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Original Article

Molecular Characterization and Antibiotic Resistance Profile of Methicillin-Resistant *Staphylococcus aureus* (MRSA) Strains Isolated From Milk Samples of Apparently Healthy Cattle in Hamedan, Iran

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Abstract

Background: *Staphylococcus aureus*, as a major food-borne pathogen, is the most commonly isolated bacterium from bovine mastitis. However, some *S. aureus* strains exhibit a high rate of antibiotic resistance, among which, methicillin-resistant *S. aureus* (MRSA) is very important. The present study was conducted to isolate, characterize, and determine the antibiotic resistance profile of MRSA strains in milk.

Methods: *Staphylococcus aureus* strains were isolated and identified from 415 milk samples collected from apparently healthy cattle in Hamedan province, Iran. Molecular characteristics of the strains were identified using multiplex polymerase chain reaction (PCR) and the antibiotic resistance profile of the isolates was determined by Kirby-Bauer disk diffusion susceptibility test.

Results: A total of 76 *S. aureus* strains were isolated and identified. The PCR results indicated that 50 (65.78%) isolates possessed *mecA* gene and were found to be MRSA strains. Twelve isolates (15.78%) showed phenotypic resistance to oxacillin in disk diffusion method. All 76 *S. aureus* isolates (100%) were resistant to penicillin and susceptible to ciprofloxacin and gentamicin.

Conclusion: The results of the present study indicated that bovine milk may contain MRSA strains and this is worrying as these isolates may transfer multi-drug resistance to the isolates that circulate among humans, animals, and food chains.

Keywords: Bovine mastitis, Milk, Methicillin-resistant *Staphylococcus aureus*, Multi-drug resistance

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Introduction

Staphylococcus aureus is an opportunistic bacterial pathogen which causes a wide range of illnesses such as impetigo, cellulitis, pneumonia, osteomyelitis, endocarditis, bacteremia, and toxic shock syndrome and food poisoning in humans, as well as mastitis, dermatitis, and arthritis in animals worldwide (1-3). Among these diseases, bovine mastitis has been known as the most prevalent and costly disease in dairy industry worldwide (4,5).

Various microorganisms including bacteria, viruses, fungi, and algae are involved in bovine mastitis, among which staphylococci, streptococci, and Gram-negative bacilli are the major etiological agents of bovine mastitis (6-9). *Staphylococcus aureus* is the most common causative agent and frequently isolated bacterium from bovine mastitis (6,10-13). In Iran, various investigations based on conventional microbiology methods and molecular techniques described some characteristics and antibiotic susceptibility profile of *S. aureus* strains as a major causative agent of bovine mastitis (14,15). However, increased resistance of *S. aureus*, isolated from mastitic cows to a wide array of commonly used antibiotics, has been reported in several studies (16-18).

Different groups of antibiotics including β-lactams, tetracycline, macrolides, and so on are commonly used to treat bovine mastitis. Hence, it is important to monitor antibiotic resistance patterns of *S. aureus* isolates, especially those which are resistant to methicillin. Methicillin is one of the recently developed antibiotics, and resistance against this antibiotic is very important. *S.*

aureus isolates are divided into two groups: methicillin-resistant *S. aureus* (MRSA) and methicillin-susceptible *S. aureus* (MSSA). Consequently, the presence of MRSA strains in milk samples could be a major concern for public health.

Resistance to methicillin in *S. aureus* is mediated by *mecA* gene which is located on the chromosome and encodes a penicillin-binding protein 2a with reduced affinity for β-lactams (19,20). Therefore, *mecA* gene can be used as a molecular marker to detect methicillin-resistant isolates. However, other factors such as *femA* operon, which contains regulatory genes, are essential for the expression of methicillin-resistance in *S. aureus* as well (21). However, *fem* genes were suggested to be specific for *S. aureus* and can be used for molecular identification of *S. aureus* isolates (22).

Given that the presence of MRSA strains, as major food-borne bacteria, in a common food source like milk is very important in terms of public health and the spread of antibiotic resistance in bacterial populations, the present study was conducted to isolate, characterize, and determine antibiotic resistance profile of MRSA strains in cattle with no clinical signs in dairy farms of Hamedan province, west of Iran.

Materials and Methods

Sampling

A total of 415 milk samples were collected from healthy cattle (the cattle with a good appetite, normal rumination, normal milk production, apparently healthy milk, normal udder tissue, and with no clinical signs of mastitis) belonging to 7 dairy farms in Hamedan province of Iran. These milk samples were collected in two seasons, warm-dry (n = 234) and cold-wet (n = 181). Microscopic somatic cell count (SCC) and California mastitis test (CMT) were carried out on the collected milk samples for direct and indirect estimation of the number of somatic cells, respectively. SCC and CMT are known as reliable indicators of chronic intramammary infection (23). Milk samples which contained over 300 000 cells/mL were considered positive cases of subclinical/chronic bovine mastitis in SCC test, while a score of one or more was considered positive in CMT (24).

Isolation and Identification of Staphylococcus aureus

Milk samples (100 µL) were inoculated on 5% sheep blood agar (Merck, Germany) at 37°C for 24 hours. Afterwards, conventional microbiological tests including microscopic examination, catalase test, coagulase test, and attributes on the mannitol salt agar and DNase agar (Merck, Germany) were carried out on the suspected colonies for the identification of *S. aureus*.

Antimicrobial Susceptibility of Staphylococcus aureus Isolates

Staphylococcus aureus isolates were tested for antimicrobial susceptibility to 9 antibiotics: penicillin (16 IU), ciprofloxacin (5 µg), oxacillin (1 µg), chloramphenicol (30 µg), gentamicin (10 µg), vancomycin (30 µg), streptomycin (10 µg), tetracycline (30 µg), and cefixime (5 µg). All antibiotic discs were purchased from Padtan Teb® (Padtan Teb Co, Iran). Antimicrobial susceptibility testing was performed on Mueller-Hinton agar (Merck, Germany) using disc diffusion method, as described in the guidelines of the Clinical and Laboratory Standards Institute (CLSI 2019). The results were categorized as susceptible, intermediate, or resistant according to the above-mentioned guidelines (25). Quality control was performed using *S. aureus* ATCC 25923 as the reference strain.

DNA Extraction

Total DNA was extracted from the phenotypically characterized bacterial isolates using the previously described method with some modifications. Briefly, the isolates were grown in nutrient broth at 37°C for 24 hours. Thereafter, 3 mL of bacterial suspension was centrifuged at 8000 rpm for 3 minutes and 200 µL of a lysis buffer (1% Triton X-100, 0.5% Tween 20, 10 mM Tris-HCl, 1 mM EDTA, pH = 8.0) was added to the pellets and microtubes were incubated in a boiling water bath (100°C) for 10 minutes, followed by centrifugation at 10 000 rpm for 2 minutes. The supernatants were transferred into clean microtubes, and 3-5 µL of each sample was used as template DNA in polymerase chain reaction (PCR) assays (26).

Multiplex Polymerase Chain Reaction

Staphylococcus aureus isolates were examined by a multiplex PCR assay which simultaneously targeted both *femA*, a species-specific gene for definite identification of S. *aureus*, and *mecA* genes to genetically detect MRSA strains using primers previously described (27). The characteristics of the primers are given in Table 1.

PCR amplifications were performed in a SimpliAmp™ Thermal Cycler (Applied Biosystems, USA). The reaction mixtures consisted of 3-5 µL of DNA template, 12.5 µL of 2X Master Mix (Ampliqon, Denmark), and 1 µL of each primer pair (25 pmol- TakapouZist, Iran), and the final volume of the reaction mixture was brought up to 25 µL using distilled deionized water. PCR program for amplification of *femA* and *mecA* genes consisted of initial denaturation at 94°C for 5 minutes, 35 cycles of amplification with denaturation at 94°C for 2 minutes, annealing at 57°C for 2 minutes, extension at 72°C for 1 minute, and a final extension at 72°C for 1 minute. The PCR products were analyzed in 2% agarose gel containing 0.5 µg/mL of ethidium bromide and subjected to electrophoresis in 1X TAE buffer. Gels

Table 1. Oligonucleotide Primers Used in the PCR Assay

Primer	Oligonucleotide Sequence (5'- 3')	Target Gene	Amplicon Size (bp)
GFEMAR-1 GFEMAR-2	AAAAAAGCACATAACAAGCG GATAAAGAAGAAACCAGCAG	*femA*	132
GMECAR-1 GMECAR-2	ACTGCTATCCACCCTCAAAC CTGGTGAAGTTGTAATCTGG	*mecA*	163

were visualized under UV light and documented using UVITEC gel documentation system (Transilluminator, France). A sample containing no DNA was used as the negative control in all PCR runs. Furthermore, standard methicillin-susceptible *S. aureus* (MSSA, ATCC 25923) and methicillin-resistant *S. aureus* (MRSA, ATCC 33591) were used as controls for PCR optimization (28).

Statistical Analysis

The data were analyzed using student's *t* test by SAS software volume 8.2, and $P<0.05$ was considered to be statistically significant.

Results

In this study, the results of SCC test and CMT on the 415 collected milk samples showed that 114 (27.47%) and 132 (31.81%) samples were positive in CMT and SCC test, respectively. There was no statistically significant difference between the results of CMT and SCC test for the collected milk samples in the two seasons ($P>0.05$). However, the correlation between the results of the two methods was high ($r=0.948$, $P<0.05$).

Staphylococcus aureus was isolated from 83 out of 415 milk samples. Seventy-six isolates (18.31%) showed the expected DNA fragment of 132 bp in PCR and were genotypically identified as *S. aureus* (Figure 1). In warm-dry and cold-wet seasons, 51 and 25 *S. aureus* isolates were identified by genotypic method, respectively.

The *mecA* gene was detected in 50 (65.78%) out of 76 isolated *S. aureus* (Figure 1), among which 12 (24%) isolates phenotypically showed resistance to oxacillin.

Meanwhile, the antimicrobial susceptibility test (disc diffusion method) was done to check the resistance of the isolates to various antibiotics including penicillin, oxacillin, ciprofloxacin, tetracycline, gentamicin, streptomycin, vancomycin, chloramphenicol, and cefixime and the results indicated that all of the 76 *S. aureus* isolates (100%) were resistant to penicillin and susceptible to ciprofloxacin and gentamicin. The detailed resistance profiles of the isolates are presented in Table 2.

Discussion

Bovine mastitis is one of the most important diseases affecting the dairy industry worldwide, and *S. aureus* is the main causative agent of bovine mastitis which responds poorly to antimicrobial therapy (29,30). The prevalence of *S. aureus* in our study was similar to previous reports from Iran and other parts of the world. Hashemi et al reported that the most frequently isolated bacteria as the cause of mastitis were coagulase-positive staphylococci in Fars province, south of Iran (31). Besides, *S. aureus* prevalence rates of 10 to 30% have been documented in mastitis-affected cattle (7). Makovec and Ruegg reported that *S. aureus* was isolated from 9.7% of cows in the United States (32).

In this study, 76 isolates were confirmed as *S. aureus* using PCR technique. Several studies suggested molecular technique as a specific, highly sensitive, and rapid method for detection and identification of *S. aureus* (33-35).

The current study results revealed that in the warm-dry season, the prevalence of *S. aureus* was higher compared to the cold-wet season, which is in accordance with the results of a study conducted by Koivula et al, indicating that S. *aureus* was more prevalent in spring and November in Southern Finland (36).

The results of this study indicated that *S. aureus* caused an increase of the SCC in the collected milk samples, which is in agreement with the findings of Jánosi and Baltay (23) and Balemi et al (37). They reported that all pathogens (including S. *aureus*) resulted in a significant increase of the SCC in individual bulk milk samples collected from dairy cows, camels, and goats.

The current study results indicated that 50 out of 76 isolates (65.78%) possessed *mecA* gene and were found to be MRSA strains. The present findings about *mecA* gene were in accordance with other studies which reported that molecular techniques such as multiplex PCR can be of great use for the diagnosis of methicillin-resistant bacteria (7,16,22,38). However, some researchers described that the *mecA* gene is carried by 95% of *S. aureus* isolates that display a phenotype of methicillin resistance (39). Results of a study performed by Pérez-Roth et al on various *S. aureus* strains isolated from patients in Spain revealed that *mecA* gene was present in 29 (58%) isolates (40).

In this study, the frequency of antibiotic resistance in the tested *S. aureus* strains was similar to that found in another study in Iran (41-43). Previous studies examining

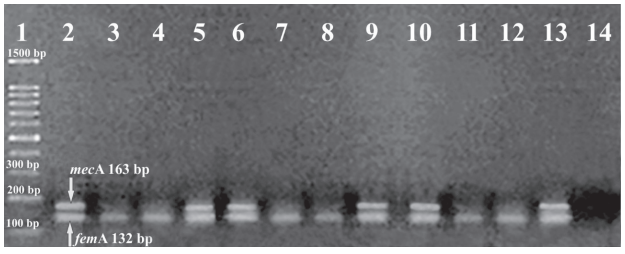

Figure 1. Agarose Gel Electrophoresis of Multiplex PCR Products of *femA* and *mecA* Genes in *Staphylococcus aureus*. Lane 1: a 100-bp ladder, Lane 2: *S. aureus* ATCC 33591, positive control for *femA* and *mecA* genes. Lanes 3-13: *femA*-positive *S. aureus* isolates. Lanes 5, 6, 9, 10 and 13: *mecA*-positive *S. aureus* isolates. Lane 14: negative control (contained no template)

Table 2. The Antibiotic Resistance Profile of the *Staphylococcus aureus* Isolates

Antibiotic Agent	Resistant No. (%)	Intermediate No. (%)	Susceptible No. (%)
Penicillin	76 (100)	0 (0)	0 (0)
Ciprofloxacin	0 (0)	0 (0)	76 (100)
Oxacillin	12 (15.78)	0 (0)	64 (84.21)
Chloramphenicol	1 (1.31)	73 (96.05)	2 (2.63)
Gentamicin	0 (0)	0 (0)	76 (100)
Vancomycin	13 (17.1)	0 (0)	63 (82.89)
Streptomycin	28 (36.84)	11(14.47)	39 (51.31)
Tetracycline	30 (39.47)	2 (2.63)	44 (57.89)
Cefixime	9 (11.84)	8 (10.52)	59 (77.63)

the antimicrobial susceptibility of *S. aureus* isolated from bovine mastitis demonstrated a high frequency of resistance to tetracycline, penicillin, and erythromycin among *S. aureus* isolates (12,16,44,45). In this study, gentamicin and ciprofloxacin were completely effective against *S. aureus* and 100% of the isolates were susceptible to the mentioned drugs. This result was in agreement with reports which indicated that gentamicin was very active against *S. aureus* (12).

Moreover, of 50 isolates containing *mecA* gene, 12 (24%) isolates phenotypically showed resistance to oxacillin. Khazaie and Ahmadi reported that among the 95 isolates of *S. aureus* from bovine subclinical mastitis in Iran, 11 (11.57%) strains were recognized as MRSA (41). In addition, various studies reported that there are some strains which are phenotypically resistant to oxacillin despite the fact that they carry *mecA* gene (46-48). These studies support our findings.

Conclusion

The present study was a survey on the molecular characterizations and antibiotic resistance profile of *S. aureus* isolated from milk samples of cattle with no clinical signs in Hamedan, north-west of Iran. The strains had a high frequency of resistance to penicillin indicating that this antibiotic is not suitable for use in dairy cattle herds in this region. However, the recommended antibiotics for the treatment of mastitis are gentamicin and ciprofloxacin. Besides, a large number of MRSA strains were identified in the present study which is absolutely a major concern for public health. Given that even apparently healthy milk may contain MRSA strains and as these isolates can circulate among humans, animals, and food chains, appropriate hygiene measures should be taken to control and decrease infections caused by these bacteria. Meanwhile, it is strongly recommended that antibiotic susceptibility of isolates should be checked before any treatment.

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Authors' Contribution

Conceptualization: Abdolmajid Mohammadzadeh, Mohamadreza Pajohi-Alamoti, and Ali Sadeghi-Nasab.
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All authors have read and approved the final version of the manuscript.

Competing Interests

The authors declare that there is no conflict of interest.

Ethical Approval

All samples were collected from industrial dairy farms and sent to the laboratory under supervision of a veterinarian.

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