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Phenotypic and Genotypic Characteristics of Methicillin-Resistant *Staphylococcus aureus* Isolated from Dairy and Meat Products in Iran

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HIGHLIGHTS

- Five out of 93 (5.37%) isolates of *Staphylococcus aureus* included *mec*A from foodstuffs in Iran.
- Two isolates of Methicillin-Resistant *S. aureus* (MRSA) were multidrug resistant.
- MRSA may be an important hygienic risk for the Iranian food consumers.

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Acronyms and abbreviations
MIC=Minimum Inhibitory Concentration
MRSA=Methicillin-Resistant *Staphylococcus aureus*
PCR-RFLP=Polymerase Chain Reaction-Restriction Fragment Length Polymorphism
SE=Staphylococcal Enterotoxin
TSST1=Toxic Shock Syndrome Toxin 1

ABSTRACT

Background: Methicillin-Resistant *Staphylococcus aureus* (MRSA) plays an important role in gastrointestinal diseases. The goal of this research was to determine phenotypic and genotypic characteristics of MRSA isolated from dairy and meat products in Iran.

Methods: Ninety-three *S. aureus* isolates were prepared which had been obtained in our previous study. Antimicrobial susceptibility testing was done using disk diffusion method. The isolates were further analyzed by *mec*A gene detection. Staphylococcal Enterotoxins (SEs) and Toxic Shock Syndrome Toxin 1 (TSST1) were screened. Biotyping and molecular typing were done by short sequence repeats of *spa* and *coa* genes.

Results: Five out of 93 *S. aureus* isolates (5.37%) included *mec*A. All five MRSA isolates were sensitive to at least six tested antibiotics and none were resistant to vancomycin. Furthermore, two isolates were multidrug resistant. Four isolates produced SEs and TSST1. Three out of 5 isolates were related to human biotype and two belonged to non-host-specific biotype.

Conclusion: Presence of MRSA in dairy and meat products may be an important hygienic risk for the Iranian consumers, especially for immunocompromised people.

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Introduction

Methicillin-Resistant *Staphylococcus aureus* (MRSA) strains are common pathogens which are the causative agents of serious infections outbreaks (Fomda et al., 2014; Guimaraes et al., 2017). These bacteria were re-

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ported for the first time in UK in 1961, two years after the introduction of methicillin. MRSA are wide spread throughout the world, being transferred to humans via various routes, especially foods (Sergelidis and Angelidis, 2017). MRSA can be transferred to human by foods originating animals which have been resistant to β-lactams through overusing (Kwok et al., 2018). Such a food contamination can also originate from food handlers during the food production process (Hamid et al., 2017; Shojaei et al., 2006). Therefore, identification of MRSA carriers for the control and prevention of outbreaks is very important (Huh and Chung, 2016).

Colonization of MRSA is a potential risk to public health (Osman et al., 2016). However, the prevalence of MRSA varies from one country to another one and is increasing in some countries (Chen and Huang, 2014; Penteado et al., 2019). The mechanisms of methicillin resistance in MRSA are different (Hwang et al., 2007). One of the most common mechanisms is producing an altered and low-affinity Penicillin Binding Proteins (PBPs). In fact, a *mec*A gene which is transferred by mobile genetic elements encodes PBP2a (Harkins et al., 2017). There are several conventional methods for characterization of MRSA, among them molecular technique using *mec*A gene detection is used as a specific and rapid method (Fomda et al., 2014; Soltan Dallal et al., 2010a).

In Iran, there are a few publications on the epidemiological aspects of MRSA in foods. In the current study, conventional and molecular methods were used to detect MRSA in food samples. The present report showed the occurrence of MRSA isolated from dairy and meat foods by phenotypic and genotypic methods. Furthermore, characterization of these isolates was carried out using production of type A–D Staphylococcal Enterotoxin (SEs) and Toxic Shock Syndrome Toxin 1 (TSST1). Biotyping and molecular typing were done by detection of short sequence repeats of *spa* and *coa* genes.

Materials and methods

Isolates

Totally, 93 *S. aureus* isolates were obtained from our previous study (Soltan Dallal et al., 2010b). The isolates had been previously obtained from 913 food samples consisting of 455 dairy products and 458 meat products in Iran. *S. aureus* isolates had been stored in laboratory of Tehran University of Medical Sciences at -20 °C in skim milk with 10% glycerol (Merck, Germany) until use.

MRSA identification

All *S. aureus* isolates were cultured on Brain Heart Infusion (BHI) broth (Merck, Germany) and incubated overnight at 37 °C, then centrifuged, and treated with 2 µl of 10 mg/ml lysostaphin (Sigma-Aldrich, USA) for 1 h at 37 ^{o}C (Normanno et al., 2007). DNA extraction was carried out using QIA amp DNA Mini Kit (Qiagen, Germany). Purified DNA was quantified using spectrophotometry and then stored at -20 °C. The presence of *mec*A gene encoding PBP2a was confirmed using Polymerase Chain Reaction (PCR) originally described by Moon et al. (2007). Amplification of this gene was carried out using forward (5′-AAAATCGATGGTAAAGGTTGGC-3′) and reverse (5′- AGTTCTGCAGTACCGGATTTGC-3′) primers, producing a 533 bp fragment. The reaction mixture consisted of 1 µl of each primer (10 pmol/µl), 0.2 mMd NTP, 1X PCR buffer, 1.5 mM $MgCl_2$, 1 U Taq DNA polymerase, and 400 ng DNA template. The ddH_2O was added to the mixture reaction up to 50 µl. The thermal reaction was the initial denaturation for 5 min at 94 °C, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min. A final extension was done at 72 °C for 10 min. The reference strain of MRSA400 was used as positive control. PCR products were then assessed using 1% agarose gel electrophoresis under UV.

Antimicrobial susceptibility tests

Antimicrobial susceptibility test for the *mec*A positive isolates was done against ceftriaxone, ciprofloxacin, clindamycin, erythromycin, tobramycin, cefoxitin, rifampicin, tetracycline, trimethoprim/sulfamethoxazole, and ampicillin (Mast, UK) using disk diffusion method. A 0.5 McFarland turbidity standard and Mueller-Hinton agar (Merck, Germany) plates was used based on the guidelines by Clinical and Laboratory Standards Institute (CLSI, 2018). *Staphylococcus aureus* ATCC 25923 strain was used as control. Minimum Inhibitory Concentration (MIC) determination of oxacillin and vancomycin was carried out according to the CLSI guidelines using agar dilution method and spot inoculums of approximately 10^4 colony forming unit/ml (1 µl) on Muller-Hinton agar (Merck, Germany) plates with 2.5% NaCl (only for oxacillin). The isolates were classified as susceptible and resistant to oxacillin with MIC of ≤2 and ≥4 µg/ml, respectively; and susceptible, intermediate, and resistant to vancomycin with MIC of ≤2, 4-8, and ≥16 µg/ml, respectively (CLSI, 2018).

Detection of SEs and TSST1

The *mec*A positive isolates were tested for *ent* and *tst* genes by PCR. PCR amplification of *ent* gene was carried out based on a protocol by Fisher et al. (2018). Strains used as positive control for SEA-SED were included *S. aureus* ATCC 25923 (SEA), *S. aureus* ATCC

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6538 (SEC), and two further SEB and SED producing strains isolated from clinical samples.

Moreover, *mec*A positive isolates were tested for TSST1 production using Reverse Passive Latex Agglutination (TST-RPLA) (Oxoid, USA) according to the manufacturer's instructions. The *tst* gene was also detected according to Fueyo et al. (2005). *Staphylococcus aureus* RN 8465 was used as positive control for *tst* gene and *S. epidermidis* ATCC 12228 was used as negative control for SEs and TSST1.

Biotyping

Kadariya et al. (2014) proposed the simplified biotyping system for *S. aureus*. The *mec*A positive isolates were biotyped according to this system using four tests, including production of staphylokinase, production of beta hemolysis, coagulation of bovine plasma during 6 h, and growth morphology on Crystal Violet agar (Merck, Germany).

The spa gene typing of MRSA isolates

The polymorphic X region of the staphylococcal protein A (*spa*) gene was amplified using oligonucleotide primers *spa*1 (5′-ATCTGGTGGCGTAACACCTG-3′) and *spa*2 (5′-CGCTGCACCTAACGCTAATG-3′) (Soltan Dallal et al., 2016). *S. aureus* COL strain and *S. epidermidis* ATCC 12228 were used as positive and negative controls, respectively. The PCR products were analyzed using agarose gel electrophoresis. Then, amplicons were digested by the restriction enzymes of *Hind*III (Roche, Switzerland) for 16 h at 37 ℃ and *Hae*II (Roche, Switzerland) for 4 h at 37 °C, separately. Digested reactions were assessed by 2% agarose gel electrophoresis. Typeability of the *spa* typing system was estimated according to a study by Hallin et al. (2007).

The coa gene typing of MRSA isolates

Typing of MRSA isolates with the target of *coa* gene was then carried out using COAG2 (5´-CGAGACCAAGATTCAACAAG-3´) and COAG3 (5´-AAAGAAAACCACTCACATCA-3´) primers utilized by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) based on the protocol by Soltan Dallal et al. (2016).

Results

Out of 93 *S. aureus* isolates, 5 isolates (5.37%) contained *mec*A gene. The *mec*A positive isolates were derived from uncooked kebabs, raw meats, and creams; two strains were obtained from traditional ice creams. The MIC values of MRSAs present in Table 1. All the isolates were sensitive to at least six antibiotics. MRSA isolates 1, 3, and 4 were resistant to oxacillin and cefoxitin by agar dilution and disk diffusion, respectively. Moreover, MRSA isolates 3 and 4 had multiple resistant. No isolate showed resistance to vancomycin (Table 2).

As show in Table 3, three isolates produced SEC, one isolate produced SEA and SEC, and no isolate produced SEB or SED; but all these four SE producing isolates made TSST1. Three isolates belonged to the human biotype and two isolates belonged to the Non-Host-Specific (NHS) biotype. Amplification of *spa* gene showed a single amplicon with three different types (A–C) with approximately 1200, 1450, and 1550 bp on the gel (not shown). Three and four distinct RFLP patterns were achieved using *Hae*II and *Hind*III restriction enzyme digestion, respectively. Consequently, four different genotypes from *Hind*III and four different genotypes from *Hae*II enzyme digestions were identified. The *spa* type B with 1450 bp was prevalent as 6%. *Nde*I restriction enzyme digestion of the *coa* amplicons generated three different RFLP patterns and five different genotypes. Furthermore, the RFLP pattern NC_1 was found in three isolates. Typeability of *spa* and *coa* typing by PCR-RFLP was calculated 100%.

Table 1: Minimum Inhibitory Concentration of Methicillin-Resistant *Staphylococcus aureus* (MRSA) isolated from dairy and meat products in Iran

Isolates	Origin	*mec*A	Minimum inhibitory concentration (µg/ml)	
			Oxacillin	Vancomycin
MRSA1	Traditional ice cream	+	16	<2
MRSA2	Uncooked kebab	+	2	<2
MRSA3	Cream	+	32	<2
MRSA4	Traditional ice cream	+	32	<2
MRSA5	Raw meat	+	2	<2

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Table 2: Patterns of antimicrobial resistance in Methicillin-Resistant *Staphylococcus aureus* (MRSA) isolated from dairy and meat products in Iran

Isolates	Antibiotics									
	T	E	TN	RP	CD	FOX	CIP	TS	CRO	AP
MRSA1	S	S	S	S	S	R	S	S	S	S
MRSA2	S	S	S	S	S	S	I	S	S	S
MRSA3	R	S	S	S	S	R	S	S	R	R
MRSA4	R	S	S	S	S	R	S	S	R	R
MRSA5	R	S	S	S	S	S	S	S	S	R

S: susceptible; I: intermediate; R: resistant
T: tetracycline; E: erythromycin; TN: tobramycin; RP: rifampicin; CD: clindamycin; FOX: cefoxitin; CIP: ciprofloxacin; TS: trimethoprim/sulfamethoxazole; CRO: ceftriaxone; AP: ampicillin

Table 3: Molecular characterization of Methicillin-Resistant *Staphylococcus aureus* (MRSA) strains isolated from dairy and meat products in Iran

Characteristics	Isolates				
	MRSA1	MRSA2	MRSA3	MRSA4	MRSA5
Origin	Traditional ice cream	Uncooked kebab	Cream	Traditional ice cream	Raw meat
SE	SEC	SEC	SEC	-	SEC+SEA
TSST1 (PCR, RPLA)	+	-	+	-	+
Biotype	Human	NHS4 (K+β+CV:A)	NHS4 (K+β+CV:A)	Human	Human
coa gene					
coa type (bp)	850 (B)	900 (C)	950 (D)	800 (A)	1000 (E)
*Nde*I RFLP pattern	NC3	NC1	NC2	NC1	NC1
*Nde*I genotype	B3	C1	D2	A1	E1
spa gene					
spa type (bp)	1250 (A)	1450 (B)	1450 (B)	1550 (C)	1450 (B)
*Hae*II RFLP pattern	*Hae*S_3	*Hae*S_2	*Hae*S_1	*Hae*S_1	*Hae*S_1
*Hind*III RFLP pattern	*Hind*S1	*Hind*S2	*Hind*S1	*Hind*S4	*Hind*S3
*Hae*II genotype	A3	B2	B1	C1	B1
*Hind*III genotype	A1	B2	B1	C4	B3

NC1: 80-170-330 bp; NC2: 80-250-330 bp; NC3: 80-330 bp; *Hae*S1: 300-500-800 bp; *Hae*S2: 300-500-900 bp; *Hae*S3: 300-800 bp; *Hind*S1: 150-550-750 bp; *Hind*S2: 150-550-850 bp; *Hind*S3: 150-550-950 bp; *Hind*S4: 550-1050 bp

Discussion

We found that of 5 from 93 *S. aureus* isolates among dairy and meat products from Iran contained *mec*A gene, indicating resistance to methicillin. The prevalence of MRSA isolates obtained from nosocomial infections in Iran is 47.6% in 2007 (Najar Peerayeh et al., 2009). However, in high risk people, MRSA isolates can reach the circulatory system and cause serious infections which may be fatal (Osman et al., 2016). Food can be considered as a main route for the entrance of pathogenic MRSA isolates within the population, especially in immunocompromised people (Rahimi, 2013). Using of antibiotics in animals resulted in transfer of resistance genes between non-pathogenic, opportunistic, as well as pathogenic bacteria, and consequently increasing the antimicrobial resistance (Asiimwe et al., 2017; Osman et al., 2016; Soltan Dallal et al., 2016). Several reasons exist for the transfer of these resistant strains to humans, including: 1) contaminated animal foods, 2) antibiotic residues in foods, and 3) food handlers as MRSA carriers (Vojkovská et al., 2017).

Similar molecular subtyping patterns may be existed between clinical isolates of *S. aureus* from hospitals and nonclinical isolates of *S. aureus* from food samples, suggesting the possibly transfer of MRSA between people in clinical and hospital environments (Soltan Dallal et al., 2016). Based on our knowledge, a few studies have detected MRSA strains in various foodstuffs (Hwang et al., 2007; Jackson et al., 2013; Osman et al., 2016; Ou et al., 2017). In the present investigation, 5 MRSA strains were identified with the target gene of *mec*A while disk diffusion of cefoxitin and MIC tests of oxacillin characterized three MRSA strains. On the other hand, the discrepancies observed between the phenotypic and genotypic results may be mainly attributed to the heterogeneous expression of resistance gene (Safarpoor Dehkordi et al., 2017). Similar with our findings, Corrente et al. (2007) found that six out of 200 (3%) *S. aureus* strains in foods of animal origin in Italy included MRSA. Jackson et al. (2013) reported only one MRSA strain in 100 (1%) beef products in Georgia, USA. Also, MRSA strains were

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found in 0.4 and 0.2% food samples from Italy (Normanno et al., 2007) and South Korea (Hwang et al., 2007), respectively. However, in a study in Jammu and Kashmir, India, 6 out of 160 milk samples (3.75%) were contaminated with MRSA (Hamid et al., 2017) which may be due to the improper hygiene, poor farm management practices, and overuse of antibiotics (Hamid et al., 2017).

It is well known that MRSA strains are resistant to beta-lactams (Normanno et al., 2007), but two *mec*A positive strains were susceptible to ampicillin in the current study. In agreement with the other previous studies (Asiimwe et al., 2017; Manukumar and Umesha, 2017; Osman et al., 2016), all MRSA strains in this study were susceptible to vancomycin, suggesting that the resistance to vancomycin is really limited to bacteria originating from hospitals. Furthermore, 4 out of 5 MRSA strains were able to produce SEs in the current work. It is worthy to note that all other SEC producing strains (except one) were TSST1 positive simultaneously. Also, the correlation between the production of SEC and TSST1 has already been reported by others (Khemiri et al., 2018; Penteado et al., 2019).

We found that most MRSA isolates belonged to the human biotype, suggesting that food handlers may be the primary source of food contamination which was also stated by Jackson et al. (2013). However, according to Capita et al. (2002), strains belonging to both NHS and human biotypes are mostly toxigenic and potentially include high risks for the public health. Only a few studies have already characterized MRSA genotypically in foods. Although, pulse field gel electrophoresis is the gold standard for genotyping of *S. aureus* strains, it is relatively a time consuming and difficult to set up method. Molecular typing based on the short sequence repeats such as X region of the protein A and 3´ end of the *coa* genes has been considered as a simple and accurate method for MRSA (Asiimwe et al., 2017; Hallin et al., 2007). Similar to genotyping of *coa* gene, *spa* types were subdivided into several subtypes, meaning that several genotypes were identified for each enzyme (Safarpoor Dehkordi et al., 2017).

Interestingly, no similar or common genotype (except one) existed for each restriction enzyme, demonstrating the genetic diversity of MRSA in the current survey. It should be indicated that RFLP pattern of *Hae*S_1 was the predominant type in this study. This finding was similar to that obtained previously from clinical MRSA strains in a study from Iran (Safarpoor Dehkordi et al., 2017), proposing that the source of these strains may be the same. In the current study, RFLP patterns of *coa* gene using *Nde*I digestion suggested that the isolated *S. aureus* transfer more than one variant of the *coa* gene. These results are similar to those reported by Mohammed et al. (2018) in Tanzania and Asiimwe et al. (2017) in South-West Uganda who worked on mastitic milk. This may be due to the coevolution of the pathogens and their host, as well as differences in reservoirs.

Conclusion

This study showed that the presence of MRSA strains in dairy and meat products may be an important hygienic risk for the Iranian consumers, especially for immunocompromised people. Molecular typing of MRSA could be considered as a useful tool for the screening of suspected outbreaks and tracking of the contamination sources. Further studies on molecular characterization of MRSA in food chain are suggested.

Author contributions

M.M.S.D. designed the project; Z.S. wrote the manuscript, M.K.S.Y. analyzed and interpreted the data; R.B. conducted the experiments; M.A. revised and edited the manuscript. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare that they have no conflict of interest.

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