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ARAŞTIRMA MAKALESİ **RESEARCH PAPER**

Virulence Determinants of Buffalo Mastitis Originated *Streptococcus agalactiae* Isolates

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Abstract: Streptococci as a cause of mastitis have become the major concern to the dairy industry worldwide due to huge economic losses. *Streptococcus agalactiae* is a major contagious mastitis pathogen and continues to be a major cause of mastitis in dairy cattle and buffaloes. The aim of the study was to investigate the virulence determinants of *S. agalactiae* strains isolated from buffalo milk.

Within the scope of the study, 24 *S. agalactiae* isolates from buffalo mastitis in Samsun were examined. Biofilm production of isolates was investigated phenotypically by CRA method. It was observed that 18 (75%) isolates were positive for biofilm production. The presence of *hylB, fnbB, scpB* and *spb1* virulence genes in *S. agalactiae* isolates were investigated by PCR. It was determined that 19 (79.17%) of the isolates were positive for *scpB* and 6 (25%) for *fnbB* virulence genes. None of the isolates were found to contain *hylB* and *spb1* virulence genes. The antibiotic resistance profiles of the isolates among kanamycin, ampicillin, enrofloxacin, erythromycin, tetracycline, trimethoprim-sulfamethoxazole antibiotic discs were determined by Kirby Bauer Disc Diffusion Method. Resistance were evaluated as 41.7 %, 45.9 %, 25 %, 12.5 %, 20.9 %, and 33.3 %, respectively. RAPD-PCR patterns of all isolates were determined using the ERIC-2 primer. The dendrograms of the RAPD patterns were plotted with the UPGMA method. It was determined that the isolates showed similarity between 59-95%.

In conclusion, the research confirms the prevalence of various virulence genes in S. agalactiae isolated from buffalo mastitis. Further studies are therefore necessary to determine the molecular epidemiology and variability of *S. agalactiae* isolated from buffaloes, with the aim of improving mastitis control programs with regard to *S. agalactiae*.

Keywords: Buffalo, *fnbB, hylB, S. agalactiae, scpB, spb1*, virulence genes.

Manda Mastitis Kökenli *Streptococcus agalactiae* İzolatlarının Virülens Belirleyicileri

Öz: Mastitisin bir nedeni olan streptokoklar, büyük ekonomik kayıplar nedeniyle süt endüstrisi için dünya çapında önemli bir sorundur. *Streptococcus agalactiae,* bulaşıcı mastitis patojeni olarak, süt sığırlarında ve mandalarda mastitisin önemli bir nedeni olmaya devam etmektedir. Bu çalışma manda sütünden izole edilen *S. agalactiae* suşlarının virulens belirleyicilerini belirlemek amacıyla gerçekleştirilmiştir.

Çalışma kapsamında Samsun'da manda mastitislerinden elde edilen 24 adet *S. agalactiae* izolatı incelendi. İzolatların biyofilm üretimi fenotipik olarak CRA yöntemiyle araştırıldı. İzolatların 18'inin (%75) biyofilm üretimi açısından pozitif olduğu görüldü. *S. agalactiae* izolatlarında *hylB, fnbB, scpB* ve *spb1* virülens genlerinin varlığı PCR ile belirlendi. İzolatların 19'unun (%79,17) *scpB*, 6'sının (%25) *fnbB* geni yönünden pozitif olduğu görüldü. İzolatların hiçbirinde *hylB* ve *spb1* virülens genleri belirlenemedi. Kanamisin, ampisilin, enrofloksasin, eritromisin, tetrasiklin, trimetoprim-sülfametoksazol antibiyotik diskleri ile izolatların antibiyotik direnç profilleri Kirby Bauer Disk Difüzyon Yöntemi ile belirlendi ve direnç oranları sırasıyla %41,7, %45,9, %25, %12,5, %20,9 ve %33,3 olarak değerlendirildi. Tüm izolatların RAPD-PCR paternleri ERIC-2 primeri kullanılarak belirlendi ve RAPD paternlerinin dendrogramları UPGMA yöntemiyle çizildi. İzolatların %59-95 arasında benzerlik gösterdiği belirlendi.

Sonuç olarak; bu araştırma ile manda mastitisinden izole edilen *S. agalactiae* izolatlarında çeşitli virülens genlerinin prevalansı belirlendi. Mandalardan izole edilen *S. agalactiae*'nin moleküler epidemiyolojisi ve değişkenliğinin belirlenmesi ve *S. agalactiae* ile ilgili mastitis kontrol programlarının geliştirilmesi amacıyla daha ileri çalışmalara ihtiyaç olduğu kanısına varıldı.

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Anahtar kelimeler: *fnbB, hylB*, manda, *S. agalactiae, scpB, spb1*, virulens belirleyicileri.

INTRODUCTION

Streptococcus agalactiae, a Group B *Streptococcus* (GBS) is considered a mammary-specific pathogen and a common cause of mastitis in cattle, but it is also a commensal of the human intestinal and genital tract and an opportunistic pathogen causing severe, potentially fatal infections, preferentially in newborns and the elderly (Uffe et al., 2019). *S. agalactiae* causes a subclinical mastitis mainly associated with high somatic cell count and a consequent reduction in production yield and milk quality. Bovine mastitis caused by GBS poses a major economic problem for the dairy industry, especially as this microorganism is highly contagious within a farm and rapidly reduces milk productivity. In recent years, some developed countries have witnessed a drastic reduction in the occurrence of GBS due to the implementation of programs specifically designed for the control of bovine mastitis. Among other things, such programs are based on the administration of antimicrobial use for the entire herd to treat and prevent the disease and have been successful because this microorganism is susceptible to most of the antimicrobial agents of choice (Keefe, 1997). However, the implementation of mastitis control programs in most developing countries has been a slow and inefficient process, leading to the historical misuse of antibiotics in the veterinary field. In addition, the farming system in such countries often includes a large number of small producers who may be unaware of proper protocols for GBS mastitis control. GBS has been virtually eradicated in many Northern European countries, but there are warnings of its re-emergence as a zoonotic threat (Tamba et al., 2022). Despite widespread control strategies, mastitis of buffaloes causes great economic losses in dairy farming.

The severity of mammary infections varies according to the virulence factors of the bacteria and the host response. For infection to occur, bacteria must first colonize the mammary gland. Colonization begins with the adhesion of mastitis agents to epithelial cells and infection is continued with various virulence characteristics of the bacteria. *S. agalactiae* colonizes by attaching to host tissue cells at the early stage of infection. While *S. agalactiae* invades tissues by binding to laminin-binding protein, C5a peptidase, fibrinogen-binding protein structures, hyaluronate lyase spreads in tissues with alpha C protein. It also causes membrane damage in cells with its virulence properties such as beta hemolysis and Christie–Atkins–Munch-Peterson (CAMP) factor. In addition to these virulence properties, structures such as Rib protein, antibiotic resistance genes, and different insertion sequences were also examined within their virulence properties.

Like other gram-positive bacteria, GBS can form biofilm-like three-dimensional structures, which may enhance its ability to colonize and persist in the host. Biofilm formation by GBS has been investigated *in vitro* and appears to be tightly controlled by environmental conditions. The biofilm facilitates the persistence of GBS under environmental stresses and enables GBS to survive in hostile environments and protect against antibiotics. It has also been reported that biofilm formation by GBS depends on the expression of various virulence factors (Rosini and Margarit, 2015; Verma et al., 2023).

Overall, dairy farms are one of the largest users of antimicrobials, including medically important antimicrobials. Some antimicrobials used in dairy farms include beta-lactams (penicillins, ampicillin, oxacillin, penicillin-novobiocin), broad-spectrum beta-lactams (third generation cephalosporins), aminoglycosides (streptomycin), macrolides (erythromycin), lincosamide (pyrlimycin), tetracycline, sulfonamides and fluoroquinolones (Dego et al., 2020). Antimicrobial resistance (AMR) occurs when microorganisms are able to overcome the effects of previously effective antimicrobials. According to EARS-Net data from 2016, AMR remains a serious threat to public health in Europe (ECDC, 2018). In addition, AMR is one of the biggest threats to global health, food security and development. Antibiotic resistance has become an important clinical and public health problem on a global scale. While it is clear that antibiotics are crucial in the selection of bacterial resistance, the spread of resistance genes and resistant bacteria also adds to the problem. Selection of resistant forms may occur during or after antimicrobial therapy. Residues of antibiotics may be present in the environment for long periods of time after treatment. Alongside antibiotics, there is increasing use of other agents aimed at destroying bacteria, namely surface antibacterials, which are now found in many household products (Levy, 2002).

Within multi-host pathogens such as *S. aureus* and *S. agalactiae*, there can be strains that are adapted to a single host species and strains that are commonly found in multiple host species (Richards et al., 2019). For example, studies based on multilocus sequence typing (MLST) have identified *S. agalactiae* ST67 and ST103 as primarily associated with cattle, while *S. agalactiae* ST23 are generalised strains that can be found in humans, cattle and other host species (Delannoy et al., 2013; Richardson et al., 2018; Zadoks et al., 2011). It seems reasonable to assume that transmission patterns differ between specific and generalist strains; bovine-adapted strains depend on bovine contacts for transmission, whereas generalist strains can be introduced from other sources. The introduction of human strains of *S. agalactiae* into dairy herds may explain its

presence in the absence of risks associated with animal movement. Studies highlighting that the same subtypes are present in both humans and cattle and confirming that some GBSs can be transmitted between cattle and humans (Botelho et al., 2018; Lyhs et al., 2016) hypothesized that transmission to humans could occur during milking, when drinking contaminated milk or through environmental contamination.

In this study, determination of some virulence determinants of *S. agalactiae* strains isolated from buffalo milk was aimed.

MATERIAL AND METHOD

***Streptococcus agalactiae* isolates:** Within the scope of the study, 24 *S. agalactiae* isolates from buffalo mastitis in Samsun were examined. *S. agalactiae* ATCC 13813 was used as the control and reference bacterial strain in all studies.

Identification of the isolates was performed with using the VITEK2 automated identification system and confirmed by species-specific PCR (Abd El-Razik et al., 2010). DNA extraction for PCR studies was performed with the Invitrogen tissue kit according to the method reported by the manufacturer. PCR mix was prepared in a final volume of 50 µl and containing 200 ng DNA, 1X PCR buffer, 3 mM $MgCl_2$, 1 µM primers (Table 1), 0.2 mM dNTP and 2 U Taq polymerase. Amplification conditions were 2 min initial denaturation at 95°C, 35 cycles of 45 sec denaturation at 94°C, 45 sec annealing at 60°C, 45 sec elongation at 72°C, and 10 min at 72°C after the last cycle. Amplification products were visualized with a UV transilluminator after 1.5% agarose gel electrophoresis containing ethidium bromide (2 µg/mL). A band of 405 bp was considered positive for *S. agalactiae*.

Investigation of biofilm production: Biofilm production of isolates was investigated phenotypically by Congo Red Agar (CRA) method (Çiftci et al., 2009). For this purpose, bacteria were inoculated with a single colony on the CRA and incubated at 37°C for 24 h. At the end of the incubation period, bacteria forming black colonies were evaluated as biofilm positive, and colorless or pink colonies were evaluated as negative. For comparative confirming the results, CRA method was performed twice.

Investigation of some virulence genes: *hylB* (Hyaluronate lyase) (Sukhnanand et al., 2005), *fnbB* (Fibrinogen binding protein), *scpB* (C5a peptidase) (Shome et al., 2012) and *Spb1* (Surface protein) (Brochet et al., 2006) virulence genes were investigated under the conditions specified in the literature. For each virulence gene, PCR mix was prepared with 1X PCR buffer, 0.5 µM of each primers, 2.5 mM of $MgCl_2$, 200 µM of dNTP and 50 ng of DNA in a volume of 25 mL. PCR amplification for each gene region was performed under the following conditions:

hylB: 95°C 5 min; 30 cycles 95°C 30 sec., 55°C 30 sec., 72°C 30 sec.; 72°C 10 min.

fnbB: 94°C 5 min; 30 cycles of 94°C 30 sec., 60°C 30 sec., 72°C 45 sec.; 72°C 5 min.

scpB: 94°C 5 min; 30 cycles of 94°C 30 sec., 58°C 30 sec., 72°C 45 sec.; 72°C 5 min.

Spb1: 94°C 5 min; 30 cycles 94°C 60 sec., 55°C 45 sec., 72°C 90 sec.; 72°C 7 min.

Amplification products were visualized with a UV transilluminator after 1.5% agarose gel electrophoresis containing ethidium bromide (2 µg/mL). The oligonucleotide primers used for the virulence factors of *S. agalactiae* isolates and the expected band sizes are shown in Table 1.

Table 1. Primers used in the identification of *Streptococcus* isolates and determination of virulence genes.

Target		Oligonucleotide primers (5'-3')	Band size (bp)	Reference
S. agalactiae	F	CGCTGAGGTTTGGTGTTTACA	405	Abd El-Razik et al. (2010)
	R	CACTCCTACCAACGTTCTTC		
hylB	F	CATACCTTAACAAAGATATATAACCCAAA	950	Sukhnanand et al. (2005)
	R	AGATTTTTTAGAGAATGAGAAGTTTTTT		
fnbB	F	TGATGCTGCAAAAGAATTGC	629	Shome et al. (2012)
	R	TTACAGCCCCTTTTTGAGGA		
scpB	F	AGTTGCTTCTTACAGCCCAGA	567	
	R	GGCGCAGACATACTAGTTCCA		
Spb1	F	TACTCAAAAAGGCGCAACCT	490	Brochet et al. (2006)
	R	GACGAGCAACAAGCACGATA		

Determination of antibiotic resistance of isolates: In order to determine the antibiotic resistance profiles of the isolates, ampicillin (10 µg), enrofloxacin (5 µg), erythromycin (15 µg), kanamycin (30 µg), tetracycline (10 µg), trimethoprim-sulfamethoxazole (25 µg) antibiotic discs were used and the susceptibilities of the isolates were determined by Kirby Bauer Disc Diffusion Method (CLSI, 2012).

Genotyping of isolates: ERIC-2 (5'-AAG TAA GTG ACT GGG GTG AGC G-3') primer was used to determine the RAPD-PCR patterns of all isolates (Versalovic and Lupski, 2002). A 25 µL master mix containing 1X PCR Buffer, 2.5 mM of $MgCl_2$, 200 µM of

each dNTPs, 2.5 U of Taq DNA polymerase, 25 pmol of primer and 5 µL of template DNA was prepared. The mixture is pre-denaturated at 94°C for 5 min followed by 1 min denaturation at 94°C, 1 min annealing at 40°C, 3 min extension at 72°C and 7 min final elongation at 72°C. Amplification products were visualized with a UV transilluminator after 1.5% agarose gel electrophoresis containing ethidium bromide (2 µg/mL).

The dendrograms of the formed Randomly Amplified Polymorphic DNA (RAPD) patterns were drawn with the UPGMA (Unweighted Pair Group Method with Arithmetic Averages) method and the genetic relationship between the isolates was determined by considering the 70% similarity coefficient.

RESULTS

S. agalactiae isolates and identification: All 24 isolates examined in the study were identified as *S. agalactiae* by VITEK2 automatic identification system. As a result of the VITEK2 automated identification system, all of the isolates were resistant to arginine dihydrolase, beta galactosidase, phosphatase, Leucine arylamidase, alanine arylamidase, polymixine B resistance, D-galactose, lactose, N-acetyl-D-glucosamine, bacitracin-D-glucosamine, D-biocintose resistance, 6.5% NaCl growth, D-mannose, saccharose/sucrose, D-trehalose, arginine dihydrolase 2 and optochin resistance. D-amygdalin, phosphatidylinositol phospholipase C, D-xylose, alpha glucusidase, Ala-Phe-Pro arylamidase, cyclodextrin, L-Aspartate arylamidase, cyclodextrin, alpha mannosidase, L-Proline arylacyl-arylamidase, beta glucuracylacylamide-lactose, beta-glucorinidase, tyrosine arylamidase, D-sorbitol, urease, D-ribose, L-lactate alkalinization, D-mannitol, methyl-B-D-glucopyranoside, pullulan, D-raffinose, O/129 resistance, catalase and salicin properties were determined as negative. All isolates formed a specific band of 405 bp in PCR and were confirmed as *S. agalactiae.*

Investigation of biofilm production: Biofilm production of isolates was investigated phenotypically by CRA method. It was observed that 18 (75 %) isolates formed black colonies on Congo red agar and were evaluated as positive for biofilm production.

Genotypic investigation of some virulence factors: The presence of *hylB*, *fnbB*, *scpB* and *spb1* virulence genes in *S. agalactiae* isolates were investigated by PCR. It was determined that 19 (79.17 %) of the isolates were positive for *scpB* and 6 (25 %) for *fnbB* virulence genes. None of the isolates were found to contain *hylB* and *spb1* virulence genes.

Determination of antibiotic resistance of isolates: Antibiograms were performed for 6 antibiotics belonging to 6 antibiotic classes in order to determine the antibiotic resistance profiles of the isolates. Antibiotic resistance profiles of the isolates are presented in Table 2.

Table 2. The antibiotic resistance profiles of the isolates.

Antibiotic discs	S		I		R	
	n	%	n	%	n	%
Kanamycin	12	50	2	8.3	10	41.7
Ampicillin	11	45.8	2	8.3	11	45.9
Enrofloxacin	11	45.8	7	29.2	6	25
Erythromycin	18	75	3	12.5	3	12.5
Tetracycline	17	70.8	2	8.3	5	20.9
Trimethoprim-Sulfamethoxazole	13	54.2	3	12.5	8	33.3

S: sensitivity; I: intermediate resistance; R: resistance.

Genotyping of the isolates: RAPD-PCR patterns of all isolates were determined using the ERIC-2 primer. The dendrograms of the RAPD patterns were plotted with the UPGMA method. It was determined that the isolates showed similarity between 59-95%. The phylogenetic relationship between the isolates was determined by considering the 70% similarity coefficient. Isolates were genotyped as 1 unique genotype (B) and 4 clusters (A, C, D, E). Genotype A contained 3 isolates and showed 77-88% similarity. The similarity rates of the isolates in cluster E (10 isolates), where the most isolates were collected, were 71-86%. However, the similarity rate of 6 isolates in genotype D, the second dominant genotype, was 74-95% and the similarity rate in genotype C, which contained 4 isolates, was 82-89%.

DISCUSSION

Streptococci as a cause of mastitis have become a major concern for the dairy industry worldwide due to large economic losses. *S. agalactiae* is recognised as an obligate intramammary pathogen that is definitely infectious in cows and buffaloes. Mastitis cases associated with this agent have been reported in 11 to 60 per cent of herds in Germany, Brazil and Uruguay (Duarte et al. 2004; Gianneechini et al. 2002; Tenhagen et al. 2006). The implementation of a standard mastitis prevention programme has eliminated *S. agalactiae* in many countries in Western Europe (Zadoks and Fitzpatrick, 2009), but infectious mastitis remains a serious problem in developing countries. Over the last few decades, the incidence of udder inflammation caused by *S. agalactiae* has decreased due to

the introduction of mastitis control programmes in developed countries. *S. agalactiae* is also the main cause of infections in human newborns and small infants. It is also an important opportunistic pathogen colonising the gastrointestinal and genitourinary tracts, throat and skin of healthy adults (An der Mee-Marquet et al., 2008). *S. agalactiae* can produce a wide range of virulence factors, including toxins and proteins that facilitate adhesion, colonization and invasion of host cells. Several regulatory proteins and surface-localized proteins identified in *S. agalactiae* have been found to play critical roles in adhesion, tissue damage and immune evasion. Genes encoding these proteins include *fbs* (fibrinogen binding protein), *bca* (α-subunit of C protein), *scpB* (C5a peptidase), *bac* (β-subunit of C protein), *lmb* (laminin binding surface protein), *rib* (protein providing resistance to proteases), *spb1* (surface protein) and *csp* (cell surface protease). In addition, *S. agalactiae* produces many toxins, including CAMP factor (cfb), hyaluronidase (hylB) and haemolysins, which help the pathogen to enter, survive and spread in host cells (Rajagopal, 2009). In many previous studies, the molecular epidemiology of putative virulence factors in GBS has been examined by analysing various virulence genes to better understand the virulence determinants of GBS isolates. Some of the genes analyzed for *S. agalactiae* (*cfb, cylE, hylB* and *fbsB*) were confirmed to be present in bovine strains in many countries such as China, Pakistan, Brazil, India, and Egypt. The frequencies of these genes were reported to be a local feature (Bangar et al. 2015; Carvalho-Castro et al. 2017; El-Behiry et al. 2015; Shome et al. 2012). However, it was also reported that *spb1* and *lmb* genes were not detected in any of the isolates analyzed (Leghari et al., 2023). Similarly, the absence of *bac* and *scp* in Chinese isolates and *cspA* in Pakistani isolates drew attention (Delannoy et al., 2013). In this study, *hylB, fnbB, scpB* and *spb1* virulence genes were investigated by PCR in *S. agalactiae* isolates. While 79.17 % of the isolates were positive for *scpB* and 25 % for *fnbB* virulence genes, none of the isolates were positive for *hylB* and *spb1* virulence genes. While the absence of the *hylB* gene in any of the isolates differed from the findings of other researchers, the absence of the *sbp1* gene in the isolates was seen as a similar finding. The fact that the *scpB* gene, which was found to be present in most of the isolates (72%) in this study, was not found in Chinese isolates was also noteworthy as a different result. On the other hand, Zastempowska et al. (2022), for the first time, determined the presence of *scpB* gene in *S. agalactiae* strains isolated from dairy cattle in Poland. In Turkey, Tuzcu et al. (2023) reported that *hylB* (83%) was the gene with the highest frequency among the virulence genes they investigated in *S. agalactiae* isolates from buffalo milk. This finding of the authors is quite different from the present study and indicates that there may be variations in strains isolated from different geographical regions in the same country. As can be seen, although there are similarities between countries in terms of the frequencies of the virulence genes analyzed, differences can also be seen. In vaccination strategies, which is a way of controlling mastitis in herds and reduces the severity of mastitis, although it does not completely prevent the occurrence of mastitis, revealing the virulence genes of the strains most frequently isolated from bovine mastitis cases in the region or country is one of the important criteria in vaccine strain selection.

Antibiotics are the main tools used to treat mastitis. However, rational use of antibiotics is essential to prevent the development and spread of antibiotic resistance. The importance of studies on antimicrobial resistance is important in terms of revealing drug-resistant pathogens and determining strategies to solve the resistance problem. Some cases where treatment is not effective despite the use of an antibiotic that has been found to be effective under *in vitro* conditions may be related to the presence of various host and bacterial factors, including the ability of bacteria to produce biofilms. Biofilm, defined as matrix-surrounded microbial accumulations that can adhere to both biological and non-biological surfaces, is an important factor in the pathogenesis of numerous diseases in both humans and animals (Preez, 2000). Boonyayatra and Pata (2016) identified the majority (81%) of *S. agalactiae* strains isolated from clinical and subclinical mastitis in Thailand as biofilm producers. Kaczorek et al. (2017) analyzed the biofilm-forming abilities of streptococcal strains isolated from bovine mastitis and found the majority of the strains (over 70 %) positive. Moreover, they demonstrated that *S. agalactiae* forms stronger biofilms than other streptococcal species. Similarly, Leghari et al. (2023) also reported that all *S. agalactiae* isolates originating from China and Pakistan formed biofilms. In this study, 75% of *S. agalactiae* strains were found to be positive for biofilm formation. In a study comparing the sensitivities of the Congo Red Agar method and the microtiter plate test, which are phenotypic tests for biofilm formation, the sensitivities were found to be 88.9 % and 100 %, respectively, and it was reported that both tests can be used to determine whether an isolate has the potential to produce biofilm (de Castro et al., 2013). The reason for the relatively low percentage of biofilm positivity in this study compared to some studies may be the use of the Congo Red Agar method. However, it has also been reported that this method is quite easy to apply, less time consuming, sensitive and specific (Jain and Agarwal, 2009).

Although antibiotic treatment is one of the most widely used practices in mastitis control, it has become ineffective today due to the emergence of pathogens that

are resistant to various antibiotics from many antibiotic classes (Oliver and Murinda, 2012). The widespread use of antibiotics in livestock, including growth stimulants, is one of the most important reasons for the development of antibiotic resistance. The fact that antibiotic resistance has reached dangerously high levels around the world causes limitation of treatment options and ultimately causes great economic losses due to increased mortality and treatment costs. Therefore, determining the antibiotic resistance profile in a mastitis-causing pathogen is critical for the development of alternative prevention and control solutions. A hypothesis about the pathways by which *S. agalactiae* can cross the interspecies barrier and be transmitted between cattle and humans is highlighted, mainly based on MLST and antimicrobial resistance systems (Botelho et al., 2018). The finding of human and bovine isolates with common genotypes and antibiotic resistance profiles supports the hypothesis of interspecies transmission of *S. agalactiae* between cattle and humans (Carra et al., 2021). There are many studies to determine the antibiotic resistance profiles of mastitis-derived *S. agalactiae* isolates. In one of these studies (Leghari et al., 2023), the antibiotics to which *S. agalactiae* strains isolated from China and Pakistan showed the highest percentages of phenotypic resistance were reported as tetracycline (94 %/95 %), erythromycin (95 %/91 %) and clindamycin (70 %/72 %). However, it was reported that the strains isolated from both countries were sensitive at high percentages to the tested beta-lactam group antibiotics (penicillin, ampicillin, amoxicillin, oxacillin, cephalothin, ceftriaxone), gentamicin and kanamycin from the aminoglycoside group, and lincomycin from the macrolide group. Nam et al. (2009) also reported that the antibiotics to which bovine mastitis-derived *S. agalactiae* isolates in Korea showed the highest resistance were tetracycline (60 %) and lincomycin (60 %), and that the resistance to penicillin and cephalothin, which are beta lactams, was at a low level. Han et al. (2022) reported that *S. agalactiae* isolates were susceptible to aminoglycosides (kanamycin, gentamicin, neomycin and tobramycin) up to 100 %, while the resistance rate to β-lactams (penicillin, amoxicillin, ceftazidime and piperacillin) was 98.1 %. In this study, the resistance rate of *S. agalactiae* isolates to tetracycline was found to be 20.9 %, while the resistance rate to ampicillin, one of the beta-lactam antibiotics, was 45.9 %. These findings are contrary to the studies in which tetracycline resistance was found to be widespread and resistance to beta lactam antibiotics was found to be lower. However, there are also studies in which very high rates of resistance to beta lactam antibiotics were detected (Han et al., 2022) and no resistance to tetracycline was detected (Gianneechini et al., 2002). β-Lactams are known to be first-line antimicrobial agents in the treatment of streptococcal breast infections. In this study, *S. agalactiae* isolates exhibiting a relatively high level of resistance to ampicillin may have a risk potential for the emergence of treatment failures. In addition, in this study, kanamycin from the aminoglycoside group was the antibiotic to which the isolates showed the highest resistance among the antibiotics tested with a rate of 41.7 %. Although this finding is not close to the 100 % resistance rate determined by Han et al. (2022) against aminoglycosides, it may be important in terms of showing that aminoglycoside resistance is one of the highest resistance percentages among the tested isolates. Hernandez et al. (2021) found 25 % and 58.3 % resistance to both macrolides (erythromycin and clindamycin) and kanamycin, respectively. In this study, the resistance percentages against both erythromycin and kanamycin were lower, and it is relatively pleasing that the resistance to erythromycin, which is one of the most commonly used antibiotics in streptococcal mastitis cases, is quite low (12.5 %).

The ability to control the infections depends on a detailed knowledge of the epidemiology of the organisms and their environment. A number of typing techniques such as MLST, pulsed-field gel electrophoresis, and RAPD have been investigated for differentiation of streptococcal strains (Phuektes et al., 2001; Sullivan et al., 2005). Tomazi et al. (2018) reported that genotyping of 89 *S. agalactiae* strains isolated from bovine clinical mastitis cases by RAPD using OPB-17 primer revealed a large genotypic diversity among the isolates. The researchers identified three clusters (Ia, Ib and II) based on 45 RAPD types and genetic similarity between genotypes. In this study, 24 *S. agalactiae* isolates were genotyped as 1 unique genotype (B) and 4 clusters (A, C, D, E) by RAPD using ERIC-2 primer and it was determined that there was genotypic diversity among the strains and the similarity was between 59-95%. Although different genotyping results can be obtained with different primers, it is obvious that there are genotypic variations among the strains.

S. agalactiae, one of the most frequently isolated causative agents of bovine mastitis, is of particular importance as it has the potential to infect humans, especially through contaminated milk. Although the role of this type of transmission in *S. agalactiae*-induced infections in humans has not been fully proven, the potential of cows with mastitis as reservoirs should not be ignored. In addition to the risk of zoonotic transmission, the antibiotic resistance profile exhibited by the agents is also an important issue that affects the success of treatment. Considering the most commonly used antibiotics in our country in bovine mastitis, the fact that resistance to ampicillin and kanamycin was found to be higher in line with the results obtained in this study shows that these antibiotics should be used more carefully in the field.

Erythromycin, to which the isolates showed the lowest percentage resistance, seems to be a preferred antibiotic among the macrolide group antibiotics. However, it should not be forgotten that regional differences in terms of antibiotic applications and choices may also affect the antibiotic resistance profile. Adding genotypic resistance profile as well as phenotypic antibiotic resistance to the scope of subsequent studies and expanding the study to include a wider region will benefit strategies related to the control of mastitis, especially epidemiologically, including vaccine development.

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