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Determination of *Staphylococcus aureus* isolation, identification and antibiotic susceptibility in raw milk

Pelin KOÇAK KIZANLIK [1,a], Melih DUYUK [2,b], Cemil ŞAHİNER [1,c*], Murat BOYACIOĞLU [3,d]

[1] Aydın Adnan Menderes Üniversitesi Veteriner Fakültesi Veterinerlik Besin Hijyeni ve Teknolojisi Anabilim Dalı, Aydın, Türkiye
[2] Aydın Adnan Menderes Üniversitesi Sağlık Bilimleri Enstitüsü Veterinerlik Farmakoloji ve Toksikoloji Anabilim Dalı, Aydın, Türkiye
[3] Aydın Adnan Menderes Üniversitesi Veteriner Fakültesi Veterinerlik Farmakoloji ve Toksikoloji Anabilim Dalı, Aydın, Türkiye

0000-0002-9824-9271 [a]; 0009-0001-6952-8637 [b]; 0000-0003-4368-4732 [c]; 0000-0001-6952-8637 [d]

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ABSTRACT

Staphylococcus aureus in raw milk poses a potential risk to public health due to its toxin-producing strains and high levels of antibiotic resistance. In this study, the presence of *S. aureus* in raw milk and the antibiotic resistance profiles of the isolated strains were examined. A total of 100 raw milk samples were analyzed, and *S. aureus* contamination was detected in 47 samples at various levels. The contamination levels ranged from 2.00 to 4.99 log cfu/ml and 45.9% of the contaminated samples were found to be in the 3-4 log cfu/ml range. The mean of contamination level for positive samples was determined as 3.63±0.77 log cfu/ml. The minimum inhibitory concentration values for the antibiotics cefoxitin, methicillin, tetracycline, tylosin, florphenicol, neomycin, ciprofloxacin, lincomycin, and polymyxin B were determined using the broth microdilution method to examine the antibiotic resistance profiles of the isolated strains. It was determined that 13 (27.6%) of the *S. aureus* isolates were resistant to all the tested antibiotic groups. Additionally, all isolates were resistant to cefoxitin, methicillin, tylosin, and lincomycin, followed by resistance rates of 95.8% to florphenicol, 89.4% to neomycin, and 87.3% to polymyxin B. Furthermore, all isolates were observed to be multidrug-resistant (MDR). This study indicates that the contamination of raw milk with *S. aureus* and the antibiotic resistance profile pose a significant public health risk. The fact that all isolates were MDR limits treatment options and complicates infection control. These findings show the need for stricter hygiene practices in milk production processes and careful management of antibiotic use.

Çiğ sütlerde Staphylococcus aureus izolasyonu, identifikasyonu ve antibiyotik duyarlılığının belirlenmesi

ÖZET

Çiğ sütte *Staphylococcus aureus* varlığı, toksin üreten suşları ve yüksek antibiyotik direnci nedeniyle halk sağlığı açısından potansiyel bir risk oluşturmaktadır. Bu çalışmada, çiğ sütlerde *S. aureus* varlığı ve izole edilen suşların antibiyotik direnç profili incelenmiştir. Araştırma kapsamında 100 çiğ süt numunesi incelenmiş, 47 örnekte çeşitli düzeylerde *S. aureus* kontaminasyonu saptanmıştır. Kontaminasyon düzeyleri 2,00 ile 4,99 log kob/ml arasında değişiklik göstermiş ve kontamine örneklerin %45,9'unun 3-4 log kob/ml aralığında olduğu belirlenmiştir. Pozitif örnekler için ortalama kontaminasyon değeri ise 3,63±0,77 log kob/ml olarak tespit edilmiştir. Çalışmada tespit edilen izolatların sefoksitin, metisilin, tetrasiklin, tilosin, florfenikol, neomisin, siprofloksasin, linkomisin ve polimiksin B antibiyotikleri için minimum inhibitör konsantrasyon değerleri broth mikrodilüsyon yöntemi kullanılarak antibiyotik direnç profilleri incelenmiştir. *S. aureus* izolatlarından 13'ünün (%27,6) incelenen tüm antibiyotik gruplarına dirençli olduğu tespit edilmiştir. İzolatların tamamının ise sefoksitin, metisilin, tilosin ve linkomisine dirençli olduğu, aynı zamanda izolatların %95,8'inde florfenikol, %89,4'ünde neomisin ve %87,3'ünde polimiksin B direnci belirlenmiştir. Ayrıca, tüm izolatların çoklu ilaç dirençli (ÇİD) olduğu gözlemlenmiştir. Bu çalışma, çiğ sütler *S. aureus* kontaminasyon düzeyinin ve bu bakterinin antibiyotik direnç profilinin, halk sağlığı açısından önemli bir risk oluşturduğunu ortaya koymaktadır. Özellikle, izolatların tamamının ÇİD olması, tedavi seçeneklerini kısıtlamakta ve enfeksiyon kontrolünü zorlaştırmaktadır. Bu bulgular, süt üretim süreçlerinde hijyen uygulamalarının sıkılaştırılması ve antibiyotik kullanımının daha dikkatli bir şekilde yönetilmesi gerektiğini vurgulamaktadır.

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* *Sorumlu Yazar e-posta adresi / Corresponding Author e-mail address:* cemil.sahiner@adu.edu.tr

1. Introduction

Staphylococcus aureus is a commensal and opportunistic pathogen that colonizes the skin, nasal passages, and mucosal membranes of both humans and animals. Due to its virulence factors, *S. aureus* can cause a wide range of diseases in humans, from minor skin infections to food poisoning, pneumonia, and toxic shock syndrome (1). Furthermore, numerous outbreaks of foodborne *S. aureus* poisoning have been linked to the consumption of contaminated milk and dairy products (2-4).

Milk and dairy products are rich in various nutrients, including proteins, lipids, carbohydrates, vitamins, and minerals, making them popular among consumers (5, 6). However, in addition to their nutritional value, milk serves as an ideal medium for microbial growth (7). Contamination of milk and dairy products with *S. aureus* can occur throughout the production chain, from milking to distribution. Moreover, *S. aureus* is a significant cause of clinical and subclinical mastitis in dairy cattle, leading to substantial economic losses (6). Factors such as poor milking hygiene, mixing milk from mastitic cows with that of healthy animals, inadequate sanitation of dairy equipment, and unclean environments further exacerbate contamination risks (8). Therefore, effective control of *S. aureus* contamination necessitates continuous monitoring of animal health (particularly udder health) alongside the implementation of stringent hygiene protocols, regular surveillance, and strict adherence to food safety standards (9).

Antibiotics play a crucial role in treating bacterial infections in both humans and animals. However, the prolonged, frequent, uncontrolled, and incorrect use of antibiotics has led to the emergence and spread of antibiotic-resistant strains. This issue has become a serious public health concern, limiting treatment options and increasing healthcare costs associated with infection management (10). Foods are effective vectors for transmitting antibiotic resistance to humans, either through antibiotic residues, transfer of resistant pathogens, or ingestion of resistant strains present in the microflora of food, potentially transferring resistance to both pathogenic and non-pathogenic bacteria in the gastrointestinal system (11). In response to this growing issue, the World Health Organization (WHO) periodically updates a list of critically important antimicrobial agents globally. This initiative aims to develop risk management strategies and prioritize resources for antimicrobial use in agricultural and veterinary practices (12-14). To address these concerns, antimicrobials are categorized into three levels of importance for human and veterinary medicine: critically important, highly important, and important. These classifications facilitate the strategic management of antimicrobial resistance on a global scale (15).

S. aureus has a remarkable ability to develop resistance to antibiotics rapidly (16). WHO classified antibiotic-resistant bacterial pathogens into critical, high, and medium priority groups to develop appropriate strategies addressing the health risks posed by antibiotic resistance in its 2024 report. *S. aureus* is categorized within the high-priority group, which includes bacteria with limited treatment options, significant treatment challenges, and high morbidity and mortality rates (17).

In the past decade, strains of *S. aureus* isolated from milk and dairy products have shown many antibiotics resistance in various countries, including Türkiye (5, 18-20). Therefore, monitoring the antibiotic resistance of *S. aureus* in raw milk, determining the rate and profile of resistance development, and ensuring the correct use of antibiotics in animal treatment are critical for food safety. This study not only provides a detailed analysis of *S. aureus* contamination in raw milk from a regional perspective but also offers novel insights into resistance patterns specific to the local strains, addressing a critical gap in the existing literature.

2. Material and Methods

In this study, 100 bulk tank milk samples obtained from farms in Muğla at different period between January 2015 and March 2015 were used as the material. The samples were collected under aseptic conditions and transported to the laboratory under a cold chain, where they were analyzed on the same day.

The isolation and enumeration of *S. aureus* were carried out as stated in the ISO method 6888-1 (21) (Table 1). Suspected colonies on Baird Park agar (Oxoid, CM275, UK) were subcultured and identified by biochemical tests

(catalase test, coagulase test, mannitol fermentation and DNase activity) and latex agglutination test (Dryspot Staphytect Test Plus, Oxoid, DR0100, UK).

One *S. aureus* isolate per positive sample was picked for determination of antibiotic resistance profile. Antibiotic susceptibility tests of the isolated strains were performed based on the broth microdilution method recommended by the Clinical and Laboratory Standards Institute (CLSI) (22). The preparation of antibiotic stock solutions followed the manufacturer's instructions. When adjusting concentrations, MIC50 values of *S. aureus* strains corresponding to the relevant antibiotics reported in the literature were considered. For each strain, the MIC was tested (22) for cefoxitin (FOX), methicillin (MET), tetracycline (TE), tylosin (TLY), florphenicol (FLO), neomycin (NEO), ciprofloxacin (CIP), lincomycin (LIN), and polymyxin B (PB). Ten serial dilutions of each antibiotic were made in cation-adjusted Mueller Hinton Broth (CAMHB; Oxoid, UK) containing NaCl (2%), with the final concentrations of antibiotics ranging from 2 to 1024 µg/mL (0.125-64 µg/mL for ciprofloxacin, 8-4096 µg/mL for lincomycin and polymyxin B). After that, 100 µL from each serial dilution was transferred into microplates. The bacterial suspension, which was prepared with 0.5 McFarland standard, was inoculated with 100 µL into each well. The last well which added MHB was used as a negative control. The plates were incubated at 37 °C for 18–24 h. Following incubation, the optical density of each well at 600 nm wavelength was measured using an ELISA reader (Mindray MR 96A, China). *S. aureus* ATCC 25923 was used as a quality control strains. The MIC mode, range, MIC50, and MIC90 of the antimicrobial agents were calculated, and antibiotic susceptibility was defined as determined by comparing the MICs with CLSI reference values (22).

Table 1: MIC values (µg/ml) for *S. aureus* (21)
Tablo 1: *S. aureus MIC değerleri (µg/ml) (21)*

Antibiotics	MIC values (µg/ml)		
	S (%)	I (%)	R (%)
Cefoxitin	≥ 8	-	≤ 4
Methicillin	≥ 16	-	≤ 8
Tetracycline	≥ 16	8	≤ 4
Tylosin	≥ 8	4	≤ 2
Florphenicol	≥ 32	16	≤ 8
Neomysin	≥ 64	32	≤ 16
Ciprofloxasin	≥ 4	2	≤ 1
Lincomycin	≥ 4	1-2	≤ 0,5
Polymixin B	≥ 8	4	≤ 2

3. Results

Among the 100 raw milk samples analyzed in this study, S. aureus contamination was undetectable in 53 samples, while the remaining 47 samples exhibited varying levels of contamination. The mean contamination level for the positive samples was calculated as 3.63±0.77 log cfu/ml. Furthermore, the contamination levels in the S. aureus-positive samples were found to range from 2.00 to 4.99 log cfu/ml, as shown in Table 2. Notably, 45.9% of the contaminated samples fell within the contamination range of 3–4 log cfu/ml.

Table 2: *S. aureus* contamination levels in raw milk samples
Tablo 2: *Çiğ süt örneklerinde S. aureus kontaminasyon düzeyi*

	Contamination levels log cfu/ml			
	0	**2-3**	**3-4**	**4-5**
n (100)	53	8	17	22
Min		2.00	3.04	4.03
Max		2.90	3.85	4.99
Mean±SE		2.51±0.4	3.42±0.25	4.40±0.26

SE: Standard error

Within the study, it was found that 13 out of the 47 isolated *S. aureus* strains (27.6%) showed resistance to all examined antibiotic groups. All isolates were found to be resistant to cefoxitin, methicillin, tylosin, and lincomycin. After that, the resistance rates for florfenicol, neomycin, and polymyxin B were 95.8%, 89.4%, and 87.3%, respectively (Table 3). Additionally, it was observed that all isolates were MDR (multidrug-resistant). The antibiotic resistance profiles of the isolates are shown in Table 4.

Table 3: Prevalence of antibiotic resistance in the *S. aureus* isolates
Tablo 3: *S. aureus izolatlarında antibiyotik direnç prevalansı*

Category	Antibiotics	Number of *S. aureus* isolates (n=47)		
		S (%)	I (%)	R (%)
Critically important	Methicillin	-	-	47 (100)
	Tylosin	-	-	47 (100)
	Neomycin	-	5 (10.6)	42 (89.4)
	Ciprofloxasin	13 (27.7)	8 (17)	26 (55.3)
	Polymyxin B	-	6 (12.7)	41 (87.3)
Highly important	Cefoxitin	-	-	47 (100)
	Tetracycline	9 (19.1)	18 (38.3)	20 (42.6)
	Florphenicol	-	2 (4.2)	45 (95.8)
	Lincomycin	-	-	47 (100)

S: Susceptible; I: Intermediate; R: Resistance

Table 4: Antibiotic resistance profiles of *S. aureus* strains isolated from raw milk (n=47)
Tablo 4: *Çiğ sütten izole edilen S. aureus izolatlarında antibiyotik direnç profili (n=47)*

Resistance Profile	No isolates (%)	Classification of isolates	
		Type of resistance	No isolates(%)
FOX, MET, TE, TLY, FLO, NEO, CIP, LIN, PB	13 (27.7)	pandrug-resistant (PDR)	13 (27.7)
FOX, MET, TE, TLY, NEO, CIP, LIN, PB	1 (2.1)	extensivedrug-resistant (XDR)	12 (25.5)
FOX, MET, TE, TLY, FLO, NEO, LIN, PB	3 (6.4)		
FOX, MET, TE, TLY, FLO, NEO, CIP, LIN	1 (2.1)		
FOX, MET, TLY, FLO, NEO, CIP, LIN, PB	7 (14.9)		
FOX, MET, TLY, FLO, NEO, LIN, PB	10 (21.3)	multidrug-resistant (MDR)	22 (46.8)
FOX, MET, TE, TLY, NEO, LIN, PB	2 (4.3)		
FOX, MET, TLY, FLO, CIP, LIN, PB	4 (8.5)		
FOX, MET, TLY, FLO, NEO, LIN	5 (10.6)		
FOX, MET, TLY, FLO, LIN, PB	1 (2.1)		

cefoxitin (FOX), methicillin (MET), tetracycline (TE), tylosin (TLY), florphenicol (FLO), neomycin (NEO), ciprofloxacin (CIP), lincomycin (LIN), and polymyxin B (PB).

4. Discussion and Conclusion

People often prefer milk and dairy products in their diet for a sustainable healthy life. The presence of *S. aureus* in raw milk and dairy products is a significant public health concern, particularly for infants, children, and immunosuppressed individuals, as they are more susceptible to staphylococcal enterotoxins (23). Since staphylococcal enterotoxin (SE) production depends on factors such as the composition of the food, temperature, antimicrobial inhibitors, and the ability of the strains to produce SE, the level of *S. aureus* required for food poisoning cannot be precisely determined. However, it has been reported that the presence of *S. aureus* at levels of 10^5-10^8 cfu/g in food is sufficient to produce a toxic dose that can cause food poisoning (24). In this study, *S. aureus* contamination was determined in 47% of the milk at a mean level of 3.63±0.77 cfu/ml. Similarly, a study conducted in the USA

found the presence of *S. aureus* in 46.6% of raw milk samples (25). Two different researches in Italy have reported *S. aureus* contamination rates of 9.1% and 40% in raw milk samples. (26, 27). Shamila-Syuhada et al. (28) reported that all the examined milk samples were contaminated with *S. aureus* at levels in the range of 2.88 to 3.41 log cfu/ml. In studies conducted in Türkiye, it was found that 50% of raw milk samples in Kayseri were contaminated with *S. aureus*. Furthermore, 50% of the positive samples had contamination levels higher than 6 log cfu/ml (23). Gündoğan and Avcı (28) detected *S. aureus* levels of 4.32-4.49 log cfu/ml in 28 out of 50 raw milk samples. In Burdur, 51.6% of raw milk samples were reported to be contaminated with *S. aureus* (7).

Differences among the studies could be attributed to factors such as the farms from which samples were collected, seasonal and regional differences, the analytical methods used, the competitive effect of the microflora present in the milk, and the inhibitory substances of the milk itself. Additionally, raw milk is considered a suitable environment for *S. aureus*, as it is for many other microorganisms. *S. aureus* is responsible for approximately 30-40% of mastitis cases. The pathogen can contaminate the milk from infected udders, but it can also be transmitted through milking equipment and personnel, environmental conditions, and storage tanks (24, 30, 31).

Due to the increasing misuse of antibiotics in livestock, the effectiveness of these antibiotics is gradually decreasing, and the number of resistant strains is on the rise (15). This situation leads to the widespread presence of pathogenic-resistant organisms in animal-origin foods, posing a potential threat to human health (32). Therefore, monitoring antibiotic use in livestock and screening animal-origin foods for antibiotic-resistant pathogens is crucial for predicting the development rate and type of antibiotic resistance and for risk assessment from a food safety perspective.

Beta-lactams, aminoglycosides, lincosamides, fluoroquinolones, and macrolides are commonly used antimicrobial agents for the treatment of *S. aureus* mastitis (33). According to the microdilution test results in this study, *S. aureus* isolates determined resistance rates of 95.8%, 89.4%, 55.3%, and 42.6% to florfenicol, neomycin, ciprofloxacin, and tetracycline, respectively. The results of the study highlight that the resistance levels are notably high. These findings indicate the urgent need for implementing an effective antimicrobial management program to prevent the overuse of these antibiotics in the region. Contrary to our results, other studies have reported overall susceptibility to ciprofloxacin (34), neomycin (35), and florfenicol (36).

Titouche et al. (34) and Deddefo et al. (6) similarly found tetracycline resistance in *S. aureus* isolates at rates of 47.8% and 46.2%, respectively. On the other hand, Can et al. (37), Keyvan et al. (38), and Hızlısoy et al. (19) reported tetracycline resistance levels of 62.5%, 30.9%, and 28.4%, respectively. The differences in tetracycline resistance levels observed may be attributed to variations in antibiotic use practices. In this study, all isolates were found to be resistant to both tylosin and lincomycin. Although macrolides and lincosamides are chemically different but both target the 50S subunit of the ribosome in bacteria. Consequently, mutations in this area may lead to resistance to both antibiotic groups (38). Furthermore, the intermediate resistance rates observed in the isolates obtained in this study highlight a critical point that warrants attention, particularly regarding the potential development of resistance in the future. The high levels of intermediate resistance suggest that resistance mechanisms may not yet be fully established but could be in the early stages of development. This situation underscores the need to reassess antibiotic usage strategies.

Methicillin-resistant *Staphylococcus aureus* (MRSA), initially associated with hospital and community-acquired infections, has also been reported in relation with livestock since 2003 (40). The CLSI (22) recommends the use of cefoxitin for the identification of MRSA strains. In our study, all isolates were phenotypically resistant to both cefoxitin and methicillin. Similarly, Tenhagen et al. (33) reported that all *S. aureus* isolates from tank milk samples in Germany were resistant to cefoxitin. Previous studies have shown lower resistance rates than our findings (6, 18, 28, 38).

The prevalence of MRSA can vary not only between countries but also among regions within the same country. This variation may be due to differences in antibiotic choices, dosage regimens of antibiotics used in clinics, and analytical methods. A limitation of the current study is that we do not know whether the phenotypically resistant isolates carry the responsible resistance genes. In homogenous methicillin resistance, all cells exhibit resistance at

high concentrations of methicillin, whereas in heterogeneous resistance, only a small proportion of the cells show resistance (41). In heterogeneous resistant strains, the responsible gene may not be synthesized, potentially leading to misleading results in phenotypic tests. Therefore, in MRSA isolates, the presence of the *mecA* gene should be investigated using PCR in addition to phenotypic tests.

The fact that all *S. aureus* isolates obtained from raw milk samples in this study are MDR, and particularly that 13 of them are PDR, raises significant concerns regarding the risk of consuming foods contaminated with resistant bacteria. The development of MDR in most of these isolates may be due to the acquisition of plasmid-mediated resistance (R factor) (42). The potentially concerning level of MDR detected in our study can be attributed to the irrational use of antibiotics in treating animal infections. The presence of resistance to nine different classes of antibiotics at varying rates found in the study is considered highly risky from a public health perspective. The potential for resistant isolates to be transmitted to humans through the processing and consumption of milk and dairy products poses a clear threat. The presence of multidrug resistance complicates infection management, increases the risk of treatment failure, and leads to significant economic losses.

The detection of *S. aureus* in nearly half of the raw milk samples indicates a widespread problem in milk hygiene practices. The average contamination level and the range of contamination levels emphasized the variability of the contamination and the potential food poisoning risk. These findings, it is imperative to implement stricter hygiene practices throughout the milk production and handling processes to minimize contamination risks. The high levels of resistance to commonly used antibiotics necessitate the reevaluation of current antibiotic use policies in the dairy industry, as this resistance further complicates the management of *S. aureus* infections. Future research should focus on effective intervention strategies to reduce *S. aureus* contamination in raw milk and developing alternative treatment options to address the challenge of MDR strains.

Conflict of Interest

The authors declare that they have no conflict of interest in this study.

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Authors' Contributions

Motivation / Concept: Murat BOYACIOĞLU, Melih DUYUK
Design: Murat BOYACIOĞLU, Pelin KOÇAK KIZANLIK
Control/Supervision: Murat BOYACIOĞLU
Data Collection and / or Processing: Melih DUYUK
Analysis and / or Interpretation: Murat BOYACIOĞLU, Melih DUYUK, Pelin KOÇAK KIZANLIK
Literature Review: Melih DUYUK, Cemil ŞAHİNER
Writing the Article: Pelin KOÇAK KIZANLIK, Cemil ŞAHİNER
Critical Review: Murat BOYACIOĞLU

Ethical Statement

An ethical statement was received from the authors that the data, information, and documents presented in this article were obtained within the framework of academic and ethical rules and that all information, documents, evaluations and results were presented in accordance with scientific ethics and moral rules.

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