



Biochemical Identification, Direct Molecular Detection, and Antimicrobial Agent Resistance of *Staphylococcus Aureus* Isolated from Raw Camel Milk in Al Madinah Region, Saudi Arabia

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Article History: 23-162

Received: 10-Apr-23

Revised: 28-Apr-23

Accepted: 01-May-23

ABSTRACT

This study aimed to isolate and identify *Staphylococcus aureus* (*S. aureus*) in raw camel milk (RCM) samples collected from various individual farms in the Al Madinah region, Saudi Arabia, and to establish an antibiotic susceptibility profile of *S. aureus* isolated from RCM. Out of 115 RCM samples cultured, 52(45.2%) were isolated and detected in the tested samples as suspected *S. aureus*. Out of the positive samples (n=52), 96.2% were confirmed as *S. aureus* by the automated method. Antimicrobial resistance profiles of the confirmed isolates to 8 common antibiotics used by veterinarians in the study area were then investigated. The study revealed that the highest resistance was observed to penicillin G as the β -lactam antibiotic group (73.1%) and tetracycline as the tetracycline antibiotic group (61.5%). In comparison, the *S. aureus* isolates showed the highest sensitivity to clindamycin as the macrolide antibiotic group (84.6%), followed by ciprofloxacin as the quinolone antibiotic group (65.4%).

Key words: Camel Milk, *S. Aureus*, Antibiotics Susceptibility, Al Madinah, KSA

INTRODUCTION

Camel milk is considered one of the most essential foods in Saudi Arabia due to its high nutritional value and health benefits. However, it may be contaminated with various microorganisms during milking, handling, transportation, and marketing, especially with pathogenic microbes such as *S. aureus*.

In the Arabian Peninsula, especially in the Kingdom of Saudi Arabia, camel milk is considered an essential nutrient with medicinal properties (FAO 2013). It is predominantly consumed raw without any thermal treatment, directly and indirectly, endangers human safety (Gitao et al. 2014). Contamination of RCM with pathogenic bacteria may occur from external environmental sources outside the udder, including *Staphylococcus* strains, which cause many outbreaks (Juma and Elhag 2015). *S. aureus* infections in camels are one of the most common causative agents of clinical and subclinical mastitis, resulting in substantial economic losses in milk yield in camel dairy farms (Gussmann et al.

2019). The pathogenicity of *S. aureus* in milk contamination and food poisoning is based on its ability to produce enterotoxins, leading to foodborne illness caused by staphylococci (Suzuki 2019). Milk and other dairy products are one of the most important sources of human exposure to enterotoxins, posing a severe public health threat (Owusu-Kwarteng et al. 2020).

In animal husbandry, antimicrobials are used to promote animal growth. Multiple drug resistance (MDR) of *Staphylococcus aureus* is increasingly recognized as a global crisis. (Jeljaszewicz et al. 2000; Ijaz et al. 2022). Because the pathogen can form an exopolysaccharide capsule that restricts antibiotic access to infected cells, which is essential for the antibiotic resistance and pathogenesis of this pathogenic agent (Begun et al. 2007; Naz et al. 2022). Isolation of *Staphylococcus aureus* (*S. aureus*) from milk and dairy products indicates that resistant microorganisms can be transmitted to humans via food, animals, or the environment (Huber et al. 2011). One of the main problems associated with *S. aureus* is its ability to rapidly develop antibiotic resistance and give

Cite This Article as: Alorainy MS, Alhajuj AM and Fathi SM, 2023. Biochemical identification, direct molecular detection, and antimicrobial agent resistance of *Staphylococcus aureus* isolated from raw camel milk in Al Madinah Region, Saudi Arabia. International Journal of Veterinary Science 12(5): 740-745. <https://doi.org/10.47278/journal.ijvs/2023.048>

rise to many antibiotic-resistant strains (Kitara et al., 2011). Nowadays, *S. aureus* strains have developed resistance to penicillin and all β -lactam drugs (Eumkeb et al. 2010; Moreno-Pérez et al. 2023). In Saudi Arabia, various antimicrobial susceptibility profiles and susceptibility trends of *S. aureus* isolates show 35% resistance to β -lactam, 50% resistance to penicillin, 65% susceptibility to ciprofloxacin, 90% susceptibility to erythromycin and clindamycin, 85% susceptibility to ceftiofur, 80% susceptibility to gentamicin, and 60% susceptibility to penicillin G, 75% susceptibility to trimethoprim, and finally 75% susceptibility to tetracycline (Farah et al. 2019). To protect consumers from microbial hazards in milk, such as foodborne staphylococcal disease, such surveillance data can help ensure food safety in the dairy value chain. This study aimed to demonstrate the identification and isolation of *S. aureus* in RCM and evaluate the susceptibility profile of *S. aureus* to antibiotics.

MATERIALS AND METHODS

All collected samples were performed with the recommended and guidelines stated by Ethical Committee of Qassim University (15 April 2021).

About 100mL raw camel milk (RCM) samples were collected (n=115) from different farms in and around the Al Madinah region, western Saudi Arabia. All preparations and manipulations were done according to ISO 7218:2007, and sampling and preparation of test samples were done according to ISO 6887-5: 2010 to ensure uniform distribution of microorganisms before analysis.

Detection and Enumeration of *S. aureus* from RCM Samples

Under aseptic conditions, about 10mL RCM was placed in a stomacher bag and mixed with 90mL Maximum Recovery Diluent (MRD) (Oxoid CM 733). Further decimal dilutions were prepared up to 10^{-7} . The technique of spreading plates on solid selective culture medium recommended by ISO 6888-1:2021 was prepared using a Baird-Parker culture medium (Oxoid DR 650) with egg yolk tellurite (Oxoid SR 54) and then incubated upside down in an incubator at $37 \pm 1^\circ\text{C}$ for 24-48 \pm 2h and then re-incubated for 24 \pm 2h. Countable plates contain all typical and atypical colonies. Typical colonies on Baird-Parker agar were identified as black or grey, shiny, and convex colonies with a clear, shiny yellow zone in the contact colonies and an opalescent ring due to proteolysis. Shiny black colonies with or without a narrow white border were also identified as typical colonies; however, the clear zone and opalescent ring were either not visible or barely visible.

Confirmation and Identification of Coagulase-Positive of *S. Aureus*

Colonies characteristic of *staphylococci* were collected for confirmation. The test was positive if the clot was more than half the original fluid volume. Controls were also performed in parallel with the test samples. The rapid coagulase test was performed using the Staphytest-plus test kit, and the result was considered positive if

agglutination of the blue test latex particles occurred within 20 seconds (ISO 6888-1:2021).

Gram Staining for Identification of *S. Aureus*

The stained smear was prepared from the pure cultures and examined under the microscope for Gram reaction, size, shape, and cell arrangement (Tripathi and Sapra 2020). The slide was examined under the microscope, and characteristic Gram-positive *Staphylococcus* spp, such as *S. aureus*, appeared as cocci in clusters or stained purple in a grape-like pattern.

Biochemical Identification of *S. Aureus*

S. aureus was confirmed through biochemical tests, including the coagulase, catalase, and salt mannitol agar tests (Fernandes et al. 2021; Koneman et al. 1997). The catalase test is a qualitative method used to detect the presence of the enzyme catalase. A positive result is indicated by the rapid development of oxygen (within 5-10 seconds) with the formation of bubbles. The coagulase test is an identification method for species of *Staphylococcus* spp. A positive result is characterized by forming a persistent plasma clot even after the tube is inserted. Salt Mannitol Agar was a selective culture medium for selecting pathogens that survive high salt concentrations. Strains of *S. aureus* showed a uniform shift in colony color from red to yellow.

Molecular Detection and Identification of *S. Aureus* (DNA Extraction)

Staphylococcal colonies were randomly selected from mannitol salt agar (MSA), and genomic DNA was extracted using a standard protocol involving enzymatic digestion and purification (Diaz et al. 2012). The identification of *S. aureus*, the specific nuclear species (nuc), was amplified using the PCR assay. DNA extracted from *S. aureus* was amplified to detect this specific gene with a molecular weight of 166 bp (Graber et al. 2007). The total volume of the mixture consisted of primer 5'-CCTGAAGCAAGTGCATTTACGA-3', reserve primer 5'-CTTTAGCCAAGCCTTGACGA-3', 2 \times Go TaqGreen Mix Master (Gold Star DNA Polymerase, dNTPs, MgCl₂, (NH₄)₂SO₄, Tris HCl (pH 8.5), blue and yellow dyes as filler and charging dyes, nuclease-free water, and template of DNA from *S. aureus*. The mixture was added to the PCR reaction tubes, the Veriti™ 96-well Fast Thermal Cycler (Applied Biosystems™4375786) was used to perform the amplification cycles, and finally, the amplicons were determined along with the DNA markers by gel electrophoresis.

Molecular Identification of *Staphylococcus* spp by PCR

A template was prepared from a volume of 10 μ L containing 4.9 μ L of DNA. The Veriti™ 96-well Fast Thermal Cycler was used to perform amplification cycles, and reaction conditions were optimized to 94 $^\circ\text{C}$ for 2min for the initial denaturation step, followed by 33 cycles at the same previous temperature level for 30 seconds, 58 $^\circ\text{C}$ for 30s, 72 $^\circ\text{C}$ for 30s, and at 72 $^\circ\text{C}$ for 5min for the final extension step (Gao et al. 2011).

Gel Electrophoresis

Electrophoresis on an agarose gel validated the DNA amplification and magnification products. Electrophoresis on agarose powder was performed at 70 volts for 45min. The result was visualized under the UV trans-illuminator (BIO-RAD), which was used to evaluate the documentation photographs (Gao et al. 2011).

Antibiotic Resistance and Sensitivity of *S. Aureus*

S. aureus isolates from RCM were evaluated for antimicrobial susceptibility and susceptibility using the disk diffusion agar method (Kirby-Bauer) in accordance with Clinical and Laboratory Standards Institute guidelines (CLIS 2021). *S. aureus* isolates were tested for antimicrobial susceptibility to 8 antimicrobial agents (antibiotics) commonly used in veterinary practice: Penicillin G (10µg), cefoxitin (10µg), clindamycin (30µg), gentamicin (10µg), ciprofloxacin (30µg), tetracycline (30µg), erythromycin (15µg), and trimethoprim-sulfamethoxazole (25µg). The inhibition zone size is inversely proportional to the minimum

inhibitory concentration (MIC), and millimeters (mm) were used as the unit of measurement for the analysis of antimicrobial drug susceptibility profiles (Hudzicki 2009).

Data Analysis and Interpretation of Results

Bacterial count results were expressed in CFU/mL and logarithmically transformed to log10 for statistical analysis; means and standard errors (SE) were calculated. Data were entered into Microsoft Excel 2016 and presented in tables and graphs accordingly.

RESULTS AND DISCUSSION

Isolation and Enumeration of *S. Aureus* from Raw Camel Milk

The results indicated that 52 (45.2%) *S. aureus* isolates out of 115 tested samples with minimum, maximum, and mean values were 0.3, 2.1, and 1.2 log10 CFU/mL, respectively. According to (GSO 1016:2015), which deals with the technical regulation of

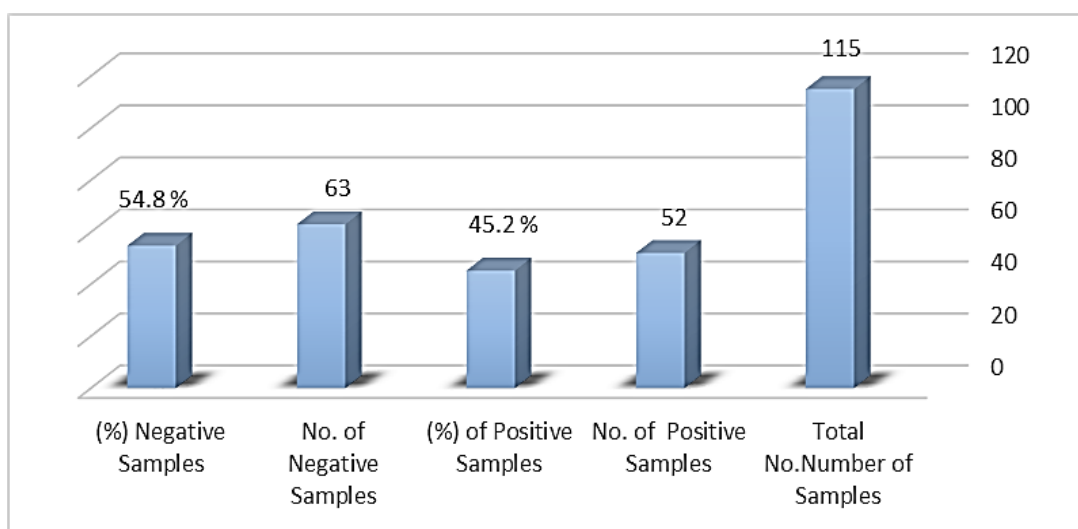


Fig. 1: Prevalence and isolation of *Staphylococcus aureus* in the examined camel RCM samples according to ISO 6888-1:2021 (n=115).

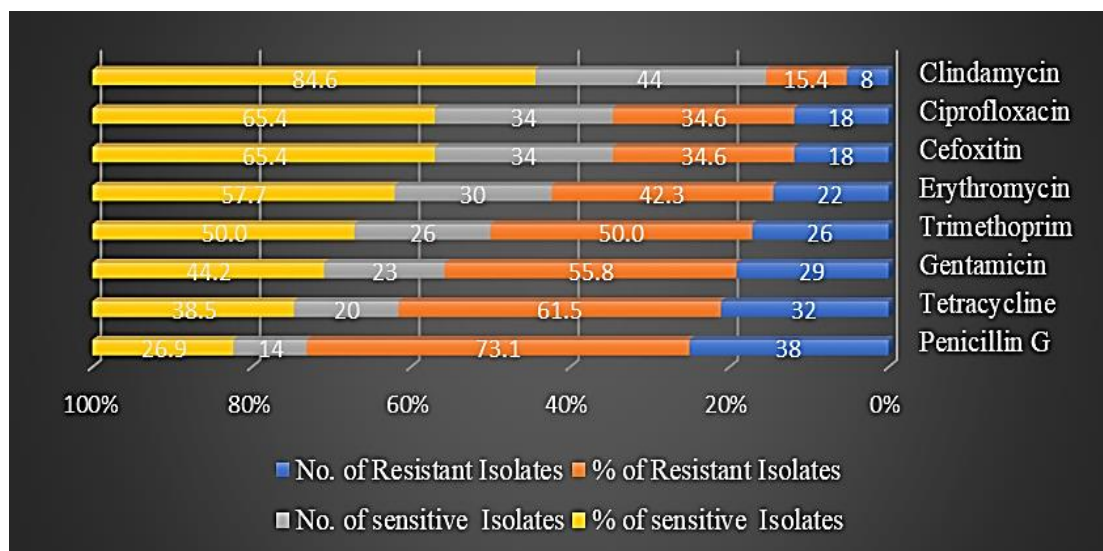


Fig 2: Overall frequency and distribution of antibiotic resistance of *Staphylococcus aureus* isolates in the examined RCM samples.

microbiological limits for some foods for human consumption, including raw camel milk, it was found that of the 52 *S. aureus* isolates, 44(84.6%) of the samples were within the acceptable limit (satisfactory), and 8(15.4%) of the samples were above the acceptable limit (unsatisfactory) ($\leq 2 \log_{10}$ CFU/mL). The results showed that 50(96.2%) of 52 *S. aureus* isolates had characteristic Gram-positive *S. aureus*, and 2(3.8%) had non-characteristic Gram-positive *S. aureus* on microscopic examination.

Biochemical Identification of *S. Aureus*

Of the 52 samples of *S. aureus* isolates, approximately 52 (100.0%) had *S. aureus* positive isolates to the catalase test, 44(84.6%) had *S. aureus* positive isolates to the coagulase test, and 39(75.0%) had *S. aureus* positive isolates to the salt mannitol agar test; a total of 45(86.5%) positive *S. aureus* isolates were identified by biochemical tests (Table 1).

Molecular identification of *S. aureus*

The PCR result showed that the nucleic (nuc) gene was identified in 50 samples (96.2%) of the isolates, with a minimum value of 143.1 (DNA conc. ng/ μ L), a maximum value of 397.5 (DNA conc. ng/ μ L), and a mean value of 217.31 (DNA conc. ng/ μ L). Moreover, 2(3.8%) were not identified as *S. aureus* isolates; these results agree with the findings of Sheet et al. (2021). The results obtained in this study show that PCR technology is considered significantly faster and more accurate than other conventional methods, which agrees with the findings of Izadpanah et al. (2018).

Antibiotic resistance and sensitivity of *S. aureus*

The results showed that a total of 52 of the *S. aureus* isolates (1.9%) did not show resistance to one antimicrobial agent, indicating multi-drug resistance (MRD) among the *S. aureus* isolates studied, (15.4%) showed resistance to one antimicrobial agent among the *S. aureus* isolates studied. *S. aureus* isolates (50%) showed resistance to two antimicrobial agents, and (98.1%) showed multi-drug resistance to the 8 antibiotics studied. The results showed the number and percentage of resistant *S. aureus* isolates for each of the antibiotic isolates studied (Table 2), where it was found that of 52 of *S. aureus* isolates, antimicrobial resistance to penicillin G (73.1%), cefoxitin (34.6%), clindamycin (15.4%), gentamicin (55.8%), ciprofloxacin (34.6%), tetracycline (61.5%), erythromycin (42.3%), trimethoprim (50%).

The traditional production and marketing of RCM in KSA need further study to evaluate such milk's quality and safety characteristics. Camel milk may become

contaminated with pathogenic and spoilage microorganisms if hygienic conditions and proper handling are not maintained during production.

The incidence of *S. aureus* in camel milk was 45.2%, which is almost in agreement with published researches (Abeer et al. 2012; El Zubeir and Ahmed 2007; Elhaj and AlSobeai 2018; Elhosseney et al. 2018). Various workers obtained lower findings (Befekadu et al. 2016; Serda et al. 2018; Verma and Prakash 2016) who isolated *S. aureus* in 12.9, 11.45, and 11.27%, respectively, from RCM. Higher results have also been reported (Asfour and Anwer 2015; Alghizzi and Shami 2021), who reported that 70% of the collected samples were contaminated with *S. aureus*. The differences may be due to different hygiene practices, such as milk handling, equipment washing, temperature control, and personal hygiene. Such conditions and handling practices have been shown to contribute significantly to substantial product losses and the spread of zoonotic diseases due to improper sanitation and unsanitary handling practices, deficiencies and weaknesses in food safety legislation and regulatory systems, inadequate funding and training of food manipulators (FAO/WHO, 2004). Moreover, the biochemical findings obtained are consistent with the findings of Fernandes et al. (2021) and Kirwa et al. (2021), who stated that *S. aureus* species are characterized by being catalase-positive, coagulase-positive, and other biochemical confirmatory tests and that molecular methods for identifying staphylococci, such as the PCR technique, are accurate methods (Martineau et al. 1996).

The results of the molecular identification of *S. aureus* agreed with the findings of Sheet et al. (2021), where the PCR technology is regarded as a much quicker and more precise technique other than conventional techniques (Izadpanah et al. 2018).

The most frequently observed antimicrobial resistance of *S. aureus* isolates was highest against penicillin G as the β -lactam antibiotic group (73.1%), followed by tetracycline as tetracycline antibiotic group (61.5%), gentamicin as aminoglycoside antibiotic group (55.8%), trimethoprim as antibiotic sulfonamide group(50%), erythromycin as macrolide antibiotic group (42.3%), cefoxitin as cephalosporin antibiotic group (34.6%), which corresponds to ciprofloxacin as quinolone

Table 1: Results of biochemical tests of positive *Staphylococcus aureus* isolates in the tested raw camel milk samples (n=52)

Test	Positive	
	Number	%
Catalase	52	100
Coagulase	44	84.6
Salt Mannitol Agar	39	75.0

Table 2: Statistical analysis results of 8 antimicrobials tested for susceptibility and sensitivity pattern of isolated *S. aureus* in the tested RCM samples.

Antibiotic class	Antibiotic name	Disc potency (μ g)	Resistant No. (%)	Intermediate No. (%)	Sensitive No. (%)
β -Lactams	Penicillin G	10	38 (73.1)	0	14 (26.9)
Cephalosporin	cefoxitin	10	18 (34.6)	5 (9.6)	29 (55.8)
Macrolide	clindamycin	30	8 (15.4)	3(5.7)	41(78.9)
Aminoglycosides	Gentamicin	10	29 (55.8)	0	23 (44.2)
Quinolones	Ciprofloxacin	30	18 (34.6)	12 (23)	22(42.4)
Tetracyclines	Tetracycline	30	32 (61.5)	0	20 (38.5)
Macrolides	Erythromycin	15	22 (42.3)	8(15.2)	22 (42.3)
Sulfonamides	trimethoprim	25	26 (50)	12 (23)	14 (2)

antibiotic group (34.6%), and finally clindamycin as macrolide antibiotic group (15.4%). The most frequently observed antimicrobial resistance of *S. aureus* isolates was the highest sensitivity to clindamycin as macrolide antibiotic group (84.6%), followed by ciprofloxacin as quinolone antibiotic group (65.4%), equal to cefoxitin as cephalosporin antibiotic group (65.4%), erythromycin as macrolide antibiotic group (57.7%), trimethoprim as antibiotic sulfonamide group (50%), gentamicin as aminoglycoside antibiotic group (44.2%), followed by tetracycline as tetracycline antibiotic group (38.5%) and finally, the least sensitive antibiotic was penicillin G as a β -lactam antibiotic group (26.9%). The results in Table 2 of 8 antimicrobials tested for susceptibility pattern of isolated *S. aureus* in the RCM samples were analyzed by disc diffusion test and classified as Resistant (R) No. (%), Intermediate (I) No. (%) and Sensitive (S) No. (%), which is in agreement with the findings of Aqib et al. (2017), who stated a total resistance of 54.7% from RCM samples with penicillin resistance (90%), cephalosporins resistance (77.5%), quinolones resistance (77.5%) and sulfonamides resistance (92.7%) (Aqib et al. 2017). These results may be attributed to the apparently healthy camels sampled in the study. Several studies have reported resistance to common antimicrobial drugs in pastoralist communities. The high resistance to beta-lactamases and aminoglycosides is due to pastoralists' widespread use and subtherapeutic doses to self-medicate their camels (Mutua et al. 2017); Omwenga et al. 2021). The growing and increasing resistance to these antimicrobial agents (antibiotics) were attributed mainly to first-line treatment without considering the appropriate dosage or withdrawal period (Omwenga et al. 2021).

Conclusion

Contamination of RCM with *S. aureus* is responsible for a significant risk to public health by producing a variety of enterotoxins. Although no cases of staphylococcal food poisoning (SFP) were reported or submitted during the study period, the permissible limit for *S. aureus* in RCM was met, indicating that most of the samples tested could be considered safe from a microbiological standpoint. Consuming RCM contaminated with multidrug-resistant (MDR) drugs may pose a potential risk for transmitting these residues between camels and humans, posing a significant public health challenge. The results suggest multidrug-resistant MRD of *S. aureus* isolates, which could be attributed to the intensive use of these drugs by veterinarians and the bucolic community. Further surveillance of antibiotic resistance is recommended as part of additional studies to prevent the spread of antimicrobial resistance (AMR).

Authors Contribution

Mohammed S. Alorainy provided the antibiotic susceptibility, and both authors designed the study, collected the samples, and performed the laboratory work.

Conflict of Interest

The authors have declared no conflict of interest.

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