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Molecular Characterization of Methicillin-Resistant and Methicillin- Susceptible *Staphylococcus aureus* Isolates Obtained from Human-Skin Samples in Iraq

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The present study is conducted to investigatethe relatedness and genomic variability between strains of methicillin-resistant and methicillin-susceptible Staphylococcus aureus.A total of 100 isolatescollected from different human skin infection and used a combination of morphological and molecular techniques to identify ten of these as Methicillin-susceptible S. aureus (MSSA), and ten as Methicillin-resistant S. aureus (MRSA). The MRSA and MSSA samples were subjected to genetic characterization with the use of the Random Amplificationof Polymorphic DNA technique (RAPD) using 10 primers.The DNA profile of the samples wereobtained and showed that the number of the bands and banding patterns varied depending upon the primers used. A two-dimensional binary matrix was constructed through scoring the presence or absence of a band at any position on the gel. A total of 142 bands were generated per the 10 primers, of which 141 ranged between 3100-100 bp. Primers OPJ-13 and OPE-16 exhibited the largest number of bands (20) with a 100% polymorphism and the greatest discriminatory index power (DI, 14.1), while primer OPI- 06 produced the lowest number of bandsand a comparably lower level of discrimination (DI, 7.09).Data in this study demonstrate the ability of the RAPD analysis to differentiate between strains at the intraspecies level. Data also suggest that this technique can be applied successfully to assess the genetic backgrounds among isolates.

Keywords: Methicillin-Resistant; Methicillin-Susceptible; Random Amplification of Polymorphic DNA; *Staphylococcus Aureus.*

Staphylococci are ubiquitous, including a dozen species occurring as part of the normal flora. The most virulent species is Staphylococcus aureus, which colonizes epithelial surfaces and causes infections in humans. Historically, Methicillin has been the treatment of choice for the S. aureus infections, but resistance is now widespread. It is believed that this resistance is related to the mec Agenes, which are carried on mobile genetic elements, and can confer resistance to most beta-lactam antibiotics and other antimicrobial classes[1,2, 3, 4]. All staphylococcal genomes are approximately 2.8 Mbp in size, and are, thus predicted to encode 2500 genes with relatively low G-C content. The

comparative analysis has revealed that most regions of the genome are well-conserved; however, several large sequence blocks showed high variability.

Lindsay and Holden first posited the concept of the 'core'; the part of the genome that is present in all strains of S. aureus, in contrast to the "accessory genome" which is variable[5,6]. The core contains housekeeping genes and many virulence genes. Its genetic plasticity facilitates the evolution of many virulent and drug-resistant strains, presenting a major clinical challenge (Holden *et al*., 2004). Most of the dissimilarity among the species of the strains is due to mobile genetic elements, such as bacteriophages or pathogenicity islands[7, 8, 9].

Gene transfer amongthe S. aureus strains is certainly much higher than among different staphylococcal species[10].

The RAPD technique makesthe study of a large proportion of target genomes possible by generating distinct banding patterns that allow for finer discrimination among the strains[11, 12]. It amplifies segments of target DNA using small arbitrary primers of unknown homology with a target sequence.

The number and size of the fragments generated during PCR are the basis for typing an isolate[13, 14, 15]. This work seeks to apply RAPD-PCR extensively to determine the relatedness and genomic variability of a collection of methicillin-susceptible (MSSA) and methicillin-resistant (MRSA) S. aureusstrains, and thus analyse their diversity.

MATERIALS AND METHODS

Isolation of Bacterial Strains and Molecular Detection using the Polymerase Chain Reaction (PCR)

The study included 100 local isolates of Staphyloccoci spp. collected from different clinical skin infections in unrelated patients. All isolates were grown at 37 °C on a mannitol salt agar (MSA) medium and were identified at the species level using their morphological characteristics and biochemical tests[16]. For the molecular determination, the MRSA and MSSA genomic DNA was extracted from the staphylococcal cultures via a heat shock/boiled-cell method that involved boiling and freeze-thawing processes as described elsewhere[17, 18, 19]. The application of the RAPD technique was preceded by the extraction of genomic DNA using the boiling method. The mecA gene was amplified to identify the MRSA strains without the need to check for antibiotic susceptibility, as discussed by Tang and Stratton, who suggested that the specificity of MRSA identification is based on the quantitative correlation of the mecA gene; this gene encodes the protein PBP2A (penicillin binding protein 2A), which has a low affinity for beta-lactam antibiotics such as methicillin). Therefore, this assay allows for a reliable identification within only six hours of sample collection.

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The purity of the extracted DNA was checked in a NanoDrop (Thermo Scientific) at the wavelengths of A260 and A280. A specificmecA -PCR primers with anamplicon size of 533bp was subjected to forward (mecA: F 5'- AAAATCGATGGTAAAGGTTGGCAG-3') and reverse (mecA: R 5'- AGTTCTGCAGTA CCGGATTTGC-3') supplied by Integrated DNA Technologies company, Canada . The amplification program was one cycle at 94 °C for one minute; thirty-five cycles of 94 °C for one minute, 63 °C for one minute, 72 °C for one minute and 72 °C for ten minutes, using the Mastercycler (Eppendorf). The amplified product was subjected to 2% agarose gel electrophoresis and was visualized under UV (Imagemaster VDS, Pharmacia Biotech, USA) after being stained with Ethidium bromide[20]. Only twenty samples were subjected to the RAPD assay after being molecularly detected as either methicillin-resistant and methicillin-sensitive strains (ten samples were mec negative=MSSA and ten were mec positive= MRSA)[21].

Random Amplified Polymorphic DNA: Reaction Mix, Program, and Detection

All isolates from samples in this research were subjected to identification at the species level using their morphological characteristics and biochemical tests; all isolates were confirmed as S. aureus. The processes of DNA amplification were performed using the mecA gene to detect the Methicillin-resistant strains.The RAPD assay was conducted twice under the same conditions to confirm the reproducibility of the method and to eliminate sporadic amplification products from the analysis.

The RAPD primers used in the studywere as follows: OPI – 06: (5'– AAGGCGGCAG-3'), OPJ-13: (5'-CCACACTACC-3'), OPE-16:(5'–GGTGA CTGTT-3'),OPQ-01: (5'-GGGACGATGG-3'),OPM-20:(5'–AGGTCTTGGG-3'),OPN-07:(5'-CAGCCCAGAG-3'),OPQ-17: (5'–GAAGCCC TTG-3'),OPD-20: (5'-ACCCGGTCAC-3'),OPB-14: (5'– TCCGCTCTGG-3'), and OPA-11: (5'-ACCCGACCTG-3') (Primer set supplied by OPT (Operon DNA Technologies Company, Canada). The reaction was conducted in a 25 µl reaction mixture containing 2 µl of DNA (50 ng), 12.5 µlGoTaq 0T® Green Master (Promega, CA), (0.5 µl) 25 mM MgCl2, 2 µl of (10 Pmol\ µl) of each primer, 2 µl of deionized and distilled water.The PCR amplifications were performed in a Labnet Thermal Cycler with the following specification: first,a denaturation cycle at 94 ºC for five minutes; forty-five cycles (segment denaturation 94 ºC for forty-five seconds; annealing 40 ºC for one minute; extension 72 ºC for forty-five seconds), and then a final extension comprised of one cycle at 72 ºC for seven minutes according to Kumar and Gurusubramanian[22, 23, 24].

The amplified products were analysed by electrophoresis in a 1.5% (w/v) agarose gel and were visualized under UV (Imagemaster VDS, Pharmacia Biotech, USA) after being stained with ethidium bromide (Kumari and Thakur, 2014). A DNA ladder (250 bp) purchased from BIONEER Co. was used to assess the size of the PCR product, which was, then, photographed using a gel documentation system.

Statistical Efficiency Discrimination

RAPD fragments were scored as (0, 1) where 1 means presence and 0 denotes the absence of a band at a specific position in the gel. The binary patterns obtained were directly compared for their similarities using the NTSYS-pc Numerical Taxonomy and Multivariate Analysis System (version 2.1, Exeter Software) as described by Kosman and Leonard[25, 26]. The data collected were analysed, and only the major bands that were consistently amplified were recorded. The polymorphism of each primer was calculated based on the following equation:

$$\text{Polymorphism\%} = (Np/Nt) \times 100$$

Where Nprefers to the number of the polymorphic bands, and Nt reflects the total number of bands of the same primer[27]. The efficiency and discriminatory powers of each primer were calculated. Primer efficiency was calculated as the number of polymorphic bands to the total number of primer bands. The discriminatory power of each primer was calculated as the percentage of the number of polymorphic bands to all of the primers[28, 29].

Genetic Distance and Relationship Estimation

Phylogenetic analysis and pairwise alignment similarities were calculated via the neighbour-joining method. The genetic distances among all pairs were calculated based on Nei and Li's similarity indices (Nei and Li, 1979) according to the following formula:

$$G.D = 1-\{2Nab/(Na+Nb)\}$$

Where Na is the total number of the fragments observed in individual 'a'; Nbisthe total number of fragments 'b' and Nab is the number of fragments shared by both individuals. Genetic relationship tree diagrams were developed to illustrate the relationships among the studied strains. The similarity was calculated based on the following formula[30, 31]

$$\text{Similarity} = 1- G.D\times 100\%$$

RESULTS

The results were analysed using band notes obtained from the PIC of primers,as illustrated in Figure 2,which included the presence or absence of bands, together with their molecular

weights that were produced by a primer across the twenty samples starting from the highest to lowest weight, as well as the total number of the amplified bands across all isolates. The bands obtained had a pattern similar to a bar code, allowing for the identification of each individual. In total, 142 bands were generated per the ten primers, of which 141 ranged between 3100 and 100 bp.

The average number of bands per primer was 14. Primers OPJ-13 and OPE-16 exhibited the most bands (20) with a 100% polymorphism, and these also had the greatest discriminatory index power (DI, 14.1). Primer OPI-06 produced the smallest number of bands (10) and had the least discriminatory power (DI, 7.09). The differences in the molecular weights of the bands reflected the number of targets for each primer site within the DNA in question. The names and sequences of the commercially-synthesized primers used to generate the PCR products (Operon Technologies, USA) along with a summary of the total number of bands and polymorphic fragments are listed in Table 1.

In respect to the percentage of polymorphism, a unique band means that the band is present in just one individual and absent in the others (as in the underlined primer OPD-20).The discriminatory index (DI) reflects the likelihood of unrelated isolates being classified as the same type. According to recent guidelines, a typing system should have DI >0.95 for a reliable assessment of the clonal relatedness of the isolates.

Analysis Of Genetic Distance

The inter-group distance is the average of the paired distances of members from both groups. The similarity measures given took values ranging from zero to unity. For identical genotypes s= 1, while for completely distinct measures s = 0. Depending onshared bands between MRSA and MSSA,the increase in bands led to a decrease inthe genetic distance, and vice versa. Table 2 illustrates the values of genetic distance. The highest similarity was obtained between strainsnumbers '11' and '12', while the lowest level was obtained between strainsnumbers '4'(MRSA) and '14'(MSSA).

Cluster Analysis of RAPD

Cluster analysis was conducted using the unweighted pair-group method, in which the samples were grouped based on similarity to construct a dendrogram. Strikingly, the twenty S. aureus isolates formed two distinct groups (Fig. 3). All MRSA strain isolates were mapped to the upper group in the phylogenetic tree. Major Cluster, I included three main groups. The first of these groups contained two sub-clusters; one contained isolate C1 and the other contained isolates C2 and C3. The second group also contained two sub-clusters; one included isolates C4 and C7 and the other included isolates C5 and C6. Likewise, there were two sub-clusters in the third group with the first cluster containing isolates C8 and C9 and the second containing isolate C10.

Table 1. Fragments Amplified by the Primers in the Twenty *S. aureus* Isolates and the % Efficiency of Amplification and Discriminatory Power

Primer Name	Sequences (5'- 3')	Number of Bands Amplified in both Strains		Polymorphism (%)	Primer Efficiency (%)	Primer Discriminatory Power (%)
		Total	polymorphic			
OPI - 06	AAGGCGGCAG	10	10	100	7.04	7.09
OPJ-13	CCACACTACC	20	20	100	14	14.1
OPE-16	GGTGACTGTT	20	20	100	14	14.1
OPQ-01	GGGACGATGG	16	16	100	11.2	11.3
OPM-20	AGGTCTTGGG	14	14	100	9.8	9.9
OPN-07	CAGCCCAGAG	13	13	100	9.1	9.2
OPQ-17	GAAGCCCTTG	11	11	100	7.7	7.8
OPD-20	ACCCGGTCAC	11	10	90.9	7.04	7.09
OPB-14	TCCGCTCTGG	12	12	100	8.4	8.5
OPA-11	ACCCGACCTG	15	15	100	10.5	10.6
Total	10	142	141	99.1	-	-

Table 2. Genetic distance of Both MRSA and MSSA Strains

	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13.	14.	15.	16.	17.	18.	19.	20.
1	0.00000																			
2.	0.32384	0.00000																		
3.	0.56972	0.28742	0.00000																	
4.	0.52774	0.56390	0.42629	0.00000																
5.	0.56573	0.55933	0.66907	0.41748	0.00000															
6.	0.72791	0.58175	0.45033	0.48715	0.49340	0.00000														
7.	0.50461	0.54077	0.49205	0.34082	0.55470	0.56185	0.00000													
8.	0.75204	0.73941	0.67117	0.88891	0.81568	0.60448	0.60125	0.00000												
9.	0.61871	0.80902	0.72639	0.86500	0.63106	0.66304	0.63931	0.35483	0.00000											
10.	0.72499	0.67018	0.49620	0.60837	0.56298	0.53091	0.51379	0.46111	0.44803	0.00000										
11.	0.84059	0.78974	0.73243	0.86146	0.68588	0.50066	0.78511	0.69469	0.64789	0.64497	0.00000									
12.	0.80057	0.79228	0.77245	0.99901	0.68508	0.65301	0.82513	0.77245	0.68791	0.64004	**0.18312**	0.00000								
13.	0.72182	0.75798	0.82327	0.82971	0.65413	0.57426	0.75335	0.86773	0.69618	0.68467	0.31399	0.21079	0.00000							
14.	0.86348	0.76611	0.78884	**0.99911**	0.82931	0.63955	0.72374	0.92238	0.74687	0.69280	0.30061	0.21892	0.24780	0.00000						
15.	0.74262	0.70182	0.76229	0.84480	0.67800	0.50583	0.85419	0.80151	0.67775	0.70992	0.35410	0.25220	0.22125	0.26860	0.00000					
16.	0.75171	0.67867	0.73649	0.76528	0.72632	0.48352	0.65564	0.81060	0.89312	0.62470	0.40241	0.30052	0.23182	0.25864	0.20488	0.00000				
17.	0.91697	0.90661	0.84233	0.99141	0.75496	0.50583	0.77723	0.80151	0.71697	0.60814	0.31243	0.21298	0.22125	0.19164	0.24512	0.22198	0.00000			
18.	0.82496	0.77411	0.75602	0.79932	0.79286	0.53633	0.84953	0.75602	0.89463	0.77777	0.37457	0.31208	0.27686	0.28499	0.29680	0.22960	0.27660	0.00000		
19.	0.80377	0.71733	0.74006	0.73557	0.69352	0.55959	0.83196	0.77928	0.69474	0.72406	0.37536	0.27080	0.21844	0.28719	0.24159	0.30895	0.29986	0.21515	0.00000	
20.	0.68913	0.72529	0.78884	0.88663	0.70148	0.58239	0.88408	0.70880	0.62426	0.69280	0.23874	0.19872	0.24780	0.29849	0.24879	0.33711	0.28880	0.26393	0.22657	0.00000

The second major cluster (Cluster II) represents the MSSA strains and included three main groups. The first group contained two sub-clusters; one contained isolates C11 and C12 and the other contained isolate C20. The second group also included two sub-clusters. The first of these contained only isolate C13, and the second contained isolates C15 and C16. The second sub-cluster, meanwhile, contained isolates C14 and C17. The third group contained only one sub-cluster which included two isolates, C18 and C19.

DISCUSSION

The taxonomy of a genus is traditionally based on morphological characteristics, but there are significant difficulties in distinguishing the closelyrelated groups such as the populations or

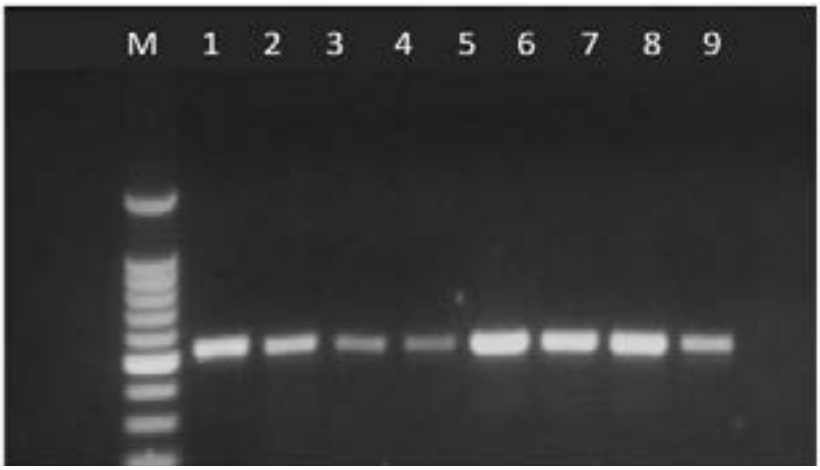

Fig. 1. DNA fragments generated by specific PCR amplification for the detection of MRSA strains. Lane (M): 100 bp DNA ladder (Bioneer), lanes (1-9): PCR product of *mecA* gene (533bp)

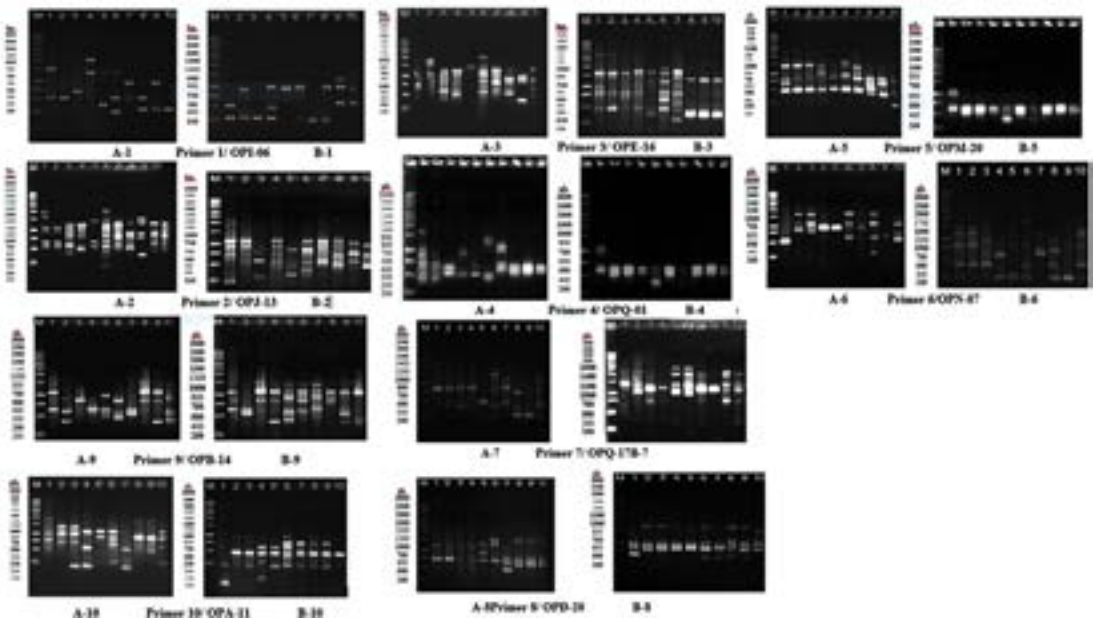

Fig. 2. MRSA primer figures, including the ten primers to the left side starting from A-1 to A-10 and, to the right, MSSA primer figures starting from B-1 to B-10 compared with a DNA ladder marker (250-3000), Lane M (marker) Other lanes contain genomic DNA of ten samples each

strains of the same species. New techniques using molecular markers, however, now allow for much easier identification of such strains[32, 33].

One of these techniques is RAPD-PCR, which depends on the amplification of DNA fragments with a single arbitrary primer resulting in the generation of amplified products that represent a multiplicity of anonymous sites that are characteristic of the studied genome[34, 35]. This method is widely recognized as a highly discriminatory tool for the molecular typing of a wide range of bacteria, due to its ability to determine polymorphisms in the entire bacterial genome [36].

The advantage of this technique is that markers are targeted to a much smaller locus within the genome, and the likelihood of identifying false positive markers is small; therefore, the RAPD analysis has been widely used for taxonomic and phylogenetic studies, for species differentiation, and for the study of phylo-geographic patterns of genetic variations [37, 38]. Furthermore, it requires only small amounts of DNA. Compared to other methods, it is simpler, very sensitive, cheaper, faster and less labour-intensive[39, 40, 41]. It can detect rearrangements, additions, or deletions of DNA by visualizing banding shifts, missing bands or the appearance of new bands[42].

The failure of some primers to amplify DNA may be attributed to their need for special requirements in terms of PCR-reagents or temperature profile since all reaction parameters were identical for all primers. The G+C content of a primer may further interfere with the PCR yield. The RAPD markers developed independently were soon used for linkage mapping and the detection of relatedness among species[43].

These markers were the simplest (as they do not require prior knowledge of the target sequences for their design), and they are less expensive and fast. In addition, these markers exhibit reasonable efficiency compared with other methods. They can anneal and prime at multiple locations throughout the genome, producing a spectrum of amplification products that are characteristic of the template[44]. Ozbey*et al*. (2004) stated that using different RAPD primers increases the differentiation power of the process. If RAPD markers are used to detect polymorphisms and genetic relationships, as in the present work, a large number of primers is recommended to make the chance of producing monomorphic bands over the species tested[45].

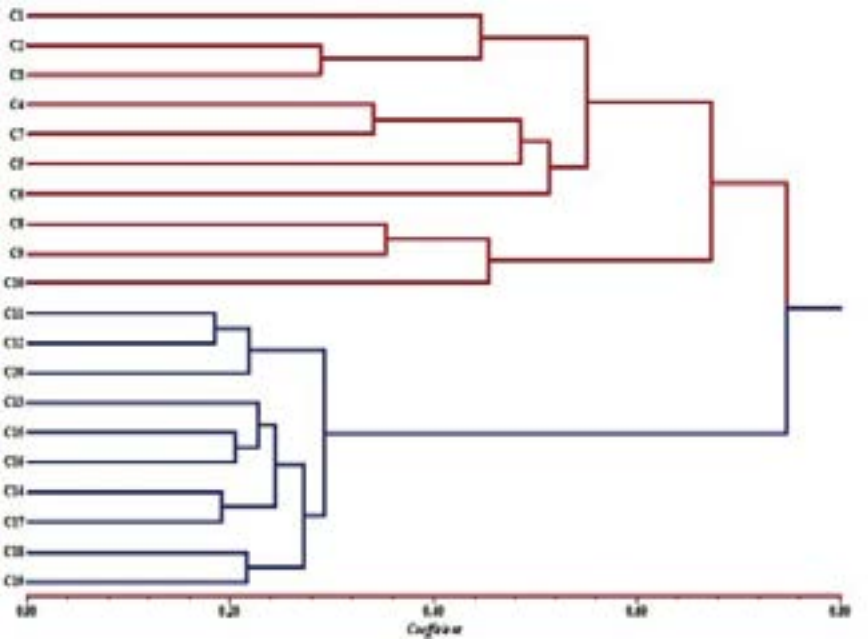

Fig. 3. Genetic Relationships of MRSA and MSSA Strains Illustrated in a dendrogram. Two major clusters have been observed. In cluster A, variation is less reported for strains (C11-C19)

In this study the differences in the RAPD profiles obtained with almost all primers revealed the presence of a great genetic heterogeneity between both strains, allowing the researchers to demonstrate the technique's ability to differentiate strains at the intra-specific level. The strategy for identifying the methicillin-resistant strains among S. aureus in this study agreed with that of Ozbey*et al*. (2004), who maintained that the RAPD analysis has been applied to identify the strains belonging to the same species.

Historically, S. aureushas been described as a variable bacterium with many pathogenic and antibiotic-resistance variants[9]. Several studies have provided evidence that MRSA strains have evolved in a relatively small number of lineages that are clonally-related, and that some MRSA strains are present in distant lineages. This has led to the prediction that the presence of the mecAgene in such widely divergent lineages is the consequence of the horizontal transfer of the mecregion into related S. aureuschromosomal backgrounds, proving that the MRSA strains have evolved several times independently[42]. Our cluster analysis is compatible with Branger's hypothesis; it demonstrates that MSSA and MRSA genomic groups are distantrelated. In Branger *et al*., the coefficient of relatedness did not exceed 45%, while in this study the coefficient was 40% with degree-interrelatedness (only 15).

The reasons behind the progressive development of genotypic strategies were the shortcomings in the previous phonotypical methods. Accordingly, RAPD has been proposed as the method of choice for typing S. aureusisolates and is considered as one of the most reproducible and discriminatory techniques[46]. Using appropriate statistical analysis is very important to make definitive identifications of genetic variation. RAPD subtyping approaches can provide useful epidemiological information regarding antibiotic resistance.

CONCLUSION

In this study, the DNA-based assays (both RAPD and conventional PCR) have provided a rapid method for the detection and characterization of methicillin-resistant Staphylococcus aureus(MRSA). The RAPD technique allows larger proportions of the genomes to be studied in the generation of the banding pattern, leading to finer discrimination among strains. This analysis has detected the genetic variation revealing that the analyzed S. aureusisolates were divided into two distinct groups of the same species, methicillin-susceptible and methicillin-resistant strains.

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