

Research Paper: Frequency of *clf-A*, *mec-A*, and *mec-C* Genes in *Staphylococcus Aureus* Strains Isolated From Nosocomial Infections and Cow's Milk

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Samira Sangarifar obtained MSc. in Biological Sciences from Islamic Azad University, Falavarjan, Iran. Her research includes virulence gene (*clf-A*) and resistance genes (*mec-A*, *mec-C*) in methicillin-resistant *Staphylococcus Aureus* strains isolated from nosocomial infections and cow's milk in Esfahan.

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ABSTRACT

Introduction: There are various virulence factors encoded by *Staphylococcus Aureus*, which enable them to cause nosocomial infections and mastitis in dairy cattle. The *clf-A* gene mediates the bacterial colonization through binding to the extracellular matrix of the host. Treatment of such infections becomes more difficult due to increased resistance to methicillin. The current study aimed at investigating the frequency of *clf-A*, *mec-A*, and *mec-C* genes in *S. Aureus* strains isolated from hospital infections and cow's milk.

Methods: A total of 280 clinical samples as well as 100 milk samples from cattle with mastitis were collected. After identification of isolates, methicillin-resistant strains were identified using agar screening and the ceftoxitin disc diffusion test. In addition, the Minimum Inhibitory Concentration (MIC) value of oxacillin was determined using the E-test method. Polymerase Chain Reaction (PCR) was used to detect *clf-A*, *mec-A*, and *mec-C* genes in the methicillin-resistant isolates. Out of 120 *S. Aureus* strains isolated from nosocomial infections, 40 were identified as Methicillin-Resistant *Staphylococcus Aureus* (MRSA), while only 1 isolate from cow's milk was MRSA.

Results: Out of the 280 studied isolates, 35 clinical strains (87.5%) carried *mec-A* gene, and the frequency of *clf-A* gene was 80% and 100% in clinical and bovine milk samples, respectively. Importantly, MRSA strains harbouring *clf-A* gene, isolated from wound samples, exhibited the highest frequency. The *mec-C* gene was not found in clinical and milk isolated strains. The high frequency of *clf-A* gene in MRSA strains isolated from wound indicated a probable role of this virulence factor in skin colonization, as well as distribution and spread of the strains.

Conclusion: Development of an appropriate method seems to be particularly useful for preventing the distribution of such strains.

Keywords:

MRSA, *mec-A*, *mec-C*, *clf-A*, Nosocomial infection, Cow's milk

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1. Introduction

S *taphylococcus Aureus* cause a wide range of diseases in human beings, ranging from skin infections to serious diseases such as sepsis, endocarditis, and pneumonia [1]. Methicillin-Resistant *Staphylococcus Aureus* (MRSA), although acquired from hospitals, is rapidly emerging among livestock [2]. MRSA can colonize and subsequently infect dogs, cats, pigs, cattle, and horses [3]. MRSA infections are generally classified into 2 major groups: Community-Acquired MRSA (CA-MRSA) and Hospital-Acquired MRSA (HA-MRSA).

CA-MRSA differs from HA-MRSA in several respects including the spectrum of disease, clinical symptoms, and antibiotic resistance patterns. CA-MRSA usually causes skin and soft tissue infections and shows resistance to beta-lactam antibiotics, while HA-MRSA strains are equipped with a wide range of antibiotic resistance mechanisms [4]. In general, CA-MRSA strains are susceptible to several non-beta-lactam antibiotics including trimethoprim-sulfamethoxazole, tetracycline, and clindamycin [4]. The *mec-A* gene, carried by a mobile genetic element called Staphylococcal Cassette Chromosome *mec* (SCC*mec*), is well-known to be responsible for methicillin resistance in *S. Aureus* [2]. Penicillin Binding Protein 2a (PBP2a), encoded by *mec-A* gene, displays a low affinity for beta-lactam antibiotics [3, 5]. Therefore, isolates harbouring *mec-A* gene are resistant to various types of beta-lactam antibiotics [6].

A new *mec-A* homologue gene, known as *mec-C* (previously called *mecALGA251*) coded in a new SCC*mec* type, was identified as type XI. The homologue gene has 70% and 63% similarities at DNA and protein levels, respectively [3]. The *mec-C* gene exists in MRSA strains isolated from human and dairy cattle samples, but its origin and epidemiology is not clear. Some studies showed that *mec-C* gene might be transmitted from cattle to humans [3]. MRSA strains harbouring *mec-C* were reported rarely in human and other hosts in some European countries, but not in Iran [7]. This new mechanism of methicillin-resistance in *S. Aureus* may be a new threat to public health. Worldwide spread of *mec-C* in humans and animals should be studied and controlled [6].

Staphylococcus Aureus has specific adhesion factors, which make it able to bind to different proteins of the host. The binding is mediated by a group of proteins called microbial surface components recognizing adhesive matrix molecules such as SpA, Fnbp A, *Clf-A* and ClfB, which are covalently bound to bacterial peptido-

glycan [8, 9]. The *Clf-A* is actually a fibrinogen-binding protein, which prevents the phagocytosis of *S. Aureus* by macrophages and inactivates C₃b component of complement and enables the bacteria to scape host immune system [10]. Studying the simultaneous presence of pathogen genes such as *clf-A* and resistance genes of *mec-A* and *mec-C* in MRSA strains can provide valuable epidemiological information to control and treat infections caused by such bacteria. The current study aimed at detecting these genes in MRSA strains isolated from clinical samples and cow's milk.

2. Materials and Methods

Sample collection

A total of 280 clinical samples including wound, blood, skin abscesses, joint fluid, sputum, and respiratory tract infections were collected from hospitals in Isfahan, Iran. Out of which, 120 strains were identified as *S. Aureus* using the conventional biochemical tests. In addition, 100 milk samples from cattle with mastitis were collected from Isfahan farms, in which 20 MRSA strains were isolated.

Disk diffusion testing

For antibiotic susceptibility testing, bacterial suspension was prepared in Muller-Hinton Broth (MHB) and adjusted to a turbidity equivalent to a 0.5 McFarland standard. Afterwards, a sterile swab was dipped in the bacterial suspension and plated onto MHA using the sweep plate method. Subsequently, a cefoxitin disk was placed on the plates, followed by incubation at 35-33°C for 24 hours. The inhibition zone diameter was measured using a particular ruler. The diameter of inhibition zone was interpreted as sensitive or resistant, according to the Clinical and Laboratory Standards Institute (CLSI) guidelines, 2014. Isolates with an inhibition zone diameter less than or equal to 21 mm, and greater than 22 mm were considered positive and negative for *mecA*, respectively [11]. Oxacillin agar screening was applied to isolate strains containing *mec-A* [11]. For this purpose, MHA medium containing 4% salt and 6 µg/mL oxacillin (Sigma) was used according to CLSI guidelines. Strains were incubated for 24 hours and a 0.5 McFarland suspension was prepared in sterile saline and inoculated with 20 µL spots. Upon the absorption of the suspension, plates were incubated at 37°C for 24 hours and then, the growth of isolates was examined.

MIC assessment

To determine the Minimum Inhibitory Concentration (MIC) of oxacillin, a suspension equivalent to a 0.5 Mc-

Farland standard was prepared for MRSA strains according to CLSI guidelines and then, the E-test Biofil Chem strips were placed onto the inoculated agar surfaces [11]. Following 24-hour incubation at 35°C, MIC was recorded for each strain. Strains with MIC ≤ 2 and >4 $\mu\text{g/mL}$ were reported as sensitive and resistant, respectively. It is important to note that ATCC 25932 and ATCC 33591 strains were used as negative and positive controls, respectively.

Polymerase chain reaction

After the identification of methicillin-resistant strains, PCR reaction was used to amplify *mec-A*, *mec-C* and *clf-A* genes.

The primer sequences were as follows: *mecA-F*, CTATCTCATATGCTGTTCCTGTATTGGC; *mecA-R*, TGGTATGTGGAAGTTAGATTGGGAT; *Clf-A-F*, CAAAATCCAGCACAAACAGGAAACGA; *Clf-A-R*, CTTGATCTCCAGCCATAATTGGTGG; *mecC-F*, TG-GCTGAACCCATTTTGGAT; *mecC-R*, CATTAAAAT-CAGAGCGAGGC [4, 12].

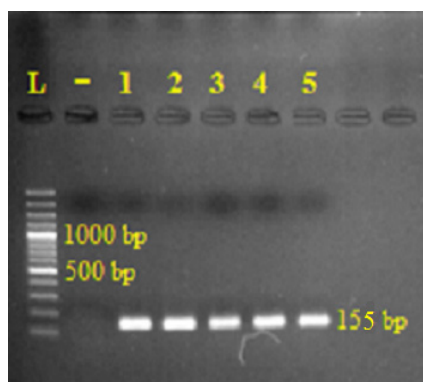
DNA was extracted using the boiling method [13, 14]. PCR amplification was performed in a final volume of 25 μL , containing 0.3 μL prime Taq premix, 10.2 μL sterile distilled water, 4 μL MgCl_2 , 2.5 μL 10X PCR-buffer, 2 μL dNTPs, 0.5 μL of forward and reverse primers, and 5 μL of DNA template. PCR amplifications were conducted on a Thermocycler under the following conditions: an initial denaturation at 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, temperatures at 56°C, 52°C and 59°C each for 45 seconds to anneal primers *mec-A*, *mec-C* and *clf-A* to the DNA template, respectively, and elongation at 72°C for

60 seconds with a final elongation at 72°C for 5 minutes. After PCR amplification, the PCR products were fractionated by electrophoresis on gel agarose. To confirm PCR fidelity, products of the expected sizes (155 bp and 638 bp corresponding to *mec-A* and *clf-A*, respectively) were sequenced, followed by analysis using the Chromas program (45.1). The results were analyzed using Chi-square and the Fisher tests with SPSS version 16.

3. Results

Staphylococcus Aureus was found in 120 and 20 isolates from 280 clinical and 100 milk samples, respectively. Surprisingly, the majority of the strains (45%) were isolated from wound specimens. Among 120 *S. Aureus* strains isolated from clinical samples, 40(32.3%) showed phenotypic resistance to oxacillin using agar screening and E-test methods. However, only 1 *S. Aureus* strain (5%) isolated from 20 milk samples exhibited resistance to oxacillin. Molecular studies showed the presence of *mec-A* gene in 87.5% and 5% of clinical and milk isolated MRSA strains, respectively (Figure 1). Frequencies of *clf-A* in clinical and milk isolated strains were 26.7% and 5%, respectively (Figure 2). Neither milk nor clinical strains carried *mec-C* gene.

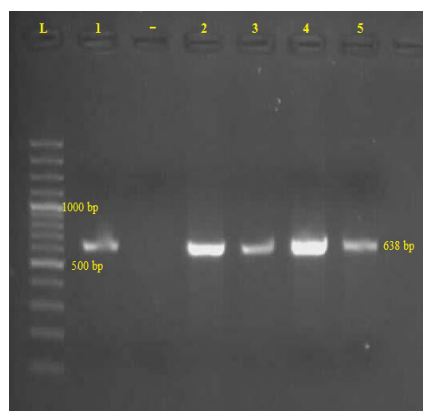
The Chi-square test showed that *mec-A* was significantly more frequent in clinical samples, compared with that of milk samples ($P=0.01$). Furthermore, the Fisher test demonstrated that *clf-A* was significantly more frequent in clinical samples, compared with milk samples ($P=0.02$). The frequency of *clf-A* was higher in strains isolated from wound samples. The frequency of this gene in MRSA strains was significantly higher than other infectious samples (Table 1).



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Figure 1. PCR amplification; *mec-A* gene was detected in MRSA isolates (155 bp in length)

Lane (L): 100-bp Ladder; Lane (-): negative control; Lanes (1-4): clinical samples; Lane (5): milk sample



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Figure 2. PCR amplification; *clf-A* gene was found in MRSA isolates (638 bp in length)

Lane (L): 100-bp Ladder; Lane (1): milk sample; Lane (-): negative control; Lanes (2-5): clinical samples

Table 1. Frequency of *mec-A* and *clf-A* in *S. Aureus* strains isolated from human infections

Infection Type	Total Number	<i>mec-A</i> Number (%)	<i>clf-A</i> Number (%)
Wound	54	13(24.07)	13(24.07)
Blood	23	19(39.1)	7(30.4)
Abscess	19	5(26.3)	4(21.2)
Synovial fluid	9	5(55.6)	5(55.6)
Trachea	9	3(33.3)	3(33.3)
Sputum	6	0(0)	0(0)
Total	120	35(29.2)	32(26.7)

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4. Discussion

Staphylococcus Aureus is one of the most common pathogens causing nosocomial and community-acquired infections. The current study aimed at evaluating the methicillin-resistance profile of *S. Aureus* strains isolated from clinical specimens and cows with mastitis. Results showed that the frequency of MRSA strains in wound samples was higher than that of other clinical samples. Consistent with the current study findings, different studies showed a high frequency of MRSA strains in wound samples [15, 16].

In the present study, the high frequency of *mecA* was detected in 35 (87.5%) clinical samples, while only 1 strain carried *mecA* gene (100%). It should be noted that among 20 strains isolated from milk, only 1 was phenotypically identified as MRSA. The methods employed in the current study, oxacillin E-Test, cefoxitin disk diffusion, and oxacillin agar screening, showed similar results, representing the potential value of the findings. According to the current study and other studies findings, MRSA strains can easily be determined by the disk diffusion method due to its high sensitivity and low cost. Numerous studies were conducted on methicillin-resistant strains so far [7, 17-19]. The frequency of MRSA strains isolated in the current study was higher than that of another study carried out in Iran, showing the increased prevalence of MRSA strains in Isfahan [20].

Findings of the current study revealed a significant difference in the frequency of *mec-A* gene between strains isolated from cow's milk and human infections. It can be due to the use of aseptic handling techniques in cattle husbandry systems, as well as the lower use of penicillin

for the treatment of MRSA infections in cattle because of high cost and difficult injections. The high frequency of *mec-A* in strains isolated from wound is also concerning. In the present study, the highest frequency of *mec-A* corresponded to the strains isolated from synovial fluid, which is inconsistent with the results of other studies. Different findings in the current study might reflect epidemiologic differences [21, 22].

The present study failed to detect *mec-C* gene. To the best of authors' knowledge, no study in Iran carried out on this gene. MRSA strains harbouring *mec-C* were rarely identified in human infections [23]. Most of *mec-C*-carrying strains in Denmark were found in rural areas; in a study conducted on 22 patients, it was demonstrated that 4 of the patients had substantial animal contact [24]. The frequency of this gene was reported 0% to 0.008%. In a study performed in Germany, *mec-C* was identified in only 2 strains out of 3207 MRSA strains (0.062%) [25]. Among 8757 *S. Aureus* strains isolated from mastitis, 4 (0.046) were identified as *mec-C*-carrying MRSA [26]. However, a study carried out in the Western Swiss showed that 565 MRSA strains lacked *mec-C* gene [27].

The main limitation of the current study to detect this gene was the low number of samples (both milk and clinical samples). The ability of *S. Aureus* to bind to fibrinogen and fibrin is an important factor in colonization and wound infection [28]. It is believed that there is a relationship between the severity and spread of the disease with virulence factors produced by *S. Aureus* [29]. The *clf-A* gene encodes a surface protein in bacteria; this protein plays a role in biofilm formation. The high frequency of *clf-A* in MRSA strains isolated from wound infections is worrying because this gene has a role in

bacterial colonization in skin and causes the spread of MRSA strains from wound infections to hospital and health care sectors. Due to the high frequency of MRSA strains in clinical samples, good practice is essential to prevent the spread of such strains.

In the present study, MRSA strains carrying *clf-A* were 32 (80%), while it was traced only in 1 cow's milk sample (100%), which is in agreement with the results of other studies [12, 30]. The highest frequency of *clf-A* was found in MRSA strains isolated from wound, whereas Painagua et al. reported that *S. Aureus* species isolates from catheter has the most frequent *clf-A* gene [31]. However, Ghasemian et al., demonstrated the highest amount of *clf-A* in blood infection [30]. In addition, Smith et al., showed that strains isolated from wound were more potential than those isolated from blood or other infections [32]. The high frequency of *clf-A* in strains isolated from wound is a real concern, because this gene has a role in bacterial colonization in skin and the spread of MRSA strains from wound infections in hospitals. As shown in the current study, clinical samples had high MRSA strains and, therefore, preventing the spread of these strains seems to be inevitable.

The present study showed the high frequency of MRSA strains in clinical samples. Due to high prevalence of antibiotic-resistant bacteria in communities and hospitals, rapid and accurate detection of MRSA is of great importance in order to administer appropriate antibiotic therapy in patients and control methicillin-resistant strains. In fact, aseptic handling techniques in livestock lead to a significant decrease in the prevalence of MRSA strains in cattle. Results of the current study showed the importance of further studies to find a relationship between genes and the pathogenicity of MRSA strains in infection.

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Conflict of Interest

The authors declared no conflicts of interest.

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