Determination of genetic relationship between methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* clinical isolates by RAPD-PCR Method[§]

RAPD-PCR yöntemi ile metisilin duyarlı ve dirençli *Staphylococcus aureus* klinik izolatları arasındaki genetik ilişkinin saptanması

Research Article

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ABSTRACT

The increase in the frequency of methicillin-resistant *Staphylococcus aureus* (MRSA) demands a quick and trustworthy characterization of isolates and identification of clonal spread within hospitals. The objective of this study is to establish variations of randomly amplified polymorphic DNA (RAPD) patterns in *S. aureus* isolates which are collected from clinical isolates and a systematic relationship between them. Identification of isolates and antimicrobial susceptibility tests were performed by automated system and conventional phenotypic methods. A total of 30 out of 47 isolates (64%) expressed methicillin-resistance. When methicillin-susceptible and resistance isolates were compared, the least active agents were rifampin, all aminoglycosides and penicillines, tetracycline and amoxicillin-clavulonic acid for methicillin-resistance isolates. RAPD-PCR method with 25 different primers was analyzed on 47 clinical isolates of *S.aureus*. All the data were employed to construct dendograms using the unweighted pair group method using arithmetic (UPGMA). In this study, it is clear that, RAPD-PCR assayed with combination of five primers can be successfully applied to assess the genetic relationship between MRSA and MSSA isolates from clinical samples.

Key Words
Antibiotyping, *Staphylococcus aureus*, methicillin resistance, RAPD-PCR

ÖZET

Metisilin dirençli *Staphylococcus aureus* (MRSA) görülme sıklığındaki artış bu suşların karakterizasyonunun hızlı ve güvenilir bir şekilde yapılmasını ve hastanelerde klonal yayılımın tanımlanmasını gerektirmektedir. Bu çalışmanın amacı, klinik örneklerden elde edilen *Staphylococcus aureus* suşlarına ait rastgele çoğaltılmış polimorfik DNA (RAPD) bant modellerindeki varyasyonu ve bunlar arasındaki sistematik ilişkiyi ortaya çıkarmaktır. Suşların tanımlanması ve antibiyotik duyarlılıkları otomatize sistem ve geleneksel yöntemler ile belirlendi. Toplam 47 izolatın 30'u (%64'ü) metisiline dirençli bulundu. Metisilin duyarlı ve dirençli izolatlar karşılaştırıldığında metisilin dirençli izolatlarda en düşük etki gösteren antibiyotikler rifampin, tüm aminoglikozidler ve penisilinler, tetrasiklin ve amoksilin-klavulonik asittir. 47 *S.aureus* klinik izolat, RAPD-PCR tekniği kullanılarak 25 farklı primer ile analiz edildi. Tüm veriler ile ağırlıksız çift grup yöntemi (UPGMA) kullanılarak dendogram çizildi. Bu çalışmada, beş primerin kombinasyonu kullanılan RAPD-PCR yöntemi klinik örneklerden izole edilen MRSA ve MSSA izolatları arasındaki genetik ilişkinin belirlenmesinde başarılı bir şekilde uygulanabildiği açıkça görülmektedir.

Anahtar Kelimeler
Antibiyotipleme, *Staphylococcus aureus,* Metisilin direnci, RAPD-PCR

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INTRODUCTION

Staphylococcus aureus caused clinically significant infections in humans, including endocarditis and deep-seated abscesses, which lead to toxic and septic shock syndromes [1,2]. Serious staphylococcal infections can frequently occur in inpatients and may lead to dire consequences, especially as to therapy with antimicrobial agents. Recently, the increase in the frequency of methicillin-resistant *S. aureus* (MRSA) as the causal agent of nosocomial infection and the possibility of emergence of resistance to multi-drug demands a quick and trustworthy characterization of isolates and identification of clonal spread within hospitals [3].

Rapid identification and classification of *S.aureus* is carried out by morphology, nutritional requirements, antibiotic resistance, phage sensitivity and more recently by DNA based methods [4,5]. Nowadays, a large number of reports describing the use of PCR for genetic typing of *S.aureus* have appeared. These include binary typing, pulsed field gel electrophoresis (PFGE), restriction fragment length polymorphism analysis (RFLP) and PCR-based methods which provide better discriminatory power than phenotyping methods [6-8]. Randomly amplified polymorphic DNA (RAPD) PCR is based on the amplification of genomic DNA with 8-10 bp primer [9, 10]. This method was reported as a useful assay for subtyping of *S. aureus* and MRSA strains and to be technically much simpler and faster [11, 12]. Some researchers have reported that RAPD-PCR typing has discriminatory power and is widely used for *S.aureus* strains [3, 13].

This study was aimed at the isolation and identification of *Staphylococcus aureus* isolates from various clinical materials in a teaching hospital during two years. These isolates were identified by RAPD-PCR analysis methods to discriminate between methicillin susceptible and resistance isolates.

MATERIAL AND METHODS

Species identification

This study was carried out at Hacettepe University Hospital, Turkey, between October 2003 and November 2005. 47 *Staphylococcus aureus* strains isolated from clinical samples including blood, tracheal aspiration, pus, wound, catheter, nose and urine. Isolates were identified by automated system (Phoenix–100, USA). These strains were characterized by conventional identification phenotypic methods: Gram staining, hemolysis of blood agar, mannitol fermentation, catalase, coagulase tests, and DNAase activity.

Antimicrobial susceptibility testing

Antimicrobial susceptibility tests were performed by automated system (Phoenix–100, USA) and using the disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) criteria. Antimicrobial agents tested included amoxicillin-clavulanic acid, gentamicin, erythromycin, oxacillin, penicillin, rifampin, streptomycin, sulbactam-ampicillin, tetracycline and vancomycin.

DNA isolation and RAPD-PCR analysis

DNA was isolated using Genomic DNA Kit (BioBasic, Canada) and stored -20°C. The PCR mixture consisted of 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 200 mM Tris HCl, 1 % Triton X-100, 1 mg/mL BSA, 2.5 mM dNTP, 2.0 mM MgSO4 and 3 U Taq DNA polymerase (BioBasic, Canada). 5 μM concentration of each of twenty five different primers. The primers used in the study are listed in Table 1. A single primer was used in each reaction. Amplification of DNA fragments was performed in a Eppendorf Thermal Cycler (Hamburg, Germany) with predenaturation for 2 min at 95°C , then 45 cycles of 1 min at 94°C, 1 min at 30°C and 2 min at 72°C, and 5 min 1 cycle of 5 min at 72°C.

PCR products were analyzed by 1.8 % agarose gel electrophoresis (Scie-Plas; UK) using TBE 1× buffer (0.9 M Tris, 0.9 M Boric acid and 20 mM EDTA, pH 8.3) at 100 V for 4 h and visualized staining ethidium bromide and using Gel Logic 200 Molecular Imaging System (Kodak; Rochester).

Phylogenetic analysis and Discriminatory power

Band patterns generated with each primer were analyzed using NTSYS-pc (version 2.1; USA) to calculate Dice coefficients of similarity. The dendogram was created by the unweighted pair group method using arithmetic (UPGMA) average clustering. Strains with a similarity coefficient

Table 1. Structure of Primers for RAPD.

Primer	Nucleotide sequence (5′-3′)	Primer	Nucleotide sequence (5′-3′)
1	AAGAGCCCGT	S21	CAGGCCCTTC
2	AGCGTCACTG	S24	AATCGGGCTG
4	AGCAGCCTGC	S31	CAATCGCCGT
6	GTGGATGCGA	S33	CAGCACCCAC
7	GCGATCCCCA	S112	ACGCGCATGT
9	TCACGATGCA	S144	GTGACATGCC
10	TCACGCTGCG	S211	TTCCCCGCGA
11	AAGTCCGCTC	S224	CCCCTCACGA
12	CAATCGCCCT	S252	TCACCAGCCA
13	CGGATCGACA	S312	TCGCCAGCCA
14	AATCGGGCAG	S1112	TCTCACCGTC
16	AATCGGGCGG	S1452	AAGAGGGCGT
S19	ACCCCCGAAG		

of 55 % or greater were grouped in a cluster for RAPD-PCR. The discriminatory power of the used primers can be calculated on the basis of the Gaston and Hunter's index D [14].

RESULTS

Antibiotic susceptibility patterns

The antimicrobial spectrum for 10 selected antimicrobial agents of different classes against methicillin sensitive and resistant *S. aureus* are summarized in Table 2. A total of 30 out of 47 isolates (64 %) expressed phenotypic methicillin resistance. 12 different antibiotype patterns were determined; 5 for MSSA with two dominant patterns-29.4 % and 7 for MRSA with two dominant patterns-26.7 % were determined.

Comparison of the percent susceptible for all *S. aureus*, MSSA and MRSA isolates are given in Figure 1. The least active agents were rifampin, all aminoglycosides, penicillines, tetracycline and

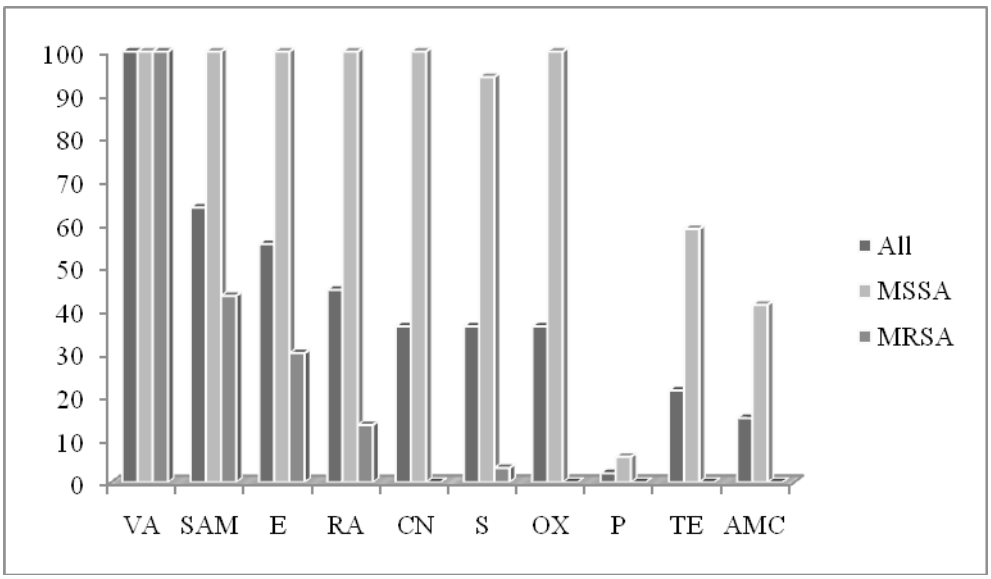

Figure 1. Antibiotic susceptibility of *Staphylococcus aureus* isolates.

MSSA: methicillin-susceptible S. aureus, MRSA: methicillin-resistant S. aureus. **AMC:** *amoxicillin-clavulanic acid, CN: gentamicin, E: erythromycin, OX: oxacillin, P: penicillin, RA: rifampin, S: streptomycin, SAM: sulbactam-ampicillin, T: tetracycline and VA: vancomycin.*

Table 2. Antibiotic resistance phenotypes of MSSA and MRSA isolates in clinical isolates

S.aureus isolates	Antibiotimicrobial agents	Total % of clinical isolates (no.)
MSSA		
S7	-	5.9 (1)
S6, S8, S9, S10, S16	P	29.4 (5)
S1, S2, S3, S4	AMC, P	23.5 (4)
S5	P, TE	5.9 (1)
S12, S13, S14, S15, S17	AMC, P, TE	29.4 (5)
S11	AMC, P, S, TE	5.9 (1)
MRSA		
R1, R10	AMC, CN, P, RA, S, TE	6.7 (2)
R5, R9, R11	AMC, E, CN, P, S, TE	10 (3)
R21	AMC, E, CN, P, S, SAM, TE	3.3 (1)
R28	AMC, E, CN, P, RA, SAM, TE	3.3 (1)
R2, R3, R13, R14, R16, R22, R24	AMC, CN, P, RA, S, SAM, TE	23.3 (7)
R4, R6, R7, R8, R12, R19, R20, R29	AMC, E, CN, P, RA, S, TE	26.7 (8)
R15, R17, R18, R23, R25, R26, R27, R30	AMC, E, CN, P, RA, S, SAM, TE	26.7 (8)

S1-S17, MSSA: methicillin-susceptible S. aureus, R1-R30, MRSA: methicillin-resistant S. aureus. **AMC:** *amoxicillin-clavulanic acid, CN: gentamicin, E: erythromycin, P: penicillin, RA: rifampin, S: streptomycin, SAM: sulbactam-ampicillin and T: tetracycline.*

amoxicillin-clavulonic acid. When MSSA and MRSA isolates were compared, it was demonstrated that MRSA isolates resistant to all antibiotics except for vancomycin and there was multiple antibiotic resistance in this isolates.

RAPD-PCR Analysis

RAPD-PCR method with 25 different primers was analyzed on 47 clinical isolates of *S.aureus* and *S.aures* ATCC 29213, *S.epidermidis* and *S.saprophyticus* as controls. All the data were employed to construct dendograms using the UPGMA. The dendogram obtained with the combination of 6, 11, 13, S31 and S144 primers data. The combination of the patterns obtained with the five primers generated a higher discriminatory power than those of single primers (0.983 for 13, 0.977 for 11, 0.972 for S31, 0.959 for S144 and 0.903 for 6).

At 55 % similarity three distinct clusters (1, 2 and 3) were detected. Cluster 1 grouped *S.epidermidis* and all methicillin susceptible *S.aureus* isolates. Cluster 2 grouped only all methicillin resistant *S.aureus* isolates. Cluster 3 contained only one *S. saprophyticus* isolate, in accordance with the data presented in Figure 2.

DISCUSSION

A number of methods are available for the comparison of clinical isolates to define clonal relatedness among isolates and epidemiology. PCR based methods are recognized as more effective than the conventional methods of phenotyping for typing different organisms. In this study, it is clear that, in order to prevent MRSA outbreaks rapid and reliable discrimination between MRSA and MSSA is extremely important.

Methicillin resistance in *S.aureus* progressively increases in many countries especially in Turkey. It is important to identify MRSA isolates rapidly and find effective treatment choice. In our study, 47 *S.aureus* from different clinical isolates, 30 (64%) is resistant to methicillin. When MSSA and MRSA were compared, it was demonstrated that MRSA isolates resistant to these antibiotics except for vancomycin and there was multiple antibiotic resistance in these isolates. This situation has become an increasing problem about treatment for MRSA infections. In none of the previous studies in Turkey, vancomycin resistant MRSA was reported; therefore vancomycin can be used for treatment [15]. Widespread use of vancomycin to treat infections caused by MRSA

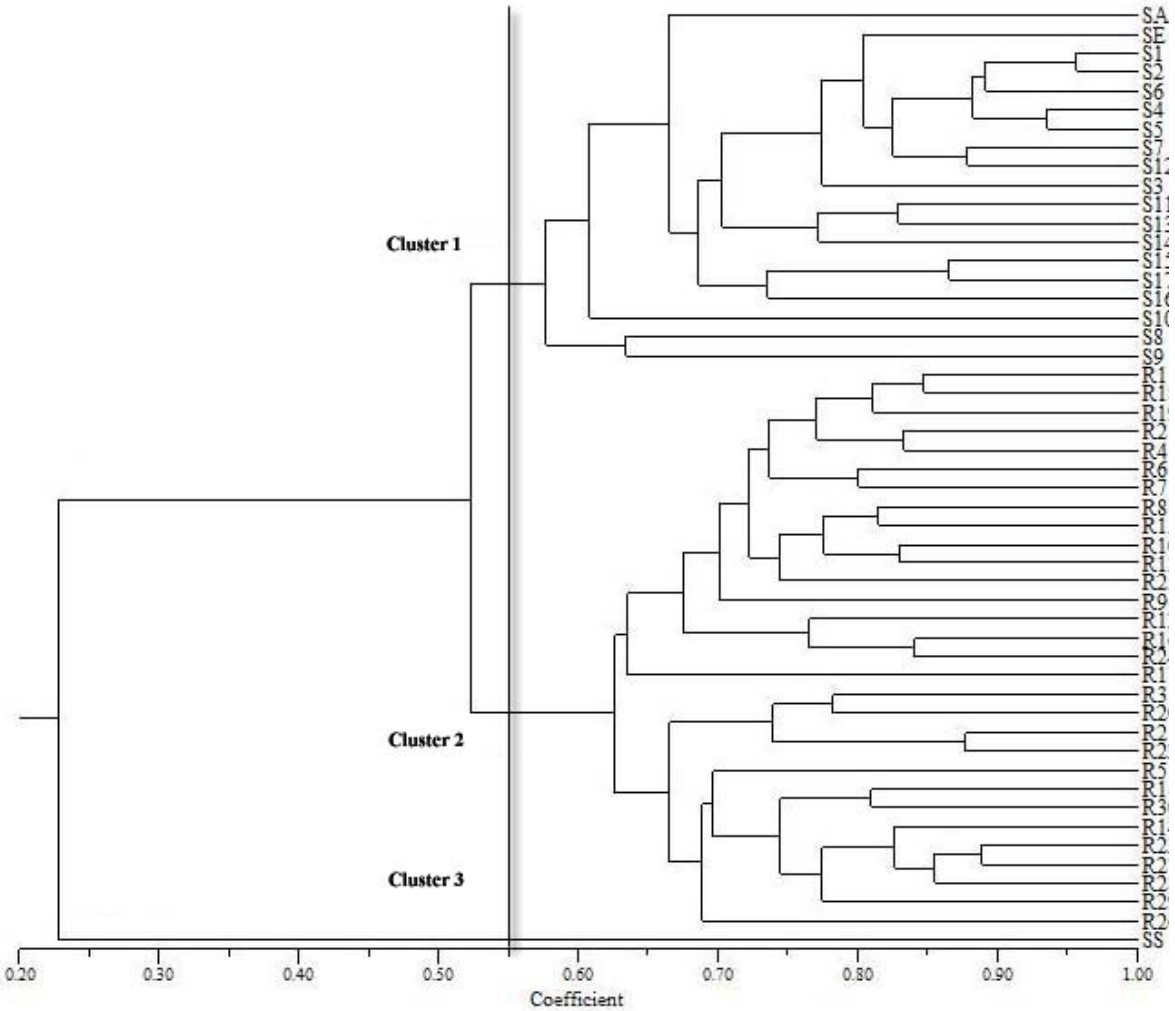

Figure 2. Dendrogram showing similarities for 47 isolates of methicillin resistant and methicillin sensitive *S.aureus* **and contro**l isolates based RAPD-PCR data using five studied primers. The RAPD-PCR profile grouping was done the NTSYS-pc 2.1 software package using Dice coefficient and unweighted pair group method with arithmetic mean (UPGMA) cluster analysis. A 55% similarity was arbitrarily chosen as a discriminating threshold to define the homogenous clusters (1-3). **SA:** *S.aures ATCC 29213,* **SE:** *S.epidermidis,* **SS:** *S.saprophyticus*, **S1-S17: methicillin-susceptible** *S. aureus* **and R1-R30: methicillin-resistant** *S. aureus*.

has been reported to result in low level resistance. The first *S. aureus* with reduced vancomycin susceptibility was isolated in 1997 and similar isolates have since been discovered in India [16-18]. Extensive use of these antibiotics, both methicillin and vancomycin high level resistant *S.aureus* are revealed inevitable in the course of time.

Currently, PCR based typing has been extensively applied for epidemiological typing *S.aureus* [12,19]. RAPD PCR typing compare to other genotyping methods such as restriction fragment length polymorphism (RFLP) and pulsed-field gel electrophoresis (PFGE), provide more fast and reliable method to typing methicillin resistance and susceptible *S.aureus* isolates [6, 20].

RAPD assay was used for subtyping of MRSA isolated from human samples in several studies [11, 12]. In our study *S.aureus* isolates were analyzed using RAPD-PCR method with five primers, each with high discriminatory power history according other studies [5,21-23]. All MRSA isolates were put in same cluster and distinguished from MSSA isolates; thus, the clonal relation was detected between these isolates by RAPD assay.

CONCLUSION

This study demonstrates that RAPD-PCR method could successfully discriminate between MRSA and MSSA isolates. Furthermore the reproducibility and rapidity of the method and its cost effectiveness make it improves identification of MRSA, thus, aids the formulation of effective control measures. It is clear that in order to prevent MRSA outbreaks rapid and reliable discrimination between MRSA and MSSA is extremely important.

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