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A molecular biological study on identification of common septicemia bacteria using 16s-23s rRNA gene spacer regions*

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Abstract: In the search for a rapid and reliable method for identification of bacteria in blood and cerebrospinal fluid, we developed a unified set of primers and used them under polymerase chain reaction (PCR) to amplify the spacer regions between the 16s and 23s genes in the prokaryotic rRNA genetic loci. Spacer regions within these loci showed a significant level of length and sequence polymorphism across most of the species lines. A generic pair of priming sequences was selected from highly conserved sequences in the 16s and 23s genes occurring adjacent to these polymorphic regions. This single set of primers and reaction conditions were used for the amplification of the 16s-23s spacer regions for 61 strains of standard bacteria and corresponding clinical isolates belonging to 20 genera and 27 species, including *Listeria, Staphylococcus and Salmonella* species, et al. When the spacer amplification products were resolved by electrophoresis, the resulting patterns could be used to distinguish most of the bacteria species within the test group, and the amplification products of the clinical isolates clustered at the standard species level. Some species presenting similar pattern were further analyzed by HinfI or AluI digestion or DNA clone and sequences analysis in order to establish the specific 16s-23s rRNA gene spacer regions map. Analysis of 42 blood specimens from septicemic neonates and 6 CSF specimens from suspected purulent meningitis patients by bacterial culture and PCR-RFLP(Restriction Fregament Length Polymorphism) showed that 15 specimens of blood culture were positive(35.7%) in the 42 septicemic neonates; 27 specimens were positive(64.2%) by PCR, and that the positive rate by PCR was significantly higher than that by blood culture($P < 0.01$). Among the 6 CSF specimens, one specimen found positive by blood culture was also positive by PCR, two found negative by blood culture showed positive by PCR; all three were *S. epidermidis* according to the DNA map. One *C. neoformans* found positive by blood culture showed negative by PCR. The remaining two specimens were both negative by PCR and blood culture. These results indicated that the method of detecting bacterial 16s-23s rRNA spacer regions using PCR and RFLP techniques was rapid, sensitive and specific in the detection of bacterial infections; and so, has very important application in the clinical diagnosis of sepsis in neonates.

Key words: 16s-23s rRNA gene, PCR, RFLP, Sequences analysis
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INTRODUCTION

Bacterial infection is very common in pediatric cases. Sepsis is the most serious stage of bacterial infection and requires rapid and accurate diagnosis and treatment. But up to now, blood culture is still the "Golden Standard" in the diagnosis of sepsis even though it is time and labor consuming. The positive rate is also very low because of low bacterial counts or prior antibiotic therapy. So pediatricians are searching for a new, sensitive, and rapid method to replace blood culture for detecting bacterial infection. PCR, as a diagnostic tool for the detection of pathogens, has been expanding during the past few years, but most PCR detection systems are only suitable for the detection of specific pathogens. When the pathogens are uncertain, many different primers and steps are required by the PCR to detect the various pathogens, thus limiting the extent of its clinical use. In work, we designed a single pair of primers based on the highly conserved sequences adjacent to the 16s-23s rRNA spacer regions. Under the same reaction condition, 61 strains of standard bacteria and corresponding clinical isolates representing 20 genera and 27 species

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were amplified by PCR and subjected to RFLP analysis. Some PCR products which had the same RFLP map were further analyzed by DNA clone and sequences analysis. Finally we applied the method to assess bacterial infection of clinical samples.

MATERIALS AND METHODS

Bacterial strain and clinical specimens

The bacterial strains used in this study are listed in Table 1. Six Gram-positive strains and 21 Gram-negative strains served as positive control. Human-genomic DNA, cytomegalovirus (CMV), hepatitis B virus (HBV), Epstein-Barr virus (EBV) and *C. neoformans* were used as negative controls. Other 35 clinical isolates including *S. epidermidis* (9 *strains*), *K. pneumoniae* (9 *strains*), *P. aeruginosa* (7 *strains*), *E. coli* (5 *strains*), *P. penneri* (3 *strains*) and *S. marcescens* (2 *strains*) were tested to assess the stability of the DNA bands. Determination of infection 42 blood specimens from septicemic neonates and 6 CSF specimens from suspected meningitis patients was achieved by bacterial culture and PCR or PCR-based RFLP analysis and 15 blood specimens from healthy neonates were tested for bacterial infection by blood culture and PCR test.

Table 1 Results of PCR amplification and RFLP with 27 types of bacteria

Organism(strain)		Bands of PCR amplification	Bands of further RFLP analysis
Gram-positive($n=6$)			
Staphylococcus aureus	(ff320)	2(1200,600bp)	
Staphylococcus epidermidis	(ff330)	2(1200,600bp)	
Streptococcus β-hemdyticus	(ff20)	4	
Enterococcus durans	(ff30)	2(3100,900bp)	(No Xma III site)
Bacillus subtilis	(ATCC6051)	3	
Listerium monocytogenes	(ff66)	1(1500bp)	
Gram-negative(21)			
Escherichia coli	(ATCC11775)	4	
Shigella dysenteriae	(ff90)	7	
Shigella flexneri	(ff91)	2(2500,800bp)	(HinfI,3)
Salmonella typhi	(ff110)	5	
Salmonella typhimurium	(ff125)	1(3200bp)	(Alu I,7)
Salmonella enteritidis	(ff138)	1(3200bp)	(Alu I,6)
Proteus vulgaris	(ff140)	1(3200bp)	(Alu I,6)
Proteus penneri	(ff152)	1(3200bp)	(Alu I,6)
Providencia stuartii	(ff160)	1(3200bp)	(Alu I,6)
Klebsiella pneumonia	(ATCC13883)	2(3100,900bp)	(Have Xma III site)
Enterobacter cloacea	(ff170)	1(480bp)	
Serratia marcescens	(ff193)	2(3150,700bp)	(HinfI,5)
Edwardsiella tarda	(ff191)	2(3150,700bp)	(HinfI,5)
Haemophilus influenzae	(ATCC33391)	1(3200bp)	(Alu I,3)
Bordetella bronchiseptica	(ff210)	4	
Pseudomonas aeruginosa	(ATCC10145)	2(3150,700bp)	(HinfI,4)
Pseudomonas putidabiovars	(ff212)	5	
Pseudomonas cepacia	(ff368)	3	
Acinetobacter calcoaceticus	(ff381)	5	
Aeromonas hydrophila	(ATCC7966)	1(350bp)	
Flavobacterium meningosepticum	(ff789)	1(1200bp)	

ATCC strains were obtaind from the American Type Culture Collection. Strains designated ff were obtained from the clinical collection of Children's Hospital, Zhejiang University, Hangzhou, China.

Synthesis of Primers

Oligonucleotide primers were designed to be complementary to conserved regions near the 3' end of the 16s regions and 5' end of the 23s regions of the ribosomal RNA operons based on a computer alignment of ribosomal RNA sequences available through Genebank. Sequences of primers are listed in Table 2.

Table 2 Sequences of primers

Name	Sequences 5' – 3'	Location
Upstream	TTGTACACACCGCCCGTC	16S rRNA(1390 – 1407)
Downstream	CCTTTCCCTCACGGTACTG	23S rRNA(456 – 474)
M13 Forward sequencing primer	T G T A A A A C G A C G G C C A G T	
M13 Reverse sequencing primer	C A G G A A A C A G C T A T G A C C	

Table 3 Comparison the result of blood culture and PCR amplification in neonatal septicemia

Items		Neonatal septicemia($n = 42$)		
		Positive	Negative	Sum
Blood culture		15	27	42
PCR-amplification	Positive	14	13	27
	Negative	1	14	15

χ^2 test, $\chi^2 = 6.857$ $P(0.01(P = 0.00883)$;

DNA isolation and PCR amplification

All bacterial strains were processed in 400 ul 5% Chelex 100 buffer (containing 0.03% SDS, 1% Tween 20 ,1% NP40), boiled for 15 min and amplified by taking 1-2 ul supernatant as template. In the lysis of the *staphylococcus*, proteinase K was added to the 5% Chelex 100 buffer, the mixture was incubated in 56 ℃ for 60 min and then boiled for 15 min. Blood samples decoagulated by heparin were processed by adding five volumes of 0.87% NH4Cl to dissolve the red blood cells. Leukocytes were pelleted; then suspended in the 200 ul 5% Chelex 100 buffer with 20 mg/ml proteinase K, incubated in 56 ℃ for 60 min and then boiled for 15 min, microfuged for 1 min, to yield supernatant suitable for PCR amplification. CSF samples were vortexed 5 min in microfuge. Then the supernatant was poured off and the cell pellets were resuspended in a mixture of 5% Chelex 100 buffer with 20 mg/ml proteinase K. The following steps were the same as those for blood samples.

PCR amplification

The PCR mixture (50 ul) contained 20 mmol/L Tris. Hcl (pH 8.0), 100 mmol/L KCl ,4 mmol/L $MgCl_2$, 55 pmol/L(each) primer, 1.0 unit of Taq polymerase(Takara Biotechnology, DALIAN, CO, LTD) and 200 – 300 ng target DNA. The PCR was performed for 35 cycles of 1 min at 94 ℃, 2 min at 72 ℃ and 30 s at 55 ℃ on a DNA thermocycler (Perkin-Elmer Cetus, Norwalk. Conn). After amplification, the samples were incubated for an additional 5 min at 72 ℃.

Restriction enzyme analysis.

DNA was recovered from the PCR system as described previously (Manceau et al., 1997); the PCR products were digested with 10 – 15 unit of Alu I or Hinf I as instructed by the manufacturer(Takara Biotech), and resolved on 2% agarose gels, which were then stained with ethidium bromide and photographed under UV light. DNA fragment sizes were compared with the DNA molecular weight marker.

DNA clone

After agarose gel electrophoresis, DNA bands of the PCR product (amplified as described above) were excised from the gel and purified using the Gene Clean Kit (Glass Milk). The purified PCR product was ligated directly into PGEM-T vector with 1 unit of T4 DNA ligase, and then incubated at 4 ℃ overnight or at 15 ℃ for 2 hours. Competent DH5a E. coli cells (50 ul; Promega) were transformed with 10 ul of the ligation mixtures according to the protocol described in Liang Wei-feng et al (2000). After the plates had been incubated overnight at 37 ℃, positive plaques were picked out and grown in LB containing ampicillin, and incubated overnight at 37 ℃ on a rocking-table (300 rev/min). The plasmid was then extracted by the phenol and chloroform method followed by ethanol precipitation. After centrifugation, the nucleic acid was suspended in distilled water. To determine the presence and size of the inserts, the plasmid DNA was used as template in PCR system using the primers which flanked the 16s-23s rRNA

spacer region (shown in Table 2).

DNA sequencing

Sequencing was done by the dideoxynucleotide method of Sanger et al. (1977) using ABI PRISM™ DYE sequencing kit (Perkin Elmer). Sequencing primers are shown in Table 2.

RESULTS

Specificity of primers and sensitivity of the PCR assay

To verify that the primers used in this study were specific for bacteria, 27 species of bacteria, human genomic DNA, CMV, HBV, EBV and *C. neoformans* were tested. Amplified products of different bands were detected only from bacterial DNA, but not from human genomic DNA, CMV, HBV, EBV and *C. neoformans*. The above tests showed that there was no cross reaction between bacteria and human DNA, viruses and fungi. The amplification method was highly specific for bacteria. To examing the lower limit for detection of bacteria, PCR was performed with diluted CFU of *P. penneri*. Serial 10-fold dilutions of CFU were added to the reaction mixture. It was possible to detect reproducibly a band in ethidium bromide stained gel with template amounts as low as 2.5 CFU.

PCR amplification

Fourteen species could be distinguished immediately by PCR. They were *L. monocytogene*, *F. meningosepticum*, *A. hydrophila*, *E. cloacae* with one band; *staphylococcus*, *P. cepacia* and *B. subtilis* with two or three bands; (*Streptococcus* β-hemolyticus, *A. calcoaceticus*, *E. coli*, *B. bronchiseptica*, *S. typhi*, *P. putidabiovars and S. dysenteriae* with two to seven bands. Thirty-five clinical isolates showed the same DNA maps as the standard bacteria accordingly. It indicated that these bands were duplicated.

RFLP results with Alu I and Hinf I

After PCR amplification, there remained 12 species that could not be distinguished immediately. Six species with one band could not be identified from each other by Hinf I digestion, but restriction of PCR products with Alu I allowed complete differentiation between all these species (shown in Fig. 1). Restriction of PCR products with Alu I did not achieve complete differentiation between the other 6 species with two bands. However, Hinf I enzyme generated multiple profiles from which four species could be identified immediately (shown in Fig. 2). But *K. pneumoniae* and *E. durans* could not be differentiated from each other by the use of these two endonucleases.

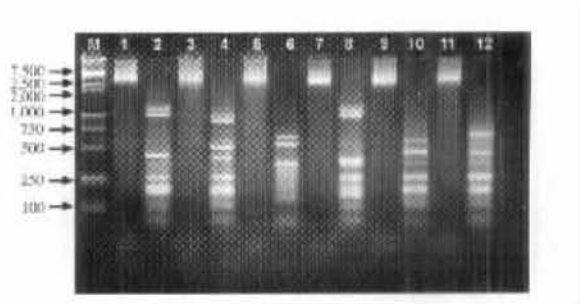

Fig. 1 Gel electrophoresis of PCR-based RFLP with Alu I for the following 6 species (before and after digestion)

1-2: *P. penneri*; 3-4: *P. vulgaris*; 5-6: *H. influenzae*
7-8: *P. stuartii*; 9-10: *S. enteritidis*; 11-12: *S. typhimurium*

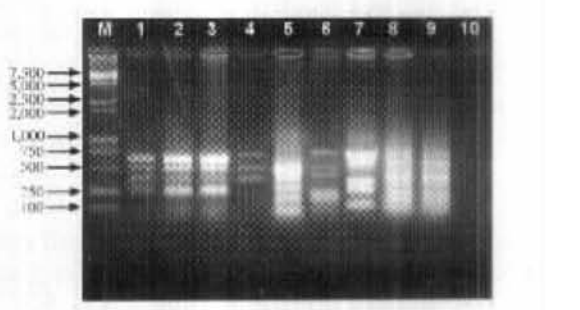

Fig. 2 Gel electrophoresis of PCR-based RFLP with Hinf I for the following 8 species

1-2: *K. pneumonia*, 3: *E. durans*, 4: *S. flexneri*, 5: *E. tarda*, 6: *P. aeruginosa*, 7: *marcescens*, 8: *S. aureus*, 9: *S. epidermidis*, 10: *Blank*

DNA sequencing

In the sequence of shorter ITS of these two species, we found that there was only one nucleotide different in length. The spacer sequence of *K. pneumonia* and *E. durans* were 908 bp and 909 bp respectively, and different only in the site of the 779th nucleotide. The former was G, and the latter was A. Assisted by computer, we found only one enzyme Xma

III that could discriminate them. *K. pneumoniae was cut into 778 bp and 130 bp, while E. durans* could not(Fig.3,4).

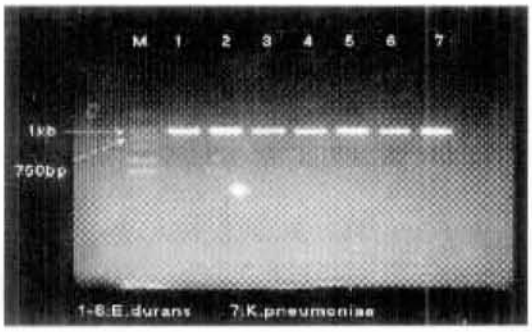

Fig.3 Clone insert target fragment
1 - 6: Target fragment of *E. durans*
7: Target fragment of *K. pneumoniae*

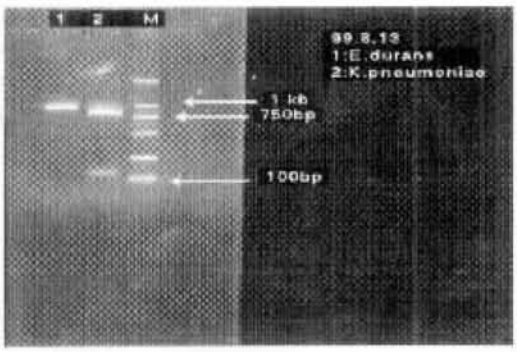

Fig.4 Target fragment of E.durans and K.pneumoiae digested with XmaIII
1: *E. durans* (After Xma III digestion)
2: *K. peumoniae* (After Xma III digestion)

Testing of clinical specimens

Fifteen specimens of blood culture were positive in 42 septicemic neonates(positive rate of 35.7%); 27 blood specimens were positive after PCR amplification and processing by Chelex 100; the positive rate was 64.29%, which was significantly higher than that by blood culture($P<0.01$). Among the 6 CSF specimens, one cultured as *S. epidermidis* was also positive by PCR, two blood specimens found negative by blood culture showed positive by PCR and were diagnosed as *S. epidermidis* according to the DNA map. One cultured as *C. neoformans* showed negative by PCR. Other two specimens were both negative by PCR and blood culture. Fifteen blood samples from healthy children were negative both by blood culture and PCR test.

DISCUSSION

The 16s-23s rDNA internal transcribed spacer (ITS) is flanked by well-conserved regions of the rRNA loci and contains both conserved and highly variable signatures, which has been suggested to be an appropriate site for identification and taxonomic classification of bacteria (Riffard et al., 1998). In our previous study (Shang shiqiang et al.,2000), we used 16s rRNA gene to detect bacterial infection in neonatal septicemia, but we could not tell which species the bacterium belonged to due to the highly conserved 16s rRNA. The evolutionary rate of the 16s-23s rDNA spacer region is 10 times greater than that of 16s rDNA (Nathalie et al., 1996), so the former is more variable and suitable for species identification of bacteria. In this work, we constructed the ITS DNA map of different bacteria at special level by PCR or PCR-based RFLP analysis.

Fourteen species could be distinguished immediately by PCR amplification of ITS, especially for the bacteria with more than 2 bands. The fact that many bacteria have multiple copies per genome of the rRNA operon and considerable variation can occur between species in both the length and sequence of this region. Therefore this poses the possibility of identification of most bacteria based on the spacer variations they display in both the length and number of the 16s-23s spacer regions. Unfortunately, there still remained some species (12 species) which could not be differentiated in ethidium bromide stained gel due to the paucity of the band and the resemblance in length. But they could be further analyzed by RFLP because of the difference in their spacer base sequences. Since sizes and sequences of 16s-23s rRNA spacer regions have not been extensively characterized, it is difficult to predict which enzyme was the most suitable one for identification of these bacteria. By reading many references, we found that Alu I and Hinf I had their digesting sites in most common bacteria. Finally, we set up the species-specific RFLP map of these species except *K. pneumoniae* and *E. durans*, which still could not be differentiated from each other by these two endonuclease

(Alu I, Hinf I).

K. pneumoniae and *E. durans* belong to different genera, the former was Gram stain negative, the latter was Gram positive, they had different antibiogrames. So we identified them by further sequencing and RFLP analysis. According to the sequence of the shorter ITS of these two species, we found that there was only one nucleotide difference in length. The spacer sequence of *K. pneumonia* and *E. durans* were 908bp and 909bp respectively, and it showed difference only in the site of the 779th nucleotide, the former was G, and the latter was A. Assisted by computer, we found only one enzyme Xma III that could discriminate them. Generally speaking, strains belonging to a species are more closely related than to members of another species. It is really hard to explain why one of the *K. pneumonia* and *E. durans*' ITS was so closely related. Nathalie et al. (1996) also found that distinct species such as *B. cuniculi* CIP 103379^T and *B. dentium* ATCC 15697^T, *B. infantis* ATCC 15697^T and *B. indium* ATCC 25912^T had high ITS sequence similarity which reached 96.3% and 97.7% respectively.

When the clinical specimens (42 blood and 6 CSF specimens) were involved, 27 blood specimens were positive after PCR amplification and processing by modified Chelex 100, the positive rate was 64.29% which was significantly higher than that of the blood culture ($P < 0.01$). Three CSF specimens were positive by PCR (*Staphylococcus*). Among them only one specimen was cultured as *S. epidermidis*. It was not only due to the high efficiency of PCR amplification but also to the fact that PCR can also amplify bacteria below the limit of routine detection, or treated with antibiotics. Among 15 culture-positive specimens, 14 were positive with PCR, and 3 were further analyzed by RFLP. The results correlated well with bacterial culture. One culture-positive sample could not be amplified by PCR. We suspect that our failure to detect the one blood sample yielding a positive culture was due to the presence of PCR inhibitors in the patients' blood. Heme and other substances found in blood are known to inhibit Taq polymerase, and different methods such as immunomagnetic separation, phenol-chloroform extraction and use of capture resins have been suggested for the inactivation of PCR inhibitors (Matto et al., 1998) However, many of these other methods are laborious and expensive. Chelex 100 sample-processing which required only one step proved to be very applicable for the detection of bacteria in clinical samples. But inhibition was still present, although to a much lesser extent with Chelex-100-treated specimens. Till now, PCR still faces the problems of false positive and false negative. It depends on how well we control them. As long as we pay more attention to the pollution of PCR, strickly obey the operating rules and inactivate the inhibitors to the greatest degree, the method of PCR is still the most effective for detecting the infecting agents in the clinical field.

Amplification and analysis of bacterial DNA in blood or in CSF can be completed in six hours or less, markedly reducing the time required for bacterial diagnosis in traditional culture. Since the 16S-23S rRNA spacer regions are highly conserved and considerably variable, PCR amplification can reveal the existence and species of bacteria, and so, can be used to direct the clinical use of antibiotics.

References

LIANG Weifeng, SHEN Yuehong, MA Yi-lin. et al. 2000. Molecular cloning and nucleotides sequence analysis of G_1 genome segment of Hantaan virus Z_{10} strain. *J. Zhejiang University (SCIENCE)*, **1**(2): 207 – 211.

Manceau C., Horvais A. 1997. Assessment of genetic diversity among strains of pseudomonas syringe by PCR-restriction fragment length polymorphism analysis of rRNA operons with special emphasis on *P. syringe Pv. tomato*. *Appl Environ Microbiol*, **63**(2): 498(505.

Matto, J., Saarela, M., Alaluusua, S. et al. 1998. Detection of Porphyromonas gingivalis from saliva by PCR by using a simple sample-processing method. *J Clin Microbio*, **136**(1): 157 – 156.

Nathalie, L. B., Herve, P., Irene, M. et al. 1996. 16S rRNA and 16S to 23S internal transcribed spacer sequence analysis reveal inter and intraspecific Bifidobacterium phylogeny. *Intern J Syst Bacteriology*, **46**(1): 102 – 111

Riffard, S., Lopresti, F., Normand, P. et al. 1998. Species identification of Legionella via intergenic 16S-23S ribosomal spacer PCR analysis. *Int J Sys Bacteriol*, **48**(3): 723 – 730.

Shang Shiqiang, Yu Xilin, Hong Wenlan et al. 2000. Detection of bacterial DNA by PCR and reverse hybridization in the 16S rRNA gene. *J. Zhejiang University (SCIENCE)*, **1**(2): 222 – 226.