Original Article

Molecular Analysis and Genotyping of Methicillin-Resistant *Staphylococcus aureus* Strains Isolated from Different Clinical Sources

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ABSTRACT

Background: Investigating genetic relatedness between methicillin-resistant *Staphylococcus aureus* (MRSA) strains from humans and different animal species may clarify the epidemiological characteristic of MRSA infections together. **Aim:** The aim of the study was to perform genotypic characterization and type strains of MRSA isolated from different clinical sources, by molecular techniques. **Materials and Methods:** The molecular characterization of the strains was performed by polymerase chain reaction (PCR), using several specific oligonucleotides. These were as follows: *S. aureus* species-specific *sau* gene, *mecA* gene coding PBP2a responsible for methicillin resistance, *femA* gene coding for a protein, which influences the level of methicillin resistance of *S. aureus*, and is universally present in all MRSA strains; *spa* gene coding for protein A; *coa* gene coding for coagulase, and *blaZ* gene coding for the production of beta-lactamase. To determine the genetic diversity of these strains, random amplified polymorphic DNA–polymerase chain reaction (RAPD-PCR) was performed. **Results:** Among the 415 *S. aureus* strains, 61 were phenotypically identified as MRSA, and confirmed as *S. aureus* by amplification of *sau* gene. However, 90.16% of the strains were *mecA* positive, while all were negative for *femA* gene. The presence and polymorphism of *coa* and *spa* genes were investigated and 83.60% and 18.03% strains were positive for *coa* and *spa*, respectively. While these strains were grouped into six *coa*-types by PCR, no polymorphism was found for *spa* gene among strains having only single 190 bp of the band. *bla* genes were found in 75.40% of strains. These strains were divided into 12 RAPD types. **Conclusions:** The results showed the relatively high heterogeneity and variation of *coa* gene among MRSA strains, while further studies on sequencing of these strains may identify which sequence type is predominant in this region.

KEYWORDS: *Methicillin-resistant Staphylococcus aureus, molecular typing, RAPD, virulence genes*

INTRODUCTION

Staphylococcus aureus (*S. aureus*) is a major cause of infections in hospitals and communities. Presently, the increasing antibiotic resistance of *S. aureus* is widely observed globally, and this complicates the treatment of associated infections, as well as control measures.[1] Since the first report of methicillin-resistant *S. aureus* (MRSA) in 1961,[2] it has gradually spread all over the world. One of the considerable features of MRSA is its resistance against an antibiotic class named beta-lactams.[3] Though in the past, MRSA almost exclusively caused hospital-associated infections, the advent of community-acquired MRSA has led to infections in people without hospital-related risk factors.[1] Methicillin resistance occurs due

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to the production of an altered penicillin-binding protein (PBP2a) with a low affinity for all penicillin classes. *mecA* gene encodes PBP2a[4] and its expression is regulated by associated repressor and inducer genes such as *mecR*, *mecI*, *ccr,* and by various other *S. aureus* genes like *fem* (factors essential for methicillin resistance) and *aux* (auxillary genes).[5] These genes have been reported to also have importance in the expression of methicillin resistance, in addition to *mecA*.[6] Considering that only *mecA* detection does not confirm the presence of *S. aureus*, several constitutive genes such as *femA*, *fem B,* and *nucA* genes are being used. However, it has been reported that there was polymorphism within these genes; therefore, some conflicts occur in confirmation of *S. aureus* species by polymerase chain reaction (PCR) targeting these genes.[7] Another mechanism for beta-lactam resistance in *S. aureus* is the production of beta-lactamases and its production is encoded by structural *blaZ* gene, so, *blaZ* has been used to determine MRSA profile as well.[8] Coagulase and protein A are important virulence factors and phenotypic determinants in *S. aureus*; thus, variations in the sequence of genes (*coa* and *spa*) coding for these two species-specific proteins have been widely used in PCR typing for *S. aureus*.[9,10] DNA-based typing methods are reportedly based on the principle that epidemiologically related bacterial isolates have genetic features that are different from those of other epidemiologically unrelated strains.[11] One of the PCR-based typing methods, random amplified polymorphic DNA (RAPD) was used to type MRSA strains isolated from different human sources.

Materials and Methods

Clinical specimens

A total of 415 *S. aureus* strains, which had been isolated from various clinical sources, were evaluated between December 2016 and December 2017 in our study in the Microbiology Laboratory of Samsun Education and Research Hospital, Turkey. Clinical samples were acquired from the following sources: blood samples [14 (22.9%)], urine samples [10 (16.3%)], wound samples [17 (27.8%)], sputum samples [8 (13.1%)], throat swab [1 (1.6%)], tracheal aspirates [6 (9.8%)], pleural fluid samples [2 (3.2%)], and abscess sample [1 (1.6%)].

Bacterial isolates

All bacterial strains were isolated by inoculating the clinical samples on Blood Agar base, supplemented with 5% sheep blood, and incubated at 37°C for 18-24 hours. After the incubation period, suspected colonies were identified by conventional methods (colony morphology, Gram staining, catalase and coagulase test, etc) as *S. aureus*.

Phenotypic determination of methicillin resistance

Methicillin susceptibilities of the *S. aureus* strains were determined by Kirby-Bauer disc diffusion method, using oxacillin discs (1 µg). Obtained results were evaluated according to the protocol of the Clinical and Laboratory Standards Institute[12] by the following criteria.

Molecular characterization of isolates

DNA extractions from MRSA determined by disc diffusion tests were performed by commercially available DNA extraction kits (PureLink Genomic DNA Kits, Invitrogen, Canada). The extractions were performed according to the manufacturers' instructions.

The molecular characterization of the strains was performed by PCR, using several specific oligonucleotides. The sequences of each primers and expected product (bp) are as presented in Table 1.

To identify and confirm *S. aureus* strains genotypically by PCR, a method, which has been reported by Abd El-Razik *et al.*[13] was modified and optimized. Amplification was performed in 50 ml reaction volumes containing 1xSPCR reaction buffer (100 mM Tris–HCl, 500 mM KCl, pH 8.3), 3 mM $MgCl_2$, 0.2 µM of each primer, 0.2 mM of each dNTP, 1 U Taq polymerase, and 5 µl of extracted DNA. The reactions were carried out under following conditions: an initial denaturation step of 94°C for 2 min, followed by 35 cycles of 94°C for 45 sec denaturation, 60°C for 45 sec annealing, 72°C for 1 min extension, and a final extension step at 72°C for 10 min. For *femA* gene, the amplification reaction was performed in 25 µl final volumes containing 1xSPCR reaction buffer, 3 mM $MgCl_2$, 0.4 mM of each primer, 0.2 µM of each dNTP, 1 U Taq polymerase, and 5 µl of template DNA. The reactions were carried out under the following conditions: 95°C for 2 min. initial denaturation step, followed by 30 cycles of 95°C for 2 min denaturation, 54°C for 1 min annealing, 72°C for 1 min extension, and a final extension step at 72°C for 7 min.[14] The amplification of *mecA* gene was performed by a modified method in 25 µl reaction volumes. The content of the reaction mixture was as follows: 1xSPCR buffer, 2.5 mM $MgCl_2$, 0.8 µM of each primer, 0.2 mM of each dNTP, 1 U Taq polymerase, and 5 µl of template DNA. The reactions were carried out under the following conditions: 94°C for 2 min. initial denaturation step, followed by 30 cycles of 94°C for 1 min denaturation, 55°C for 1 min annealing, 72°C for 2 min extension, and a final extension step at 72°C for 7 min.[14] To determine the presence and polymorphism of *coa* gene, a PCR protocol, which has been used by Aslantas *et al.*[15] was modified and optimized. The reaction was carried

out in a 50 µl final volume. The reaction mixture was prepared as follows: 1xSPCR buffer, 2.5 mM $MgCl_2$, 1 µM of each primer, 0.2 mM of each dNTP, 2 U Taq polymerase, and 5 µl of template DNA. The reactions were carried out under the following conditions: 95°C for 2 min. initial denaturation step, followed by 30 cycles of 95°C for 30 sec denaturation, 58°C for 2 min annealing, 72°C for 4 min extension, and a final extension step at 72°C for 10 min.

To determine the presence and polymorphism of *spa* gene, a protocol that has previously been used by Montesinos *et al*.[11] was modified and optimized. We used primers, which were designed to amplify the polymorphic X region that contains a variable number of 24 bp tandem repeats of the *spa* gene coding for protein A. The reaction was carried out in a 50 µl final volumes containing 1xSPCR reaction buffer, 2.5 mM $MgCl_2$, 0.25 µM of each primer, 0.2 mM of each dNTP, 1.5 U Taq polymerase, and 5 µl of extracted DNA. The reactions were carried out under following conditions: an initial denaturation step of 95°C for 3 min, followed by 30 cycles of 94°C for 1 min denaturation, 60°C for 1 min annealing, 72°C for 1 min extension, and a final extension step at 72°C for 10 min.

The amplification of *blaZ* gene was carried out in a 25µl reaction volume containing 1xSPCR reaction buffer, 2,5 mM $MgCl_2$, 0.2 µM of each primer, 0,2 mM of each dNTP, 0,5 U Taq polymerase, and 5 µl of extracted DNA. The reactions were carried out under following conditions: an initial denaturation step of 95°C for 5 min, followed by 30 cycles of 94°C for 1 min denaturation, 54°C for 1 min annealing, 72°C for 1 min extension, and a final extension step at 72°C for 10 min.[16]

The amplification products of all genes were detected by electrophoresis in 1.5% agarose gel, and DNA was visualized by staining with ethidium bromide.

Molecular typing of strains by RAPD-PCR analysis

To determine RAPD-PCR patterns of each MRSA strains, M13 (5′- GAG GGTGGC GGT TCT- 3′) oligonucleotide was used. Amplification was carried out by modifying a method reported by Verselovic *et al*.[17] The reaction mixture was prepared in a total volume of 25 µl containing 1x PCR reaction buffer, 2.5 mM of $MgCl_2$, 200 µM of each dNTP, 2.5 U Taq DNA polymerase, 25 pmol of universal M13 primer, and 5 µl of template DNA. The amplification program was started with an initial denaturation at 94°C for 5 min. Following the initial denaturation, denaturation at 94°C for 1 min, annealing at 40°C for 1 min, and extension at 72°C for 3 min were repeated 40 times. A final extension was performed at 72°C for 7 min. The amplification products were detected by electrophoresis in 1.5% agarose gel, and DNA was visualized by staining with ethidium bromide.

Similarities between RAPD patterns were determined based on the Dice similarity coefficient. A dendrogram that graphed genetic relatedness between MRSA strains was created using "Unweighted Pair Group Method with Arithmetic Averages (UPGMA)" by Quantity One and Software.

Reproducibility, discriminatory power, and confidence intervals

To determine the reproducibility of RAPD-PCR typing by inter-assay analysis, analysis of isolates was repeated on five consecutive days. To determine the discriminatory indices of RAPD-PCR typing method, the formula described previously[18] was used. The confidence intervals (CIs) of this method were calculated according to the formula described previously.[10]

Results

Phenotypic determination of methicillin resistance

A total of 415 *S. aureus* strains, which had been isolated from various clinical sources were investigated and 61 (13.5%) of them were found as resistant to oxacillin according to Kirby-Bauer tests.

Molecular characterization of isolates

All 61 *S. aureus* strains, which were determined as methicillin-resistant, produced 1318 bp band and confirmed as *S. aureus* by PCR. These isolates were selected to perform initial molecular characterization. Fifty-five (90.16%) of all *S. aureus* strains were positive for *mecA* gene (310 bp), which is responsible for intrinsic resistance to methicillin. All (100%) strains were negative for the *femA* gene occurring naturally in *S. aureus*, which is essential for the expression of methicillin resistance. Forty-six (75.40%) strains were given 173 bp band and determined as *bla*-positive. Fifty-one (83.60%) of the strains were *coa* gene-positive. Polymorphism was found among *coa*-positive strains and they were grouped into six *coa*-types (C1-C6).

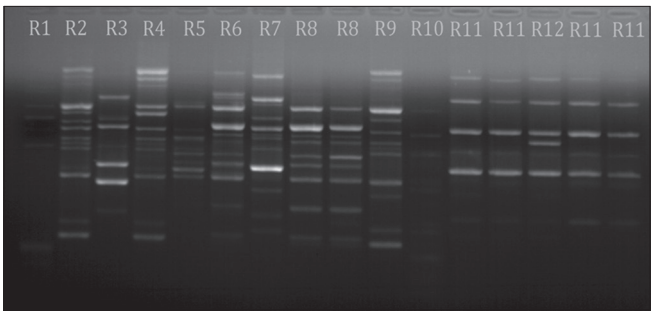

Figure 1: RAPD patterns of *S. aureus*

The strains gave one or two bands with eight different sizes, ranging between 180-480 bp. While 32 (62.74%) of the strains gave only one band, the remaining strains produced two bands. *coa*-types and the band profiles of the strains are shown in Table 2. The most predominant *coa*-type was C6, which included 22 strains having single band. These constituted 43.13% of all *coa*-positive strains, as other types included nine or less number of strains. Eleven (18.0%) strains produced a single 190 bp band, which were *spa* gene-positive. No polymorphism was found among these strains.

Table 1: Oligonucleotid primer sequences of primer sets

Primer	Oligonucleotid sequence (5′-3′)	Expected product (bp)
mecA (F)	GTA GAA ATG ACT GAA CGT CCG ATA A	310
mecA (R)	CCA ATT CCA CAT TGT TTC GGT CTA A	
spa (F)	TCA AGC ACC AAA AGA GGA AGA	Variable
spa (R)	GTT TAA CGA CAT GTA CTC CGT TG	
fema (F)	CTT ACT TAC TGC TGT ACC TG	684
fema (R)	ATC TCG CTT GTT ATG TGC	
bla (F)	ACT TCA ACA CCT GCT GCT TTC	173
bla (R)	TGA CCA CTT TTA TCA GCA ACC	
coa (F)	CGA GAC CAA GAT TCA ACA AG	Variable
coa (R)	AAA GAA AAC CAC TCA CAT CA	
sau 327 (F)	GGA CGA CAT TAG ACG AAT CA	1318
sau 327 (R)	CGG GCA CCT ATT TTC TAT CT	
M13	GAG GGT GGC GGT TCT	Variable

Table 2: The results of *coa genotyping* of *S. aureus* isolates

coa genotyping		*n*	Percentage
C1	230+480 bp	8	15.68
C2	190 bp	6	11.76
C3	390+450 bp	2	3.92
C4	220+440 bp	9	17.64
C5	450 bp	4	7.84
C6	180 bp	22	43.13

Molecular typing of strains by RAPD-PCR analysis

The genetic diversity among MRSA strains isolated from various human sources was investigated by RAPD-PCR using M13 primer. All strains gave bands with M13 primer [Figure 1].

Among the strains, genetic diversity was observed at 70.0% similarity, and strains were grouped into 12 RAPD-types (R1-R12), including three clusters and five unique types. Dendrogram derived from RAPD-PCR data is shown in Figure 2. The most predominant RAPD type of strains, R11, included 20 (32.78%) strains of all isolates. Other types included less number of strains than R1. While R6, R7, and R9 included two strains, R4 included only one strain. Similarity matrices of RAPD data are summarized in Table 3.

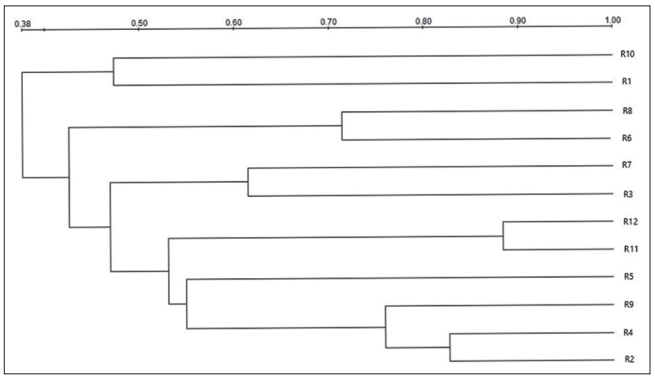

Figure 2: RAPD patterns of *S. aureus* and dendrogram obtained by UPGMA

Table 3: Dendrogram obtained by UPGMA and phylogenetic closeness of *S. aureus*

	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11	R12
R1	**100.0**	38.5	53.0	28.0	26.3	37.5	31.1	33.3	27.2	40.5	29.2	26.2
R2	38.5	**100.0**	43.6	75.7	45.4	40.1	47.5	35.5	73.8	41.9	32.3	38.8
R3	53.0	43.6	**100.0**	36.0	30.8	31.6	53.1	35.0	34.0	34.4	18.1	15.4
R4	28.0	75.7	36.0	**100.0**	42.7	43.7	46.4	39.5	96.2	32.2	37.9	38.1
R5	26.3	45.4	30.8	42.7	**100.0**	44.1	40.3	48.0	41.5	43.7	37.9	49.2
R6	37.5	40.1	31.6	43.7	44.1	**100.0**	45.6	76.3	42.0	40.2	55.5	47.6
R7	31.1	47.5	53.1	46.4	40.3	45.6	**100.0**	43.6	50.7	37.4	43.2	43.1
R8	33.3	35.5	35.0	39.5	48.0	76.3	43.6	**100.0**	37.6	41.2	41.5	41.3
R9	27.2	73.8	34.0	96.2	41.5	42.0	50.7	37.6	**100.0**	31.5	36.8	36.5
R10	40.5	41.9	34.4	32.2	43.7	40.2	37.4	41.2	31.5	**100.0**	44.3	49.1
R11	29.2	32.3	18.1	37.9	37.9	55.5	43.2	41.5	36.8	44.3	**100.0**	84.1
R12	26.2	38.8	15.4	38.1	49.2	47.6	43.1	41.3	36.5	49.1	84.1	**100.0**

Reproducibility, discriminatory power, and confidence intervals: The reproducibility of RAPD-PCR was 100%. The discriminatory indices (D) and confidence intervals for *coa* typing and RAPD-PCR analysis performed in the strains originated from different human sources were 0.802 (78.0-81.0%) and 0.852 (84.0-85.0%), respectively.

Discussion

MRSA was reported in the early 1960s and then ultimately spread worldwide over the next several decades. MRSA is now endemic in healthcare facilities in virtually, although recent data indicate a decrease in the number of invasive MRSA infections.[19] Community-acquired/associated MRSA appeared inexplicably in the 1990s and is currently a major problem in many countries worldwide.[19,20] Unlike healthcare-associated MRSA infections, which occur in individuals with predisposing risk factors, community-acquired/associated MRSA typically causes disease in otherwise healthy individuals.[20]

Two species-specific proteins (coagulase and protein A), which are also important virulence factors have been widely used to characterize *S. aureus* strains, based on variations in the sequence of *coa* and *spa* genes that code for these proteins.[9] MRSA is conferred by the presence of *mecA* gene coding for an alternative target (altered) protein PBP2a. Although this gene as a marker for the detection of methicillin resistance is generally used, *mecA* alone has been reported not to solely confer methicillin resistance. It has been shown that *fem* (factors essential for methicillin-resistance) or the auxiliary genes like *fem A/B/X* are also important in the expression of methicillin resistance.[7] In this study, 61 phenotypically resistant *S. aureus* strains from various clinical cases were investigated for *mecA* gene, and also for the presence of *blaZ* gene coding for beta-lactamase, which is responsible for beta-lactam antibiotic resistance. All the phenotypically methicillin-resistant strains were found as 90.16% *mecA* positive and 75.40% were *blaZ* positive. However, in the report of Chikkala *et al*.[8], none of the strains was *fem* gene-positive, although variations existed, even in the genomic sequences around *femA*. Also, 22 strains of *S. aureus* were found negative for both *femA* and *nuc* genetic markers, possibly since it has been suggested that likely mutations or deletions in the *nuc* gene were present. They were also first to report the absence of *femA*, and also probable variations in the sequences around the *femA* gene in clinical *S. aureus* strains in India. In Turkey, Fındık *et al*.[14] earlier found that no MRSA strain was positive for *fem* genes. In this study, 9.83% of MRSA strains were negative both for *mec* gene and *femA* gene. Although *mecA* and *femA* genes are specific markers for methicillin resistance, it has been suggested that methicillin resistance in *S. aureus* may be significantly regulated by other genes, such as, *mecR1* and *mecI* genes or some other unidentified factors, rather than *femA* gene.[21]

In this study, MRSA strains identified both phenotypically (oxacillin susceptibility in agar disc diffusion test) and genotypically (*mecA* and *fem* gene amplifications) were typed using *coa*- and *spa*-PCR analysis. As *coa* gene was identified in 83.60% of the MRSA strains, a polymorphism among MRSA strains was observed, and six *coa*-types (C1-C6), which were observed in different band patterns varying between 180 and 480 bp were also identified. The majority of strains (32/51) gave two bands, while 37.25% showed single band pattern. However, the most predominant *coa*-type was C6, and the strains belonged to this type that showed a single band (180 bp). In several studies, different numbers of *coa*-types that showed various sizes of band patterns have been identified.[22-24] In Turkey, four patterns were identified among 120 MRSA strains. The difference in band patterns and *coa*-types may be considered to depend on the presence of different allelic forms of *coa* gene and geographical variation.[25]

Some researchers have reported that the X region of the *spa* gene of *S. aureus* was polymorphic, due to a variable number of repeats, and the number of these repeats was suggested to be used in strain discrimination.[26] In this study, however, *spa* gene was selected as a discriminatory marker for the subtyping of MRSA strains. In several studies, various sizes of the PCR products, which were reflecting the number of 24 bp repeat units contained in the *spa* gene have been reported.[9,27] Whereas, no band polymorphism was observed among MRSA strains in this study. Furthermore, the majority (82.0%) of the MRSA strains were *spa* gene negative and 18.0% of the MRSA strains produced a single band (190 bp). Shakeri *et al*.[27] have reported that 3.4% of MRSA strains had no *spa* gene (no band), and the majority (83.0%) of MRSA strains gave one band (different sizes) of *spa* gene. These results were, however, lower than that those found in this study. In addition to the more prevalent strains without *spa* gene, there was no diversity in *spa* gene among the MRSA strains identified in this study. It was, therefore, considered that the MRSA strains were typed into strains without *spa* band but with a single band.

PCR-based methods are simple, cheap, and useful techniques for epidemiological studies, and some of which like RAPD-PCR, is an easy, fast, and

economically affordable method, which has been widely used for typing of *S. aureus* strains.[1,27]

MRSA strains isolated from different human samples in this study were typed by RAPD-PCR, and all the strains were typeable. According to a good discriminator power (0.852) of RAPD-PCR in this study, a total of 12 RAPD types were grouped into three clusters and five unique types. The most predominant type, R11 included 20 MRSA strains isolated from different sources. A total of 50.0% of the 20 strains of R11 type MRSA in C6 type, were the most predominant *coa* type. However, there is a need to work with a larger number of MRSA strains, to verify that predominant strains were involved in similar *coa* and RAPD types. In several studies, *S. aureus* strains have been subtyped by RAPD using different primers, to investigate the genetic relatedness among the MRSA strains.[28,29] Generally, it has been found that the MRSA strains isolated from each hospital were grouped into the same cluster.[29,30] In this study, although the majority of the MRSA strains were grouped into one predominant RAPD type, the remaining were included in other types. There was a genetic diversity in MRSA strains. None of the samples was from in-patients, as they were samples from out-patients admitted to the hospital, from different regions of Samsun. Therefore, MRSA strains had different RAPD profiles, according to their different geographical origin. The same observation may also true for *coa* types of MRSA in this study.

Conclusions

Recently, identification, monitoring, and controlling of MRSA strains both in hospitals and in the community have become very important. Rapid, easy, cheap, and accurate diagnostic tools and typing methods make it possible to take well-timed measures for prevention against MRSA outbreaks, and also to monitor the spreading of strains and transmission routes. In this study, all MRSA strains were characterized genetically by some PCR-based molecular typing methods. While a genetic diversity was found among these strains using *coa*-PCR and RAPD-PCR, they could not be typed by *spa*-PCR, which has previously been reported to be a successful method for this purpose. It was concluded that coa- and RAPD-PCR typing were useful for epidemiological studies of MRSA but the differences in polymorphism of *spa* gene among MRSA strains should be considered.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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