

Genotyping of *coa* and *aroA* Genes of Methicillin-Resistant *Staphylococcus aureus* Strains Isolated From Nasal Samples in Western Iran

Parviz Mohajeri,¹ Samira Azizkhani,² Abbas Farahani,^{3,4,*} and Baharak Norozi⁵

¹Department of Microbiology, School of Medicine, Kermanshah University of Medical Sciences, Kermanshah, IR Iran

²Department of Microbiology, Islamic Azad University, Lahijan Branch, Lahijan, IR Iran

³Department of Microbiology, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, IR Iran

⁴Student Research Committee, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, IR Iran

⁵Department of Microbiology, Student Research Committee, Kermanshah University of Medical Sciences, Kermanshah, IR Iran

*Corresponding author: Abbas Farahani, Department of Microbiology, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, IR Iran. Tel: +98-9188495574, Fax: +98-8633353132, E-mail: Farahani.a@ajums.ac.ir

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Abstract

Background: Methicillin-resistant *Staphylococcus aureus* (MRSA) is a bacterial pathogen frequently isolated in both hospital and community environments. Methicillin-resistant *Staphylococcus aureus* is considered a major nosocomial pathogen that causes severe morbidity and mortality.

Objectives: The main objective of this study was to determine the genotypes of MRSA strains isolated from the nares of hospitalized and community patients in Kermanshah Hospital, western Iran, by PCR-restriction fragment length polymorphism (PCR-RFLP).

Materials and Methods: Of 1387 patients, 1217 patients were screened for more than 48 hours after admission in hospital wards and 170 patients were screened in the hemodialysis unit of Kermanshah Hospital, which is the largest hospital in western Iran. *S. aureus* was identified by standard biochemical tests, including colonial morphology, production of coagulase, and DNase and the API20 Staph test. Methicillin-resistant *Staphylococcus aureus* was identified by the Oxacillin strip test.

Results: In total, 258 *S. aureus* isolates were recovered from 1387 samples, of which 96 isolates were MRSA, 82 were hospital acquired, and 14 were community acquired. Digestion of the *aroA* gene revealed only one distinctive RFLP pattern in the 258 isolates.

Conclusions: Methicillin-resistant *Staphylococcus aureus* is an increasingly common cause of nosocomial infections. Our results are in agreement with those of other studies reporting that a few specialized clones are responsible for most cases of MRSA nasal carriage. In this study, MRSA strains isolated from different wards of hospital were closely related when analyzed by coagulase gene typing. Identifying patients colonized with MRSA during hospitalization and rapidly typing them with these methods may facilitate detection of outbreaks and prevention of the spread of organisms in hospitals.

Keywords: Restriction Fragment Length Polymorphisms, Methicillin-Resistant *Staphylococcus aureus*, *Coa* gene, *aroA* gene, *Staphylococcus aureus*

1. Background

Staphylococcus aureus could be considered a major cause of human diseases, particularly in the hospital setting. It is one of the most feared pathogens because of its ability to cause overwhelming sepsis and death (1). Methicillin-resistant *Staphylococcus aureus* (MRSA) is a bacterial pathogen frequently isolated and presented in community and hospital environments (2). Since the first report of MRSA in 1961, it has been considered an important pathogen in hospitalized patients that causes severe morbidity and mortality worldwide and is a major problem for public health and clinical settings (3, 4). Although hospital-acquired (HA) MRSA infections were initially reported in the 1980s, immediately after the introduction of methicillin in the hospital settings and they have continued to increase in last year's (5).

The potential for HA infections increases when patients with unidentified community colonization are placed in healthcare-associated environments. In an active sur-

veillance program, a patient is screened for carrying a selected organism, regardless of whether or not the patient is exhibiting signs and symptoms of infection at that time (6). The rate of nasal carriage of *S. aureus* isolates was found to vary from 10% to up 90% (7-9). Molecular typing plays an important role in epidemiological studies. Methods such as polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) typing are alternatives to more expensive techniques, such as pulsed-field gel electrophoresis (PFGE) (9). Pulsed-field gel electrophoresis is a labor-intensive and time-consuming technique and can be performed only in reference research laboratories with technically demanding but expensive than the present typing techniques such as coagulase gene restriction profile (CRP) analysis and require only optimized and standard protocols (10).

A PCR-based typing systems, such as rep-PCR, PCR-RFLP analysis of the *coa* and *aroA* gene would be appropriate for

detection and typing of *S. aureus* isolates, because these are simple, inexpensive, rapid, reproducibility and easy in applicable settings and also ideal typing method for analysis of large numbers of Methicillin-resistant *Staphylococcus aureus* isolates in many outbreaks result from these organisms (11). The *aroA* gene encodes the enzyme 5-enolpyruvylshikimate 3-phosphate synthase. *S. aureus* isolates can be confirmed with PCR amplification of the *aroA* gene (12). On the other hand, coagulase is produced by all isolates of *S. aureus*. Coagulase (*coa*) gene typing has been used to identify and compare *S. aureus* genotypes, as it is a simple, specific, discriminatory, and reproducible method for the typing of *S. aureus* isolated from plain sources (13, 14).

2. Objectives

The main objective of this study was to determine genotypes of MRSA strains isolated from the nares of hospitalized and community patients in Kermanshah Hospital, western Iran, by PCR-RFLP.

3. Materials and Methods

This cross-sectional study was conducted from October 2012 to August 2013, in 1387 patients at the Imam Reza hospital, which is a major referral hospital in western Iran with 514 tertiary care beds and 10 health wards, including Emergency, Pediatrics, Internal Medicine, Intensive Care Unit (ICU) Infectious diseases, Critical Care Unit, and Pediatrics. In total, 1217 patients in different wards were screened more than 48 hour after admission for HA isolates, that is, for nosocomial infections defined as infections noted 48 hour after admission. Another 170 patients in the hemodialysis unit were screened for community-acquired isolates without gathering any admission history. Exclusion criteria for patient enrollment included the following items: antibiotic therapy in the previous week, hospitalization during the previous month, history of *Staphylococcus* infections, and known immune deficiency.

Nasal samples (n = 1387) were taken using cotton sterile swabs moiled with sterile saline. Sampling was performed using methods described by Mohajeri et al. (8). All swabs were transported in the Amies medium and collected by the same researcher during the study. The swabs were directly inoculated on Mannitol salt agar (Merck, Germany). Each distinctive morphotype of the mannitol-fermenting colony was selected from the plate, subcultured on a blood agar plate (Merck, Germany), and incubated at 37°C in an incubator under humidified conditions. *S. aureus* isolates were identified by standard phenotypic tests such as colonial morphology, coagulase production, and DNase and the API 20 staph test (bioMerieux, France). MRSA was identified by the Oxacillin strip test (Mast, UK) (15).

3.1. DNA Extraction

Staphylococcus aureus genomic DNA was extracted using the standard phenol-chloroform method. PCR for the *aroA* and *coa* genes and RFLP for *aroA* were performed according

to the method described by Dastmalchi Saei et al. (Table 1), with minor modifications (16). After optimizing PCR reactions for the amplification of the *aroA* gene, the reaction was carried out in a DNA thermal cycler (BioRad C1000, USA) for obtaining a single amplicon with an anticipated size of 1153 bp. Each PCR reaction was carried out in a final volume of 50 µL reaction containing 2 µL of template DNA, 0.5 mM of each primer, the four deoxynucleoside triphosphates (each at 200 mM), PCR buffer 10 × 1.5 mM of MgCl₂, and 1.5 Units of TaqDNA polymerase (Sinaclone, Iran).

Staphylococcus aureus ATCC 25923 was used as the positive control for the *aroA* and *coa* genes and sterile water was used as the negative control. Amplified products were detected by electrophoresis at 60 V for 20 minutes and at 45 V for 70 minutes and photographed by Gel Doc (Bio-Rad XR+, Hercules, CA, USA). When the *aroA* gene was amplified, 10 µL of the PCR product of this reaction was incubated with 16 µL of sterile water, 3 µL of 10 × buffer, and 1 µL (20 U/µl) of HaeIII restriction enzyme (Fermentas, EU) at 37°C for 2.5 hours. The digested PCR product was electrophoresed on a 1.5% agarose gel at 80 V for 60 minutes. Coagulase gene typing was performed as described by Dastmalchi Saei et al. with some modifications (16). The method is based on the heterogeneity of a region including tandem repeats with 81-bp size at the 3' end of the *coa* gene. After confirming the amplification of the variable region of the *coa* gene, 10 µL of the PCR product of this gene was incubated with 16 µL of sterile water, 3 µL of 10 × buffer, and 1 µL (20 U/µl) of HaeIII restriction enzyme (Fermentas, EU) at 37°C for 2.5 hour. The digested PCR products were electrophoresed on a 1.5% agarose gel at 80 V for 60 minutes. The products were stained with ethidium bromide and the images were obtained by a gel documentation (BioRad XR +, Hercules, CA, USA) system.

4. Results

In this study, 258 *S. aureus* isolates were recovered from 1387 samples: 96 isolates were MRSA, which included 82 HA isolates and 14 community-acquired isolates. The 96 isolates were collected from different wards: 6 (6.2%), ICU; 11 (11.5%), surgery; 20 (20.8%), internal medicine; 7 (7.3%), gynecology; 5 (5.2%), infection; 1 (1%), heart; 21 (21.87%), pediatrics; and 25 (26%), hemodialysis. Eight age groups were defined (Table 2). The *aroA* gene was successfully amplified in all MRSA isolates tested (Figure 1). Digestion of the *aroA* gene yielded one distinctive RFLP pattern. All the isolates generated approximately 300- and 850-bp bands (Figure 1). In contrast, PCR amplification of *coa* produced bands of five different sizes, ranging from 490 bp to 810 bp (Figure 2). RFLP analysis of the *coa* gene showed five distinctive patterns (Figure 3). Table 3 shows the genotype distribution of HA and community-acquired isolates, and Table 4 lists the patterns seen within the different wards in our hospital. Table 3 also shows *coa* gene typing and PCR-RFLP patterns detected using restriction enzyme AluI.

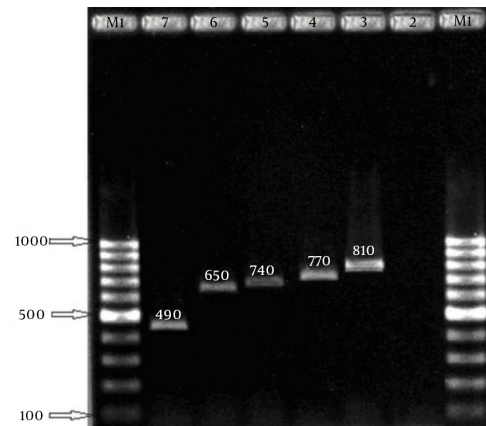
Table 1. Primers to *aroA* and *Coa* Used in Polymerase Chain Reaction

Primer Name	Sequence	Product Size (base pair)
<i>aroA F1</i>	5'-AAG GGC GAA ATA GAA GTG CCG GGC-3'	1153
<i>aroA R2</i>	5'-CAC AAG CAA CTG CAA GCA T-3'	
<i>coa1</i>	5'-ATA GAG ATG CTG GTA CAG G-3'	850
<i>coa2</i>	5'-GCT TCC GAT TGT TCG ATG C-3'	

Table 2. Distribution of Patients by Age

Age Group	Frequency, %
< 10	20 (20.8)
10 - 19	3 (3.1)
20 - 29	5 (5.2)
30 - 39	9 (9.4)
40 - 49	7 (7.3)
50 - 59	20 (20.8)
60 - 69	16 (16.7)
≥ 70	16 (16.7)
Total	96 (100.0)

Figure 2. Agarose gel Electrophoresis of the *coa* Gene PCR Amplification Products and the PCR Amplified *aroA* Gene



Amplification products. Left to right lanes 1 and 8: 100 bp digested. Left to right lane 1: 100 bp DNA ladder, DNA ladder. Lane 2: negative control. Lanes 3 - 7: 490 bp, Lane 2: *aroA* gene PCR 1153 bp. Lane 3: *aroA* gene 650, 740, 770, 810 bp. Digestion: 850 and 300 bp, Lane 4: negative control.

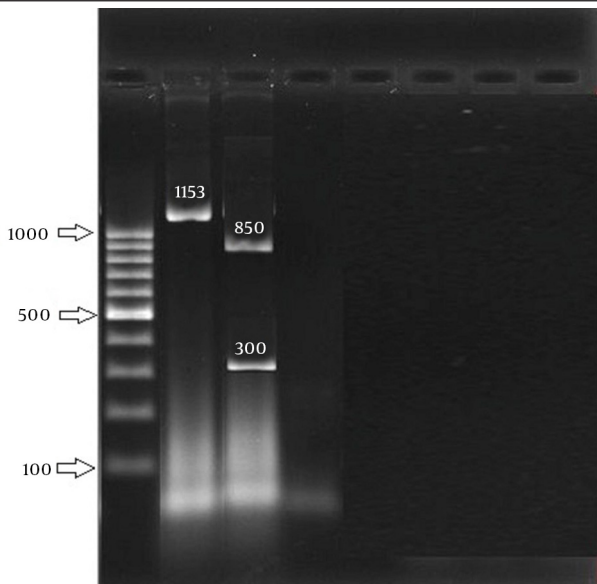
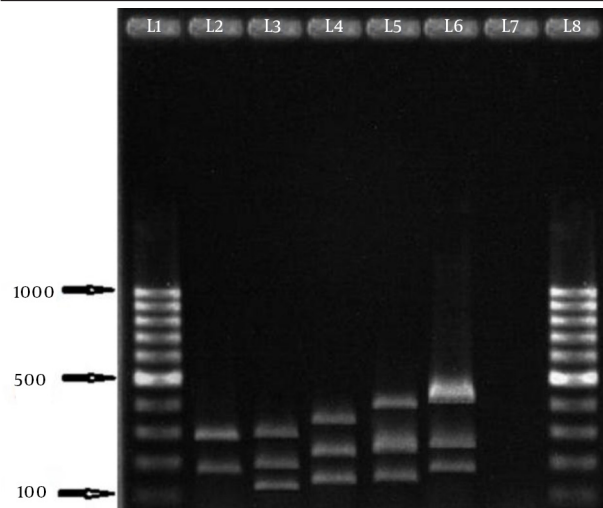


Figure 1. Agarose gel Electrophoresis of the *aroA* Gene PCR Amplification Products

Figure 3. PCR-amplified *coa* Gene Digested (RFLP) With HaeIII Restriction Endonuclease



Left to right lanes 1 and 8: 100 bp DNA ladder. Lanes 2 - 6: digested PCR products (490 - 810 bp). Lane 7: negative control.

Table 3. Genotypes Distribution of Hospital-acquired and Community-acquired Isolates and Their RFLP Patterns

Genotype Code	PCR Product, bp	No. of Isolates	No. of Hospital-acquired Isolates	No. of Community-acquired Isolates	RFLP Patterns, bp
I	810	15	6	9	360, 260, 190
II	770	13	9	4	350, 260, 160
III	740	29	25	4	320, 260, 160
IV	650	27	22	5	300, 200, 190
V	490	12	9	3	300, 190

Table 4. Distribution of HaeIII Restriction Patterns Within Different Hospital Wards

Ward	No. of Isolates	Genotype Code				
		I	II	III	IV	V
ICU	6	1	1	1	3	0
Surgery	11	1	2	4	2	2
Internal medicine	20	0	4	5	6	5
Gynecology	7	1	1	2	3	0
Infection	5	0	1	0	4	0
Heart	1	0	0	1	0	0
Pediatrics	5	1	0	3	1	0
Infants	16	2	0	9	3	2
Hemodialysis	25	9	4	4	5	3

5. Discussion

As the model pathogen, we selected MRSA because this multidrug-resistant nosocomial pathogen has previously been studied genetically worldwide (17, 18). Methicillin-resistant *Staphylococcus aureus* isolates are suitable as the best model for investigating infection control and outbreak in hospitals and community (12). Methicillin-resistant *Staphylococcus aureus* is an increasingly common cause of nosocomial outbreaks with several morbidity and mortality worldwide, and more than 50% of all *S. aureus* diseases occurred in some part of hospitals. Another study reported that detection of hospital and community isolates of MRSA in nasal samples and their treatment may be an important modality in the prevention of infections by these isolates (19, 20).

In this regard, molecular typing provides valuable information on the genetic background of MRSA isolates. These techniques have been useful for differentiation of MRSA isolates and are further used as tools for epidemiological purposes (10). In the present study, PCR products of five different sizes were obtained from amplification of the *coa* gene. Similarly, studies by other researchers in different parts of world (12) conducted using the same primer pairs also showed distinct coagulase gene types in some isolates. This variation and polymorphism among some isolates of *S. aureus* is unclear, but some studies attribute it to the insertion or deletion mutations that occur at the 3' end of the *coa* gene; these mutations

change the *coa* gene size and also change the antigenic properties of the coagulase enzyme in serological tests. This region of the *coa* gene may play an important role in the antigenic variation of coagulase and its escape from the inhibitory effect of anticoagulase agents (17).

Our results and those of other studies have shown that some specialized clones are responsible for the outbreak, most cases of MRSA nasal carriage, and skin colonization (16, 21). The 3' end of the *coa* gene contains a series of tandem repeats 81-bp in size, which is distinctive among *S. aureus* isolates within their number or in the location of AluI restriction sites. The production of *coa* is important for the future of epidemiology typing and molecular identification of *S. aureus* (13). Classification of *S. aureus* isolates based on the *coa* gene is a simple and accurate method for molecular typing (13, 18, 22). Detection and isolation of carriers is crucial for controlling the spread of nosocomial infection with MRSA isolates in a hospital setting. During nosocomial outbreaks, a rapid MRSA screening test based on molecular epidemiology typing can be used for detecting the DNA of epidemic strains in colonized sites of patients. Potential targets for genotyping of MRSA isolates based on the detection of conserved sequences include specific nucleotide sequences such as 81-bp tandem repeats in the 3' end of the *coa* gene, which is considered effective in identifying outbreaks. In this study, MRSA strains isolated in the different wards of the

hospital were closely related when analyzed by *aroA* gene typing.

In the present study, HaeIII enzyme, which has been used in previous studies (23, 24) for generating RFLP patterns of the *coa* gene of *S. aureus*, did not digest all PCR products. RFLP analysis for the *coa* gene showed five distinctive patterns in these isolates. A possible reason for this finding might be alterations in the polymorphic repeated region of the *coa* gene because of point mutations (23, 24). As mentioned before, isolates with 740- and 650-bp PCR products were the predominant types, and interestingly all isolates with the 740- and 650-bp PCR products yielded the same RFLP pattern, indicating no heterogeneity in HaeIII recognition sites among this predominant type (25). Aarestrup et al. reported that *coa* gene polymorphism is useful for classifying *S. aureus* strains (26), because of its high typability, reproducibility, and good discriminatory power, and also because it can be used in epidemiological investigations and for molecular typing of *S. aureus* isolated from different origins (27). In contrast to our findings in human isolates, Dastmalchi Saei et al., in their study in northwest Iran, observed that 58 *S. aureus* isolates from bovine mastitis were distributed over nine RFLP different patterns with 490- to 850-bp fragments from digestion with HaeIII enzyme (16). Each of these patterns differed in frequency, with the highest frequency observed for pattern I (37%) followed by pattern VIII (24%).

5.1. Conclusion

Colonization by a microorganism, an essential step leading to infection with that microorganism, may persist for months or years. Patients with MRSA colonization serve as a reservoir for MRSA transmission in hospitals. Nasal carriage of *S. aureus* is a substantial source of human infections in some wards of hospitals. Identifying patients colonized with MRSA during hospitalization and rapidly typing them with the methods presented in this paper may facilitate detection of outbreaks and prevention of the spread of the organism in hospitals. Our results showed that RFLP types may differ between hospitalized and community patients in hospitals.

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Footnotes

Authors' Contribution: Parviz Mohajeri, Samira Azizkhani, and Abbas Farahani contributed to study design, data collection, statistical analysis, data interpretation, manuscript preparation, literature search, and fund collection; Baharak Norozi contributed to statistical analysis,

data interpretation, manuscript preparation, and literature search.

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