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Antimicrobial resistance properties, virulence characters and RAPD-PCR typing of Methicillin-resistant *Staphylococcus aureus* isolated from raw milk and dairy samples

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ABSTRACT

Methicillin-resistant *Staphylococcus aureus* portion as an important food-borne pathogen is unmoving unidentified. This survey assessed the antibiotic resistance properties, molecular typing, and virulence characters of MRSA isolated from raw milk and dairy samples. Totally, 350 raw milk and dairy samples were examined for *S. aureus*. MRSA bacteria were identified using disk diffusion based on cefoxitin and oxacillin. Phenotypic and genotypic patterns of antibiotic resistance were assessed using disk diffusion and PCR, respectively. The PCR assessed the distribution of virulence characters. MRTSA typing was done using RAPD-PCR. Forty-five out of 350 (12.85%) raw milk and dairy samples were contaminated with *S. aureus*. Its distributions amongst the raw milk and dairy samples were 9.72% and 16.36%, respectively. Twenty-six strains out of 45 (57.77%) isolated *S. aureus* bacteria were determined as MRSA. These bacteria revealed the uppermost rate of resistance against cefoxitin (100%), ceftaroline (100%), penicillin (100%), tetracycline (92.30%), gentamicin (80.76%), erythromycin (69.23%), and trimethoprim-sulfamethoxazole (69.23%). *BlaCTX-M* (100%) and *blaZ* (100%) were detected in all MRSA isolates. *TetK* (73.07%), *aacA-D* (69.23%), *dfrA1* (50%), *vanA* (42.30%), *ermA* (42.30%), and *msrA* (42.30%) were the most routinely detected antibiotic-resistance determinants. *PVL* gene was detected in 69.23% of MRSA isolates. *Coa* (61.53%), *hla* (42.30%), and *fnbA* (38.46%) were also the most routinely detected virulence characters. MRSA isolates had a lower than 80% similarity pattern and were characterized in the same group. Simultaneous presence of virulence and antibiotic- resistance determinants amongst the MDR-MRSA bacteria suggests an imperative threat rendering contaminated raw milk and dairy consumption.

Keywords: Methicillin-resistant *Staphylococcus aureus*, RAPD-PCR typing, Virulence genes, Antibiotic-resistance, Raw milk, Dairy.
Article type: Research Article.

INTRODUCTION

The use of food, including food from animal and plant sources, as well as food supplements, is very important in human health (Dehbandi *et al.* 2019; Oboodiat *et al.* 2021; Al-Musawi 2022; Abed Almjalawi *et al.* 2022; Abdullah *et al.* 2023). Raw milk and dairy, as vigorous foods with high antioxidant contents, are nutrient components packages with extremely helpful effects on human health (Grażyna *et al.* 2017). Rendering their high vitamins, proteins, and minerals contents, they are considered as high-consuming food stuffs. Nevertheless, they largely can transfer a hefty number of food-borne microorganisms (Grace *et al.* 2020; Al-Noman *et al.* 2022). Contaminated raw milk and dairy consumption may cause severe food-borne diseases with a substantial economic weight (Ranjbar *et al.* 2018 a, b). *Staphylococcus aureus* is a Gram-positive, catalase-positive, and cocci-shaped bacterium isolated from the human upper respiratory tract and skin (Safarpoor Dehkordi *et al.* 2017; Safarpoor Dehkordi *et al.* 2018; Mohammadrezaei

Khorramabadi *et al*. 2022; Naderi *et al*. 2022; Shahmoradi *et al*. 2023). It is mainly responsible for unadorned nosocomial infections and food-borne diseases (Mohammadrezaei Khorramabadi *et al*. 2022; Naderi *et al*. 2022; Shahmoradi *et al*. 2023). *S. aureus* food-borne disease is basically documented with nausea and vomiting, abdominal cramps, weakness, partly diarrhea, and toxic shock syndrome (TSS; Safarpoor Dehkordi *et al*. 2017; Safarpoor Dehkordi *et al*. 2018). Food consumption portion, particularly raw milk and dairy, in *S. aureus* transmission and subsequent food-borne diseases have been documented well (Momtaz *et al*. 2013; Jamali *et al.* 2015; Hasanpour Dehkordi *et al.* 2017; Alghizzi *et al.* 2021; Machanlou *et al*. 2022). High resistance rate of *S. aureus* toward antimicrobial agents is one of hottest topic in the last decade (Gajdács *et al*. 2019). At this moment, methicillin-resistant *S. aureus* (MRSA) has transformed a thoughtful issue in hospitals and the community (Turner *et al*. 2019; Machanlou *et al*. 2022). MRSA bacteria harboured the high resistance rate and are arguable for the plain and complicated clinical diseases with higher morbidity, mortality, and economic loss (Klevens *et al.* 2007). They basically resist to all penicillin and cephalosporin agents (Klevens *et al*. 2007). Nevertheless, reports exhibiting the high rate of MRSA resistance toward other antimicrobial types, particularly aminoglycosides, macrolides, quinolones, tetracycline, and penems, are abundant (Klevens *et al*. 2007; Turner *et al*. 2019). Genetic-based antibiotic-resistance determinants encoded resistance toward specific types of antimicrobial agents possess a boost portion in epidemiological examinations. The genes that encode resistance toward penicillins (*blaZ*), glycopeptides (*vanA*), cephems (*blaCTX-M*), streptogramins (*vatA*), aminoglycosides (*aacA-D*), tetracyclines (*tetM* and *tetK*), folate pathway inhibitors (*dfrA1*), macrolides (*msrA* and *ermA*), ansamycins (*rpoB*), and ansamycins (*rpoB*), possess significant distributoin amongst the MRSA bacteria (Otarigho *et al*. 2018). Besides the role of antibiotic resistance in the MRSA epidemiology, the activity of diverse genetic-based virulence factors is noteworthy (Bukowski *et al*. 2010; Jenul *et al*. 2019). Toxic shock syndrome toxin-1 (*TSST-1*), Coagulase (*coa*), hemolysin (*hla*), exfoliative toxin A (*eta*), and fibronectin-binding protein (*fnbA*) are the most substantial MRSA virulence factors in the pathogenesis of infections (Bukowski *et al*. 2010; Jenul *et al*. 2019). Panton-Valentine leukocidin (*PVL*) as cytotoxic and leukocidin agent is another substantial MRSA virulence factor with the significant portion in the infections pathogenicity and treatment inactivation (Shallcross *et al*. 2013). Genotyping based on molecular techniques is practical and novel method to originate the genetic connotation among bacteria isolated from plentiful sources. Randomly Amplified Polymorphic DNA (RAPD)-PCR, a modest, precise, and fast method, has been epidemiologically applied to evaluate the genetic distinction and proceed strain-specific fingerprints (Zare *et al*. 2019). The meticulous character of virulent and antibiotic-resistant-MRSA on the food-borne diseases occurrence have not been exactly acknowledged. Accordingly, the contemporary examination was accomplished to assess the incidence, virulence and antibiotic resistance characters, and RAPD-PCR-based molecular typing of MRSA bacteria isolated from raw milk and dairy samples.

MATERIALS AND METHODS

Samples

A total of 350 raw milk (n = 185) and dairy (n = 165) samples were haphazardly collected from Isfahan Province retail centres, Iran from August 2020 to February 2021. Dairy samples were collected from home-based dairy producing companies. A total of 50 g were collected from each raw milk and dairy using a sterile laboratory tube. Sampling was performed using sterile hygienic procedure without any cross contamination between and within samples. Samples were roughly transferred to the laboratory by means of cool bags.

Isolation and identification of S. aureus

All microbiological media were purchased from Merck, Germany. Totally, 25 g of each raw milk and dairy sample were blended with 225 mL buffered peptone water. Then Stomacher (Interscience, Saint-Nom, France) was applied for sample hemogenization. Thereafter, 5 mL of the hemogenized solution were transferred into 50 mL Trypticase Soy Broth (TSB) accompanied with sodium pyruvate (1%) and NaCl (10%). Media were incubated at 35 °C for 18 h. Afterward, a culture loopful was shifted into egg yolk tellurite emulsion-supplemented Baird-Parker agar. Media were then incubated at 37 °C for 24 h. Black colonies with shiny entrance sorrounding with noteworthy zones were recognized using biochemical tests, including Gram staining, catalase, coagulated and oxidase tests, bacitracin resistance examination, urease, phosphatase, and deoxyribonuclease (DNase) activities, voges-proskaver test, nitrate

reduction, blood agar hemolysis, and carbohydrate (mannitol, glucose, sucrose, xylose, fructose, trehalose, lactose, maltose, and mannose) fermentation tests (Fijałkowski *et al*. 2016).

MRSA identification

Antibiotic susceptibility test was applied for MRSA identification rendering *S. aureus* resistance assessment toward cefoxitin (30 µg) and oxacillin (1 µg) antibiotic disks. Clinical and Laboratory Standards Institute (CLSI) guidelines were applied (CLSI 2007). *S. aureus* isolates simultaneously resist toward both cefoxitin and oxacillin disks were considered MRSA, which were confirmed using the *mecA* gene PCR-based detection (Fijałkowski *et al*. 2016).

MRSA phenotypic evaluation of antibiotic resistance

MRSA phenotypic pattern of antibiotic resistance was assessed by the disk diffusion method. The Mueller–Hinton agar was applied as basic culture medium. CLSI-guiding principles were applied as reference (CLSI, 2018). Cefoxitin (30 µg $disk^{-1}$), penicillin (10 units $disk^{-1}$), ceftaroline (30 µg $disk^{-1}$), gentamicin (15 µg $disk^{-1}$), azithromycin (15 µg $disk^{-1}$), vancomycin (5 µg $disk^{-1}$), ciprofloxacin (5 µg $disk^{-1}$), erythromycin (15 µg $disk^{-1}$), tetracycline (30 µg $disk^{-1}$), levofloxacin (5 µg $disk^{-1}$), nitrofurantoin (300 µg $disk^{-1}$), doxycycline (30 µg $disk^{-1}$), rifampin (5 µg $disk^{-1}$), trimethoprim-sulfamethoxazole (1.25/23.75 µg $disk^{-1}$), and quinupristin-dalfopristin (15 µg $disk^{-1}$) (Oxoid, UK) were employed. MRSA isolates were cultured on media; disks were also located on the surface, and all were incubated at 37 °C for 24 h. Growth inhibition zone of MRSA for each antibiotic was measured and compared to CLSI zones (CLSI 2018). MRSA ATCC 43300 was used as control.

MRSA genotypic evaluation of antibiotic resistance and of virulence characters

PCR ingredients were purchased from Ermo Fisher Scientific Co (St. Leon-Rot, Germany). At first, MRSA DNA was extracted, then one-night MRSA cultures on TSB was used as the source of bacteria. MRSA Genomic DNA was extracted using kit regarding the guiding principle. MRSA DNA purity (A260/A280; NanoDrop, Thermo Scientific, Waltham, MA, USA) and quality (electrophoresis on 2% agarose gel) were assessed. Table 1 depicts the PCR ingredients, thermal cycles and volumes (Xuehan *et al*. 2018; Pagani *et al*. 2003). Eppendorf Mastercycler device (No 5330, Eppendorf-Nethel-Hinz GmbH, Hamburg, Germany) was employed. Electrophoresis (120 V/208 mA) in 2.5% agarose gel was performed for the PCR products visualization. Gel staining was prepared by the ethidium bromide (0.1%, 0.4 µg mL^{-1}). The PCR results analysis was performed using UVI doc gel documentation systems (Grade GB004, Jencons PLC, London, UK).

MRSA RAPD-PCR

MRSA RAPD-PCR molecular typing was accomplished affording to the beforehand documented method. OLP6 (5'-GAG-GGA-AGA-G-3'), OLP11 (5'-ACG-ATG-AGC-C-3'), and OLP13 (5'-ACC-GCC-TGC-T-3') (60–70% of G-C content) were applied in amplification. Thermal cycles were included one cycle of 5 min at 94 ºC, 40 cycles of 60 s at 93 ºC, 90 s at 37 ºC, and 60 s at 72 ºC, with final extension of 72 ºC for 8 min. Electrophoresis was carried out using agarose gel (1.5%). GelWorks 1D software (version 3.00, UK) was applied for gel analysis. Similarity rate (%) was inspected. Similarity matrices cluster analysis was performed using unweighted pair group method with arithmetic averages (UPGMA). The NTSYS-pc software (version 2.01e, Applied Biostatistics, USA) was applied. Analysis was accomplished using the approaches labelled beforehand (Reinoso *et al*. 2004).

Data analysis

Data analysis was performed using the SPSS 21.0 statistical software (SPSS Inc., Chicago, IL, USA). Chi-square test and Fisher's exact two-tailed test were applied to evaluate any significant relationship between the collected data. The student's t-test was applied to measure statistical significance between RAPD clusters. p-value < 0.05 was measured as a statistical significant level.

RESULTS

***S. aureus* and MRSA distribution**

Table 2 presents the *S. aureus* and MRSA bacteria incidence between examined raw milk and dairy samples. Forty-five out of 350 (12.85%) raw milk and dairy samples were contaminated with *S. aureus* which its distributions amongst the raw milk and dairy samples were 9.72% and 16.36%, respectively. A significant difference was obtained for the incidence of *S. aureus* between raw milk and dairy samples ($p < 0.05$). Twenty-six out of 45 (57.77%) *S. aureus* isolates were simultaneously resistant toward oxacillin and cefoxitin agents and were recognized as MRSA. Additionally, all of them harboured the *mecA* gene. MRSA distributions amongst the *S. aureus* bacteria isolated from raw milk and dairy samples were 50% and 62.96%, respectively ($p < 0.05$).

Table 2. *S. aureus* and MRSA incidence amid examined raw milk and dairy samples.

Samples	N. samples	N. positive for *S. aureus* (%)	MRSA distribution out of *S. aureus* isolates (%)
Raw milk	185	18 (9.72)	9 (50)
Dairy	165	27 (16.36)	17 (62.96)
Total	350	45 (12.85)	26 (57.77)

MRSA phenotypical assessment of antibiotic resistance

Table 3 depicts the MRSA phenotypic pattern of antibiotic resistance. MRSA bacteria revealed the uppermost rate of resistance against cefoxitin (100%), ceftaroline (100%), penicillin (100%), tetracycline (92.30%), gentamicin (80.76%), erythromycin (69.23%), and trimethoprim-sulfamethoxazole (69.23%). Nevertheless, they harboured the lowest rate of resistance toward rifampin (34.61%), doxycycline (38.46%), and quinupristin-dalfopristin (38.46%). MRSA bacteria isolated from dairy samples harboured a higher incidence of resistance toward all examined antibiotic agents than those of raw milk ($p < 0.05$).

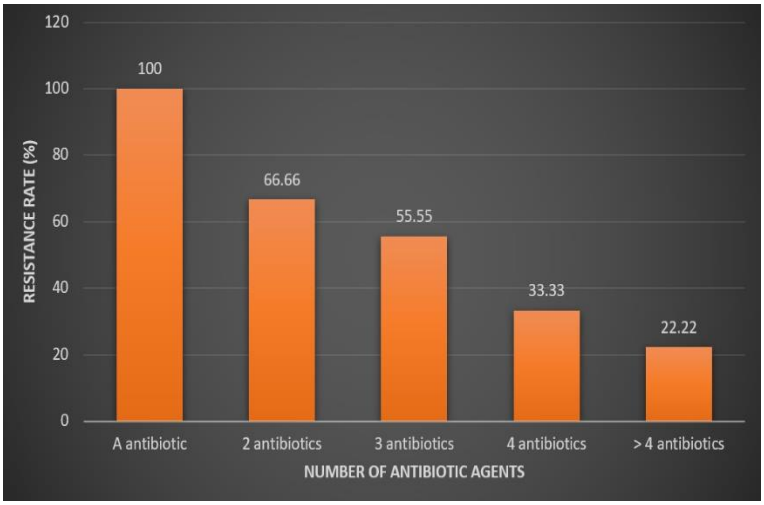

Fig. 1. MDR distribution amongst the MRSA isolates of raw milk samples.

Fig. 1 illustrates the multi-drug resistance (MDR) distribution amongst the MRSA isolates of raw milk. All isolates exhibited at least resistance against 1 antibiotic agent. However, only 22.22% of MRSA isolates harboured resistance to more than 4 antibiotic agents.

Table 1. Characters of the PCR reactions.

Target genes	Encoding antibiotic agent	Sequences (5'-3')	Size (bp)	Thermal cycles	Volume (50 µL)
aacA-D	Aminoglycosides	F: TAA-TCC-AAG-AGC-AAT-AAG-GGC R: GCC-ACA-CTA-TCA-TAA-CCA-CTA	227	**1 cycle** 94 °C, 5 min. **25 cycles** 94 °C, 60 s 55 °C, 70 s 72 °C , 60 s **1 cycle** 72 °C, 10 min	10X PCR buffer: 5 µL MgCl2: 1.5 mM dNTP: 200 µM Primer F: 0.5 µM Primer R: 0.5 µM Taq DNA polymerase: 1.25 U DNA template: 2.5 µL
ermA	Macrolides	F: AAG-CGG-TAA-ACC-CCT-CTG-A R: TTC-GCA-AAT-CCC-TTC-TCA-AC	190		
tetK	Tetracycline	F: GTA-GCG-ACA-ATA-GGT-AAT-AGT R: GTA-GTG-ACA-ATA-AAC-CTC-CTA	360		
grlA	Fluoroquinolones	F: ACT-TGA-AGA-TGT-TTT-AGG-TGA-T R: TTA-GGA-AAT-CTT-GAT-GGC-AA	618		
tetM	Tetracycline	F: AGT-GGA-GCG-ATT-ACA-GAA R: CAT-ATG-TCC-TGG-CGT-GTC-TA	158	**1 cycle** 94 °C, 6 min. **34 cycles** 95 °C, 50 s 55 °C , 70 s 72 °C, 60 s **1 cycle** 72 °C, 8 min	10X PCR buffer: 5 µL $MgCl_2$: 1.5 mM dNTP: 200 µM Primer F: 0.5 µM Primer R: 0.5 µM Taq DNA polymerase: 1.25 U DNA template: 2.5 µL
gyrA	Fluoroquinolones	F: AGT-ACA-TCG-TCG-TAT-ACT-ATA-TGG R: ATC-ACG-TAA-CAG-TTC-AAG-TGT-G	280		
msrA	Macrolides	F: GGC-ACA-ATA-AGA-GTG-TTT-AAA-GG R: AAG-TTA-TAT-CAT-GAA-TAG-ATT-GTC-CTG-TT	940	**1 cycle** 94 °C, 6 min.	10X PCR buffer: 5 µL $MgCl_2$: 1.5 mM

dfrA1	Folate pathway antagonists	F: CTC-ACG-ATA-AAC-AAA-GAG-TCA R: CAA-TCA-TTG-CTT-CGT-ATA-ACG	201	**34 cycles** 95 °C, 60 s 50 °C, 70 s 72 °C, 70 s **1 cycle** 72 °C, 8 min	dNTP: 200 µM Primer F: 0.5 µM Primer R: 0.5 µM Taq DNA polymerase: 1.25 U DNA template: 2.5 µL
blaZ	Penicillin	F: TGA-ACC-GTA-TGT-TAG-TGC R: GTC-GTG-TTA-GCG-TTG-ATA	681	**1 cycle** 94 °C, 6 min. **30 cycles** 95 °C, 60 s 59 °C, 60 s 72 °C, 60 s **1 cycle** 72 °C, 10 min	10X PCR buffer: 5 µL $MgCl_2$: 1.5 mM dNTP: 200 µM Primer F: 0.5 µM Primer R: 0.5 µM Taq DNA polymerase: 1.25 U DNA template: 2.5 µL
rpoB	Ansamycins	F: ACC-GTC-GTT-TAC-GTT-CTG-TA R: TCA-GTG-ATA-GCA-TGT-GTA-TC	460	**1 cycle** 94 °C, 5 min **40 cycles** 94 °C, 40 s 45.5 °C, 40 s 72 °C, 90 s **1 cycle** 72 °C, 8 min	10X PCR buffer: 5 µL $MgCl_2$: 1.5 mM dNTP: 200 µM Primer F: 0.5 µM Primer R: 0.5 µM Taq DNA polymerase: 1.25 U DNA template: 2.5 µL

vatA	Streptogramins	F: TGG-TCC-CGG-AAC-AAC-ATT-TAT R: TCC-ACC-GAC-AAT-AGA-ATA-GGG	268	**1 cycle** 94 °C, 6 min **34 cycles** 95 °C, 50 s 55 °C, 70 s 72 °C, 60 s **1 cycle** 72 °C , 8 min	10X PCR buffer: 5 µL $MgCl_2$: 1.5 mM dNTP: 200 µM Primer F: 0.5 µM Primer R: 0.5 µM Taq DNA polymerase: 1.25 U DNA template: 2.5 µL
vanA	Glycopeptides	F: ATG-AAT-AGA-ATA-AAA-GTT-GC R: TCA-CCC-CTT-TAA-CGC-TAA-TA	1032	**1 cycle** 98 °C, 2 min **35 cycles** 98 °C, 10 s 50 °C, 60 s 72 °C, 90 s **1 cycle** 72 °C, 10 min	10X PCR buffer: 5 µL $MgCl_2$: 1.5 mM dNTP: 200 µM Primer F: 0.5 µM Primer R: 0.5 µM Taq DNA polymerase: 1.25 U DNA template: 2.5 µL
coa	Coagulase	F: CGA-GAC-CAA-GAT-TCA-ACA-AG R: AAA-GAA-AAC-CAC-TCA-CAT-CA	970	**1 cycle** 95 °C, 2 min **30 cycles** 95 °C , 30 s 58 °C , 2 min 72 °C , 4 min **1 cycle** 72 °C, 7 min	10X PCR buffer: 5 µL MgCl2: 1.5 mM dNTP: 200 µM Primer F: 0.5 µM Primer R: 0.5 µM Taq DNA polymerase: 1.25 U DNA template: 2.5 µL
blaCTX-M[a]	Cephems	F: ATG-TGC-AGY-ACC-AGT-AAR-GT R: TGG-GTR-AAR-TAR-GTS-ACC-AGA	593	**1 cycle** 94°C, 7 min. **35 cycles**	10X PCR buffer: 5 µL $MgCl_2$: 1.5 mM dNTP: 200 µM

				94 °C, 50 s 50 °C, 40 s 72 °C, 60 s **1 cycle** 72 °C, 5 min	Priumer F: 0.5 µM Primer R: 0.5 µM Taq DNA polymerase: 1.25 U DNA template: 2.5 µL
TSST-1	Toxic Shock Syndrome Toxin	F: ATG-GCA-GCA-TCA-GCT-TGA-TA R: TTT-CCA-ATA-ACC-ACC-CGT-TT	350	**1 cycle** 94°C, 6 min. **30 cycles** 94 °C, 2 min 55 °C, 2 min 72 °C, 1 min **1 cycle** 72 °C, 8 min	10X PCR buffer: 5 µL $MgCl_2$: 1.5 mM dNTP: 200 µM Primer F: 0.5 µM Primer R: 0.5 µM Taq DNA polymerase: 1.25 U DNA template: 2.5 µL
eta	exfoliative toxins A	F: CTA-GTG-CAT-TTG-TTA-TTC-AA R: TGC-ATT-GAC-ACC-ATA-GTA-CT	119		
pvl	*PVL*	F: ATC-ATT-AGG-TAA-AAT-GTC-TGG-ACA-TGA-TCC-A R: GCA-TCA-AST-GTA-TTG-GAT-AGC-AAA-AGC	433	**1 cycle** 94 °C, 5min **30 cycles** 94 °C, 30 s 56 °C, 30 s 72 °C, 1 min **1 cycle** 72 °C, 10 min	10X PCR buffer: 5 µL MgCl2: 1.5 mM dNTP: 200 µM Primer F: 0.5 µM Primer R: 0.5 µM Taq DNA polymerase: 1.25 U DNA template: 2.5 µL

fnbA	Fibronectin-binding protein	F: GTG-AAG-TTT-TAG-AAG-GTG-GAA-AGA-TTA-G R: GCT-CTT-GTA-AGA-CCA-TTT-TTC-TTC-AC	643	**1 cycle** 94 °C, 5min **30 cycles** 94 °C, 30 s 57 °C, 40 s 72 °C, 1 min **1 cycle** 72 °C, 10 min	10X PCR buffer: 5 µL $MgCl_2$: 1.5 mM dNTP: 200 µM Primer F: 0.5 µM Primer R: 0.5 µM Taq DNA polymerase: 1.25 U DNA template: 2.5 µL
hla	hemolysin	F: CTG-ATT-ACT-ATC-CAA-GAA-ATT-CGA-TTG R: CTT-TCC-AGC-CTA-CTT-TTT-TAT-CAG-T	209	**1 cycle:** 94 °C, 5min. **30 cycle** 94 °C, 30 s 58 °C, 15 s 72 °C, 1 min **1 cycle** 72 °C, 10 min	10X PCR buffer: 5 µL $MgCl_2$: 1.5 mM dNTP: 200 µM Primer F: 0.5 µM Primer R: 0.5 µM Taq DNA polymerase: 1.25 U DNA template: 2.5 µL

[a]R is A or G; Y is C or T; S is G or C.

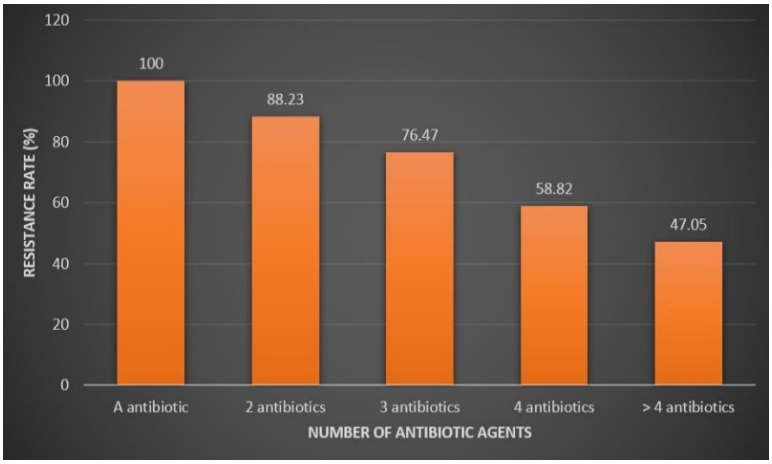

Fig. 2. MDR distribution amongst the MRSA isolates of dairy samples.

Fig. 2. indicates the MDR distribution amongst the MRSA isolates of dairy samples. All isolates displayed at least resistance against 1 antibiotic agent. However, 47.05% of MRSA isolates harboured resistance to more than 4 antibiotic agents.

MRSA genotypical assessment of antibiotic resistance

Table 4 depicts the MRSA genotypic pattern of antibiotic resistance. *BlaCTX-M* (100%) and *blaZ* (100%) were detected in all MRSA isolates. *TetK* (73.07%), *aacA-D* (69.23%), *dfrA1* (50%), *vanA* (42.30%), *ermA* (42.30%), and *msrA* (42.30%) were the most routinely detected as antibiotic-resistance determinants. Nevertheless, *tetM* (23.07%) and *rpoB* (23.07%) harboured the lowest distribution amongst all examined genotypic determinants of antibiotic resistance. MRSA bacteria isolated from dairy samples harboured a higher incidence of antibiotic resistance genes than raw milk ($p < 0.05$). A significant difference was found between the distribution of *tetK* and *tetM* ($p < 0.05$) antibiotic-resistance determinants.

MRSA distribution of virulence factors

Table 5 described the MRSA virulence characters. *PVL* gene was detected in 69.23% of MRSA isolates of raw milk and dairy samples. The *coa* (61.53%), *hla* (42.30%), and *fnbA* (38.46%) were also the most routinely detected MRSA virulence characters. *Tsst-1* was only detected in 15.38% of isolates. MRSA bacteria isolated from dairy samples harboured a higher incidence of antibiotic resistance genes than milk ($p < 0.05$).

Table 5. MRSA virulence characters.

Type of samples (N. MRSA)	N (%) MRSA harboured each virulence factors					
	eta	*PVL*	*coa*	*tsst-1*	*fnbA*	*hla*
Raw milk (9)	2 (22.22)	4 (44.44)	3 (33.33)	-	2 (22.22)	2 (22.22)
Dairy (17)	6 (35.29)	14 (82.35)	12 (70.58)	4 (23.52)	8 (47.05)	9 (52.94)
Total (26)	8 (30.76)	18 (69.23)	16 (61.53)	4 (15.38)	10 (38.46)	11 (42.30)

MRSA RAPD-PCR typing

Fig. 3 exhibits the RAPD-PCR molecular typing of MRSA strains isolated from raw milk and dairy samples. All MRSA isolates displayed a similarity lower than 80% (except for isolate No. 10) and were categorized in the same group. MRSA isolate No. 10 revealed a 100% genetic difference with other isolates. Among other isolates, 15.4% to 87.5% similarities were observed.

Table 3. MRSA phenotypic pattern of antibiotic resistance.

Type of samples (N. MRSA)	N (%) isolates resistant to each antibiotic														
	Streptogramins	**Macrolides**		**Cephems**		**Aminoglycosides**	**Fluoroquinolones**		**Folate pathway antagonists**	**Penicillin**	**Ansamycin**	**Glycopeptide**	**Tetracyclines**		**Nitrofurantoins**
	Qun-Dlf*	**Azi**	**Ery**	**Cfx**	**Cft**	**Gen**	**Cip**	**Lev**	**Tr-sul**	**P10**	**Rif**	**Van**	**Tet**	**Dox**	**Nit**
Raw milk (9)	3 (33.33)	5 (55.55)	6 (66.66)	9 (100)	9 (100)	7 (77.77)	5 (55.55)	5 (55.55)	6 (66.66)	9 (100)	3 (33.33)	4 (44.44)	8 (88.88)	3 (33.33)	3 (33.33)
Dairy (17)	7 (41.17)	10 (58.82)	12 (70.58)	17 (100)	17 (100)	14 (82.35)	10 (58.82)	10 (58.82)	12 (70.58)	17 (100)	6 (35.29)	8 (47.05)	16 (94.11)	7 (41.17)	6 (35.29)
Total (26)	10 (38.46)	15 (57.69)	18 (69.23)	26 (100)	26 (100)	21 (80.76)	15 (57.69)	15 (57.69)	18 (69.23)	26 (100)	9 (34.61)	12 (46.15)	24 (92.30)	10 (38.46)	9 (34.61)

*Qun-Dlf: quinupristin-dalfopristin (15 µg/disk), Azi: azithromycin (15 µg/disk), Ery: erythromycin (15 µg/disk), Cfx: cefoxitin (30 µg/disk), Cft: ceftaroline (30 µg/disk), Ge: gentamicin (15 µg/disk), Cip: ciprofloxacin (5 µg/disk), Lev: levofloxacin (5 µg/disk), Tr-Sul: trimethoprim-sulfamethoxazole (1.25/23.75 µg/disk), P10: penicillin (10 units/disk), Rif: rifampin (5 µg/disk), Van: vancomycin (5 µg/disk), Tet: tetracycline (30 µg/disk), Dox: doxycycline (30 µg/disk), and nitrofurantoin (300 µg/disk).

Table 4. MRSA genotypic pattern of antibiotic resistance.

Type of samples (N. MRSA)	N (%) isolates harboured each antibiotic resistance gene												
	vatA	***ermA***	***msrA***	***blaCTX-M***	***aacA-D***	***gyrA***	***grlA***	***dfrA1***	***blaZ***	***rpoB***	***vanA***	***tetK***	***tetM***
Raw milk (9)	2 (22.22)	3 (33.33)	2 (22.22)	9 (100)	5 (55.55)	2 (22.22)	2 (22.22)	3 (33.33)	9 (100)	1 (11.11)	4 (44.44)	5 (55.55)	1 (11.11)
Dairy (17)	6 (35.29)	8 (47.05)	9 (52.94)	17 (100)	13 (76.47)	9 (52.94)	7 (41.17)	10 (58.82)	17 (100)	5 (29.41)	7 (41.17)	14 (82.35)	5 (29.41)
Total (26)	8 (30.76)	11 (42.30)	11 (42.30)	26 (100)	18 (69.23)	11 (42.30)	9 (34.61)	13 (50)	26 (100)	6 (23.07)	11 (42.30)	19 (73.07)	6 (23.07)

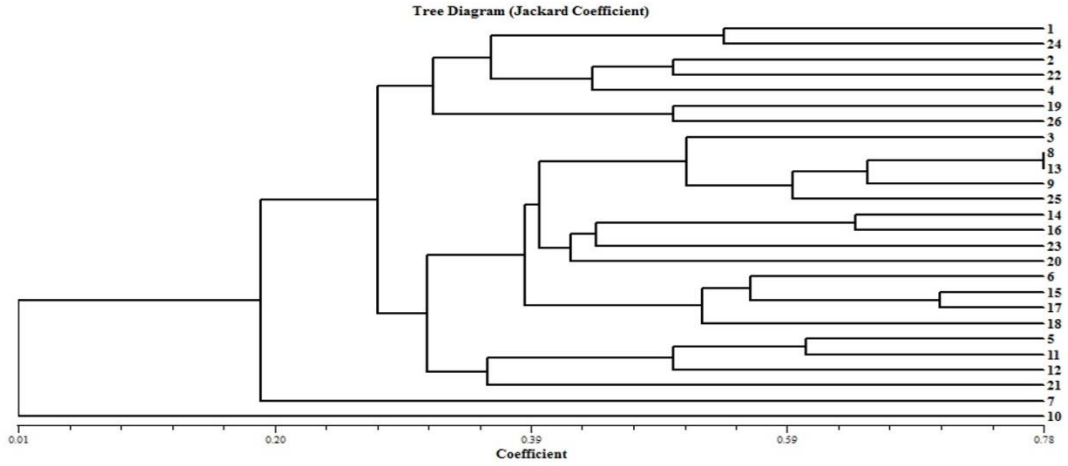

Fig. 3. RAPD-PCR typing of MRSA strains isolated from raw milk and dairy samples. All of the 26 MRSA isolates were considered.

DISCUSSION

It is crucial to differentiate the meticulous routes of antibiotic resistant-bacteria transmission to the human population from the epidemiological perspective. Foods possess a challenging portion in transmitting some types of antibiotic resistant-bacteria to humans (Rahimi *et al*. 2014; Dehkordi *et al*. 2014). Raw milk and dairy samples are two of the most important and highly-consumed foodstuff in the world (Velázquez-Ordoñez *et al*. 2019). They are measured as an omnipresent source of antibiotic-resistant *S. aureus* (Al-Ashmawy *et al*. 2016). An existing survey was aimed to assess the molecular typing as well as phenotypic and genotypic assessment of antibiotic resistance along with virulence factors of the MRSA bacteria isolated from raw milk and dairy samples. Total incidence of MRSA bacteria amongst the raw milk and dairy samples was 4.86% (9/185) and 10.30% (17/165), respectively. Yakubu *et al*. (2020) from Nigeria reported that the *S. aureus* prevalences amongst the bulk and fresh milk samples were 7.14% and 2.94%, respectively. In comparison with our survey, they reported lower distribution without MRSA analysis. Oliveira *et al.* (2022) in Portugal reported that 53% of raw milk samples were contaminated with *S. aureus*, a higher distribution than that of our findings. However, they reported only 8.10% distribution for MRSA strains, which was lower than that in our report. Compared to these studies (Dai *et al*. 2019; Lienen *et al*. 2021), the MRSA prevalence in the present survey was higher, which may reflect the lower hygienic conditions. MRSA presence in raw milk and dairy samples may have two different origins of primary (animal origin) and secondary (human origin after manipulation and dairy processing). Survival of bacteria from farm to fork and transmission of MRSA bacteria from contaminated milk to dairy samples or from contaminated staffs of milking halls or dairy producing companies to dairy samples are probable reasons for the higher incidence of bacteria in the examined dairy samples. Additionally, both raw milk and dairy samples have optimum growth circumstances for *S. aureus* (pH 4.8 – 9.3) and temperatures (7-43 ^{o}C). Thus, it is not surprising that 12.85% of samples were contaminated by *S. aureus* with high prevalence of MRSA strains. Similar to our report, Al-Ashmawy *et al.* (2016) reported the high MRSA distribution (53% of *S. aureus* isolates) amongst the examined raw milk and dairy samples. According to their study, MRSA prevalence amongst the raw milk, cheese, ice cream, and yogurt samples were 75.00%, 40.00-65.00%, 50.00%, and 35.00%, respectively. The present study revealed that MRSA bacteria isolated from raw milk and dairy samples harboured high resistance toward cefoxitin, ceftaroline, penicillin, tetracycline, gentamicin, trimethoprim-sulfamethoxazole, and erythromycin, which was assisted with a high incidence of *blaCTX-M*, *blaZ*, *aacA-D*, *tetK*, *dfrA1*, and *vanA* antibiotic-resistant genes. Otherwise, phenotypic assessment of antibiotic resistance of MRSA bacteria was confirmed by the genotypic evaluation of the resistance gene. Furthermore, the presence of multidrug resistant-MRSA was found in some isolates. Irregular and unauthorizing antibiotic prescription is the probable reason for the high incidence of resistance and high distribution of antibiotic-resistant genes. Findings suggest that some MRSA bacteria isolated from dairy samples exhibited higher incidences of resistance toward antibiotic agents used for treatment of human clinical infections, which can indirectly signify that they may transmit from infected staff and workers. Rendering the literature searches, the current study is one of the first and most comprehensive reports on phenotypic and genotypic assessments of antibiotic resistance amongst the MRSA bacteria isolated from raw milk and dairy

samples. Shrestha *et al.* (2021) reported that *S. aureus* strains isolated from cattle milk in Nepal were sensitive to cefazolin (75.90%) and tetracycline (48.30%), while all were resistant to ampicillin (100%), indicating that 96.60% of isolates were multidrug-resistant (MDR). Alembo *et al*. (2023) from Ethiopia reported that the resistance rate of *S. aureus* strains isolated from raw cow milk samples against penicillin, cefoxitin, gentamicin, erythromycin, tetracycline, ciprofloxacin, sulfamethoxazole, clindamycin, and chloramphenicol were 84.70%, 76.30%, 50.90%, 37.30%, 30.50%, 3.40%, 3.40%, 5.10%, and 8.50%, respectively. Similarly, in a study in Turkey (Keyvan *et al*. 2020) the resistance rate of *S. aureus* strains isolated from raw milk samples against oxacillin, penicillin, clindamycin, and cefoxitin were 71.15%, 69.82%, 67.93%, 67.31%, respectively, In Africa (Titouche *et al*. 2019; Ghaderi *et al*. 2021), resistance rates of *S. aureus* isolates from raw animal milk samples against penicillin, cefoxitin, oxacillin, kanamycin, neomycin, tobramycin, erythromycin, spiramycin, lincomycin, tetracycline, clindamycin, ofloxacin, norflocxacin, fosfomycin, bacitracin, and fusidic acid were 91.30%, 15.90%, 15.90%, 1.40%, 1.40%, 2.90%, 2.90%, 1.40%, 1.40%, 47.80%, 1.40%, 15.90%, 15.90%, 1.40%, 4.30%, and 1.40%, respectively. Similar resistance rates of *S. aureus* and MRSA strains isolated from milk and dairy samples against tetracyclines, cephalosporins, aminoglycosides, macrolides, penicillins, quinolones, penems, and other routine antimicrobial agents have been reported in the studies carried out on Iran (Titouche *et al*. 2019), United States (Patel *et al*. 2021), China (Liu *et al*. 2022), Australia (Rowe *et al*. 2023), and Germany (Schnitt *et al*. 2020). Assessment of antibiotic-resistant genes amongst the MRSA strains isolated from raw milk and dairy samples is scarce in the literature. Dehkordi *et al.* (2017) reported that the incidence of *aacA-D*, *tetK*, *tetM*, *msrA*, *ermA*, *ermC*, *vatA*, *vatB*, *vatC* and *linA* amongst the MRSA bacteria isolated from hospital food samples were 62.16%, 72.97%, 27.02%, 64.86%, 72.97%, 27.02%, 45.94%, 18.91%, 5.40%, and 43.24%, respectively. Huang *et al*. (2023) reported that the distribution of *mecA*, *blaI*, *lnuB*, *lsaE*, *fexA*, *ermC*, *tetL*, and *dfrG* amongst the *S. aureus* strains isolated from raw milk over 10 years in China were 14.15%, 70.21%, 5.85%, 5.75%, 6.83%, 4.39%, 9.27%, and 5.85%, respectively. Similar to our report, Rahi *et al*. (2020) reported that the incidence of *blaZ*, *aacA-D*, *ermA*, *ermB*, *msrA*, *msrB*, *mefA*, *tetK*, *tetM*, *gyrA*, *grlA*, *linA*, *dfrA1*, *cfr*, and *rpoB* amongst the MRSA bacteria isolated from raw milk samples were 100%, 67.85%, 50%, 25%, 35.71%, 10.71%, 35.71%, 85.71%, 35.71%, 42.85%, 28.57%, 28.75%, 71.42%, 25%, and 10.71%, respectively. Our findings were also released a higher incidence of the phenotypic profile of resistance than the genotypic pattern. For instance, all of the glycopeptides-resistant MRSA bacteria did not harbour *vanA* antibiotic-resistant genes. This matter also existed for other antibiotics and resistance genes, since antibiotic-resistant genes are one of the known procedures for the occurrence of antibiotic resistance in MRSA strains. Otherwise, numerous mechanisms have been recognized to induce antibiotic resistance in bacteria, including efflux antibiotic's active pumps to out of the bacterial cell, reduced permeability of bacteria to antibiotics, inactivation of antibiotics through hydrolysis or alterations in their structure, change in the antibiotic target site and access of bacteria to the secondary metabolic pathways that compensate the antibiotic-inhibited reactions and occurrence of genetic mutations. Discoveries also showed the high incidence of multidrug resistant-MRSA strains amongst examined samples, particularly dairy. In the same way, high incidence of multidrug-resistant bacteria has been reported in herbal product samples in Egypt (Eid *et al*. 2020) Turkey (Ektik *et al*. 2018), and Tanzania (Mohammed *et al*. 2018). Altogether, high incidence of antibiotic resistant-MRSA which was accompanying with the high distribution of antibiotic-resistant genes and presence of multidrug resistance, revealed a pressing public health issue regarding the consumption of raw milk and dairy samples. Given these antibiotics have been progressively utilized in human and animal treatments and exchange of antibiotic-resistant genes by the mobile genetic elements, it is not astonishing that resistant-bacteria become more mutual nowadays. Nevertheless, the high antimicrobial resistance of MRSA observed in this study should receive much attention. Furthermore, controlled administering antimicrobials would limit the emergence of drug-resistant bacteria. Findings described the considerable incidence of virulence factors amongst the MRSA bacteria isolated from raw milk and dairy samples. Alpha-hemolysin (*hla*) toxin is the most emphasized and characterized virulence factor of the *S. aureus*, which is considered as a vaccine candidate to inhibit the dissemination of infections (Zhang *et al*. 2018). Most MRSA bacteria recovered from clinical infections, and more recently, food samples harboured the *coa* factor (Dallal *et al* 2016). It mainly acts as a blood coagulase factor in the pathogenesis of staphylococcal infections (Dallal *et al*. 2016). *PVL* is cytotoxin responsible for severe tissue necrosis and leukocyte destruction (Gao *et al.* 2019). *PVL*, *coa*, and *hla* virulence factors also exhibited a high incidence amongst the *S. aureus* bacteria isolated from food and clinical samples collected from Egypt (Elsayed *et al*. 2015), Myanmar (Aung *et al*. 2016), China (Zhang *et al*. 2018), Iran (Tahbaz *et al*. 2019), and Brazil

(Rodrigues *et al*. 2017). The high incidence of identified virulence factors in the MRSA bacteria of the present study may show high virulence and pathogenicity of MRSAS bacteria which poses an imperative public health hazard rendering the consumption of contaminated raw milk and dairy samples. Assessing the distribution of *PVL* gene is one of the important practical methods to find the presence of healthcare-associated (HA-) or community-associated (CA) MRSA bacteria. Findings of epidemiological investigations revealed that the CA-MRSA bacteria mainly carry the *PVL* gene (Aung *et al*. 2016). In the present study, 44.44% of MRSA bacteria isolated from raw milk samples and 82.35% of those of dairy samples harboured the *PVL* gene. Thus, majority of MRSA bacteria isolated from raw milk samples may categorized as HA-MRSA, while majority of those of dairy samples may categorized as CA-MRSA. However, assessment of the presence of Staphylococcal Cassette Chromosome *mec* (SCC*mec*) may clear the exact type of MRSA isolates. Molecular typing of MRSA bacteria showed a similarity lower than 80% (except for isolate No. 10), which may show that they possess the same genetic cluster. This matter may show the expected contamination of raw milk and dairy samples by MRSA bacteria with the same molecular cluster. High similarities between the Staphylococcal isolates of other types of food samples was reported by Mohammed *et al.* (2018), Ning *et al.* (2023) and Basanisi *et al.* (2017).

CONCLUSION

In conclusion, MRSA presence in examined samples, accompanied by the high incidence of resistance toward diverse classes of antibiotic agents and different antibiotic-resistant genes. Hence, virulence factors was reported in the present survey. According to our search, the current study is one of the first and comprehensive reports assessing antibiotic-resistant properties, virulence characters, and molecular typing amongst the MRSA bacteria recovered from raw milk and dairy samples. MRSA bacteria recovered from dairy samples displayed a higher incidence of antibiotic resistance, virulence factors, and antibiotic resistance genes. Simultaneous attendance of virulence factors and antibiotic-resistance amongst the MRSA bacteria pose an imperative menace, rendering the role of consuming raw milk and dairy samples on the transmission of antibiotic-resistant and virulent MRSA bacteria to the human population. Incidence of resistance toward human-based antibiotics can indirectly show the origin of MRSA isolates. It seems that cefoxitin, ceftaroline, penicillin, tetracycline, gentamicin, trimethoprim-sulfamethoxazole, and erythromycin are not effective therapeutic agents in the cases of MRSA food-borne diseases in Iran. According to RAPD-PCR, all isolates exhibited lower than 80% similarities and were categorized in the same cluster, showing their common contamination source. Put together, the findings of the present survey showed that raw milk and dairy samples were significant sources of virulence and resistant MRSA bacteria in the community. According to the distribution of the *PVL* gene, most MRSA bacteria isolated from raw milk may be characterized as HA-MRSA, while most of those of dairy samples as CA-MRSA. However, further investigations may determine the exact role and characters of MRSA bacteria amongst the raw milk and dairy samples.

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CONFLICT OF INTEREST

No conflict of interest declared.

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