Original article

Analysis of genomic fingerprint patterns of coagulase-negative *Staphylococci* strains isolated from pediatric patients blood cultures using repetitive sequence-based PCR

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Abstract

Introduction and objective: Coagulase negative *Staphylococci* (CoNS) are often isolated from blood cultures which may represent either contamination or bacteremia. Repetitive sequence-based PCR (rep-PCR) as a suitable and potential tool permit differentiation of isolates to species, subspecies and strain level. The aim of this study was analysis of DNA fingerprint patterns and detection of similarity or differentiation of CoNS strains isolated from pediatric patients blood cultures.

Materials and methods: In this study, coagulase-negative *Staphylococci* isolated from hospitalized pediatric patients, were examined. DNA was extracted by using a DNA extraction kit and then diverse-sized DNA fragments consisting of sequences between the repetitive elements were amplified by rep-PCR. Amplified PCR products in different sizes were separated in agarose gels.

Results: Forty-seven strains of CoNS were differentiated by DNA fingerprints generated by rep-PCR. Rep-PCR generated 2-12 different-sized PCR products visible on ethidium bromide stained agarose gels and 29 different fingerprint patterns. A unique fragment was identified in multiple blood cultures from each of seven different patients. Three patients each had two isolates that were closely related, one patient had two isolates that were possibly different, and three patients each had two isolates that were different.

Conclusion: Rep-PCR is a rapid and suitable technique for epidemiological studies and this method with high discrimination could detect similarity or differentiation of strains isolated from bacteremic patients consequently.

Keywords: Coagulase-negative *Staphylococci*, Rep-PCR, DNA fingerprint, Bacteremia

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Introduction

Coagulase- negative *Staphylococci* (CoNS) are part of normal skin flora which mostly cause nosocomial bacteremia in children especially in those with central venous catheters [1-3]. Sometimes also, pseudobacteremia refers to the isolation of bacteria from blood cultures that originate outside the patient's bloodstream [4]. If two consecutive isolates of CoNS recovered from the blood of patient are different strains, they are considered as the most contaminants [5,6].

One of the molecular epidemiologic techniques which can identify differences in bacterial isolates is repetitive sequence-based PCR (rep-PCR) method [7,8]. This is a simple and rapid method to distinguish closely related strains to explain phylogenetic relationships between strains [9]. Three families of repetitive sequences have been studied, including repetitive extragenic palindromic (REP), enterobacterial repetitive intergenic consensus (ERIC), and box element sequences [10].

The complementary oligonuclotide primers of repetitive DNA sequences have been designed to be used in rep-PCR method and thereby they can produce DNA fragment patterns which are specific for different strains of bacteria, are studied [9]. The aim of this study was using rep-PCR method for the analysis of genomic fingerprints of coagulase-negative *Staphylococci* that were isolated from children with bacteremia.

Materials and methods

Isolation of coagulase negative Staphylococci

Blood culture isolates of coagulase-negative were obtained from the clinical microbiology laboratory of Pittsburg Children's Hospital in USA. All isolates were purified by three serial transfers on sheep blood agar plates. Isolates were identified as CoNS by colonial morphology, appearance on Gram stain, negative mannitol test and a negative test tube coagulase reaction [11]. Suspensions of the purified bacteria were stored at -80°C.

Purification of DNA

DNA extraction was performed based on modified method of Killgore *et al*. [12]. Briefly, a loopful of each stocked suspension of the purified bacteria was subcultured overnight on a sheep blood agar. One fourth of a loopful (10µl) of growth from an overnight culture was suspended in 180µl of cell lysis buffer (20 mM Tris HCl (pH 8.0), 2mM EDTA, 1.2% Triton X-100, 200 µg/ml lysozyme, and 20 µg/ml lysostaphin) and incubated at 37ºC for 1.5h. 20µl of Qiagen Proteinase K and 200µl buffer AL were added to lysis reactions, and the reactions were incubated at 56ºC for 30min and then at 95ºC for 15 min. DNA was then purified from the lysates by a spin column method and following the instructions for the tissue protocol (QIAamp DNA Blood Mini Kit; Qiagen, Valencia, Calif.).

Rep-PCR

Repetitive sequence-based PCR was performed using the U-prime E rep-PRO Kit (Bacterial Bar Codes, Inc., Houston, Tex. USA) according to the instructions provided by the manufacturer. This kit uses a single oligonuclotide primer that is complementary to the ERIC sequence in bacteria. Briefly, one microliter of each DNA sample was added to 24µl of reaction mixture (master mix) into each of the PCR tube consisting 5µl of PCR buffer, 0.2µl of BSA, 2.5µl of Dimethyl sulfoxide (DMSO), 3.125µl of dNTPs, 1.0µl of rep-PCR primer, 0.4µl of Taq polymerase and 11.77µl of high performance liquid chromatography (HPLC) H_2O. Also, one

microliter of DNA sample as a template was added to positive control and 1µl of HPLC H_2O used in negative control tubes.

After mixing of the contents, the PCR tubes were inserted into thermal cycler (ABI Prism 7700 Sequence Detection System) and amplification was performed based on the following program: initial denaturation 95ºC for 2min, then 31 cycles including of 94ºC for 3 Sec and 92ºC for 30 Sec (Denaturation), 40ºC for 1min (Annealing) and 65ºC for 8min (extension). A single of final extension was 65ºC for 8min (Bacterial Barcode Inc.). So, the PCR amplification protocol was completed in about 5h and 6min.

Analysis of rep-PCR products

Prepare the gel: Agarose-gel 1.5% in 1X TAE buffer mixed with 3.0µg/ml of ethidium bromide. The gel covered with 1X TAE buffer after solidification and removing of the comb. Then 10µl of each of the amplified products mixed with 2µl loading buffer into microfuge tube and then 10µl of each tube loaded onto the gel. The PCR products were separated by electerophoresis for 3.5h at 80 volts. Finally fingerprints were compared by visual examination and classified according to the number of band differences without regard to band intensity as follows: 0=indistinguishable; 1=closely related; 2= possibly different and 3 or more=different [13].

Results

A total of 47 isolates of CoNS recovered from blood cultures 28 pediatric patients were studied (Table 1). Out of 47 isolates of CoNS, 14 isolates from single blood culture of 14 patients (related to approximately one-half of the patients), were assessed by testing of the DNAs which showed that number of the bands per fingerprint was between 8 to 11 (Table 2).

Table 1: Distribution of single and multiple isolates of coagulase negative *Staphylococci* from the blood culture of pediatric patients

No. of isolates from each patient	No. of patients	Total no. of isolates
1	14	14
2	11	22
3	1	3
4	2	8
Total	28	47

Table 2: Distribution of fingerprint patterns from 14 isolates obtained from different patients with a single isolate

Fingerprint pattern	No. of patients	No. of bands per fingerprint
A	5	8
B	2	9
C	1	10
D	1	10
E	1	9
S	1	9
A1	1	11
B1	1	11
C1	1	11

Also, interastrain reliability of these isolates was confirmed by duplicate PCR reactions carried out on each one of 14 isolates which produced identical banding patterns (Fig. 1).

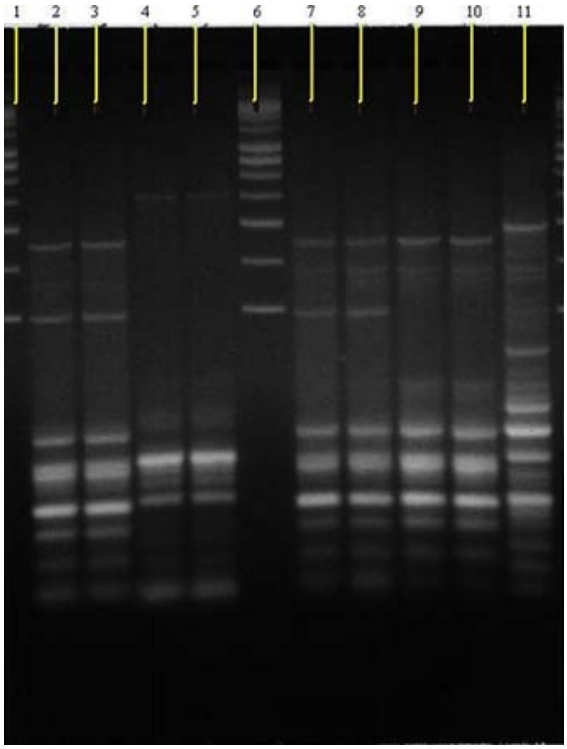

Fig. 1: rep-PCR fingerprints generated by ERIC-PCR from 4 isolates of the CoNS. Identical banding patterns in duplicate wells 2 & 3, 4 & 5, 7 & 8 and 9 & 10 are related to 4 single isolates from 4 patients. Each patient fingerprint is different from others. Well # 11 is positive control. Wells 1 and 6 are DNA ladder (1kb)

The number isolates from the remainder patients with more than one blood culture were 2, 3 and 4 isolates (Table 1). Fingerprint patterns related to 33 strains, isolated from patients with at least two isolates, were "indistinguishable" in 7 patients, "Closely related" in 3 patients, "possibility different" in one and "different" in 3 patients (Table 3 and Fig. 2).

Table 3: Fingerprint patterns observed in 14 patients with multiple isolates of coagulase negative *Staphylococci* from blood culture

Patient no.	No. of isolates	No. of identical bands	No. of different bands	Fingerprint pattern(s)	Interpretation
1	2	13	0	F	Indistinguishable
2	2	7	6	G,H	Different
3	2	8	1	I,J	Closely related
4	2	9	0	K	Indistinguishable
5	2	7	2	L,Q	Possibly different
6	2	8	3	T,R	Different
7	2	2	1	U,W	Closely related
8	2	3	0	U	Indistinguishable
9	2	2	4	V,X	Different
10	2	7	0	M	Indistinguishable
11	2	6	1	N,Y	Closely related
12	3	7	0	O	Indistinguishable
13	4	6	0	Z	Indistinguishable
14	4	7	0	P	Indistinguishable
Total	33				

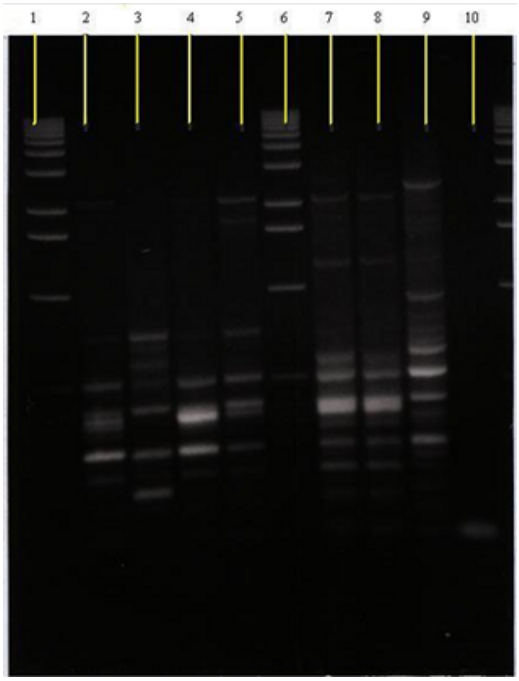

Fig. 2: rep-PCR fingerprints generated by ERIC-PCR from 6 isolates of the CoNS. Wells 2 and 3 are related to one patient which are different at least in 2 bands. Wells 4 and 5 are related to another patient with different strains at least in 2 bands. Wells 7 and 8 are related to one patient with indistinguishable fingerprints. Well 9 and 10 are positive and negative Controls. Wells 1 and 6 are DNA ladder (1kb)

Although indistinguishable isolates in comparison with each other had no different band, but the number of identical bands of them were different in patterns F, K, U, M, O, Z and P, (Table 3). The different rep-PCR fingerprint patterns generated in this study showed the range of 2 to 12 bands among 47 strains of CoNS, but frequency distribution of these bands indicated that the most common isolates (23.4%) had 7 bands in each fingerprint. In these conditions the least of CoNS isolates (2.1%), showed 2 and 5 bands in each fingerprint (W and X patterns, Table 4).

Table 4: Frequency distribution of number of bands generated by rep-PCR for 47 isolates of coagulase negative *Staphylococci*

No. of bands/per fingerprint	No. of isolates	% of isolates	Fingerprint patterns (no. of isolates)
2	1	2.1	W(1)
3	4	8.6	U(3),V(1)
4	0	0.0	N.A.[a]
5	1	2.1	X(1)
6	5	10.6	Y(1), Z(4)
7	11	23.4	L(1), M(2), N(1), O(3), P(4)
8	5	10.6	A(5)
9	10	21.3	B(2), E(1), H(1), I(1), K(2), Q(1) R(1), S(1)
10	5	10.6	C(1), D(1), G(1), J(1), T(1)
11	3	6.4	A1(1), B1(1), C1(1)
12	2	4.3	F(2)
	47	100	29 patterns

[a] N.A., not applicable.

Discussion

Prolonged intravascular catheters which used for treatment or parenteral nutrition will dispose patients particularly infants or children to CoNS infections [2,14]. CoNS bacteremia also is an important cause of mortality and morbidity, especially in pediatric patients [2]. If a blood culture is positive for CoNS bacteremia, sampling of the patient and inoculation to blood culture will usually be repeated and clinician start the patient antibiotic therapy, empirically. If the second blood specimen is also reported as positive for CoNS, it is presumed that the first and second isolated strains are the same and so it is considered as a true bacteremia.

In this study, we used rep-PCR technique to demonstrate differentiation or similarity between coagulase-negative *Staphylococcus* strains which were isolated from blood culture of pediatric patients with bacteremia. Fourteen single CoNS isolates, each one from a patient produced identical banding patterns by duplicate PCR reactions, although the number of bands in each fingerprint was different from 8 to 11 (Table 1). So, these results confirmed reproducibility of fingerprint patterns which obtained from each one of the isolates [15]. Out of 47 CoNS isolates obtained from 28 patients, rep-PCR generated 2 to 12 different sized PCR products and 29 different fingerprint patterns on the agarose gels (Table 4), which showed diversity of CoNS isolates among the studied patients (Fig. 2).

The indistinguishable rep-PCR profiles showed that distribution source of CoNS in seven patients which had at least two isolates, were probably similar and so the episode in these patients represents true bacteremia. Closely-related fingerprint patterns of I & J, U& W and N & Y (in six isolates) related to three patients (Table 3) which had at least one different DNA band also demonstrated these strains were probably uniform in their that parents phylogenic, but possibly have altered during a long time. Some factors such as: activation of trasposons, mutations due to deletion or replacement of nucleotides, developed mutation caused by defect in DNA repairing system and genomic recombinant could be caused by genomic alteration [16].

In table 3, six isolates with at least three different bands related to the fingerprint patterns of G & H, T & R, and V & X have been shown. This means that different strains of CoNS could be causative agents of bacteremia in some of the patients. Although the species of CoNS were not determined in this study, but some of the reports demonstrated that the majority of the isolates among CoNS were *S. epidermidis.* Babay *et al*. [2] showed out of 173 Gram-positive bacteria which were isolated from 230 pediatric patients with bloodstream infections, 55.4% belonged to *S. epidermidis*, while 9.5% isolates were *S. aureus* [2]. Widerstrom *et al*. [17] in a molecular epidemiology study also demonstrated out of 140 meticillin-resistant coagulase-negative *Staphylococci*, 120 isolates were *S. epidermidis* [17].

Conclusion

We concluded that rep-PCR is a suitable method for epidemiological study of CoNS and also, this method with high discriminately and intra-reproducibility could detect similarity or differentiation of strains isolated from bacteremic patients.

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