Investigation of Various Toxigenic Genes, Antibiotic and Disinfectant Resistance Profiles in Staphylococcus aureus Originating from Raw Milk

Gulay Merve Bayrakal * and Ali Aydin

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Article

Investigation of Various Toxigenic Genes, Antibiotic and Disinfectant Resistance Profiles in *Staphylococcus aureus* Originating from Raw Milk

Gulay Merve Bayrakal * and Ali Aydin

Department of Food Hygiene andTechnology, Faculty of Veterinary Medicine, İstanbul University-Cerrahpasa, Avcilar, İstanbul 34500, Türkiye

Abstract: This study investigated the toxigenic genes and antimicrobial resistance profiles *Staphylococcus aureus* strains isolated from 260 raw milk samples collected from dairy farms in Türkiye. Results indicated that 60.7% of staphylococcal enterotoxin genes (*sea*, *seb*, *sed*, *seg*, *sei*, *sej*, *sek*, *seq*, *sem*, *seo*, and *seu*) and 21.4% of the *tst* and *eta* genes were positive, with most enterotoxin-positive samples carrying more than one gene. The genes *sec*, *see*, *seh*, *sel*, *sen*, *sep*, and *etb* were not identified in any samples. The prevalence of antibiotic resistance genes (*mec*A, *bla*R, *bla*I, *bla*Z, *van*A, *erm*T, *tet*K, *aac/aph*, *ant*, *dfrA*, *tca*R, *IS256*, and *IS257*) was high at 89.2%, with bla being the most frequently detected gene (75%). The *mecA* gene was present in 14.2% of samples, while *tcaR* was detected in 78.5%. Nevertheless, the *mecC* was not identified. Disinfectant resistance genes (*qac*A/B, *qac*C, *qac*J, *smr*) were detected in 21.4% of the samples. The results of the disc diffusion test showed that 64% of strains were resistant to penicillin G and ampicillin, with additional resistance found for cefoxitin, teicoplanin, levofloxacin, norfloxacin, and other antibiotics. These findings highlight a significant public health and food safety risk associated with raw milk due to the presence of *S. aureus* strains with toxigenic genes and high antimicrobial resistance.

Keywords: antibiotic resistance; disinfectant resistance; raw milk; *Staphylococcus aureus*; toxigenic genes

1. Introduction

Milk is a nutritionally complete food, providing all the nutrients required for growth and healthy development in humans [1]. Nevertheless, raw milk is also an optimal source of nutrition for the cultivation of pathogens and spoilage organisms [2]. As the demand for raw milk, perceived to be more nutritionally beneficial by the public, continues to rise, a corresponding increase in health concerns has been observed. *Staphylococcus aureus* (*S. aureus*) is one of the most significant pathogenic microorganisms found in milk and is responsible for several serious diseases in humans [3,4]. *S. aureus* is a significant pathogen for dairy farms, due to its virulence and antimicrobial resistance properties.

S. aureus is the most frequently identified causative agent of clinical and subclinical mastitis in dairy cattle [4,5]. It is worth noteworthy that, in addition to mastitis in animals, *S. aureus*, which can be transmitted from farm personnel to milk, has been identified as a causative agent of food poisoning in humans [6,7]. In contrast to other toxins secreted by *S. aureus*, a relatively small quantity is sufficient for enterotoxins to exert their toxic effects. Staphylococcal food poisoning represents a significant cause of foodborne illness globally, attributed to the presence of enterotoxins with enhanced tolerance to environmental agents [8,9].

S. aureus possesses a variety of virulence factors that contribute to its pathogenicity [3]. The known secretory virulence factors include toxins (staphylococcal enterotoxins, toxic shock syndrome toxin-1 (*tst*), haemolysins, and exfoliative toxins A and B (*eta* and *etb*), as well as enzymes (coagulase, staphylokinase, DNAase, phosphatase, lipase, and phospholipase) [3,10]. Due to its virulence properties, *S. aureus* can overcome the host defense system, thereby facilitating the occurrence of disease, and prolonging the course of treatment.

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Food represents a significant vector for the transmission of antimicrobial resistance [7]. The term "antimicrobial resistance" encompasses both disinfectant and antibiotic resistance. In recent years, there has been an alarming increase in the severity of infections caused by S. *aureus*, largely due to the emergence of antibiotic resistance [11]. The emergence of penicillin, vancomycin, and methicillin resistance is frequently observed because of excessive antibiotic treatment [12]. Methicillin-resistant *S. aureus* (MRSA) represents a significant global public health threat on a global scale, particularly in humans and animals, and is a common cause of severe infection [13,14]. The primary cause of hospital-acquired infections, MRSA, exhibits resistance to numerous known antibiotics, rendering the fight against the disease even more challenging [15].

Quaternary ammonium compounds (QACs) are a commonly used disinfectant in the dairy industry, employed for the disinfection of milking equipment and udder disinfection, particularly for the prevention of mastitis [16]. In the absence of the requisite conditions, including the correct selection of disinfectant, appropriate dosage, and pre-cleaning, the efficacy of disinfectants is diminished, leading to the emergence of resistant strains [17]. This situation represents a significant public health concern that requires urgent attention.

The objective of this study was to investigate the virulence characteristics and antimicrobial resistance profile of *S. aureus* strains isolated from raw milk. The antimicrobial resistance profiles of *S. aureus* strains isolated from raw milk were investigated by analyzing the presence of antibiotic and disinfectant resistance genes. Moreover, agar diffusion assays were performed to evaluate the antibiotic susceptibility of the samples against nine distinct antibiotic groups. To identify the virulence properties, an investigation was conducted into the presence of SEs genes responsible for enterotoxin production, exfoliative toxin genes (*eta* and *etb*), and the toxic shock syndrome toxin-1 gene (*tst*). Furthermore, the milk to be analyzed in the study was collected from the Thrace region, which represents a border area with Europe. The objective was to examine a region that is significant in terms of disease control.

2. Materials and Methods

2.1. Isolation and Strain Identification

A total of 260 raw milk samples were collected from various dairy farms in the Thrace region and plated on Baird Parker agar (BPA, Oxoid, CM275, Basingstoke, UK) and *S. aureus* was isolated. The isolation of *S. aureus* from food samples was conducted by the EN ISO 6881-1 standardized procedures documented by the International Organization for Standardization [18]. The strains were identified by biochemical tests, including gram staining, catalase testing, latex agglutination testing, growth on mannitol fermentation (using Mannitol Salt Agar, Oxoid CM0085B), and DNase activity testing (using DNase agar, Oxoid CM0321). The identifications were subsequently confirmed by PCR following DNA extraction [7]. To confirm the identity of the *S. aureus* strains, an analysis was conducted to determine the presence of the thermonuclease gene (*nuc*), the coagulase gene (*coa*), and the production of the surface protein A gene (*spa*) [7,19,20].

2.2. Detection of Toxigenic Genes in S. aureus Isolates

The presence of staphylococcal enterotoxin genes *sea*, *seb*, *sec*, *sed*, *see* [21], *seg*, *seh*, *sei*, *sej*, *sek*, *sel*, *sep*, *seq* [22], *sem*, *sen*, *seo* [23], and *seu* [24] were detected using multiplex and monoplex PCR. Moreover, the presence of the exfoliative toxins' genes (*eta*, *etb*) [21], and the toxic shock syndrome toxin-1 gene (*tst*) [25] were investigated.

2.3. Determination of Antimicrobial Susceptibility of S. aureus Isolates

The antimicrobial susceptibility of the isolates was determined by the agar disk diffusion method, in accordance with the guidelines set forth by the EUCAST (European Committee on Antimicrobial Susceptibility Testing) [26]. The following antibiotic disks were used: penicillin G (Oxoid-CT0043, 10 U), ampicillin (Oxoid-CT0003, 10 μg), gentamicin (Oxoid-CT0024,10 μg), tobramycin (Oxoid-CT0056, 10 μg), teicoplanin (Oxoid-CT0647, 30 μg), ceftaroline (Oxoid-CT1942, 5

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μg), cefoxitin (Oxoid-CT0119, 30 μg), tetracycline (Oxoid-CT0054, 30 μg), erythromycin (Oxoid-CT0020, 15 μg), levofloxacin (Oxoid-CT1587, 5 μg), ofloxacin (Oxoid-CT0446 5 μg), norfloxacin (Oxoid-CT0434, 10 μg), fusidic acid (Oxoid-CT0023, 10 μg), trimethoprim-sulfamethoxazole (Oxoid-CT0052, 1.25 μg/23.75 μg), linezolid (Oxoid-CT1649, 10 μg). The antibiotics were selected from nine preferred antibiotic groups, namely penicillins, aminoglycosides, glycopeptides and lipoglycopeptides, cephalosporins, tetracyclines, macrolides, lincosamides and streptogramins, fluoroquinolones, miscellaneous agents, and oxazolidinones. Antimicrobial discs were placed on Mueller-Hinton agar (MHA, CM 337 Oxoid) containing *S. aureus*. Following the incubation period, the diameter of the inhibition zone was measured and evaluated. MDR isolates were defined as those exhibiting resistance to at least three distinct antimicrobial classes.

2.4. Detection of Antimicrobial Resistance Genes in S. aureus Isolates

Multiplex and monoplex PCR was used to detect the presence of antibiotic resistance gene; methicillin resistance genes (*mecA* [27] *and mecC* [28]), penicilin resistance genes (*blaR*, *blaI*, *blaZ* [*blaZ*F-R]) [29], and (*blaZ*1-2) [30], aminoglycoside resistance genes (*aac/aph*, *aph*, *ant*) [31], teicoplanin associated locus (*tcaR*) [32], vancomycin resistance gene (*vanA*) [33], trimethoprim resistance gene (*dfrA*) [29], erythromycin resistance gene (*ermT*) [34], tetracycline resistance gene (*tetK*) [29], and insertion sequence (IS) elements (*IS256*, *IS257*) [29]. Isolates were classified as multidrug-resistant when they exhibited an inability to respond to at least three distinct drug classes. To detect disinfectant resistance, which is the other responsible factor for the formation of antimicrobial resistance, the presence of the QAC resistance gene *qac*AB and *smr* [35], *qac*C [29], and *qac*J [36] was determined by monoplex PCR.

The PCR products were analyzed by horizontal electrophoresis in a system containing 1xTris-acetate-EDTA (TAE) buffer, 1.5% (w/v) agarose gel, and 5% (v/v) fluorescent DNA dye (SafeView Classic, Applied Biological Materials Inc. Richmond, Canada). The gels were imaged using the Infinity Gel Imaging System (Vilber Lourmat, Marne-la-Vallée, France). All the PCR experiments were done twice for each strain.

3. Results

3.1. Toxigenic Genes (Virulence Genes)

In this study, 260 raw milk samples were collected, and 28 strains (10.7%) of *S. aureus* were confirmed by PCR following microbiological analysis. The investigation of the presence of virulence and antimicrobial resistance genes in these strains is presented in Tables 1–3 respectively.

Table 1. The results of the virulence gene profile analysis.

Strain Number (n=28)	Virulence Gene	
	Enterotoxin Producing Gene	Toxic Shock Syndrome Toxin-1 Gene and Exfoliative Toxin Genes
2		*tst*
4		
10		
18	*seo*	*tst*
22		
23	*seo*, *seu*, *seq*	
24	*sed*, *sei*, *sej*, *sek*, *seo*, *seq*	
25	*seu*, *seq*	
26	*sei*, *seo*	*tst*
29	*sei*, *seu*	
43		
44	*seo*	
46	*sea*, *sed*, *sek*, *sem*, *seq*	*tst*

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47	*sea, sed, seq*	
97		
100		
101	*seb, sed, seg, sei, sem, seu*	
102		
112	*seb, seg, sei, sem, seo, seu*	
114	*seb, seg, sei, sem, seu,*	
123	*seb, seg, sei, sem, seo, seu*	
124	*seb, seg, sei, sem, seu*	
131		*tst*
167	*seo*	
168		
190		
194	*seg, seo*	*tst, eta*
218	*sed, seg, sem, seo, seu*	
Total Strain Number	**17/28**	**6/28**

Table 2. Distribution of antibiotic-resistant strains of *S. aureus.*

Antibiotic Groups	**Name of Antibiotics**	**Distribution of *S. aureus* Strains According to EUCAST**	
		R (%)	S (%)
Penicillins	Penicillin G 10 µg	18 (%64)	10 (%35)
	Ampicillin 10 µg	18 (%64)	10 (%35)
Aminoglikozid	Gentamicin 10µg	1 (%3.5)	27 (%96)
	Tobramycin 10µg	2 (%7.1)	26 (%92)
Glycopeptides and Lipoglycopeptides	Teicoplanin 30 µg	6 (%21)	22 (%78)
Cephalosporins	Ceftaroline 5 µg	0 (%0)	28 (%100)
	Cefoxitin 30 µg	8 (%28)	20 (%71)
Tetracycline	Tetracycline 30µg	2 (%7.1)	26 (%92)
Macrolides, lincosamides and streptogramins	Erythromycin 15µg	2 (%7.1)	26 (%92)
Fluoroquinolones	Levofloxacin 5 µg	6 (%21)	22 (%78)
	Ofloxacin 5 µg	4 (%14)	24 (%85)
	Norfloxacin 10 µg	5 (%17)	23 (%82)
Miscellaneous Agents	Fusidic Acid 10 µg	4 (%14)	24 (%85)
	Trimethoprim-Sulfamethoxazole (1.25 µg/23.75 µg)	0 (%0)	28 (%100)
Oxazolidinones	Linezolid 10 µg	0 (%0)	28 (%100)

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Table 3. The results of the antimicrobial resistance gene and antimicrobial susceptibility test.

Strain Number (n=28)	Antimicrobial Resistance Gene		Antimicrobial Susceptibility Test
	Disinfectant Resistance Gene	Antibiotic Resistance gene	
2		*BlaI*, *BlaZ*1-2, *tcaR*, *IS256*	Penicillin G, Ampilcillin
4		*BlaI*, *BlaZ*1-2, *tcaR*, *IS256*	Penicillin G, Ampilcillin
10		*BlaR*, *BlaI*, *BlaZ*1-2, *BlaZ*F-R, *tcaR*, *IS256*	Ampilcillin, Cefoxitin, Fusidic Acid
18			
22		*BlaI*, *BlaZ*1-2, *BlaZ*F-R, *tcaR*, *vanA*	Penicillin G, Ampilcillin, Levofloxacin, Norfloxacin
23	*qac*C, *qac*J, *smr*	*BlaR*, *BlaI*, *BlaZ*1-2, *BlaZ*F-R, *tcaR*, *vanA*, *dfrA*, *ermT*, *IS256*	Penicillin G, Ampilcillin, Cefoxitin, Erythromycin, Levofloxacin
24	*qac*J, *smr*	*BlaR*, *BlaI*, *BlaZ*1-2, *BlaZ*F-R, *tcaR*, *IS256*	Penicillin G, Levofloxacin, Ofloxacin, Norfloxacin
25		*BlaI*, *BlaZ*1-2, *BlaZ*F-R, *tcaR*, *dfrA*	Penicillin G, Levofloxacin, Ofloxacin, Norfloxacin
26		*BlaI*, *BlaZ*1-2, *BlaZ*F-R, *ant*, *tcaR*, *IS256*	Cefoxitin, Fusidic Acid
29		*BlaR*, *BlaI*, *BlaZ*1-2, *BlaZ*F-R, *tcaR*, *IS256*	Penicillin G, Ampilcillin, Levofloxacin, Norfloxacin
43		*BlaI*, *BlaZ*1-2, *BlaZ*F-R, *aac/aph*, *tcaR*, *IS256*	Penicillin G, Ampilcillin
44	*qac*J, *smr*	*BlaI*, *BlaZ*1-2, *vanA*, *IS256*	Penicillin G, Ampilcillin
46	*qac*AB, *qac*J, *smr*	*mecA*, *BlaR*, *BlaI*, *BlaZ*1-2, *BlaZ*F-R, *tcaR*	Penicillin G, Ampilcillin, Levofloxacin, Ofloxacin, Norfloxacin
47		*Bla*I, *BlaZ*1-2, *BlaZ*F-R, *tcaR*	Penicillin G, Ampilcillin, Cefoxitin
97		*BlaI*, *BlaZ*1-2, *ant*, *tcaR*, *IS256*	Cefoxitin, Fusidic Acid
100		*BlaI*, *BlaZ*1-2, *BlaZ*F-R, *tcaR*, *IS256*	Tetracycline
101	*qac*AB, *smr*	mec*A*, *BlaI*, *BlaZ*1-2, *BlaZ*F-R, *tcaR*	Penicillin G, Ampilcillin, Teicoplanin
102		*mecA*, *BlaR*, *BlaI*, *BlaZ*1-2, *BlaZ*F-R, *tcaR*, *vanA*, *tetK*, *IS256*	Penicillin G, Ampilcillin, Teicoplanin, Tetracycline, Erythromycin
112		*BlaI*, *BlaZ*1-2, *BlaZ*F-R, *tcaR*	Penicillin G, Ampilcillin
114		*BlaI*, *BlaZ*1-2, *BlaZ*F-R, *tcaR*	Penicillin G, Ampilcillin
123		*BlaI*, *BlaZ*1-2, *BlaZ*F-R, *tcaR*	Penicillin G, Ampilcillin
124		*BlaI*, *BlaZ*1-2, *BlaZ*F-R, *tcaR*	Penicillin G, Ampilcillin, Gentamicin, Tobramycin, Cefoxitin, Ofloxacin, Fusidic Acid
131		*IS257*	Teicoplanin, Cefoxitin
167		*tcaR*, *IS256*	Teicoplanin
168		*IS256*	Cefoxitin
190			Teicoplanin
194	*qac*C, *qac*J, *smr*	*mecA*, *tcaR*, *vanA*, *IS256*	Penicillin G, Ampilcillin, Tobramycin, Teicoplanin
218			Ampilcillin
Total Strain Number	**6/28**	**25/28**	**27/28**

The research findings indicated that at least one gene encoding for enterotoxin was present in 60.7% of the *S. aureus* samples obtained from raw milk. Fourteen of the 17 enterotoxin-positive

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samples demonstrated the presence of two or more distinct enterotoxin genes. The most prevalent gene observed in the samples was the *seo* gene (35.7%), and the gene observed in three samples carrying a single gene was also the *seo* gene. The percentages of the *sea*, *seb*, *sed*, *seg*, *sei*, *sej*, *sek*, *sem*, *seo*, *seu* and *seq* enterotoxin genes that were isolated from the 28 samples were found to be 7.1%, 17.8%, 17.8%, 25%, 28.5%, 3.5%, 7.1%, 25%, 35.7%, 32.1% and 17.8%, respectively. The genes *sec*, *see*, *seh*, *sel*, *sen,* and *sep* were not identified in any samples.

Regarding the virulence genes examined, the *tst* gene was identified in 21.4% of the samples. Among the exfoliative toxin genes, the *eta* gene was identified in a single sample (3.5%), while the *etb* gene was not detected.

3.2. Antimicrobial Susceptibility

The antimicrobial susceptibility of the isolates was determined by the agar disk diffusion method. The results of the disc diffusion test indicated that the highest resistance was observed against penicillin G and ampicillin (64%), followed by cefoxitin (28%), teicoplanin (21%), levofloxacin (21%), and norfloxacin (17%). The prevalence of resistance to ofloxacin and fucidic acid was observed to be 14%, while the prevalence of resistance to tobramycin, tetracycline, and erythromycin was observed to be 7.1%. The lowest resistance was observed in the gentamicin treatment group. All isolates demonstrated susceptibility to ceftaroline, trimethoprim-sulfamethoxazole, and linezolid.

3.3. Antimicrobial Resistance Genes

The present study demonstrated a high prevalence (89.2%) of antimicrobial resistance genes in *S. aureus* strains isolated from raw milk. The results of the analysis of antibiotic resistance genes revealed the presence of the *mecA* gene, responsible for resistance to the methicillin antibiotic, at a rate of 14.2%. In contrast, the *mecC* gene was not detected in any of the examined strains. The most frequently identified antibiotic resistance gene was the *bla* gene, which is responsible for resistance to beta-lactam antibiotics. This gene was identified in 75% of the samples analyzed. In the present study, the *aac/aph* and *ant* genes, which are responsible for the development of resistance to the aminoglycoside antibiotic group, were identified to be present in 3.5% and 7.1% of the strains, respectively. The *aph* gene was not detected in any of the strains. *The tcaR gene, which is responsible for resistance to the teicoplanin antibiotic, was identified in 78% of the isolates. The vanA gene, which is responsible for vancomycin resistance, was analyzed at a rate of 17%, while the dfrA gene, which is responsible for resistance to trimethoprim antibiotics, was analyzed at a rate of 7.1%.* The *ermT* gene, which is responsible for resistance to the erythromycin antibiotic from the lincosamide group, and the *tetK* gene, which is responsible for tetracycline resistance, were identified in a single sample each. *The insertion sequence (IS) elements IS256 were identified in 53.5% of the cases, while IS257 was analyzed in 3.5%.*

The QAC genes responsible for QAC disinfectant resistance were identified in 21.4% of the samples. The prevalence of disinfectant resistance genes *qac*AB, *qac*C, *qac*J, and *smr* was determined to be 7.1%, 7.1%, 17.8%, and 21.4%, respectively.

4. Discussions

4.1. Toxigenic Genes

Epidemiological studies have demonstrated that *S. aureus* strain agents in milk produce a group of virulence factors and have indicated a correlation between the severity of infection and the virulence factors produced by *S. aureus* [3]. The most significant virulence factor of *S. aureus* is the production of enterotoxin. SEs are regarded as a significant public health concern [37]. A number of studies have investigated the occurrence of enterotoxin genes in strains of *S. aureus* isolated from food sources, reporting a range of prevalence rates for different genes. In their study, Zhang et al. (2022, a) reported the detection of the *sea* (17.45%), *seb* (16.44%), *sec* (7.38%), and *sed* (1.68%) enterotoxin genes, while the *see* gene was not detected. In their 2020 study, Titouche et al. reported that 62.5% of the samples tested positive for at least one gene encoding SEs. The most prevalent genes were *sei* and *seg* (47.69%), followed by *seb* (23.08%). It is noteworthy that, none of the isolates carried

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the *sed* and *see* genes. In another study, the *seg*, *sei* and *sec* genes were identified as positive in 35% of raw milk samples, while the *sea* and *sei* genes were found to be negative [39]. In a study conducted by Khemiri et al. (2019), at least one enterotoxin gene was identified in 87.5% of *S. aureus* strains isolated from raw milk samples. Of these, the *sed* gene was the most frequently analyzed. Consistent with previous studies [3,38,39], our analysis revealed the presence of at least one enterotoxin gene in the strains under investigation, while the *sec* and *see* genes were not identified. In contrast with the findings of the present study, Fursova et al. (2018) examined the *sea* gene in 53.30% and the *sec* gene in 50% of *S. aureus* strains isolated from food sources. Furthermore, the present study identified the presence of the *seb*, *sed* (17.8%), and *sei* (28.5%) genes, which were not detected in the studies conducted by Adame-Gómez et al. (2020) and Pereira et al. (2009). One of the primary causes for the disparate outcomes observed in the study was attributed to the distinct characteristics exhibited by the strains in varying geographical regions. As observed in our study, the *see* gene was not detected, while the *sed* gene was among the most prevalent in a similar study conducted in the Thrace region by Papadopoulos et al. (2019). The findings of our study are consistent with those of a similar investigation conducted in our **neighboring** country, in the Thrace region. In the study, the *see* gene was not detected, while the sed gene was identified with high frequency [43].

In addition to food poisoning, *S. aureus* is a causative agent of a range of acute and chronic diseases, including septicemia, pneumonia, endocarditis, and respiratory tract illnesses, as well as autoimmune diseases. These diseases have a high morbidity rate in a variety of hosts. One of these diseases is toxic shock syndrome [44]. In their study, Zhang et al. (2022, a) identified the presence of the *tst* gene at a rate of 23.50%, while Dan et al. (2019) reported a rate of 26.5%. As in the aforementioned studies, the *tst* gene was identified in 21.4% of cases in our own investigation. In contrast with the findings of our study, the *tst* gene was not identified in other studies conducted on milk [3,42,46].

Exfoliative toxins (*eta*, *etb*) are virulence factors secreted by staphylococci. These toxins are responsible for the degradation of keratinocytes in both human and animal skin, which can result in the development of skin infections [47]. In the present study, the *eta* gene was identified as positive, whereas the *etb* gene was identified as negative. In other studies, Tegegne et al. (2021) analyzed the *eta* gene in 22.05% of *S. aureus* strains isolated from milk. Similarly, Kot et al. (2016) analyzed the *eta* gene in 5.6% of *S. aureus* strains isolated from milk. The studies conducted by Dan et al. (2019), Gharsa et al. (2019) and Chenouf (2021) did not identify the presence of the *eta* and *etb* genes in any of the samples. The findings demonstrate that *S. aureus* strains may encode a range of virulence factors and that the distribution of these factors varies between different genotypes. These results indicate that *S. aureus* virulence is not exclusive to the strain level but is also influenced by factors such as the origin and genetic background of the strain [45].

4.2. Antimicrobial Susceptibility

Upon examination of the antibiotic resistance profiles, it was observed that the results of the disc diffusion and resistance genes assays were consistent for the beta lactam group. In the course of our investigation, it was found that 75% of the isolates encoding the *bla* gene and 64% of the isolates were resistant to penicillin G. The *aac*/*aph* and *ant* genes responsible for resistance to the aminoglycoside group were detected in 3.5% and 7.1% of isolates, while resistance to gentamicin and tobramycin was 3.5% and 7.1%, respectively. The findings of our study are in accordance with those of other researchers indicating that the highest resistance was observed in the penicillin group [39,45,51]. As observed by Dan et al. (2019), the current study revealed that the isolates did not demonstrate resistance to linezolid. However, in contrast to the findings of this study, the isolates exhibited high resistance to teicoplanin. As previously observed by Pereira et al. (2009), a minor percentage of isolates exhibited resistance to gentamicin, erythromycin, and tetracycline. The current study reports a lower prevalence of norfloxacin-resistant isolates than that observed by Kotb and Gafer (2020).

The data presented here are similar to those reported by Dan et al. (2019), in that all MRSA isolates demonstrated resistance to penicillin G and ampicillin. Of the analyzed samples, 78% showed resistance to more than one antibiotic. The findings of our study indicate that some isolates encoding

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resistance genes (*tcaR* and *dfrA*) did not develop resistance to the antibiotic. These findings indicate that, despite the presence of antimicrobial resistance genes, their expression may be insufficient to confer a resistant phenotype. Nevertheless, these strains have the potential to transform into resistant strains under certain conditions and may therefore have epidemiological importance in the transmission of antibiotic resistance [45].

4.3. Antimicrobial Resistance Genes

The increasing prevalence of antimicrobial resistance in *S. aureus* represents a significant global public health concern [4]. As a consequence of the excessive and inappropriate usage of antimicrobials, microorganisms develop resistance to these substances, thereby transferring this resistance to other microorganisms through the transfer of genes [53]. This situation gives cause for concern about both food safety and public health. The range of treatment options for life-threatening infections caused by MDR strains of *S. aureus* is limited. Considering these considerations, the World Health Organization (WHO) classified *S. aureus* as the most significant bacterial pathogen exhibiting antibiotic resistance in 2017 [54].

Methicillin-resistant *S. aureus* is one of the most significant antibiotic-resistant bacteria. Isolates of MRSA are frequently multidrug resistant, which results in increased costs, longer treatment durations, and higher rates of hospitalization and comorbidity. The *mecA* gene, which encodes the production of penicillin-binding proteins, is responsible for methicillin resistance in the MRSA chromosome. It is therefore considered the most reliable method for the detection of methicillin resistance [4,55]. The present study revealed the presence of the *mecA* gene in 14.2% of the samples. A review of the literature on the detection of the *meC* gene in milk revealed that the findings of Dan et al. (2019) were consistent with those of the present study. The results of the *mecA* analysis in the present study were found to be lower than those reported by Ganai et al. (2016), Riva et al. (2015), and our neighboring country [43], but higher than those observed by Mahanti et al. (2020), Chenouf et al. (2021), and Pereira et al. (2009). In studies conducted in Türkiye, the prevalence of strains carrying the *mecA* gene was found to be 6.3% [59] and 1.70% [60], which is lower than the data obtained in the present study. In numerous additional studies conducted in Türkiye, the presence of the *mecA* gene was not detected in *S. aureus* strains isolated from food sources [51,61,62]. It is also noteworthy that, as with the present study, the *mecC* gene was not detected in studies conducted in Brazil [63] and Greece [43].

The primary mechanism of resistance to penicillin and penicillin derivatives is the *blaZ* gene, which encodes the production of beta-lactamases that hydrolytically break down beta-lactams [4,55]. Elevated levels of the *blaZ* gene have been reported in milk [63 (74.07%), 64 (94.6%), 65 (95.7%)]. In this study, the *blaI* and *blaR* genes, which are responsible for the production of beta-lactamase, were also subjected to analysis. A review of the literature revealed no previous analysis of these genes in raw milk in Türkiye. The *blaI* and *blaR* genes are responsible for the regulation of the expression of the *blaZ* gene [*66*]. *In a study conducted by Kreausukon et al. (2012), the presence of the blaI, blaR, and blaZ genes was identified in all MRSA strains. Additionally, the study demonstrated the presence of elevated levels of the blaI and blaR genes. Furthermore, the investigation revealed that the blaI and blaZ genes were present in all isolates that also carried the blaR gene.*

The bifunctional enzyme AAC/APH encoded by the aac/aph gene, the APH enzyme encoded by the aph gene, and the ANT enzyme encoded by the ant gene are responsible for resistance to aminoglycosides [68]. The present study identified the presence of the aac/aph and ant genes in various strains, while the aph gene was not detected in any strains. In other studies, the presence of these genes in milk was confirmed [68,69]. The Teicoplanin-associated locus (tcaR) gene, which is associated with teicoplanin resistance, was analyzed for the first time in Türkiye and the results demonstrated that it was present in 78.5% of the samples examined. In contrast, the tcaR gene was identified in clinical isolates in a study conducted in India [70].

Vancomycin, a glycopeptide antibiotic, has been employed in the treatment of MRSA for several decades. This has resulted in the emergence of vancomycin-resistant S. aureus strains [71]. In the present study, the prevalence of the vanA resistance gene was determined to be 17%, which is

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consistent with the findings of Kou et al. (2021). The dihydrofolate reductase protein, which confers resistance to trimethoprim (dfrA), was identified at a prevalence of 7.1% in our study. Nevertheless, the dfrA gene was not detected in raw milk in Egypt [73] and Mozambique [74], nor in chicken, fish, and red meat in Türkiye [75].

The resistance to lincosamides, including erythromycin and clindamycin, is a consequence of the methylation of the receptor-binding site on the ribosome. Methylation is a process that is mediated by an enzyme called methylase and is encoded by erm genes. During our investigation, the ermT gene was isolated from a single isolate. Nevertheless, other studies have indicated that the ermT gene was not identified [50]. The efflux pump system encoded by the tetK gene and carried by plasmids represents one of the resistance pathways to tetracyclines, a broad-spectrum antibiotic [71]. The prevalence of the tetK gene, which was identified in a single isolate in our study, was found to vary significantly in other studies [50 (30%), 45 (3.2%), 4 (48.66%)].

The insertion sequence (IS) elements IS256 and IS257, which are associated with multiple antibiotic resistance, were identified as being present in the study. As observed in our data, studies by Zhang et al. (2022, b) and Miao et al. (2018) similarly identified the presence of IS257 and IS256, respectively. Araújo et al. (2017) did not identify the presence of IS256 and IS257 in their samples. The present study revealed that 64.2% of S. aureus strains demonstrated multidrug resistance (MDR), predominantly to beta-lactams, methicillin, and teicoplanin (Table 3). The findings indicated a high prevalence of MDR S. aureus in raw milk, which represents a significant public health concern. Absent the implementation of necessary preventive measures, the probability of encountering far more significant difficulties in the future is considerable. To prevent the development and transmission of antibiotic-resistant strains, it is recommended that different groups of antibiotics be preferred over this resistance group of antibiotics, particularly when antibiotics are used on dairy farms.

A further component of antimicrobial resistance is disinfectant resistance. To investigate the potential for disinfectant resistance, this study examined the presence of the efflux pump gene (*qac* and *smr*), which is responsible for resistance to QACs. The *qac* and *smr* genes resulted in 21.4% positive results. The findings of Kroning et al. (2020), Kotb and Gafer (2020) and Ergun et al. (2017) indicate a lower prevalence of the *qac* gene than that observed in our data. Furthermore, the *qacJ* gene, identified as 17% positive in our study, was found to be negative by Kroning et al. (2020). Upon analysis of the results of the study, it was observed that all samples exhibiting disinfectant resistance genes also carried at least one antibiotic resistance gene. Three of the four MRSA strains were found to carry a disinfectant resistance gene. This was regarded as an additional risk associated with MRSA strains. Furthermore, previous research has indicated a genetic correlation between disinfectant and antibiotic resistance genes [80,81]. The co-detection of genes responsible for both disinfectant and antibiotic resistance in strains is indicative of the presence of antimicrobial-resistant strains. Despite the disparate mechanisms of action exhibited by the two groups of genes, genetic studies have revealed striking similarities in their genetic systems and the locations of their genes on the same mobile genetic elements [80]. Moreover, research has indicated that the probability of the pathogen developing antibiotic resistance increases with prolonged exposure to the disinfectant [82].

5. Conclusions

This study represents the first comprehensive investigation into the antimicrobial resistance levels and virulence characteristics of raw milk in Türkiye. The high prevalence of virulence and antimicrobial genes in *S. aureus* in our study indicates a potential risk associated with raw milk. **The necessary measures must be** taken to eliminate this situation, which presents a significant risk to both food safety and public health. To prevent the development of MDR bacteria, which represents an important potential health threat in the future, it is essential to focus on the selection of antibiotics and the utilization of novel, advanced-generation antibiotics. To prevent the development of enterotoxins and disinfectant resistance, it is essential to implement hygiene and sanitation procedures, as well as to ensure the correct selection of disinfectants. **Further** studies **are necessary**

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to determine the risks associated with food products and to identify the precautions to be taken before these risks occur.

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