

Full Length Research Paper

Genomic fingerprints analysis of coagulase-positive and negative *Staphylococci* isolated from patients with bacteremia by repetitive sequence based PCR method

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Staphylococci are important organisms involved in many infections, including bacteremia or septicemia. Repetitive sequence-based PCR (rep-PCR) is a useful method for detection of staphylococcal DNA fingerprint patterns, especially when the origins of these organisms are unknown. Staphylococcal positive blood cultures were collected from patients with bacteremia hospitalized in four hospitals. The patients who had two positive blood cultures out of three samples were considered as subjects. After isolation of *staphylococci* on blood agar medium, the species of isolates were determined by standard biochemical tests. DNA was extracted from bacterial cells and genomic fragments were amplified by rep-PCR. Furthermore, relationship of strains was determined based on the similarities between DNA fingerprints by using Jaccards coefficient. In this survey, 88 cases of bacteremia caused by coagulase positive *Staphylococcus aureus* (36 cases), and coagulase negative strains (52 cases), were studied. Extracted DNA from staphylococcal isolates generated multiple fingerprints in sizes ranging between 600(61%) and 2642 bp (87.5%) by rep-PCR method. The fingerprint patterns of *S. aureus* (33 strains), *S. epidermidis* (32 strains) and *S. lugdunensis* (7 strains) were 31, 30 and 7 types, respectively. This study demonstrated only a few of *Staphylococci* strains that displayed similarity or that are closely related in the DNA fingerprint patterns. We concluded that rep-PCR is a rapid, simple and suitable method for epidemiological studies. The results of our study also showed that most of *Staphylococci* isolated from bacteremic patients produced "different" genomic fingerprint patterns by rep-PCR and so, at present study, dissemination source of infection is different.

Key words: rep-PCR, fingerprint patterns, *Staphylococci*, bacteremia.

INTRODUCTION

Staphylococci are important pathogenic organisms involved in many of human infections particularly in bacteremia or septicemia. Staphylococcal bloodstream infection is an important cause of morbidity and mortality, especially in pediatric patients (Babay et al., 2005; Hakim et al., 2007). These bacteria are ubiquitous and isolated frequently from surfaces of skin and mucous membranes. Risk factors such as prolonged hospitalization, prematurity, indwelling catheterization, remaining a foreign objective in wound, mechanical ventilation and longed antimicrobial therapy are causes of nosocomial infections

(Babay et al., 2005).

Isolation of gram positive in comparison to gram-negative bacteria from hospital-acquired infections has increase during 2 decades ago. The role of coagulase negative *Staphylococci* (CONS) such as *Staphylococcus epidermidis* in producing nosocomial infections, bacteremia, endocarditis, surgical wounds, UTI and associated infections to CSF, artificial joints, intravenous catheters have been approved (Agraharkar et al., 2003; Babay et al., 2005; Gaudio de Allori et al., 2006). Methicillin-resistant *Staphylococcus aureus* (MRSA) is also one of the coagulase-positive bacteria, which has been reported in many of medical researches (Liu Y et al., 2009; Salaripour et al., 2006; San et al., 2007). It has been estimated that the rate of nosocomial blood infections is 14% by some species of CONS, in hospital different units

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and 37.3% in the intensive care unit (ICU). This rate has been reported as 12.6% by *S. aureus* (Gonzalez et al., 2005; Hacek et al., 1999).

Because of worldwide dissemination of staphylococci, it is necessary that molecular techniques be used for exact identification of nosocomial infection sources, detection of transportation ways of bacteria between persons, drug resistant strains, phylogenetic development relationships between organisms and other epidemiological studies.

Versalovic et al. (1991) introduced a PCR- amplification of repeat DNA sequences found in many bacteria. They used oligonucleotide primers, which were complementary of repeat DNA sequences in a rep- PCR method.

By repetitive sequence based on PCR method, DNA fragment patterns will be produced which are specific for different strains of pathogenic bacteria, such as *S. aureus* (Del Vecchio et al., 1995; Weinstein, 1996).

The objective of this study was detect and analyse genomic fingerprints produced by rep-PCR method which are related to the coagulase- positive and negative *Staphylococci* isolated from patients with bacteremia.

MATERIALS AND METHODS

Bacterial strains

Staphylococcal positive blood cultures were collected from the clinical laboratories in Taleghani, Razi, Golestan, and Shafa hospitals, affiliated with Ahvaz, Jundishapur University of Medical Sciences, Ahvaz, Iran. These blood cultures belonged to patients with bacteremia, who were hospitalized in four mentioned hospitals. The patients with 2 positive cultures out of 3 samples were considered for this study. After transporting the samples to the Microbiology Laboratory in Medical School, those were again subcultured on blood agar medium (Merck, Germany) two times, in order to prepare isolated colonies (Weinstein, 1996).

Isolates identification

Prepared smears from isolated colonies were stained by gram's method, and the pure isolates, which were morphologically confirmed by microscopy, were identified by biochemical standard tests (Forbes et al., 2002; Weinstein, 1996; Wistreich, 1997). These tests include catalase and coagulase tests, susceptibility to bacitracin and novobiocin (Boden and Flock, 1989; NCCLS, 2003) urea and VP (Voges-Proskuer) (Forbes et al, 2002) tests and carbohydrate fermentation tests, such as saccharose, maltose, mannose, manitol, trehalose and xylose (Merck, Germany). The identified *staphylococci* were stocked in -70°C until DNA extraction.

DNA extraction

A loop full of the overnight *staphylococci* grown on blood agar was subcultured into tripticase soy broth (TSB-Merck, Germany). The TSB tubes were incubated at 37°C for 24^h and after centrifugation of the tubes, DNA was extracted from sediment based on the methods which were explained before (Del Vecchio et al., 1995; Sambrook & Russell, 2001). The DNA vials were placed in 4°C overnight and DNA quantities then were measured by photobiometer (Eppendorf, Germany) in 260/280 nm UV long waves

(Sambrook and Russell, 2001).

Rep-PCR amplification

Following a pilot study, we obtained suitable conditions for rep-PCR method with a brief modification in the procedures which have been described before (Del Vecchio et al., Van der, Zee et al 1995;). In this method master mixture was prepared in a final volume of 25ul based on the following components: 2.5 ul of 10X PCR buffer (50mM Kcl, 10 mM Tris-Hcl, Triton X-100)-(final 1X), 200 uM of mixed dNTPs (Roche), 2.5% DMSO(Roche), 0.5 mM of Mgcl₂, 2 ul of Tag DNA polymerase (Roche), 2 pmol/ul of each primer (Metabion, Germany) with 2.5 ul of ERIC1-R: 5'-ATG TAA GCT CCT GGG GAT TCA C-3' and 2.5 ul of ERIC2: 5'-AAG TAA GTG ACT GGG GTG AGC G -3' (Versalovic et al., 1991; Wieser and Busse, 2000) and 100 ng of phenol-chloroform-extracted DNA(1 ul). Deionized sterile water was added to 13.85 ul. Master mixture preparation was vortexed briefly and distributed into reaction vials in 25 ul volumes. Reactions were amplified by a thermal cycler (Techne Co. UK) based on the following programs: an initial denaturation for 7 min at 95°C and then denaturation (1 min, 94°C), annealing (1 min, 45°C) and extension of DNA molecule (8 min, 65°C) in which each of them were repeated for 30 cycles. A final extension (16 min, 65°C) was also carried out in the end (Del Vecchio et al., 1995; Van der, Zee et al., 1999).

Electrophoresis

PCR products were electrophoresed based on the published methods (Del Vecchio et al., 1995; Sambrook and Russell, 2001). Band sizes were determined by comparison with 100-bp DNA ladder (Roche). PCR fingerprints of staphylococcal strains were visualized in a gel documentation equipped with a trans-illuminator and photographed with a polaroid film.

RESULTS

In this survey, 88 patients with bacteremia caused by coagulase positive *Staphylococcus aureus* (36 cases) and coagulase negative strains (52 cases) were studied. These patients were hospitalized in four hospitals affiliated with Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran. These hospitals were geographically apart of each other in Ahvaz city and the patients were afflicted with infection before hospitalization.

Out of a total 88 staphylococcal isolates, forty-one and fifty-nine percent belonged to coagulase positive and coagulase negative strains, respectively. Frequencies of these isolates in the four hospitals are shown in Table 1.

Bacterial identification by standard biochemical tests revealed different species of *staphylococci*, which were isolated from the studied patients (Table 1).

Following DNA extraction, amplification of genomic regions was inserted between ERIC elements by rep-PCR method, and then PCR products electrophoresis was performed. After which, the genomic fingerprint patterns were determined for predominant species of *staphylococci* in each hospital. Figures 1 and 2 represent DNA fingerprint profiles of staphylococcal species that were isolated from the patients.

Table 1. Frequency of 88 staphylococcal species isolated from the patients with bacteremia in four hospitals.

Hospital name	Staphylococcal Spp					
	COPS ¹ (41%)			CONS ² (59%)		
	<i>aureus</i>	<i>epidermidis</i>	<i>lugdunensis</i>	<i>haemolyticus</i>	<i>cohnii</i>	<i>xylosus</i>
Taleghani	10	8	0	0	0	1
Razi	15	7	1	1	0	0
Golestan	8	9	2	2	1	0
Shafa	3	8	7	3	1	1
Total(88)	36	32	10	6	2	2

¹Coagulase positive *Staphylococci*; ²Coagulase negative *Staphylococci*.

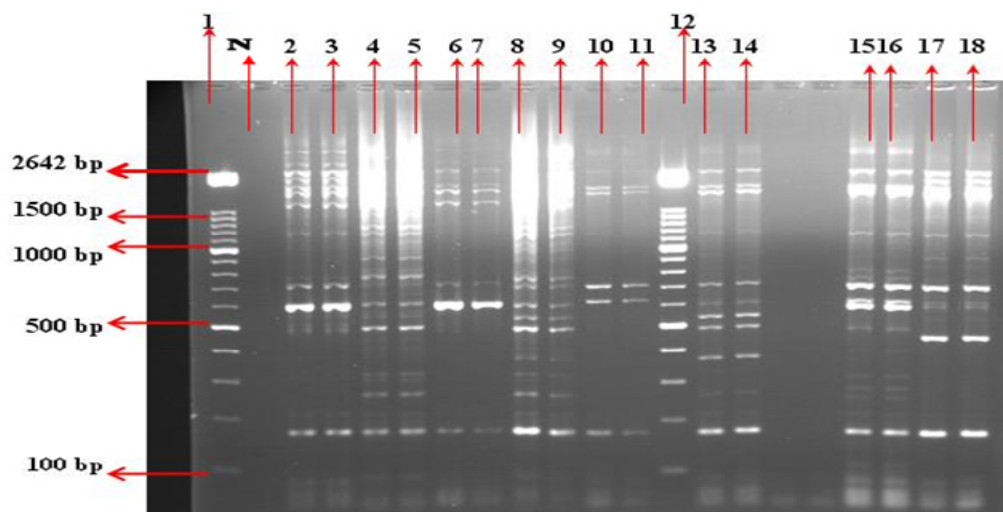


Figure 1. DNA fingerprints based on rep-PCR of *S. aureus* isolated from the patients in Taleghani Hospital. (Each of the two lanes is related to a single isolate which was tested in duplicate). Lanes: (2,3), (4,5), (6,7), (8,9), (10,11), (13,14), (15,16) and (17,18) represent 8 isolates. Lanes 1 and 12 are DNA size markers. (N) is negative control.

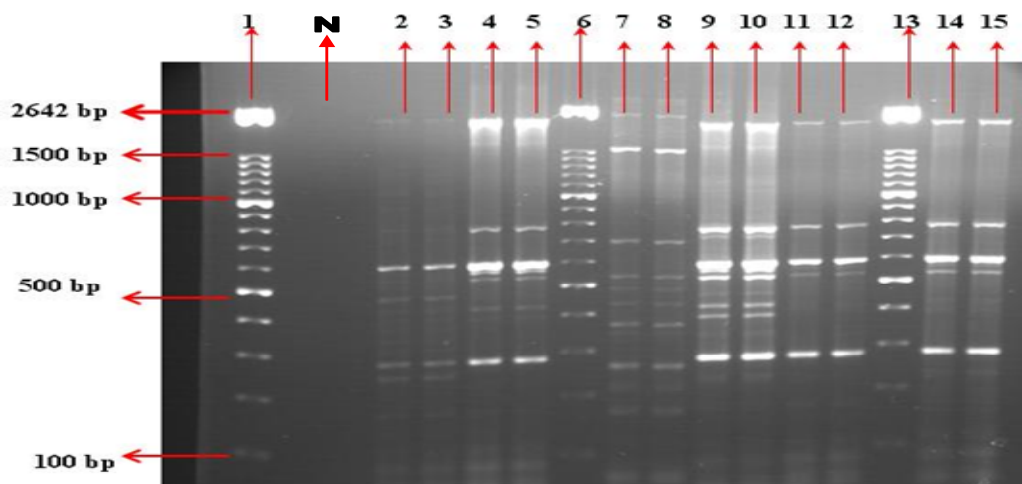


Figure 2. DNA fingerprints based rep-PCR of *S. epidermidis* isolated from the patients in Taleghani hospital. (Each of the two lanes is related to a single isolate which was tested in duplicate). Lanes: (2,3), (4,5), (7,8), (9,10), (11,12) and (14,15) represent 6 different isolates. Lanes of 1, 6 and 13 are DNA size markers. (N) is negative control.

Table 2. Frequency of bands and different binding patterns of 32 *S. epidermidis* isolates.

No. of bands	Total no. of isolates	% of isolates	Fingerprint patterns (no. of isolates)
4	2	6.3	A(2)
5	3	9.4	B(1) , C(1) , D(1)
6	5	15.6	E(2) , F(1) , G(1),H(1)
7	4	12.5	I(1) , J(1) , K(1) ,L(1)
8	2	6.3	M(1), N(1)
9	2	6.3	O(1), P(1)
10	2	6.3	Q(1), R(1)
11	5	15.6	S (1) T(1) U(1) , V(1) , W(1)
12	1	3.1	X(1)
13	1	3.1	Y(1)
15	3	9.4	Z(1) ,Z ₁ (1) , Z ₂ (1)
16	1	3.1	Z ₃ (1)
17	1	3.1	Z ₄ (1)
Total	32	100	

In this study, the relationship of staphylococcal strains was determined based on the similarities between DNA fingerprints by using Jaccards coefficient (Rodrigues-Barrads et al., 1995). Also, the number of different bands in each fingerprint was considered for comparison of *staphylococci* species based on the following criteria:

"Indistinguishable" (No different band), "Closely related" (with 1 different band) "Possibility different" (with two different bands), "Different" (three or more different bands).

So in this survey different banding patterns were observed for predominant species of *staphylococci*, such as rep-PCR profiles A to Z₅ (31 patterns) for *S. aureus* (33 strains), A to Z₄ (30 patterns) for *S. epidermidis* (32 strains) and A to G (7 patterns) for *S. lugdunensis* (7 strains).

These results also showed that most isolates (5 out of 33 strains) of *S. aureus* (15.2%) had six bands in each one of E, F, G and H pattern, while a less number of them (range: 3-12%) were different in both fingerprint pattern and the number of bands. However, among the *S. epidermidis* isolates, 15.6% had 6 and 11 bands in each fingerprints of E, F, G, H and S, T, U, V, W, respectively. Table 2 shows frequency number of bands and different binding patterns produced based on rep-PCR for 32 *S. edidermidis*, which were isolated from the four hospitals. In addition, frequency of some common bands in isolated *staphylococci* was determined as below: 2642 bp (61%), 1500 bp (55.5%), 700 bp (38.9%) and 600 bp (87.5%).

DISCUSSION

Staphylococcal bacteremia is an important cause of mortality and morbidity, especially in pediatric patients (Babay et al., 2005; Hakim et al., 2007). Many reports show that infections due to methicillin-resistant *S. aureus*

(MRSA) and coagulase negative *Staphylococci* (CONS) have increased in recent years (Liu Y et al., 2009; Hakim et al., 2007; San et al., 2007). The results of this study demonstrated higher frequency of bacteremia (59%) due to CONS compared to (41%) coagulase positive *staphylococci* (COPS). In some studies, this ratio has been reported three times more for CONS (Diekema and Michael, 2003).

The rate of *S. epidermidis* was the highest (36%) among the CONS, which were isolated from all the studied hospitals. This species is one of the important bacteria in skin normal flora, which has been reported as causative agent of bacteremia (Arrecubieta et al., 2007; Gaudio de Allori et al., 2006; Milisavljevic et al., 2005). So, it seems this species spread easy to blood stream during transfusion or using a health aid device in a hospital.

In addition, isolation of uncommon CONS such as species of *lugdunensis* (7 strains from 7 patients), *haemolyticus*, *cohnii* and *xylosus* from the hospitalized bacteremic patients in four studied hospitals, could be important. These bacteria, which have been isolated from other infections such as meningitis, wound infections, septic arthritis, endocarditis and peritonitis (Arrecubieta et al., 2007; Hellbacher et al., 2005; Renzulli et al., 2000; Kloos et al., 1994), showed that without suitable health conditions and rapid treatment of patients, *S. lungdunensis* infections might produce server complication in the patients.

In this study, we showed the different genomic profiles of isolated *staphylococci* by repetitive sequence-based PCR (rep-PCR) method. Rep-PCR is a molecular typing method that utilizes one or more primers directed at a repetitive bacterial DNA target, such as enterobacterial repetitive intergenic consensus sequence (ERIC). There is some evidence that these sequence elements also

exist in the genomes of gram-positive bacteria (Wieser and Busse, 2000). Van Belkum et al. (1993) used ERIC primers at low stringency PCR conditions and showed reliable differentiation of strains of *S. aureus*. Also, based on data collected by Wieser and Busse (2000), ERIC-like sequences exist in the genomes of *staphylococci*. Therefore, in this study we used the primers corresponding to ERIC sequence which was the same as the others used (Wieser and Busse, 2000).

The results of our study generally demonstrated that among 72 examined *staphylococci* isolates, genomic fingerprint patterns of only 4 isolates were "Indistinguishable" (2 isolates in each of aureus and epidermidis species), 8 isolates were "closely related" but remainder of them were possibility or completely "Different".

The indistinguishable rep-PCR profiles showed that distribution source only a few of *staphylococci* isolates were probably similar. Closely related rep-PCR profiles by only one different DNA band also demonstrated that such strains probably were uniform in their parents phylogenic, but might be altered during a long time. The following factors could have effect on genomic alteration: mutations due to deletion or replacement of nucleotides, activation of motile elements such as transposons, developed mutation caused by defect in DNA repairing system and genomic recombinant (Olive and Been, 1999).

Although Wieser and Busse (2000) demonstrated that banding patterns generated after ERIC-PCR displayed a high degree of similarity among *S. epidermidis* strains collected from different sources, however in our study, the most rep-PCR fingerprint patterns belonged to isolates of *staphylococci* (aureus, epidermidis and lugdunensis spp.) which were "possibility different" or "different". For example, thirty different fingerprint patterns A to Z4 were detected among 32 isolates of *S. epidermidis* (Table 2), which showed that different strains of one species of epidermidis could be causative agents of bacteremia in the examined patients.

There are also many reports from different strains of the other pathogenic bacteria that show no similarity between rep-PCR fingerprint patterns. Del Vecchio et al. (1995) showed 8 different banding patterns from A to H among 170 MRSA isolates collected from various sources. Alam et al. (1999) likewise demonstrated that among 72 *Streptococcus viridans* isolates, only 2 isolates with 93% and 5 isolates with 85% similarity coefficients were closely related. In addition, Van der Zee et al. (1999), using the rep-PCR method for typing of *S. aureus* isolates showed that, out of 59 strains isolated from a hospital, only 2 strains were "similar", four strains "closely related" and the remainder of them were "different" in genomic fingerprint patterns.

Frequency of some bands with the specific sizes such as 2642 and 600bp among isolated strains were relatively high (87.5 and 61%, respectively). Some studies by rep-PCR have shown that all of MRSA strains, which were

isolated in their study, had the common bands of 600 and 980 bp. This means that we can simultaneously detect a specific gene (for example mec gene) and *staphylococci* strains by rep-PCR, or determine relationship between a gene and the bands, which have specific fingerprint patterns (Del Vecchio et al., 1995).

Conclusion

Detection of DNA fingerprint patterns of staphylococcal strains by rep-PCR and their comparison to other genotypic and phenotypic properties is a suitable method for epidemiological studies. By this simple and rapid method, we showed that there was no similarity between the most *staphylococci* strains, which caused bacteremia.

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