

Assessing *Staphylococcus aureus* as an indicator of microbial quality and its antibiotic resistance characteristics in raw donkey milk

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ABSTRACT

Background and Objectives: In mammals, milk provides the nutrients required for growth and development, including proteins, fats, carbohydrates, vitamins, and essential minerals. Donkey milk has recently attracted increasing interest as an alternative to cow's milk, particularly for infants with cow's milk protein allergy, due to its favorable nutritional composition. However, limited information is available on the microbiological quality of donkey milk based on molecular confirmation of *Staphylococcus aureus* and the detection of antimicrobial resistance genes, particularly in Iran. The present study assessed the microbiological quality of raw donkey milk through conventional bacteriological culture, PCR confirmation of *Staphylococcus aureus*, antimicrobial susceptibility testing, and molecular detection of the *mecA* and *blaZ* resistance genes.

Materials and Methods: Between January and April 2023, a total of 50 raw donkey milk samples were collected and subjected to conventional bacteriological examination. Presumptive *Staphylococcus aureus* isolates were identified by culture-based methods and confirmed by PCR targeting the 23S rRNA gene. PCR assays were also performed to detect the *coa*, *blaZ*, and *mecA* genes. Antimicrobial susceptibility testing was conducted using the CLSI disk diffusion method with ten antimicrobial agents.

Results: Of 12 bacterial isolates, 8 (16%) were confirmed as *S. aureus*. The *blaZ* gene, was present in all isolates (100%), while *mecA*, was absent. Antibiotic susceptibility testing showed variable resistance among isolates, with notable resistance against penicillin, with all isolates showing resistance (100%). The second highest resistance rate was observed for trimethoprim-sulfamethoxazole, where 25% of the isolates showed resistance.

Conclusion: These findings indicate the presence of β -lactam resistance determinants despite the absence of molecular evidence of methicillin resistance. The study provides baseline data on the occurrence and antimicrobial resistance characteristics of *S. aureus* in raw donkey milk in Iran and highlights the value of combining phenotypic methods with PCR for microbiological surveillance of dairy products.

Keywords: Donkeys; Milk; *Staphylococcus aureus*; Drug resistance; Bacterial; Polymerase chain reaction

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INTRODUCTION

Milk represents the primary source of early-life nutrition for mammalian offspring, providing essential macronutrients and micronutrients, including proteins, lipids, carbohydrates, vitamins, and minerals. Following animal domestication, humans began consuming animal milk, which has contributed to human nutrition, particularly growth and bone development (1). In recent decades, interest in non-cow milk has increased worldwide. Donkey milk (DM) has been consumed traditionally in various regions and has also been recognized for its potential medicinal properties (2). Recently, DM has attracted increasing scientific attention due to its unique nutritional characteristics and potential applications in human nutrition (3).

Milk from equids, including mares (*Equus caballus*) and donkeys (*Equus asinus*), possesses distinctive nutritional and functional properties, particularly for infants with cow's milk protein allergy (CMPA) (1). The composition of equine milk, including protein, fat, lactose, ash, and water content, shows similarities to human milk (4). Donkey milk contains several bioactive compounds, including functional proteins, polyunsaturated fatty acids, vitamins, polar lipids, and phytosterols. Similar to human milk, it is characterized by a high lactose content and relatively low levels of protein and ash, which contribute to its suitability as an alternative for individuals with cow's milk protein allergy (1, 2).

Although donkey milk is often considered microbiologically safer than milk from dairy ruminants due to its high concentrations of antimicrobial components such as lysozyme and lactoferrin, its microbial quality remains influenced by udder health, milking hygiene, and post-harvest handling practices (2, 5). Recent studies have reported low total bacterial counts and a low prevalence of foodborne pathogens in raw donkey milk; however, opportunistic microorganisms, including *Staphylococcus aureus*, may still be isolated from individual milk samples, particularly when hygienic conditions are inadequate or intramammary infections occur (2, 6). In contrast, *S. aureus* is a major mastitis-associated pathogen in bovine, ovine, and caprine milk and is frequently linked to milk contamination, economic losses, and staphylococcal food poisoning (7-9). Furthermore, antimicrobial-resistant *S. aureus*, particularly methicillin-resistant *S. aureus* (MRSA), represents

an increasing concern in dairy production because food-producing animals may act as reservoirs of resistant bacteria and resistance genes with zoonotic potential (10, 11). While antimicrobial resistance in *S. aureus* from ruminant milk has been extensively investigated, comparable information regarding donkey milk remains limited, particularly in developing countries.

The presence of antimicrobial-resistant *Staphylococcus* species in dairy environments has become a significant public health concern due to the potential transmission of resistant bacteria and resistance determinants from animals to humans. Among dairy-associated *Staphylococcus* isolates, commonly reported resistance genes include *mecA*, *blaZ*, *tetM*, *tetK*, *ermA*, *ermC*, *msrA*, and *msrB*, which are associated with resistance to β -lactams, tetracyclines, and macrolides (11). Therefore, monitoring antimicrobial resistance in bacteria isolated from dairy products is essential for evaluating potential food safety risks.

Previous studies investigating the microbiological quality of donkey milk have primarily focused on culture-based isolation and phenotypic characterization of bacterial contaminants, while relatively few have incorporated molecular approaches for confirmation of *S. aureus* or detection of antimicrobial resistance genes (2, 5, 6). Consequently, data regarding the molecular epidemiology of *S. aureus* in donkey milk remain limited, particularly in Iran and neighboring countries.

Therefore, the present study aimed to evaluate the microbiological quality of raw donkey milk through conventional bacterial isolation, molecular confirmation of *S. aureus*, antimicrobial susceptibility testing, and detection of the *mecA* and *blaZ* resistance genes. This integrated phenotypic and molecular approach provides baseline data regarding the occurrence and antimicrobial resistance characteristics of *S. aureus* in raw donkey milk in Iran.

MATERIALS AND METHODS

Sample collection. Between January and April 2023, a total of 50 raw donkey milk samples were collected from four donkey-rearing facilities in three regions of Yazd Province, Iran (Yazd, Meybod, and Khizrabad). The participating facilities varied in size and maintained approximately 10-15 donkeys each. Some farms operated under semi-industrial condi-

tions for commercial milk production, and the produced milk and dairy products were marketed for human consumption. Only clinically healthy lactating donkeys without visible signs of mastitis or systemic illness were included in the study. One aseptic milk sample was collected from each selected animal.

To minimize microbial contamination from the skin, udder, teat, and surrounding farm environment, all samples were collected under aseptic conditions. Before sampling, before sample collection, the udder was thoroughly cleaned and disinfected with 70% ethanol, and the first few streams of milk from each teat were discarded. Milk samples were then aseptically collected into sterile, pre-labeled 15-mL tubes and transported to the laboratory in an insulated ice box maintained at approximately 4°C for subsequent microbiological analyses.

The study protocol was reviewed and approved by the Ethics Committee of Ardakan University, Ardakan, Iran (Approval No. IR.Ardakan.REC.1404.024).

Isolation and presumptive identification of *Staphylococcus aureus*. Presumptive *Staphylococcus aureus* colonies were isolated by directly streaking milk samples onto Baird-Parker agar (Ibresco, Iran) supplemented with egg yolk tellurite emulsion, followed by incubation at 37°C for 24-48 h. Plates were examined for colonies with morphological characteristics suggestive of *S. aureus*. Presumptive colonies were subsequently subcultured onto Mannitol Salt Agar (Ibresco, Iran) and incubated at 37°C for an additional 24-48 h. Pure cultures were obtained by further subculturing onto freshly prepared nutrient agar. Presumptive isolates were characterized by Gram staining (Gram-positive cocci) and catalase testing, as described by Quinn et al. (12). Molecular confirmation of *Staphylococcus aureus* was subsequently performed by PCR.

DNA extraction. Genomic DNA was extracted from presumptive *S. aureus* colonies grown overnight. The colonies were suspended in a microtube containing 1 mL of distilled water and boiled for 10 min in a water bath. Following boiling, the suspension was centrifuged for 5 min at 170 × g. A volume of 100 µL of the supernatant was then stored at -20°C for further testing (13).

Molecular Identification of *S. aureus*. The *Staphylococcus aureus* isolates were molecularly con-

firmed by PCR amplification of the species-specific 23S rRNA gene, as previously described by Neelam et al. (14). The primers used to amplify the 23S rRNA gene are listed in Table 1. For each PCR assay, genomic DNA from *Staphylococcus aureus* ATCC 25923 was used as the positive control, while nuclease-free water served as the no-template negative control.

PCR was performed in a final reaction volume of 25 µL, containing 3 µL of DNA extract, 0.5 µM of each primer, 12.5 µL of 2× Taq DNA Polymerase Master Mix RED (Ampliqon, Odense, Denmark), and distilled water to adjust the final volume. PCR products were visualized by electrophoresis on a 1.5% agarose gel containing Safe DNA Gel Stain (Genex, Iran) and documented using a Gel Doc 1000 imaging system (Arash Teb Pishro Pars, Iran).

The thermal cycles for PCR contains: an initial denaturation of DNA at 95°C for 5 minutes, followed by 35 cycles consisting of 94°C for 30 seconds, 55°C for 45 seconds, and 72°C for 1 minute and then a final extension step of 10 minutes at 72°C

PCR assay for the detection of coagulase and antibiotic resistance. The isolates confirmed as *Staphylococcus aureus* by species-specific PCR were further screened by PCR for the presence of the coagulase gene (*coa*) as a virulence-associated marker, as well as the β-lactam resistance gene (*blaZ*) and the methicillin resistance gene (*mecA*). The primer sequences used in this study are presented in Table 1.

The reaction mixture and thermal cycling conditions were identical to those described in the previous section, except for *blaZ* amplification, for which an annealing temperature of 50°C was used.

Antimicrobial susceptibility testing of *S. aureus*. Antimicrobial susceptibility testing of *Staphylococcus aureus* isolates was performed using the disc diffusion method on Mueller–Hinton agar with commercially available antimicrobial discs (PadtanTeb, Iran). Ten antimicrobial discs, including penicillin (10 units), doxycycline (30 µg), ofloxacin (5 µg), ciprofloxacin (5 µg), azithromycin (15 µg), gentamicin (10 µg), chloramphenicol (30 µg), tetracycline (30 µg), erythromycin (15 µg), and trimethoprim-sulfamethoxazole (1.25/23.75 µg), were used in this study. The isolates were categorized as susceptible, intermediate, or resistant according to the interpretive criteria of the U.S. Clinical and Laboratory Standards Institute (CLSI) guidelines (16).

Table 1. Sequences of the used primers used in the study

Factor	Gene	Sequence	Amplicon size (bp)	Reference
Species-specific	23S rRNA	GGACGACATTAGACGAATCA CGGGCACCTATTTTCTATCT	1319	(14)
Coagulase	<i>coa</i>	ACCACAAGGTACTGAATCAACG TGCTTTCGATTGTTTCGATGC	750	(14)
Antibiotic resistance genes	<i>mecA</i>	AAAATCGATGGTAAAGGTTGGC AGTTCTGCAGTACCGGATTTC	533	(14)
	<i>blaZ</i>	ACTTCAACACCTGCTGCTTTC TGACCACTTTTATCA GCAACC	173	(15)

RESULTS

Bacterial isolation and identification. A total of 12 bacterial isolates were identified as *Staphylococcus* spp. based on colony morphology on mannitol salt agar and Baird–Parker agar, along with microscopic observation of Gram-positive cocci arranged in clusters. The staphylococcal isolates were further confirmed by species-specific PCR, of which eight isolates were identified as *Staphylococcus aureus*.

PCR results. The frequency of *S. aureus* isolates was 8 (16%-95% CI: 7.2-29.1) (Fig. 1). The coagulase gene was not detected in any of the isolates. The β -lactam resistance gene *blaZ* was detected in all *Staphylococcus aureus* isolates (100%) (Fig. 2). However, the *mecA* gene, which confers methicillin resistance, was not detected in any of the *S. aureus* isolates.

Antimicrobial susceptibility testing of *S. aureus*. Antimicrobial susceptibility testing of *Staphylococcus aureus* isolates revealed varying levels of resistance to different antimicrobial agents across the tested antibiotic classes (Table 2). The highest resistance was observed against penicillin, with all isolates showing resistance (100%). The second highest resistance rate was observed for trimethoprim- sulfamethoxazole, where 25% of the isolates showed resistance. The other isolates were completely sensitive to the antibiotic discs used (Table 3).

DISCUSSION

Beyond its role as a basic dietary component, milk provides a complex nutritional matrix containing proteins, lipids, carbohydrates, and bioactive com-

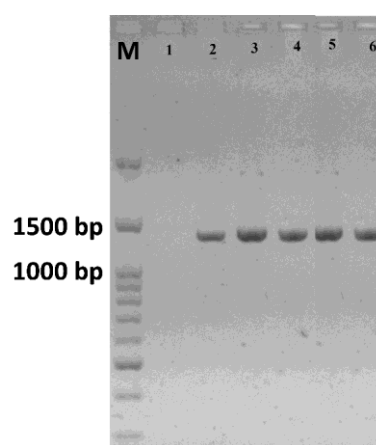
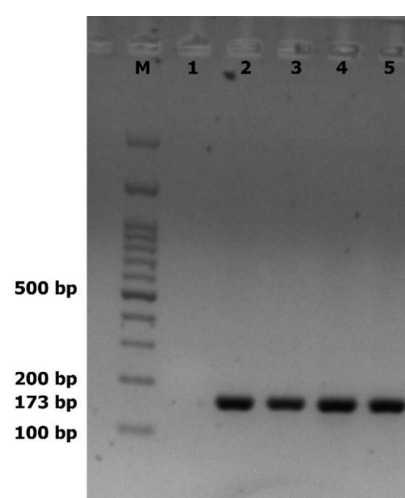
**Fig. 1.** Agarose gel electrophoresis of PCR products targeting the 23S rRNA gene (1319 bp) confirming *S. aureus* isolates. Lane M: 100 bp DNA ladder; Lane 1: negative control; Lane 2: positive control; Lanes 3-6: tested isolates.**Fig. 2.** Agarose gel electrophoresis showing amplification of the *blaZ* gene (173 bp) in *S. aureus* isolates from raw donkey milk. Lane M: 100 bp DNA ladder; Lane 1: Negative control; Lanes 2–5: tested isolates

Table 2. Individual inhibition zone diameters (mm) of all *Staphylococcus aureus* isolates tested by the Kirby–Bauer disk diffusion method, along with the mean $\pm$ standard deviation (SD) values for each antimicrobial agent.

Isolate ID	P*	CIP	DOX	GEN	CL	TE	E	AZM	SXT	OFX
S1	20	30	27	15	18	22	24	27	0	24
S2	28	25	28	20	20	19	23	25	0	21
S3	23	21	18	17	19	21	28	20	32	24
S4	19	25	28	22	22	20	30	18	24	32
S5	21	30	30	15	21	23	32	20	23	33
S6	22	24	32	18	19	19	28	19	16	26
S7	26	22	29	20	20	22	33	21	19	32
S8	21	26	30	21	18	24	27	22	20	20
Mean $\pm$ SD	22.5 $\pm$ 3.07	25.4 $\pm$ 3.29	27.75 $\pm$ 4.23	18.5 $\pm$ 2.67	19.6 $\pm$ 1.41	21.25 $\pm$ 1.83	28.1 $\pm$ 3.52	21.5 $\pm$ 1.83	22.33 $\pm$ 5.54	26.5 $\pm$ 5.18

• Abbreviations: PEN, penicillin; AMP, ampicillin; DOX, doxycycline; CIP, ciprofloxacin; OFX, ofloxacin; GEN, gentamicin; CHL, chloramphenicol; TET, tetracycline; ERY, erythromycin; SXT, trimethoprim–sulfamethoxazole. Zone diameters are expressed in millimeters (mm).

Table 3. Antimicrobial susceptibility profile of *Staphylococcus aureus* isolates recovered from donkey milk

Antimicrobial agent	Antimicrobial susceptibility		
	Resistant n (%) [95% CI]	Intermediate n (%) [95% CI]	Sensitive n (%) [95% CI]
Penicillin	8 (100) [67.6%-100%]	-	-
Ciprofloxacin	-	-	8 (100) [67.6%-100%]
Azithromycin	-	-	8 (100) [67.6%-100%]
Deoxycycline	-	-	8 (100) [67.6%-100%]
Gentamicin	-	-	8 (100) [67.6%-100%]
Tetracycline	-	-	8 (100) [67.6%-100%]
Erythromycin	-	-	8 (100) [67.6%-100%]
Chloramphenicol	-	-	8 (100) [67.6%-100%]
Ofloxacin	-	-	8 (100) [67.6%-100%]
Trimethoprim–sulfamethoxazole	2 (25) [7.2%-59.1%]	-	6 (75) [40.9%-92.8%]

pounds important for human health. Although milk from ruminant species is the most commonly consumed worldwide, equine milk also plays an important nutritional role in countries such as Russia, Kazakhstan, Kyrgyzstan, Tajikistan, Uzbekistan, and Mongolia (17). Historical records indicate that Hippocrates and Pliny the Elder were among the first to recognize the health benefits of donkey milk (18). Donkey milk has attracted increasing attention because of its nutritional composition, which closely resembles that of human milk. Equine milk contains approximately 40–45% casein, while whey proteins account for more than half of the total protein content (19). Furthermore, donkey milk contains considerably higher concentrations of lysozyme than bovine

milk and has a whey protein profile that makes it a suitable alternative for infants with cow's milk protein allergy when breastfeeding is not possible (1).

The prevalence of *Staphylococcus aureus* in raw donkey milk has been investigated less frequently than in bovine milk, and available data remain limited. In the present study, *Staphylococcus aureus* was detected in 16% (8/50) of raw donkey milk samples. Differences in the reported prevalence among studies may be attributed to geographical location, herd management, hygienic milking practices, animal health status, sample size, and the microbiological and molecular methods used for bacterial identification. In a longitudinal investigation, Pilla et al. isolated *S. aureus* from only five of 101 half-udder

milk samples collected from healthy donkeys, supporting the generally low prevalence of this pathogen in donkey milk (5). Similarly, reviews on the microbiological quality of donkey milk have reported that *S. aureus* is detected only sporadically in healthy dairy donkeys when good hygienic practices are maintained (2). In contrast, *S. aureus* is one of the major contagious mastitis pathogens in dairy cattle and is reported much more frequently in bovine milk worldwide (20). These comparisons should be interpreted cautiously because differences in host species, udder physiology, herd management, antimicrobial use, and study design may influence the reported prevalence.

One possible explanation for the relatively low prevalence of *Staphylococcus aureus* observed in the present study is the intrinsic antimicrobial properties of donkey milk, which have been previously reported. These properties may be attributed to the presence of several bioactive components, including lysozyme, lactoferrin, and certain free fatty acids (21, 22). Although lactoferrin is present at relatively low concentrations, it exhibits antibacterial, antiviral, antifungal, and antiparasitic activities (23). Moreover, α -lactalbumin and the natural microbiota of donkey milk, including *Lactobacillus plantarum*, have also been reported to contribute to its antimicrobial activity. Fermented donkey milk has demonstrated greater inhibitory activity against foodborne pathogens than raw milk, particularly against Gram-positive bacteria (24). Nevertheless, because the antimicrobial activity and concentrations of these bioactive compounds were not evaluated in the present study, their contribution to the observed low prevalence of *S. aureus* remains speculative and warrants further investigation.

Pasteurization effectively eliminates *S. aureus*; however, thermostable staphylococcal enterotoxins may remain active after heat treatment (1). In the present study, species identification was based on selective culture, biochemical characterization, and PCR amplification of a species-specific region of the 23S rRNA gene. Although none of the isolates yielded amplification of the targeted *coa* gene fragment, phenotypic coagulase testing was not performed. Therefore, the absence of *coa* amplification should not be interpreted as evidence that the isolates were coagulase-negative staphylococci (CoNS). Rather, it indicates only that the targeted *coa* gene fragment was not amplified with the selected primer pair,

which may reflect sequence variation within the *coa* gene. Confirmation using an additional species-specific marker such as *nuc* or *femA*, or DNA sequencing, would further strengthen species identification in future studies. To our knowledge, this study represents one of the first comprehensive investigations of *Staphylococcus aureus* in raw donkey milk in Iran, integrating phenotypic identification, molecular confirmation, antimicrobial susceptibility testing, and resistance gene detection. These findings provide important baseline data for future monitoring programs and contribute to improving the microbiological safety of donkey milk intended for human consumption.

CONCLUSION

In conclusion, this study provides new data on the occurrence of *Staphylococcus aureus* in raw donkey milk and the antimicrobial susceptibility profiles of the recovered isolates. Although the prevalence of *S. aureus* was relatively low, the detection of antimicrobial-resistant isolates and the presence of the *blaZ* gene highlight the importance of continued microbiological surveillance and prudent antimicrobial use in dairy animal production. The absence of the *mecA* gene suggests that methicillin-resistant *S. aureus* (MRSA) was not detected among the isolates examined. These findings contribute to the limited knowledge regarding the microbiological quality of raw donkey milk. Future studies should include larger and more geographically diverse sample collections, sequence-based confirmation of bacterial identification, screening for staphylococcal toxin genes, and assessment of routine microbiological hygiene indicators to provide a more comprehensive evaluation of the microbiological quality and public health significance of raw donkey milk.

Limitation. This study has several limitations. First, the relatively small sample size and the inclusion of samples from a single geographical region may limit the generalizability of the findings. Second, although PCR identification was performed using previously validated primers and published protocols with appropriate negative controls, reference positive control strains were not available to further confirm assay performance. Third, antimicrobial susceptibility testing reflected the CLSI criteria available and rou-

tinely applied in the laboratory during the study period. Moreover, because the original *Staphylococcus aureus* isolates were no longer available after completion of the study, additional testing, including ceftiofur screening for methicillin-resistant *S. aureus* (MRSA) and determination of vancomycin minimum inhibitory concentrations (MICs), could not be performed retrospectively. Finally, the study did not assess staphylococcal toxin genes or routine hygiene indicators, such as total viable counts (TVC), coliforms, and yeast/mould counts, which would have provided a more comprehensive evaluation of the microbiological quality and safety of raw donkey milk.

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