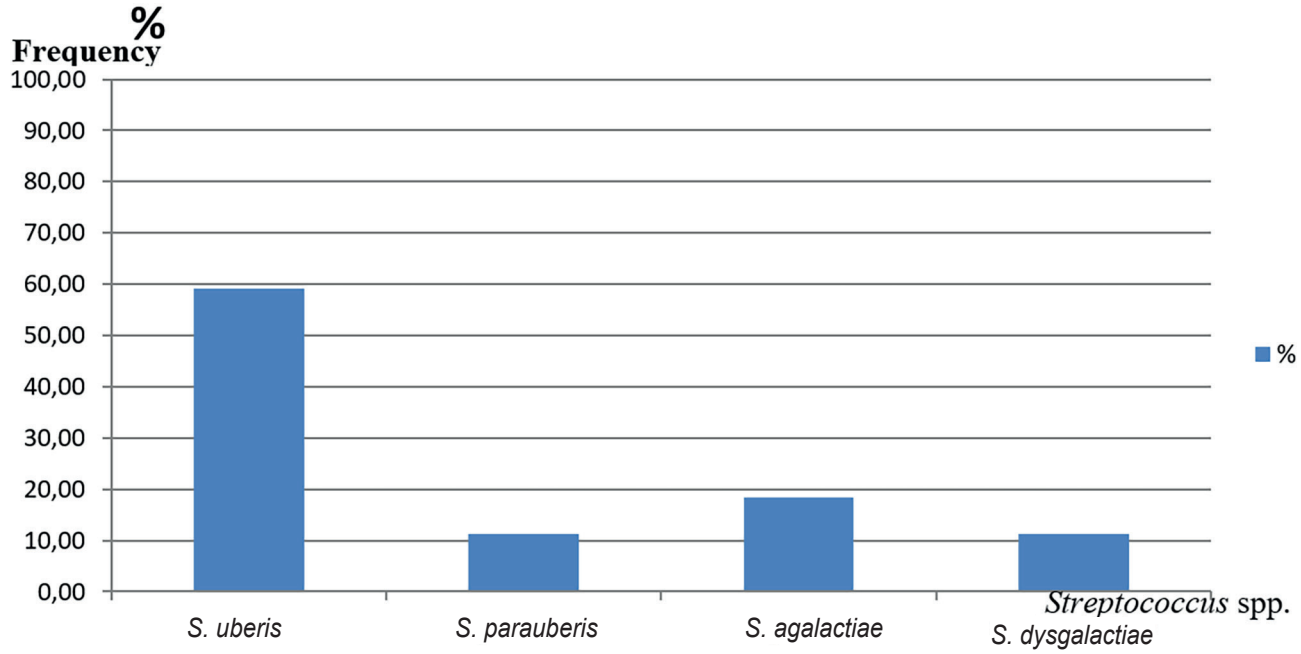

Figure 3. Identified streptococcal species

Table 3. Biofilm forming capacities of isolates.

Tür (n, %)	NB (%)	BF	WB	MB	SB
S. uberis (n=42, 59.1%)	13 (30.9)	29 (69.1)	12 (28.6)	13 (30.9)	4 (9.6)
S. parauberis (n=8, 11.3%)	2 (25.0)	6 (75.0)	2 (25)	3 (37.5)	1 (12.5)
S. agalactiae (n=13, 18.3%)	2 (15.3)	11 (84.7)	7 (53.8)	3 (23.0)	1 (7.7)
S. dysgalactiae (n=8, 11.3%)	3 (37.5)	5 (62.5)	4 (50.0)	1 (12.5)	0 (0%)
Total (n=71, 100%)	**20 (28.2)**	**51 (71.8)**	**25 (35.2)**	**20 (28.2)**	**6 (8.4)**

status of all streptococcal isolates in the study are displayed in Table 6.

Statistical Analysis

The relationship between biofilm production (BP) and multiple antibiotic resistance in *Streptococcus* spp. isolates from cow milk with mastitis is shown in Table 5.

A significant correlation was found between multi-antibiotic resistance and biofilm forming capacity of the isolates (P=0.016).

DISCUSSION

During infection, streptococci can form biofilms, which are thought to be associated with bacterial pathogenicity (9). In this study, it was aimed to investigate the effect of biofilm forming abilities of bovine mastitis-associated streptococcal isolates on multi-antibiotic resistance.

In studies conducted in Türkiye in recent years, streptococcal isolation rates were 30.9%-61.6% (24, 25). Although the isolation rate (25.2%) obtained in this study was slightly lower than the isolation rates in the studies in Türkiye, it is similar to the rates obtained in other studies in the world (23-50%) (14).

It is important to determine the prevalence of both environmental and contagious mastitis pathogens in the control of mastitis (14). In farms with good hygienic milking practices, contagious streptococcal mastitis (*S. agalactiae*) was greatly reduced. On the other hand, streptococcal mastitis has been found to be predominantly (40-70%) caused by environmental (*S. uberis*, *S. parauberis*) streptococci in enterprises where hygienic conditions are not good (14). *S. dysgalactiae* has the unique feature of being recognized as both an infectious and environmental pathogen (3). These organisms can

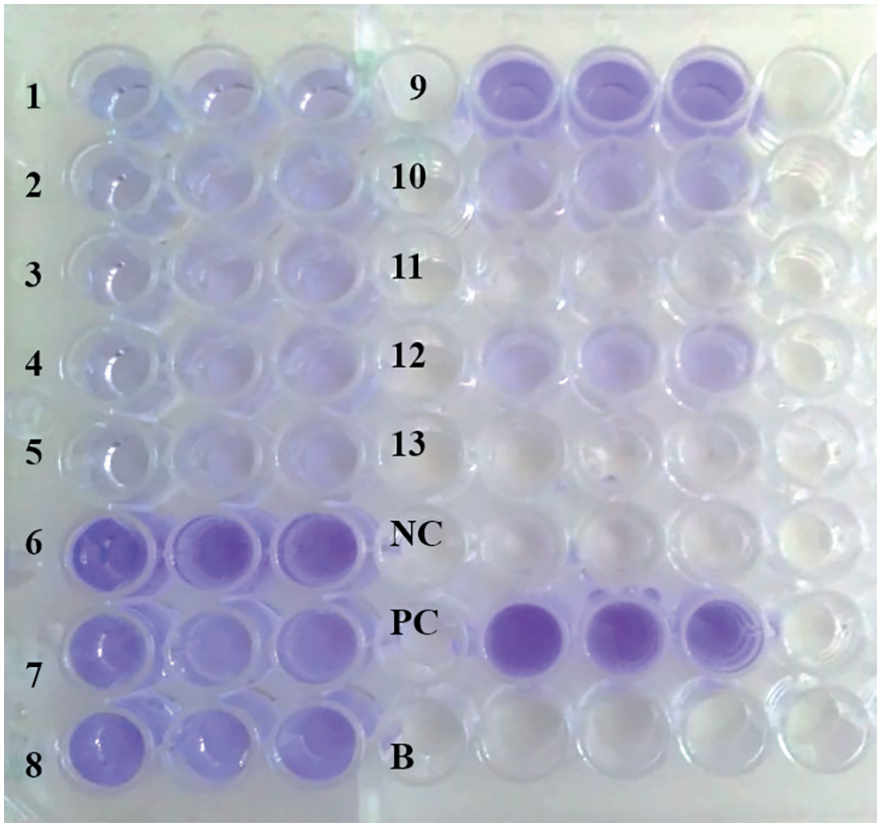

Figure 4. Biofilm assay for *S. agalactiae* isolates using 96-well microtiter plate method. Wells 1-5, 10,12: WB producer isolates, Wells 6: SB producer isolates, Well 7-9: MB producer isolates. Wells 11,13: NB producer isolates NC: Sterile MHB, PC: *S. aureus* 25923, B: Blank,

Table 4. MAR index and resistance phenotype of isolates.

Number of isolate (%)	Number of resistant antibiotic	MAR index	Resistance Phenotype
9 (12.7)	0	0	NMDR
18 (25.4)	1	0.11	NMDR
13 (18.3)	2	0.22	NMDR
11 (15.4)	3	0.33	MDR
5 (7.0 %)	4	0.44	MDR
6 (8.4 %)	5	0.55	MDR
6 (8.4 %)	6	0.66	MDR
3 (4.2 %)	7	0.77	MDR

Table 5. The relationship between biofilm production and multiple antibiotic resistance in *Streptococcus* spp. isolates.

		R Phenotype (MDR)		P	χ^2
		(+)	(-)		
Biofilm production	(+)	27	24	0.016	6.337
	(-)	4	16		

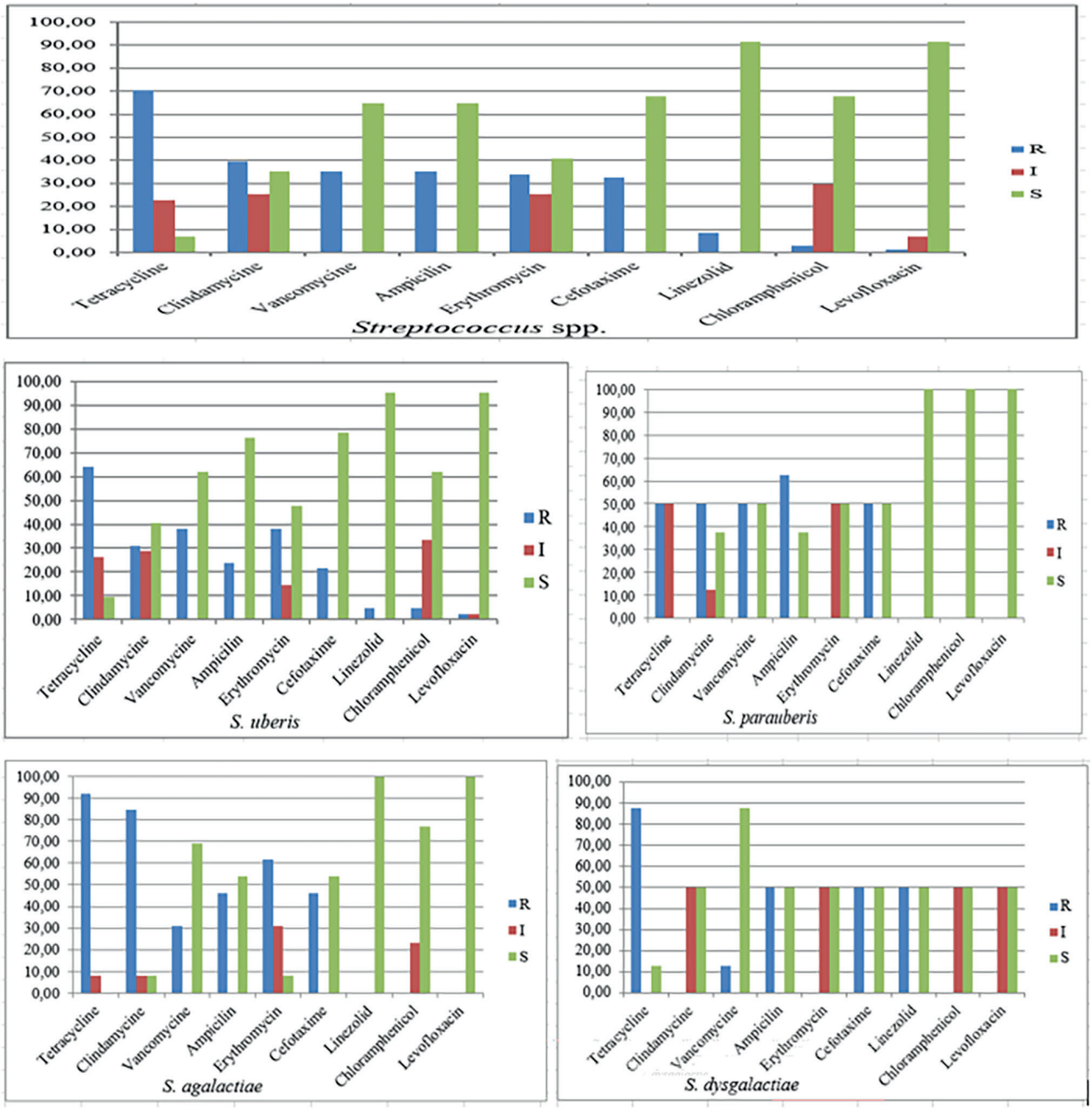

Figure 5. Antimicrobial susceptibility and resistance profiles of *Streptococcus* isolates.

spread from cow to cow at milking time and are commonly found in the environment of the cow (3, 14).

Although infectious and environmental streptococci cause bovine mastitis worldwide, their distribution also varies. *S. uberis* has been found to be the majority of streptococcal mastitis cases in Europe, Australia, and New Zealand and was estimated to be responsible for approximately 64% and 62% of infections, respectively (14). In contrast, *S. agalactiae* was documented to be responsible for most mastitis infections in Africa and Asia with 49% and 40%, respectively (14). *S. uberis* and *S. dysgalactiae* (47% and 40%) in North America and *S. uberis* and *S. agalactiae* (34% and 35%) in South America were reported as the predominant streptococcal mastitis pathogens (14).

In this study, 18.3% of *S. agalactiae*, was accepted as the contagious mastitis agent. Environmental mastitis agents *S. uberis* (59.1%) and *S. parauberis* (11.3%) were isolated at an especially high rate (70.4%) and *S. dysgalactiae* with a rate of 11.3%. In this study, milking was done by machine in all of the farms where material was collected. According to these findings, it can be considered that environmental hygiene was not taken seriously into account in the farms where milk samples are taken. Environmental pathogens are oppurtinistic agents that enter the udder canal through manure, litter, soil, plant material, and water (2). Especially environmental streptococcal species appear as an important problem even in well-managed dairy farms where precautions are taken against contagious mastitis agents. In the past, infectious pathogens such as *S. agalactiae* were much more important than today (1, 14).

Rapid and specific identification of streptococci is required for monitoring mastitis. Traditionally, the identification of mastitis pathogens was carried out according to classical phenotypic microbiological methods (2). However, these methods have many disadvantages such as laborious, time-consuming and limited reproducibility (6). In addition, typing at the species level is not always possible (5). The development of PCR-based genotypic methods is a valuable option for rapid and reliable identification of bacteria (5, 6). A PCR method has been developed to quickly, sensitively and specifically identify the main pathogens that cause intramammary infections in cows, including *S. agalactiae, S. dysgalactiae, S. uberis*, and *S. parauberis* (6). In this study, the primers used for the detection of *S. agalactiae* and *S. dysgalactiae* were based on 16S rRNA, and the primers used for the detection of *S. uberis* and *S. parauberis* were based on the 23S rRNA gene (6). The results of another study showed that these specific primers were able to distinguish closely phylogenetic bacterial species (6). The specificity of the primers was also tested with DNA from different bacterial species. Therefore, it was not possible to have false positive results in our study.

In this study, isolates identified as *Streptococcus* spp. from subclinical mastitis cases were at the same time identified by PCR at genus and species level molecularly. All 71 isolates obtained as a result of cultural identification were *Streptococcus* spp. were confirmed by genus-specific PCR. Moatamedi *et al.* (2007) reported that there was 91.6% agreement between PCR and culture methods in the diagnosis of streptococcal mastitis (27). However, in this study, researchers carried out bacterial identification by PCR and DNA amplification from milk samples (27). All six *S. hongkongensis* isolates obtained after PCR using 16S universal primers were typed as *S. uberis* by species-specific PCR. When the 16S rRNA sequence analysis and species-specific PCR results were compared, it was found that the results were most frequently (91.6%) compatible with each other. It was concluded that 16S rRNA sequence analysis could successfully identify streptococcal species at both genus and species levels.

Mastitis is one of the most common infectious diseases in dairy cattle and is a compelling reason for the use of antimicrobial drugs in dairy cows (1). Overall, antimicrobial resistance rates in mastitis-causing streptococci are high (28). In order to detect such changes in susceptibility as early as possible, antimicrobial resistance in streptococci should be regularly investigated. In this study, in parallel with previous studies, resistance to tetracycline, lincosamides and macrolides was detected most frequently (29, 30, 31). Resistance rates for antibiotics tested in studies conducted in France have been reported to be more or less stable for ten years, except for tetracycline resistance, which increased from 15.7% to 20.4% between 2006 and 2016 (29). Lincosamides with relatively low toxicity and side effects, used to treat mastitis caused by Gram positive cocci in dairy cows, are approved for veterinary use and can only be administered intramammary (31). Combined resistance to macrolides and lincosamides was found in eighteen isolates in this study. Resistance to macrolides and lincosamides is common among streptococci, and there is a possibility of horizontal transfer of resistance genes (32). Resistance to vancomycin, an antibiotic approved for use only in human medicine, has also been reported in animals in Germany and Türkiye (33, 34). This antibiotic was included in our study after it was reported that vancomycin resistance was found to increase progressively in studies conducted in India and China (16, 30). In this study, vancomycin resistance was found to be lower (57.1%) than the study conducted in India (16), but higher (35.2) than the study conducted in China (8%) (30). In a study conducted in Germany, streptococcal species were resistant to tetracyclines, erythromycin and pirlimycin and furthermore it has been reported to be sensitive to penicillin and ampicillin (31). In a recent study, it was found that streptococci, in addition to having a relatively high MDR, are fully resistant to penicillin and highly resistant to vancomycin (16). In this study,

it is considered that a significant resistance to vancomycin started to be seen in penicillin-resistant streptococci and in the near future this may be the cause health problems. Cephalosporins are used in veterinary medicine to treat bacterial infection (35). In a study conducted in China, cephalosporin (cephalexin) resistance (19.0%) was reported at lower rates (25.3%) compared to this study (30). In a study conducted in India, cefotaxime resistance was reported at very high rates (100.0%) (16). In the present study, moderate resistance (25.3%) was recorded in streptococcal species against cephalosporins (cefotaxime), which are widely used in the treatment of bovine mastitis. The degree of resistance to antibiotics may depend on the strains of streptococci isolated, antibiotics used in isolated farms, growth stimulants and prophylactic agents used in animal husbandry. Overall, the dynamics of resistance acquisition in streptococci is slower than that experienced in *Enterobacteriaceae*, probably due to much lower horizontal transfer rates of resistance genes (36). Evaluating all strains of streptococci isolated in this study, the most common was tetracycline resistance, followed by clindamycin, vancomycin, and ampicillin.

Multiple antibiotic resistant bacteria are an important public health problem. This is since mobile genetic elements can spread antibiotic resistance genes to commensal and enteric bacteria through horizontal gene transfer and cause foodborne diseases (1, 12). In a study conducted in China, 21% (21/101) of the isolates were reported to have MDR (30). In this study, 43.7% of the isolates were MDR. When the results of this study were evaluated at the species level, 84.6%, 50.0%, 50.0% and 28.6% of *S. agalactiae, S. dysgalactiae, S. parauberis* and *S. uberis* isolates were MDR, respectively.

The MAR index is an effective, valid and cost-effective method used to trace the source of antibiotic-resistant organisms (23). The MAR index was calculated as the ratio of the number of antibiotics to which an isolate is resistant to the total number of antibiotics to which the organism was exposed. A MAR greater than 0.2 indicates that the source of high-risk contamination was where antibiotics are frequently used (23).

A better understanding of the mechanisms of the biofilm forming abilities of bacteria is of great importance for successful treatment. In previous studies, the effect of atmospheric conditions (aerobic and microaerophilic environments) on the formation of streptococcal biofilms was investigated. As a result of this study, it was reported that the microaerophilic atmosphere could induce the expression of genes encoding biofilm formation in *S. agalactiae* and as a result greater rate of biofilm formation was detected than in the aerobic environment (11). Therefore, microaerophilic media was used in this study. Although the mechanism that causes a higher biofilm production under CO_2 atmospheric conditions has not yet been clarified. Previous studies have already shown increased production in other bacterial species such as *Staphylococcus aureus* and *S. epidermidis* (37). Similarly, we performed our study under incubation conditions at 37°C, as it was shown that the best biofilm formation occurs under cow host conditions (37°C) (38). Since it was reported in previous studies that carbohydrates (glucose and lactose) negatively affect biofilm formation (38, 39), we used the culture medium without the addition of carbohydrates in this study. It has been shown that biofilm production begins to occur soon after the start of incubation (between 2 and 5 hours), but greater biofilm production occurs at 48 hours. On the other hand, mature biofilms can be separated after 48 hours (38). Therefore, we used the 48-hour incubation period in our study. In previous studies, it has been shown that streptococci can form biofilms at high rates (90.3%, 70%) (15, 40) as in this study (71.8%). In a study conducted in Poland, researchers found that 16.4% of their isolates did not form biofilms (10). They reported that biofilm formation occurred in 83.6% (65.8 weak, 14.1 medium and 53.7% strong) (10). In this study, while 28.2% of our isolates did not form biofilm we found that 71.8% formed a biofilm. However, 8.4% of our isolates were weak, 28.2% were medium and 35.0% were strong biofilm forming. The higher rate of strong and moderate biofilm-forming isolates compared to the study conducted in Poland may be due to the relatively longer incubation period (48 hours) used in our study. However, we propose that the high prevalence of biofilm-producing streptococci in the geographic area we are investigating posed a serious challenge to both dairy farmers and veterinarians. As mentioned earlier, the production of this specific structure by bacteria can have a significant impact on treatment efficiency (7, 8).

In this study, which was carried out with the aim of investigating the effect of biofilm forming abilities of streptococcal isolates obtained from bovine mastitis, on multi-antibiotic resistance: a significant relationship was found between multi-antibiotic resistance and biofilm forming capacity of the isolates. The rates of multiple resistance to various antibi-

otics in streptococcal isolates were higher in biofilm positive strains than in negative strains. The ability of multi-antibiotic resistant isolates to form biofilms at high rates (87.0%) may indicate the high pathogenic potential of these isolates.

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