

Molecular typing of methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* isolates from Shiraz teaching hospitals by PCR-RFLP of coagulase gene

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ABSTRACT

Background and Objectives: To investigate coagulase gene polymorphisms of MRSA and MSSA isolates from Shiraz teaching hospitals from 2011 to 2012.

Materials and Methods: A total of 302 isolates of *Staphylococcus aureus* were collected from clinical specimens in three major teaching hospitals and confirmed on the basis of morphological characteristics and biochemical tests. The isolates were subjected to molecular typing on the basis of coagulase enzyme gene polymorphism by PCR-RFLP.

Results: There were 27 and 28 different RFLP patterns for *AluI* and *HaeIII* restriction enzymes respectively. This study showed that the discriminatory power of coagulase gene typing by *Hae III* enzyme was more than that of *Alu I* enzyme.

Conclusion: PCR-RFLP method is rapid, reproducible, simple and efficient for typing *Staphylococcus aureus* isolated from clinical specimens. This study showed that *Hae III* discriminatory power is better than *Alu I* for typing *Staphylococcus aureus* isolates.

Keywords: Antibiotic resistance, MRSA, PCR, RFLP, Coagulase gene

INTRODUCTION

Staphylococcus aureus is the most common cause of nosocomial infections that causes skin and soft tissue infections such as boils, carbuncle, cellulitis and abscesses (1, 2). Drug-resistant strains of the bacteria are rapidly being developed; thus, the treatment of this organism is difficult. Methicillin resistant *Staphylococcus aureus* (MRSA) infection is a major cause of increasing morbidity and mortality (1).

The prevention and control of *Staphylococcus*

aureus infections depends initially on detection of the risk factors in individuals exposed to *S. aureus*, analysis of the isolates by discriminatory DNA typing methods, and understanding the transmission of the bacterial infection (1). Molecular typing can play an important role in the epidemiological study of nosocomial infections, such as methicillin-resistant *Staphylococcus aureus* (MRSA) infection (3). In many countries, *Staphylococcus aureus* genotyping methods have become a part of the upcoming health care system and also the study of the strain origin, clonal relatedness and the epidemiology of the infection (2).

In the past two decades, a variety of molecular typing methods including pulsed-field gel electrophoresis (PFGE)(4), multilocus enzyme electrophoresis (MLEE), multilocus sequence typing (MLST)(5), *spa* typing(6), and coagulase genotyping(7) have developed to differentiate the related strains from

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Table 1. The percentages of the studied isolates from various wards of the hospitals.

Ward	Percentage	ward	Percentage
Surgery	6.76%	Internal	9.47 %
PER	1.01 %	CCU	0.72 %
OR	2.74 %	Internal ICU	21.28 %
Out Patient	16.93 %	Pediatric Internal ICU	3.1%
Neurology	0.28%	Skin	14.8 %
Neonatal Emergency	3.32 %	Internal Neonatal	0.73 %
Surgery ICU	5.6 %	Emergency	12.54
KT	0.72 %		

PER: Pediatric Emergency, OR: Operation Room, ICU: Intensive Care Unit, KT: Kidney Transplantation, CCU: Cardiac Care Unite.

unrelated ones (8) and to identify and compare the genotypes of *S. aureus* (9). Genotyping methods are relatively stable in natural conditions and produce reproducible results. These methods are rapid, do not require laboratory culture. The ability for typing by these methods is nearly 100% (2). Pulsed-field gel electrophoresis and multilocus sequence typing are the most reliable and the highest discriminative typing methods, but are very hard, difficult, time-consuming and expensive to be used in a clinical microbiology laboratory (1). Coagulase gene (*coa*) typing is a simple, accurate, reproducible enough, easy to interpret and discriminatory method for typing *Staphylococcus aureus* isolates from various sources (2, 9-11).

It is well-known that coagulase enzyme is produced by most of the strains of *S. aureus*. Today, the ability to produce the coagulase in the clinical microbiology laboratory is used to detect *S. aureus* in human infections. It has been demonstrated that coagulase is an important virulence factor during the infection process (1,11,12). There are many different allelic forms of the *S. aureus coa* gene, each isolate produces one or more than one of these forms (11, 13, 14).

The discriminatory power of coagulase gene typing depends on the variability of the region containing the 81 bp tandem repeats at the 3' coding region of the coagulase gene that differs both in the number of tandem repeats and the location of *AluI* and *HaeIII* restriction sites among different isolates (1-3,11,12,15-17). So, different *S. aureus* isolates could be discriminated using the coagulase gene typing method (11, 18).

In Iran, there is a little information about the genetic differences between *aureus* isolates from various hospitals and in particular, no information

about coagulase gene polymorphisms. In present study, we have used coagulase gene typing method on the basis of the PCR-RFLP to discriminate *S. aureus* strains obtained from different specimens in teaching hospitals in Shiraz, Iran. Also, the ability of *AluI* and *HaeIII* restriction enzymes, for differentiating *S. aureus* isolates in PCR-RFLP method was also evaluated (1).

MATERIALS AND METHODS

Cultivation and identification of bacteria. From August 2011 to July 2012, 302 clinical isolates of *Staphylococcus aureus* were collected from three major teaching hospitals in Shiraz, Iran. *S. aureus* strains were identified based on morphological characteristics and biochemical tests. Of all bacterial isolates, 39% were from female and 61% from male subjects. The percentage of the isolates from different wards of hospitals and various samples are presented in Tables 1 and 2.

Antibiotic susceptibility test. Disks containing cefoxitin (30 µg, Mast, UK) was used to discriminate the MRSA from MSSA isolates according to CLSI guideline recommendations. MHA (Muller Hinton Agar) plates were cultured with 0.5 McFarland standard of the bacterial broth culture and antibiotic disks were placed on the plates. Then this plate was incubated for 18 h at 37°C (1). There are several studies reporting that cefoxitin disk is preferable to oxacillin for detection of MRSA strains (19-23).

Extraction of Genomic DNA. Bacterial whole DNA was extracted from isolates by using the small-scale phenol-chloroform extraction method and used as template in PCR (24).

Table 2. The percentages of the studied bacterial isolates from various samples

Sample	Percentage	Sample	Percentage
Inguinal	0.3%	Wound	9.4%
Sterile Fluid	2.68%	Urine	14.7%
Eye	3.13%	Throat	4%
Ear	0.74%	Sputum	26.2%
Lesion	0.74%	Skin	11.5%
CSF	1.9%	Peritoneal	0.35%
Blood	15%	Plural	0.3%
Axillary	0.85%	Nasal	7.81%
Abdominal	0.4%		

Polymerase chain reaction. This method was carried out with a little difference from the method described by Himabindu *et al.* The forward primer sequence for *coa* gene was 5'CGAGACCAAGATTCAACAAG and the reverse primer sequence was 5'AAAGAAAACCACTCACATCA-3' (1). PCR conditions were as follows: 94°C for 5 min, followed by 30 cycles of 95°C for 30 sec, 55°C for 45 sec and 72°C for 1.5 min, followed by a final extension of 72°C for 7 min. The PCR products were separated by 1.5% agarose gel electrophoresis.

Restriction Fragment Length Polymorphism (RFLP) analysis. *Alu* I (Fermentas, Lithuania) digestion of the products was performed by adding 15 µL of PCR product to 15 µL of the mixture that contained 2 U of *Alu* I, 3 µL of buffer enzyme and 8.11 µL of distilled injection water. Then, the reaction mixture was incubated at 37°C for 16 h. *Hae* III (Fermentas, Lithuania) digestion of the product was performed by adding 15 µL of PCR product to 15 µL of the mixture that contained 5 U of *Hae* III, 3 µL of buffer enzyme and 5.11 µL of distilled injection water. Then, the reaction mixture was incubated at

37°C for 16 h. To prevent evaporation of reaction mixture, 20 µL of sterile PCR oil was added. The restriction digest fragments were detected by 3% agarose gel electrophoresis. In this study, *S. aureus* strain 25923 was used as positive control.

Discriminatory power. The ability of a typing method to discriminate different types of the unrelated strains sampled from the hospital was assessed according to the Hunter-Gaston formula (1, 25). This is also called discriminatory index (D).

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^s n_j(n_j-1)$$

D= Numerical index of discrimination, N= The total number of isolates in the sample Population, s= The total number of types obtained, n_j= The number of isolates belonging to the jth type contains (1, 25).

RESULTS

Coagulase gene amplification: The size of PCR products produced after coagulase gene amplification was separated into 6 different banding patterns in electrophoresis (Fig. 1, Table 3). The majority of isolates grouped in pattern 3 (729bp). We

Table 3. Band classes of PCR coagulase gene products in 3 hospitals.

The size of PCR products (bp)	Hospital			Total
	Faghihi	Ghotbodini	Namazi	
567	4(1.3%)	0(0.0%)	1(0.3%)	5(1.7%)
648	34(11.3%)	0(0.0%)	14(4.6%)	48(15.9%)
729	77(25.5%)	4(1.3%)	56(18.5%)	137(45.4%)
810	32(10.6%)	1(0.3%)	27(8.9%)	60(19.9%)
891	6(2.0%)	1(0.3%)	3(1.0%)	10(3.3%)
972	8(2.6%)	0(0.0%)	5(1.7%)	13(4.3%)
Total	161(58.9%)	6(2.1%)	106(38.8%)	273(100.0%)

Table 4. Band patterns by *AluI* restriction enzyme digestion of PCR products

	Hospital			Total
	Faghihi	Ghotbodini	Namazi	
81-162-486	4(1.5%)	0(0.0%)	0(0.0%)	4(1.5%)
162-324-486	5(1.8%)	0(0.0%)	4(1.5%)	9(3.3%)
81-162-405	23(8.4%)	0(0.0%)	12(4.4%)	35(12.8%)
324-405	55(20.1%)	3(1.1%)	45(16.5%)	103(37.7%)
162-243-405	11(4.0%)	0(0.0%)	6(2.2%)	17(6.2%)
162-567	2(0.7%)	0(0.0%)	3(1.1%)	5(1.8%)
243-405	3(1.1%)	0(0.0%)	0(0.0%)	3(1.1%)
81-324-405	12(4.4%)	0(0.0%)	11(4.0%)	23(8.4%)
81-162-567	2(0.7%)	0(0.0%)	1(0.4%)	3(1.1%)
81-243-324	4(1.5%)	0(0.0%)	3(1.1%)	7(2.6%)
324-486	1(0.4%)	1(0.4%)	0(0.0%)	2(0.7%)
405-486	3(1.1%)	0(0.0%)	1(0.4%)	4(1.5%)
81-162-324	2(0.7%)	0(0.0%)	0(0.0%)	2(0.7%)
162-243-324	1(0.4%)	0(0.0%)	3(1.1%)	4(1.5%)
243-567	1(0.4%)	0(0.0%)	1(0.4%)	2(0.7%)
162-405	1(0.4%)	0(0.0%)	1(0.4%)	2(0.7%)
162-486	3(1.1%)	0(0.0%)	0(0.0%)	3(1.1%)
162-648	2(0.7%)	0(0.0%)	2(0.7%)	4(1.5%)
Others*	4(1.6%)	1(0.4%)	3(1.2%)	8(3.2%)
Undigested	22(8.1%)	1(0.4%)	10(3.7%)	33(12.1%)
	161(59.0%)	6(2.2%)	106(38.8%)	273(100.0%)

*Others are the patterns that produced only by one sample including 81-162-324-405, 243-486, 81-243-567, 81-243-405, 81-405-486, 81-162-243-405, 162-729, 162-324-405

found that 2.4% of the isolates with the negative tube coagulase test, was positive for coagulase by PCR method, indicating the superiority of the molecular methods on phenotypic methods for typing of the *Staphylococcus aureus*. However, because the discriminatory power on the basis of the PCR alone was 0.68, which was very low, this typing method could not be used for *S. aureus* typing.

PCR product digestion by *AluI* enzyme: After digesting of PCR products with *AluI* and analysis of digestants on 3% agarose gel, 1 to 4 bands for each isolate were observed. The size of the band produced by enzyme digestion was divided into 12 different band classes (81, 162, 243, 324, 405, 486, 567, 648, 729, 810, 891 and 972 base pairs) each containing multiples of 81bp tandem repeat units.

After RFLP analysis of all the isolates, based

on the number and size of the produced bands, 27 different patterns were observed (Table 4). The pattern 4 “324-405” was found to be the predominant type. The isolates grouped in pattern 27, produced only 1 band, indicating that this PCR product is not digested by *AluI*. This probably indicates the lack of *AluI* restriction sites amongst them. Discriminatory power was detected as 0.82 that indicated a good discriminatory power of this method for typing of the *aureus*. As a result, this method can be used for typing *aureus*.

PCR product digestion by *HaeIII* enzyme. After digestion of PCR products with *HaeIII* enzymes and analysis of amplicons in 3% agarose gel, 1 to 4 bands were observed for every isolate. The digestants were divided into 12 different band classes (81, 162, 243, 324, 405, 486, 567, 648, 729, 810, 891 and 972 base

Table 5: Band patterns by *HaeIII* restriction enzyme digestion of PCR products

<i>HaeIII</i>	Hospital			Total
	Faghihi	Ghotbodini	Namazi	
162-567	30(11.0%)	1(0.4%)	19(7.0%)	50(18.3%)
81-162-243-324	5(1.8%)	0(0.0%)	8(2.9%)	13(4.8%)
162-324-486	2(0.7%)	0(0.0%)	1(0.4%)	3(1.1%)
162-486	24(8.8%)	0(0.0%)	11(4.0%)	35(12.8%)
324-405	28(10.3%)	1(0.4%)	22(8.1%)	51(18.7%)
81-324-405	13(4.8%)	0(0.0%)	6(2.2%)	19(7.0%)
81-243-324	6(2.2%)	0(0.0%)	1(0.4%)	7(2.6%)
81-162-243-486	3(1.1%)	0(0.0%)	3(1.1%)	6(2.2%)
81-162-567	10(3.7%)	0(0.0%)	9(3.3%)	19(7.0%)
243-486	1(0.4%)	0(0.0%)	1(0.4%)	2(0.7%)
81-162-486	6(2.2%)	2(0.7%)	12(4.4%)	20(7.3%)
162-243-324	12(4.4%)	0(0.0%)	4(1.5%)	16(5.9%)
81-162-405	3(1.1%)	0(0.0%)	2(0.7%)	5(1.8%)
243-324-405	3(1.1%)	0(0.0%)	0(0.0%)	3(1.1%)
324-486	3(1.1%)	1(0.4%)	0(0.0%)	4(1.5%)
162-324-405	1(0.4%)	1(0.4%)	1(0.4%)	3(1.1%)
324-567	2(0.7%)	0(0.0%)	0(0.0%)	2(0.7%)
162-405	3(1.1%)	0(0.0%)	1(0.4%)	4(1.5%)
81-243-486	1(0.4%)	0(0.0%)	1(0.4%)	2(0.7%)
405-486	2(0.7%)	0(0.0%)	0(0.0%)	2(0.7%)
81-162-648	0(0.0%)	0(0.0%)	2(0.7%)	2(0.7%)
Others*	2(0.8%)	0(0.0%)	2(0.8%)	4(1.6%)
Undigested	1(0.4%)	0(0.0%)	0(0.0%)	1(0.4%)
Total	161 59.0%	6 2.2%	106 38.8%	273 100.0%

*Others are the patterns that produced only by one sample including 162-243-567, 81-243-405, 162-648, 81-162-243-405

pairs) consisting of multiples of 81bp tandem repeat units. After RFLP analysis of all the isolates, based on the number and size of produced bands, 26 different patterns were observed (Table 5). The pattern 5 “324-405” was found to be the predominant pattern. The isolates that belonged to pattern 26 produced only 1 band which indicates that this PCR product is not digested by *HaeIII*. This probably indicates the lack of *HaeIII* restriction sites amongst them. The discriminatory power was detected as 0.90.

DISCUSSION

Typing can be used to prevent or reduce an epidemic infection, reducing costs and hospital infection rates

in hospitals (1). In this study, the polymorphism of coagulase gene among MRSA and MSSA isolates were investigated using PCR-RFLP analysis. The results of typing can be used to discriminate the strains within a given species (26). If strains from two patients have the same fingerprint, it indicates that both of them are infected from the one source. Discriminatory power of coagulase gene typing is high. It is an appropriate method for epidemiologic investigation of *Staphylococcus aureus* infection since it is applicable for typing of large number of strains in a short time.

he discriminatory power of this method is lower than PFGE and MLST but it is quicker and less costly (27). RFLP method can help to trace the source of

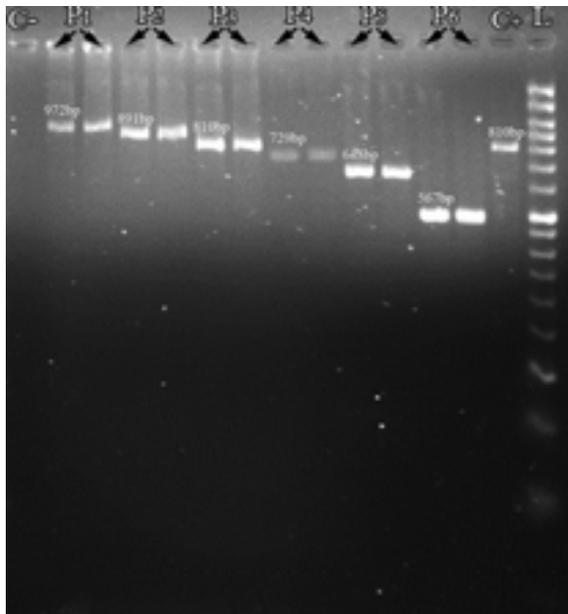


Fig. 1. Representative agarose gel electrophoresis image of coagulase gene PCR products. L: 50bp DNA ladder, C-: Control negative, C+: Control positive, P1: 972, P2:891, P3:810, P4:729, P5: 648, P6: 567 bp bands

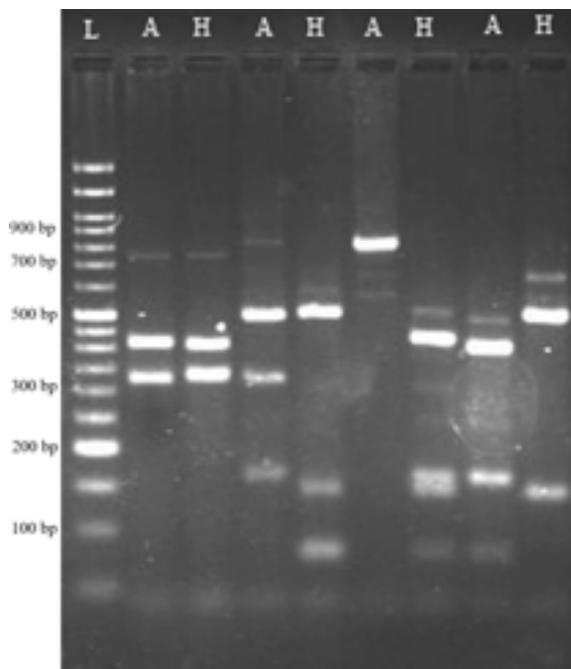


Fig. 2. Representative agarose gel electrophoresis image of *AluI* and *HaeIII* restriction enzyme digestion products. L: 50bp DNA ladder, A: *AluI* restriction digested products, H: *HaeIII* restriction enzyme digestion products

infection and transmission; thus, this typing method can be used to prevent and control the spread of infection (1).

The purpose of this study was to investigate genetic variation in coagulase gene of *aureus* strains isolated from teaching hospitals in Shiraz, Iran. Although, MRSA strains are important cause of the nosocomial infections, no data are available on molecular typing of MRSA or even MSSA isolates by PCR-RFLP of coagulase gene in hospital isolates from Iran. There are few studies on this subject in Iran and one of them was in veterinary practice (9). However, in a study in Urmia, Iran, 26 *S. aureus* isolates from urine and skin in two hospitals were analyzed by PCR-RFLP using *HaeIII* enzyme. PCR products ranged from 490-790 bp in size and 6 distinct RFLP banding patterns were observed after the digestion of PCR products (2).

The discriminatory index of coagulase gene typing by PCR-RFLP on the basis of *HaeIII* enzyme is more than of this method based *AluI*. Thus, *HaeIII* enzyme is better than *AluI* enzyme for typing *aureus*.

In conclusion, our study proved that, of PCR-RFLP of coagulase gene is a rapid, simple and efficient method for typing *S. aureus* strains isolated from different clinical specimens in Shiraz teaching hospitals. This typing method can be used for tracing the source and transmission route of *S. aureus* infections and helps to prevent and control those related infections in our hospitals.

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