Comparision of Two PCR-Based Methods in Typing of Clinical Staphylococcal Strains

Klinik Stafilokokal Suşların Tiplendirilmesinde İki Farklı PCR Temelli Yöntemin Karşılaştırılması

Research Article

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ABSTRACT

The present study was carried out to investigate usefulness and effectiveness of randomly amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) and repetitive element sequence-based polymerase chain reaction (REP-PCR) in differentiating of staphylococcal strains and to compare the results of these methods with those obtained by antibiotyping. Staphylococcal strains, obtained from various clinical samples and collected from different wards, were characterized phenotypically by susceptibility testing and genotypically by using RAPD-PCR and REP-PCR methods. It was found that there was no significant association between genotypes obtained from RAPD and REP-PCR. Strains with a similarity coefficient of 80% and 70% or greater were grouped in a cluster for RAPD-PCR and REP-PCR, respectively. RAPD-PCR was found to be very efficient with the discriminatory index (DI) of 0.91 whereas discrimination index (DI) of REP analysis was found to be 0.88 with RW3A primer and combination of REP1R-I, REP2-I primers. The findings of this study indicate that RAPD-PCR reliably distinguish ward and source-related clustering. The RAPD primers provide to discriminate MRSA, MSSA and CNS strains whereas REP analysis could not be as discriminative as RAPD. Therefore, RAPD-PCR, evidenced to be inconsiderably more discriminatory than REP-PCR, is well suited for fast and accurate strain identification.

Key Words
Staphylococcal strains, antibiotype, RAPD-PCR, REP-PCR.

ÖZET

Bu çalışmada stafilokokal suşların ayrımında RAPD-PCR (Rastgele Çoğaltılmış Polimorfik DNA-Polimeraz Zincir Reaksiyonu) ve REP-PCR (Tekrarlayan Ekstragenik Elementlerin Polimeraz Zincir Reaksiyonu ile Amplifikasyonu) yöntemlerinin etkinliğinin ve kullanılabilirliğinin araştırılmasını ve elde edilen sonuçların antibiyotipleme ile karşılaştırılması amaçlanmaktadır. Çeşitli klinik örneklerden izole edilen ve farklı servislerden toplanan stafilokokal suşlar fenotipik olarak antibiyotik duyarlılık testi, genotipik olarak RAPD-PCR ve REP-PCR yöntemleri ile karakterize edilmiştir. RAPD ve REP-PCR ile elde edilen genotipler arasında önemli ölçüde benzerlik bulunamamıştır. RAPD-PCR ve REP-PCR için sırasıyla %80 ve %70 olarak belirlenen benzerlik katsayısı ile suşlar gruplandırılmıştır. RAPD-PCR'ın 0.91 oranında ayrım gücü ile oldukça etkin olduğu tespit edilirken, RW3A primeri, REP1R-I ve REP2-I primerlerinin kombinasyonu ile elde edilen REP analizinin ayrım gücü 0.88 olarak bulunmuştur. Çalışmanın bulguları RAPD-PCR yönteminin klinik örnek ve servisler ile ilişkili kümelemede güvenilir olduğunu göstermektedir. RAPD primerlerinin MRSA, MSSA ve CNS suşların ayrımını sağlayabildiği, REP analizinin RAPD kadar ayırt edici olmadığı bulunmuştur. Böylece RAPD-PCR'ın REP-PCR'dan daha ayırt edici olduğu ve suşların tanımlanmasında hızlı ve doğru bir yöntem olarak uygunluğu kanıtlanmıştır.

Anahtar Kelimeler
Stafilokokal suşlar, antibiyotip, RAPD-PCR, REP-PCR.

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INTRODUCTION

Staphylococci are frequent etiological agents of several infections [1]. In the past years, only coagulase-positive species (*Staphylococcus aureus*) recognized as a major cause of nosocomial infections [2]. Although coagulase-negative staphylococci (CNS) have been considered as non-pathogen for a long time, they have emerged as important causative agents of some nosocomial infections recently [3, 4]. The increase in resistance of staphylococcal species to a wide range of antibacterial agents along with recognizing the increase in prevalence as a nosocomial pathogen is of major concern. Therefore, the identification of these strains plays a key role in investigating the epidemiology in the hospital [5]. In this respect, molecular characterization is extensively used to track the spread of clonal dissemination [2].

PCR-based techniques, identified for typing of many bacteria including Staphylococci, have contributed significantly to recent advances in tracking the spread of these strains [6]. Randomly amplified polymorphic DNA (RAPD)-PCR, is one of these methods, based on the use of short oligonucleotide primers with a random sequence which is designed without any prior sequence information concerning the target DNA. As an alternative to this approach, amplification of highly conserved regions by using primers leads to differentiating DNA fingerprints [7]. A technique, called as Repetitive element sequence based (REP)-PCR, in which primers derived from the REP sequences were used, is relied on the amplification of regions between non-coding repetitive sequences [8]. However, the application of these methods alone and using one primer are not effective enough to differentiate strains within species, at least two or more typing systems with many primers should be used for the identification.

The present study was carried out to investigate usefulness and effectiveness of RAPD-PCR and REP-PCR in differentiating of staphylococcal strains and to compare the results of these methods with those obtained by antibiotyping.

MATERIALS AND METHODS

Bacterial strains and identification

Forty-six staphylococcal strains were obtained from various clinical specimens including catheter, blood, pus, material of surgical operation, tracheal secretion, bronchia-alveolar lavage, were collected from different wards of a university hospital (Table 2). All strains, identified by conventional techniques [9] were stored in BHI (Brain Heart Infusion) broth with 10% glycerol.

Antibiotic susceptibility testing

The susceptibility testing of staphylococcal strains to 11 antibiotics was determined by Kirby-Bauer disk diffusion method. The antimicrobial agents tested included ampicillin (10 μg), amoxicillin-clavulanic acid (20/10 μg), ciprofloxacin (5 μg), clindamycin (2 μg), eritromycin (15 μg), gentamicin (10 μg), oxacillin (1 μg), penicillin (10 unit), rifampicin (5 μg), trimethoprim-sulfamethoxazole (1.25 μg), vancomycin (30 μg). The susceptibility test results were interpreted according to Clinical and Laboratory Standards [10]. Antibiotyping of strains was relied on the resistance patterns to selected antimicrobial agents. *S. aureus* ATCC 29213 was used as a standard control strain.

DNA extraction

Prior to amplification by PCR method, bacterial genomic DNA was extracted from an overnight culture of each strain by using bacterial Genomic DNA extraction kit (BioBasic, Canada) and stored at -20°C.

Randomly amplified polymorphic DNA(RAPD) PCR finger printing

RAPD-PCR has been applied using ten primers: A1: CAAGGCATCCACCGT; A2: AAGACGCCGT; A3: AGCAGCCTGC; A4: AGGCCGCTTA; A5: AGCGG-GCCAA; A6: GTAACGCC; A7: GGTTGGGTGAGAATT-GCACG; A8: CAATCGTCCGT; A9: AGTTCTGCAGTAC-CGGATTTGC; A10: ACGGCCGACC. PCR mixture (50 μl) contained 50 ng of template DNA, 1 x PCR buffer (50 mM KCl, 10 mMTris-HCl, 1.5 mM $MgCl_2$), 2.5 mM dNTP, 1.5 mM $MgCl_2$, 5 μM primer, 2U Taq DNA polymerase (Roche Diagnostics, Germany).

Negative controls for each primer contained all components except template DNA. PCR was carried out in a thermal cycler (Eppendorf Thermal Cycler, Hamburg) programmed for 2 min at 95°C, followed by 42 cycles each consisting of 1 min at 95°C, 1 min at 32 or 34°C, and 2 min at 72°C and a final extension period of 5 min at 72°C.

Repetitive element sequence-based (REP) PCR finger printing

Three primers were screened to amplify DNA for REP-PCR genotyping. Each REP-PCR reaction mixture contained 5 μM of each primer RW3A-TCGCTCAAAACAACGACACC, REP1R-I-IIIICGICGIC-ATCIGGC, REP2-I-ICGICTTATCIGGCCTAC. The PCR mixture consisted of 50ng template DNA and of the master mix reported before. The amplification reaction initiated by an initial denaturation step (95°C for 2 min) followed by 42 cycles of denaturation (94°C for 1 min), annealing (54, 38 or 46°C for 1 min with RW3A, REP1R-I or REP2-I primers, respectively), extension (72°C for 2 min) and a final extension step (72°C for 5 min).

Gel electrophoresis and data analysis

The amplification products of the RAPD and REP assay were separated by electrophoresis (Scie-Plas; UK) in a 1.8 % (w/v) agarose gels in TBE 1× buffer (Tris-borate-EDTA buffer) for 4.5 h at 90 V. A 1.5 kb DNA ladder (Roche Diagnostics, Germany) was included for each gel which was photographed by Gel Logic 200 Molecular Imaging System (Kodak; Rochester). The genomic fingerprints obtained from each primer were analyzed using NTSYS-pc (version 2.1; USA). Three dendograms for cluster analysis of all the strains, were constructed by the unweighted pair group method with arithmetic mean (UPGMA) using Dice coefficients of similarity. Strains with a similarity coefficient of 80% and 70% or greater were grouped in a cluster for RAPD-PCR and REP-PCR, respectively. The discriminatory power of each typing method was compared using the Hunter-Gaston discriminatory index [11].

RESULTS

In this study, staphylococcal strains, obtained from various clinical samples and collected from different wards, were characterized phenotypically by susceptibility testing and genotypically by using RAPD-PCR and REP-PCR methods. Of the 46 strains, 26 were diagnosed as MRSA whereas 15 were MSSA and 5 were CNS (Table 2). The characteristics, antibiotic resistance patterns, RAPD-PCR and REP-PCR genotypes of the investigated strains are shown in Table 2.

Eight different antibiotypes (designated A-H) were identified on the basis of antibiotic susceptibility profiles (Table 1). Three of the antibiotypes (A, B and C) were only present among MRSA strains and antibiotype B was predominantly found antibiotype. The predominant antibiotype (D) among the MSSA and CNS strains represent resistance to ampicillin and penicillin.

Dendograms constructed with each primer indicated that several strains could not be distinguished from each other. Therefore, dendogram analyses were examined by the combination of two primers used for RAPD-PCR. In Figure 1, the cluster analysis of the profiles obtained by RAPD-PCR are shown by using combined results of A3 and A4 primers generating 2 major groups with a similarity of 50% and analyzing the dendogram with a similarity coefficient of 80 %, 14 clusters were formed. In Figure 2 and Figure 3, REP cluster analysis results are shown by using RW3A and combined results of REP1R-I and REP2-I-primers, respectively. The discriminatory power by RAPD was 0.91, slightly higher than that by REP-PCR with RW3A (0.88) and combination of REP1R-I and REP2-I (0.88) primers for all tested strains.

Table 1. Antibiotic resistance patterns and antibiotype profiles of tested strains.

Antibiotic resistance patterns	Antibiotype
AM,AMC,CIP,GM,OX,P,RA	A
AM,AMC,CC,CIP,E,GM,OX,P,RA	B
AM,CC,CIP,E,GM,OX,P,RA	C
AM,P	D
AM,CIP,P,RA	E
AM,E,P	F
AM,AMC,CIP,OX,P	G
AM,AMC,CC,CIP,E,GM,OX,P	H

AM = Amphicilin; AMC = Amoxicillin - clavulanic acid; CC = Clindamycin; CIP = Ciprofloxacin; E = Eritromycin; GM = Gentamicin; P = Penicilin; RA = Rifampicin OX = Oxacillin.

Table 2. Characterization of staphylococcal strains used in this study.

Strain No	Wards	Clinical specimen	Antibiotype	RAPD pattern (A3, A4)	REP pattern (RW3A)	REP pattern (REP1R-I, REP2-I)
R1	GENS	PF	A	1	1	1
R2	GENS	Pus	A	1	2	1
R3	EMS	TA	B	2	2	1
R4	PS	Pus	B	1	6	2
R5	URO	Other	B	1	1	2
R6	PS	Pus	B	1	1	2
R7	GENS-ICU	Pus	B	2	7	2
R8	GENS	Catheter	A	2	7	2
R9	IM-ICU	Blood	A	2	7	2
R10	IM-ICU	TA	B	3	2	2
R11	BNS-ICU	SM	C	5	6	3
R12	IM-ICU	Other	A	2	7	3
R13	GENS-ICU	Catheter	A	2	7	3
R14	PS	Pus	B	3	7	3
R15	GENS	Blood	A	3	7	3
R16	PLC	TA	B	2	7	3
R17	BNS	TA	A	2	7	3
R18	IM-ICU	Catheter	A	2	7	3
R19	ORT	BAL	A	2	2	3
R20	BNS-ICU	SM	B	3	6	3
R21	NEU	TA	B	3	3	3
R22	PS	Other	B	3	6	3
R23	IM	Pus	A	3	6	3
R24	IM	Blood	A	3	6	4
R25	IM	Blood	B	3	4	4
R26	NEU-ICU	TA	B	4	6	4
S1	OPT	Pus	D	5	8	4
S2	IM	Pus	D	6	8	5
S3	PLC	Pus	D	6	8	6
S4	IM	Blood	D	11	8	6
S5	PLC	Pus	D	6	8	7
S6	PLC	Pus	D	7	10	7
S7	ORT	SM	D	10	11	7
S8	PS	Pus	D	5	9	7
S9	PLC	Pus	D	8	8	8
S10	IM	Pus	E	9	11	8
S11	PLC	Blood	D	9	8	8
S12	ORT	Pus	D	8	9	8
S13	ORT	SM	D	9	9	9
S14	ON-ICU	Pus	D	7	9	10
S15	ORT	SM	F	8	10	10
C1	BNS	Pus	D	13	4	11
C2	DERM	Pus	D	14	4	12
C3	GENS	Pus	G	13	5	13
C4	GYN	Pus	H	12	5	14
C5	BS	Blood	D	13	5	14

R= Methicillin resistant *Staphylococcus aureus*, S= Methicillin resistant *Staphylococcus aureus*, C= Coagulase negative *Staphylococcus sp.*, BS= Burn Service, BNS = Brain and Neuro Surgery Service; EMS = Emergency Service; DERM=Dermatology GENS = General Surgery; IM = Internal Medicine; GYN= Gynecology, NEU= Neurology, ON= Oncology, OPT= Ophthalmology Service, ORT= Orthopaedy PS= Plastic Surgery PLC= Polyclinic; URO = Urology; BAL = Bronchoalveolar lavage; PF=Prostatic Fluid, SM= Surgery Material TA = Tracheal aspirate; REP = Repetitive extragenic palindromic elements; ERIC = enterobacterial repetitive intergenic consensus sequences; R = REP genotype; E = ERIC genotype.

DISCUSSION

Nosocomial infections, caused by *Staphylococcus sp.* are frequently difficult to treat, are of great concern in many health settings because of increasing resistance. Therefore, rapid and sensitive molecular typing methods for accurate strain identification are necessary to prevent these infections due to the obvious limitations in classification of clinical strains with phenotypic methods.

All of the staphylococcal strains were susceptible to trimetoprim-sulphametoksazol and vancomycin while penicillin and ampicillin were the least effective antimicrobial agents. It was noted in previous studies that methicillin resistance was associated with resistance to other antibiotics [5,12,13]. It is actually observed that there was a relationship between methicillin and aminoglycoside resistance in *S. aureus* in this study [5,13,14]. All of MRSA strains were resistant to gentamicin and whereas none of MSSA strains were susceptible to this aminoglycoside. Resistance to rifampicin by MSSA was less than 1% while MRSA resistance was 100%. It was found that there was no significant association between genotypes obtained from RAPD and REP-PCR and antibiotype profiles. As already noted in other studies, some of strains in the same genotype showed different antibiotype profiles, and vice versa [1]. However in some cases, MRSA strains belong to the same RAPD genotype have the same antibiotype profile.

RAPD typing was discriminatory in differentiating ward-related and source-related strains and able to distinguish unrelated strains. All strains except R5 in genotype 1 had been admitted to surgery wards. Three strains in genotype 2 were isolated from the same clinical sample and six strains shared the same antibiotype. In genotype 3, two of the strains were isolated either same source or same department, three strains had been admitted to internal medicine and also two strains were isolated from tracheal aspirate. All strains in genotypes 5, 6, 7 and 8, except S15 were isolated from pus and included antibiotype D. In addition, genotype 4 (R26) was detected only in the neurology-ICU and genotype 14 (C2) was detected only in dermatology department.

As a supporting method to RAPD-PCR, another fingerprinting method, REP-PCR was applied for typing. Although characteristic banding patterns were obtained by both of these fingerprinting methods it was found that there were different similarity degrees between clusters in RAPD and REP-PCR analyses. Due to the small number of bands generated, the discriminating power of REP-PCR was lower than RAPD-PCR. The results obtained from RAPD revealed that different banding patterns were generated using different primers, allowing the genotyping of studied strains. Some primers were not useful in terms of distinguishing differences between strains. Therefore, dendogram were constructed using the primers A3 and A4. These primers, used for RAPD analysis, provide to discriminate MRSA, MSSA and CNS strains whereas REP analysis could not be as discriminative as RAPD. Furthermore, RAPD patterns were more associated with ward-related and source-related clustering than those of REP. The most common genotypes were genotype 2 and 3 in RAPD analysis among MRSA and genotype 2 was also appeared to be most common in surgery departments.

On the other hand, the results obtained from REP-PCR in this study were clearly different from a previous research, in which strains including *S.aureus* were analyzed by REP-PCR using REP1R-I and REP2-I primers. The results showed no band for *S.aureus* whereas one to two bands were observed in other staphylococcal strains and it was noted that genome of *S.aureus* does not include sequences which are complementary to the REP primers. The observed banding patterns were not discriminative and REP-PCR was not found as suitable to be employed for identification of staphylococcal strains [2]. Our findings indicated that REP-PCR analysis yielded ranging from 1-10 bands, 2-10 bands and 3-12 bands for REP1R-I, REP2-I and RW3A primers, respectively. It can be concluded that the amplification conditions may have a significant effect on yielding strain or species-specific patterns.

A great number of studies in which discriminatory power of RAPD-PCR compared with other methods have given rise to different conclusions. Van Belkum et al. (1993) reported that 23 genotypes were genetared by RAPD analysis, whereas 13 different phage types could be generated.

In conclusion, the antibiotic susceptibility testing provides information for routine surveillance. However, supplementary typing systems should be performed. For the purpose of genetical analysis, it is of great acceptation to apply techniques with high discriminatory power. In this study, using two different selected primers in the RAPD amplification reactions resulted in the highest degree of discrimination. RAPD-PCR was found to be an excellent screening method for differentiating MRSA, MSSA and CNS. The findings of this study also indicate that RAPD-PCR reliably distinguish ward and source-related clustering in MRSA, especially. Therefore, RAPD-PCR, evidenced to be inconsiderably more discriminatory than REP-PCR, is well suited for fast and accurate strain identification.

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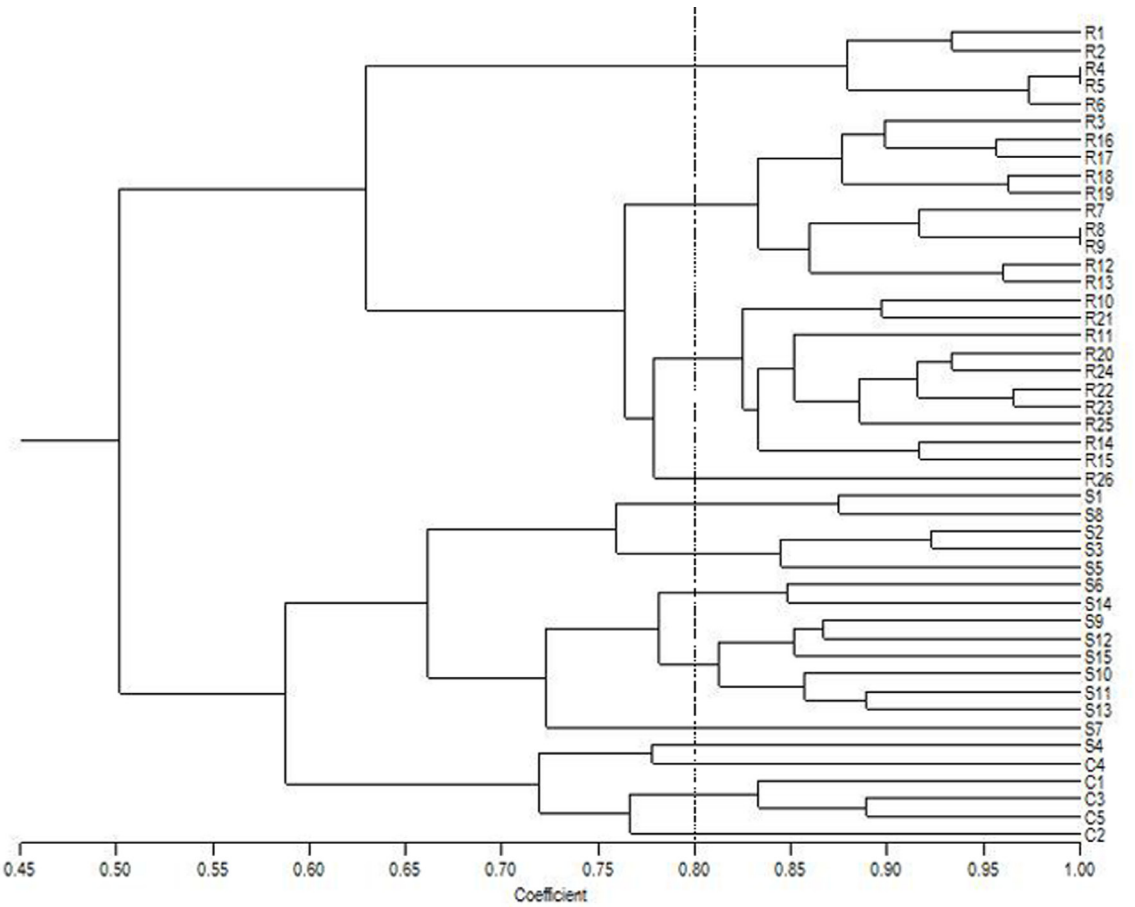

Figure 1. Cluster analysis of the profiles obtained from the Staphylococcal strains by RAPD-PCR analysis. A similarity coefficient of 80 % was chosen to guarantee species differentiation.

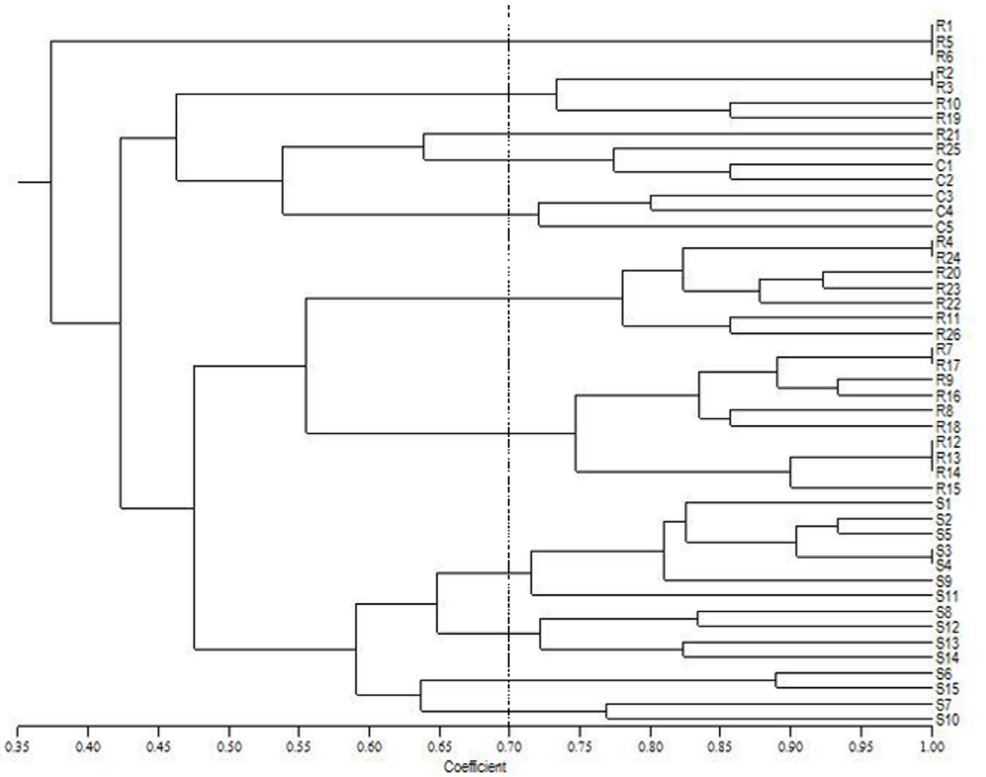

Figure 2. Cluster analysis of the profiles obtained from the Staphylococcal strains by REP-PCR (RW3A primer) analysis. A similarity coefficient of 70 % was chosen to guarantee species differentiation.

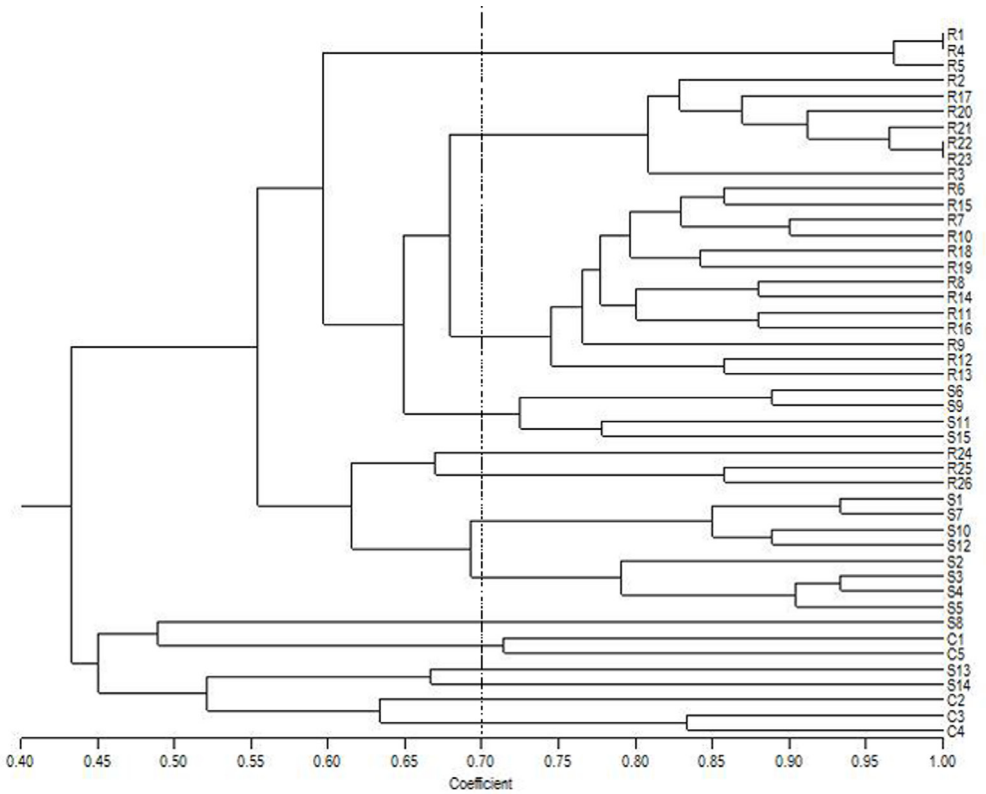

Figure 3. Cluster analysis of the profiles obtained from the Staphylococcal strains by REP-PCR (combination of REP1R-I, REP2-I primer) analysis. A similarity coefficient of 70 % was chosen to guarantee species differentiation.

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