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Investigation of antimicrobial resistance genes in *Staphylococcus aureus* from bovine mastitis milk in Southwest Turkey[1]

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ABSTRACT.- Akgüç Çöl N, Ercan M. **Investigation of antimicrobial resistance genes in *Staphylococcus aureus* from bovine mastitis milk in Southwest Turkey**. *Pesquisa Veterinária Brasileira 46:e07607, 2026*. Department of Laboratory Animals, Division of Basic Science, Veterinary Faculty, Muğla Sıtkı Koçman University, Milas, Muğla 48277, Türkiye. E-mail: nihanakguc@mu.edu.tr

One of the most important pathogens of bovine mastitis, *Staphylococcus aureus*, often shows antibiotic resistance due to the widespread use of antibiotics in dairy herds. Here, we analyzed *S. aureus* isolates recovered from milk samples of mastitis-affected cows from various dairy farms for the presence of specific antibiotic resistance genes (*mecA*, *blaZ*, *aacA-aphD*, *tetK*, *tetM*, *vanA*, *cfr*, *fusB*, and *ileS*). To isolate and identify *S. aureus*, milk was inoculated onto Baird-Parker agar plates. The thermonuclease gene (*nuc*) was used for molecular identification of each *S. aureus* isolate. The chosen resistance genes were found in 14 verified isolates. The *blaZ* and *tetM* genes were found in all isolates (100%), whereas the *aacA-aphD*, *mecA,* and *cfr* genes were found in 42.9%, 14.3%, and 7.1% of the isolates, respectively. *TetK, vanA*, *fusB*, and *ileS* genes were not found in any of the isolates. Every isolate carried at least *blaZ* and *tetM*, demonstrating the widespread co-carriage of several genes. The findings show that *S. aureus* from bovine mastitis in this area exhibits broad genotypic resistance to β-lactams and tetracyclines, and emphasize the need for ongoing regional surveillance of resistance determinants.

INDEX TERMS: Bovine, mastitis, *Staphylococcus aureus*, antibiotic resistance genes.

RESUMO.- [Investigação de genes de resistência antimicrobiana em isolados de *Staphylococcus aureus* do leite de vacas com mastite no sudoeste da Turquia.] *Staphylococcus aureus*, um dos patógenos mais importantes da mastite bovina, frequentemente apresenta resistência aos antibióticos devido ao uso generalizado de antibióticos em rebanhos leiteiros. Analisamos isolados de *S. aureus* recuperados de amostras de leite de vacas afetadas por mastite de várias fazendas leiteiras para a presença de genes específicos de resistência aos antibióticos (*mecA*, *blaZ*, *aacA-aphD*, *tetK*, *tetM*, *vanA*, *cfr*, *fusB*, e *ileS*). Para isolar e identificar *S. aureus*, o leite foi inoculado em placas de ágar Baird-Parker. O gene termonuclease (*nuc*) foi usado para a identificação molecular de cada isolado de *S. aureus*. Os genes de resistência escolhidos foram encontrados em 14 isolados verificados. Os genes *blaZ* e *tetM* foram encontrados em todos os isolados (100%), enquanto os genes *aacA-aphD*, *mecA* e *cfr* foram encontrados em 42,9%, 14,3% e 7,1% dos isolados, respectivamente. Os genes *TetK*, *vanA*, *fusB* e *ileS* não foram encontrados em nenhum dos isolados. Todos os isolados carregavam pelo menos *blaZ* e *tetM*, demonstrando o transporte conjunto generalizado de vários genes. Os resultados mostram que *S. aureus* da mastite bovina nesta região tem ampla resistência genotípica aos β-lactâmicos e tetraciclinas, e enfatizam a necessidade de vigilância regional contínua dos determinantes de resistência.

TERMOS DE INDEXAÇÃO: Bovinos, mastite, *Staphylococcus aureus*, genes de resistência aos antibióticos.

INTRODUCTION

The most significant infectious disease affecting dairy cattle globally is still bovine mastitis, which causes significant financial losses due to decreased milk production, discarded milk, medical expenses, and early culling (Sharun et al. 2021). The illness can be broadly classified as either clinical or subclinical, with the latter being especially troublesome due to its

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silent nature and extended persistence in herds. *Staphylococcus aureus* is acknowledged as one of the main infectious agents causing both clinical and, particularly, subclinical intramammary infections among the many pathogens involved (El-Ashker et al. 2015, Lozano et al. 2016).

The main strategy for treating and managing bovine mastitis is still antibiotic therapy. The most commonly used classes in dairy practice are aminoglycosides, tetracyclines, and β-lactams, especially penicillin (Bakir et al. 2011, Li et al. 2023). However, resistant *S. aureus* populations have been strongly selected for due to the extensive, prolonged, and frequently indiscriminate use of these antimicrobial agents. As a result, strains resistant to one or more of these drug classes, including multidrug-resistant (MDR) isolates, are now frequently found in dairy farms on various continents (Saidi et al. 2019, Rana et al. 2022, Naranjo-Lucena & Slowey 2023, Yang et al. 2023).

The majority of *S. aureus* resistance is caused by acquired genetic determinants. The genes that are most commonly reported are *blaZ* (penicillin resistance), *mecA* (methicillin/oxacillin resistance), *aacA-aphD* (aminoglycoside resistance), *tetK* and *tetM* (tetracycline resistance), *vanA* (vancomycin resistance), *cfr* (resistance to phenicols, lincosamides, and streptogramin A), *fusB* (fusidic acid resistance), and *ileS* (high-level mupirocin resistance) (Jensen & Lyon 2009, Kumar et al. 2011). There have been reports of regional variations in the occurrence of these resistance genes and in phenotypic resistance profiles, which are probably due to differences in local antibiotic usage patterns, farm management techniques, and selective pressure (Qu et al. 2019, Zhang et al. 2022, Naranjo-Lucena & Slowey 2023, Rodríguez et al. 2023).

The distribution of resistance genes in *S. aureus* causing subclinical mastitis in Turkey is still poorly understood, especially at the provincial level, despite growing global data. This study was conducted to ascertain the presence and frequency of nine specific antibiotic resistance genes (*blaZ*, *mecA*, *aacA-aphD*, *tetK*, *tetM*, *vanA*, *cfr*, *fusB*, and *ileS*) in *S. aureus* isolates recovered from milk samples of cows with subclinical mastitis in the Milas district of Muğla Province, Turkey.

MATERIALS AND METHODS

Ethical approval. The experimental protocols and handling of experimental animals in this study were approved by the "Muğla Sıtkı Koçman University" Experimental Animals Application and Research Center Local Ethics Committee for Animal Experiments, under file number 34/22, approval number: 2022/05.

Sample collection and preparation. From September 2023 to May 2024, 170 milk samples were obtained from individual udder quarters of dairy cows in the district of Milas and its neighboring villages in Muğla Province, Turkey. The Milas district was chosen because it has one of the highest numbers of dairy cows in Muğla Province and supplies a large share of the region's milk and dairy product consumption. At different stages of lactation, cows with at least one calving and without antibiotic treatment in the last three months were sampled and subjected to the California mastitis test (CMT). CMT was used to identify cases of subclinical mastitis, and the test evaluation (+1, +2, +3, suspicious, and negative) was carried out as previously documented by Kandeel et al. (2018). The presence of abnormalities in the milk, such as pus and blood streaks, and positive CMT results for subclinical mastitis (Abdalhamed et al. 2018). After evaluation, the first milk was discarded before samples were collected, and the udders of animals showing positive, suspicious, or negative reactions to CMT were cleaned with water and dried with 70% alcohol. Subsequently, 10 ml of milk from each udder was stored in sterile tubes and transported to the laboratory via cold chain as quickly as possible.

Bacterial isolation and identification. Ten µL of each milk sample collected under aseptic conditions was sown on Baird-Parker agar (Oxoid, UK) and incubated at 37 °C for 24–48 h (Corry et al. 2003). After incubation, colonies with a black or gray, transparent zone-forming, shiny and smooth appearance were evaluated as *Staphylococcus aureus*. To ensure the purity of the bacterial isolates, each colony that appeared dark gray to black, shiny, and convex with entire margins was restreaked onto fresh Baird-Parker agar. A single colony was selected for further analysis. The purity of the isolates was then confirmed through microscopic examination. Pure Gram-positive colonies exhibiting a typical grape-like cluster under the microscope were identified based on biochemical tests, including catalase and coagulase tests. Isolates that were both catalase-positive and coagulase-positive were considered *Staphylococcus aureus* and stored in 30% glycerol stocks for further processing. For DNA isolation, bacteria were inoculated into tubes containing 5 mL of tryptic soy broth (TSB) (Oxoid, UK) from pure colonies after single colony cultivation on tryptic soy agar (TSA) (Oxoid, UK) and incubated for 24 h at 37 °C under aerobic conditions. DNA isolation was performed using a commercial genomic DNA purification kit (One-Tube Bacteria Genomic DNA Isolation Kit, BIOBASIC, UK). Obtained template DNA suspensions were stored at -20 °C for the next step. Diagnosis of *S. aureus* isolates at the species level was performed by polymerase chain reaction (PCR) amplification using primers *nuc* forward primer, 5'-ATATGTATGGCAATCGTTTCAAT-3' and *nuc* reverse primer, 5'-GTAAATGCACTTGCTTCAGGAC-3', which contain a 395 bp fragment specific for the thermostable nuclease gene (*nuc*) in *S. aureus* (Gao et al. 2011). *S. aureus* ATCC 25923 reference strain and molecular-grade water were used as positive and negative controls, respectively.

Determination of antimicrobial resistance genes. The oligonucleotide sequences, band sizes and primer design references of the primers used for the detection of *S. aureus*-specific genes (*mecA, blaZ, aacA-aphD, tetK/M, vanA, cfr, fusB, ileS*) involved in antimicrobial resistance are listed in Table 1. All PCR reactions were performed using Xpert Fast Hotstart DNA Polymerase (Grisp, Porto, Portugal) according to the manufacturer's instructions. PCR amplifications were performed using a 25 µL reaction mixture containing 3 µL template DNA, 10 pmol of each primer listed in Table 1, and Xpert Fast Hotstart Mastermix (2X) (Grisp, Porto, Portugal). The PCR thermal cycling protocol consisted of an initial denaturation at 95 °C for 3 minutes, followed by 40 cycles of denaturation at 95 °C for 15 seconds, annealing at the optimized temperature described in Table 1, and extension at 72 °C for 15 seconds, with a final extension at 72 °C for 3 minutes using a thermal cycler (Techne TC-512, UK). The amplification products were stained with Xpert Green DNA Stain (5 µl) (Grisp, Porto, Portugal) and electrophoresed in 1% agarose gel at 120 volts and constant current for 40 minutes and observed under UV illumination.

RESULTS

Results of isolation and identification

From September 2023 to May 2024, 170 individual milk samples were initially taken from dairy cows in the Milas district of Muğla Province, Turkey, that showed symptoms of subclinical mastitis. The California mastitis test (CMT) was the initial screening

method used on the farm for all samples. Samples with a CMT score of ≥ 1 (trace or higher) were regarded as having subclinical mastitis. Fifty (29.4%) of the 170 samples tested positive for CMT and were chosen for additional bacteriological testing. These 50 milk samples that tested positive for CMT were cultivated on Baird-Parker agar. A total of 38 colonies with a characteristic black, shiny appearance and clear zones were selected as presumed *Staphylococcus* species following a 24–48 hour incubation period at 37 °C (Fig. 1). Out of these 38 suspected isolates, 14 (36.8% of cultured samples; 28% of all CMT-positive samples; 8.2% of the original 170 samples) were definitively identified as *Staphylococcus aureus* by PCR amplification of the *S. aureus*-specific thermonuclease (*nuc*) gene, which produced the anticipated 395-bp product (Fig. 2). As a result, 14 confirmed *S. aureus* isolates made up the final study population.

Determination of antimicrobial resistance genes

Among the 14 isolates tested for antibiotic resistance genes, all carried *blaZ* and *tetM* genes, followed by *aacA-aphD* in six isolates. Two isolates contained the *mecA* gene, and only one contained the *cfr* gene. None of the isolates was detected as harboring *fusB*, *tetK, ileS*, or *vanA*. A total of five resistance gene combination patterns were found.

It was also determined which classes of antimicrobial-related genes were present in the isolates. All isolates (100%) had penicillin resistance genes (*blaZ*) (Fig. 3) and tetracycline resistance genes (*tetM*) (Fig. 4), aminoglycoside resistance genes (*aacA-aphD*) in six isolates (42.9%) (Fig. 5), methicillin resistance genes (*mecA*) in two isolates (14.3%) (Fig. 6), and chloramphenicol, florfenicol, and clindamycin resistance genes (*cfr*) in just one isolate (7.1%) (Fig. 7).

DISCUSSION

The most notable discovery of the current investigation was the consistent presence of *tetM* and *blaZ* in every *Staphylococcus aureus* isolate from subclinical bovine mastitis. According to earlier reports from Turkey and other nations, *blaZ* and *tetM* have become the predominant resistance determinants in bovine *S. aureus* populations. This full carriage of both genes is in striking agreement with those findings (Rychshanova et al. 2022, Zhang et al. 2022, Yang et al. 2023, Cantekin et al. 2024).

The bifunctional aminoglycoside-modifying gene *aacA-aphD* was present in six isolates (42.9%). Reported prevalence varies widely worldwide, from 10–30% in many European countries (Naranjo-Lucena & Slowey 2023) to 80–100% in some studies from China (Qu et al. 2019, Zhang et al. 2022). The frequency observed here falls in the upper half of the published range.

The methicillin-resistance gene *mecA* was detected in two isolates (14.3%), whereas the multiline-resistance gene *cfr* was found in only one isolate (7.1%). The remaining targeted genes (*tetK*, *vanA*, *fusB* and *ileS*) were not detected. These findings align with previous Turkish surveys, in which *mecA* prevalence ranged from 0–30%, and *cfr* was either absent or extremely rare (Buyukcangaz et al. 2013, Cantekin et al. 2024, Pehlivanoglu & Yardimci 2012).

Co-occurrence of multiple genes in the same isolate was common: all 14 isolates carried at least two genes (*blaZ* +

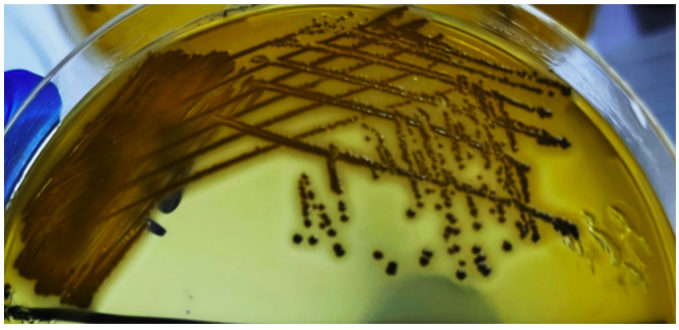

Fig. 1. Typical colonies of *Staphylococcus aureus* on Baird-Parker agar after 48 h of incubation at 37 °C.

Table 1. *Staphylococcus aureus* specific antibiotic resistance genes involved and their oligonucleotide primers for the polymerase chain reactions

Target gene	Primer sequences (5'- 3')	Length of amplified product (bp)	Annealing temperature (°C)	Reference
blaZ	F- TAAGAGATTTGCCTATGCTT	377	48	Olsen et al. (2006)
	R- TTAAAGTCTTACCGAAAGCAG			
mecA	F- GTGAAGATATACCAAGTGATT	174	57	Choi et al. (2003)
	R- ATGCGCTATAGATTGAAAGGAT			
aacA-aphD	F- TAATCCAAGAGCAATAAGGGC	227	55	Strommenger et al. (2003)
	R- GCCACACTATCATAACCACTA			
cfr	F- TGA AGT ATA AAG CAG GTT GGG AGT CA	746	48	Kehrenberg & Schwarz (2006)
	R- ACC ATA TAA TTG ACC ACA AGC AGC			
fusB	F- ATTCAATCGGAAACCTATATGATA	292	48	O'Neill et al. (2004)
	R- TTATATATTTCCGATTTGATGCAAG			
ileS	F- TATATTATGCGATGGAAGGTTGG	458	50	Anthony et al. (1999)
	R- AATAAAATCAGCTGGAAAGTGTTG			
tetK	F- GTAGCGACAATAGGTAATAGT	360	48	Strommenger et al. (2003)
	R- GTAGTGACAATAAACCTCCTA			
tetM	F- AGTGGAGCGATTACAGAA	158	48	Strommenger et al. (2003)
	R- CATATGTCCTGGCGTGTCTA			
vanA	F- GGGAAAACGACAATTGC	732	50	Dutka-Malen et al. (1995)
	R- GTACAATGCGGCCGTTA			

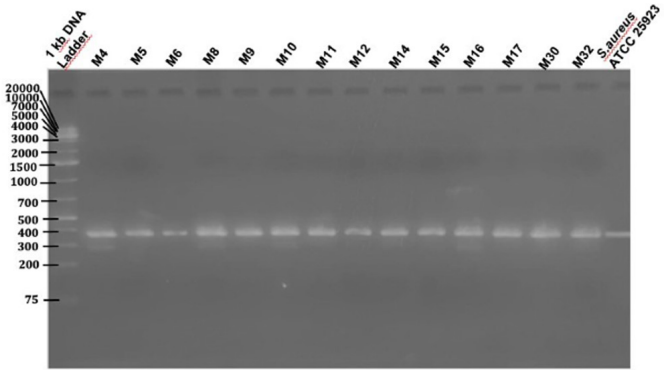

Fig. 2. Results of PCR amplicons (395-bp) of *Staphylococcus aureus* using *nuc* gene primers. M (4, 5, 6, 8, 9, 10, 11, 15, 16, 17, 30, 32) = *S. aureus* strains isolated from bovine mastitis milk.

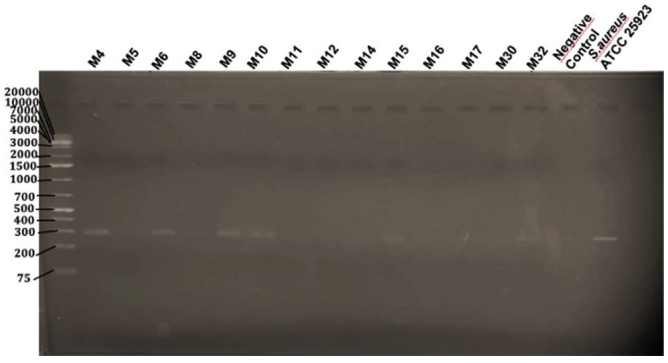

Fig. 3. Results of PCR amplicons (227-bp) of *Staphylococcus aureus* using *aacA-aphD* gene primers. M (4, 5, 6, 8, 9, 10, 11, 15, 16, 17, 30, 32) = *S. aureus* strains isolated from bovine mastitis milk.

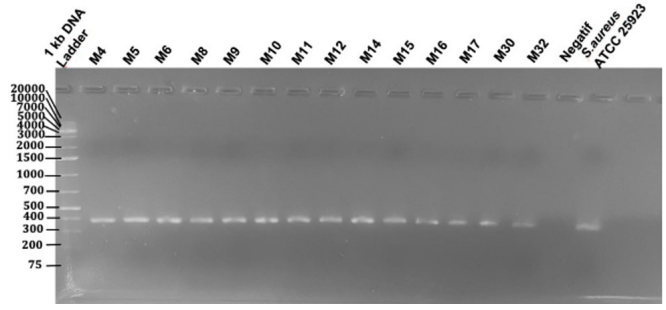

Fig. 4. Results of PCR amplicons (377-bp) of *Staphylococcus aureus* using *blaZ* gene primers. M (4, 5, 6, 8, 9, 10, 11, 15, 16, 17, 30, 32) = *S. aureus* strains isolated from bovine mastitis milk.

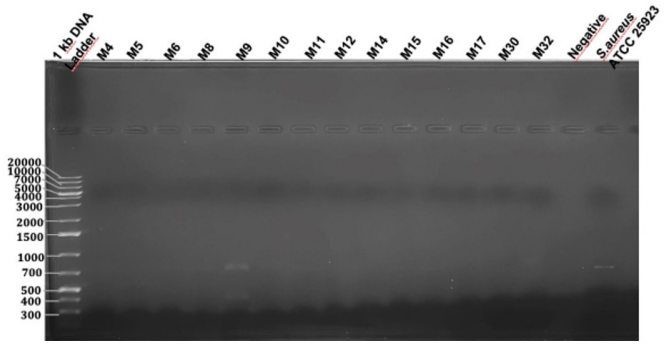

Fig. 5. Results of PCR amplicons (746-bp) of *Staphylococcus aureus* using *cfr* gene primers. M (4, 5, 6, 8, 9, 10, 11, 15, 16, 17, 30, 32) = *S. aureus* strains isolated from bovine mastitis milk.

tetM), eight carried three genes (*blaZ* + *tetM* + *aacA-aphD*), and three carried four genes (*blaZ* + *tetM* + *aacA-aphD* + *mecA*). A single isolate additionally harboured the *cfr* gene. A more comprehensive picture of expressed antibiotic resistance could be obtained by combining phenotypic antimicrobial susceptibility testing results with the study's exclusive focus on genotypic analysis.

Reported resistance gene profiles of mastitis pathogens exhibit marked regional variation, and these profiles are likely influenced by local antibiotic use and farm management practices (Rodríguez et al. 2023). These high carriage rates, especially for *blaZ*, *tetM*, and *aacA-aphD*, indicate the importance of province-level surveillance in Turkey to better guide treatment guidelines and antimicrobial stewardship.

In summary, *S. aureus* isolates from subclinical bovine mastitis in southwestern Turkey uniformly carried *blaZ* and *tetM* genes, showed moderate carriage of *aacA-aphD* (42.9%) and *mecA* (14.3%), rare carriage of *cfr* (7.1%), and complete absence of *tetK*, *vanA*, *fusB* and *ileS*. These results contribute to the growing genotypic evidence on antimicrobial resistance determinants in bovine mastitis and highlight the need for continued regional monitoring, followed by phenotypic susceptibility testing, whole-genome sequencing, and correlation of genotypes with expressed resistance to track the dissemination of successful resistance strains.

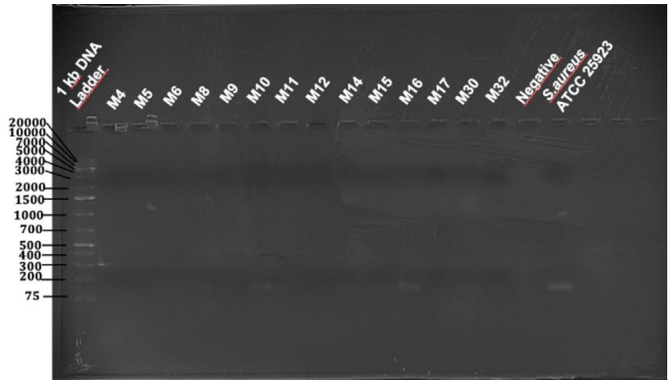

Fig. 6. Results of PCR amplicons (174-bp) of *Staphylococcus aureus* using *mecA* gene primers. M (4, 5, 6, 8, 9, 10, 11, 15, 16, 17, 30, 32) = *S. aureus* strains isolated from bovine mastitis milk.

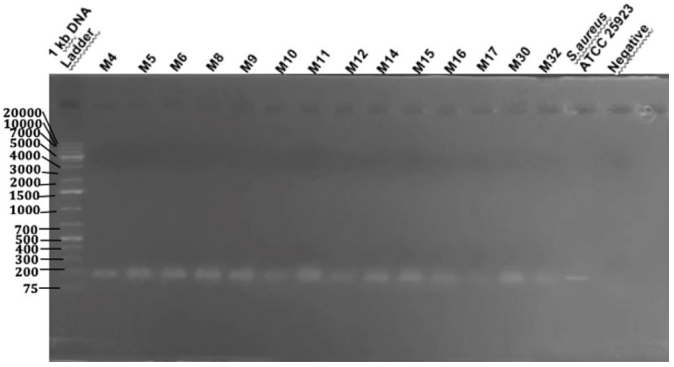

Fig. 7. Results of PCR amplicons (158-bp) of *Staphylococcus aureus* using *tetM* gene primers. M (4, 5, 6, 8, 9, 10, 11, 15, 16, 17, 30, 32) = *S. aureus* strains isolated from bovine mastitis milk.

CONCLUSION

In this study, a unique genotypic resistance profile in *Staphylococcus aureus* isolated from subclinical bovine mastitis in southwest Turkey was documented, with *blaZ* and *tetM* present in all isolates, *aacA-aphD* in more than half, *mecA* in a significant proportion, and *cfr* only sporadically; *tetK, vanA*, *fusB*, and *ileS* were not detected at all. The detected resistance gene profile is primarily directed against β-lactams, tetracyclines, and aminoglycosides, the antimicrobial classes most commonly used in Turkish dairy mastitis therapy. Complementary phenotypic antimicrobial susceptibility testing would help elucidate the clinical expression of the detected determinants and solidify conclusions regarding actual resistance patterns. The findings add valuable regional data to the increasing body of knowledge of the prevalence of antimicrobial resistance genes in bovine *S. aureus* and emphasize the need for continued monitoring.

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Data availability statement.- All data generated or analyzed during this study are included in this published article.

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