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혈액 배양 액체배지에서 세균성 병원균 검출을 위한 16S rDNA PCR의 평가

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배 경 : 혈류 감염에 연관된 사망률은 매우 높기 때문에 환자 치료에 있어서 혈액내 병원균을 신속하게 검출하는 것은 중요하다. 임상 검사실에서 혈액배양에서 다양한 병원균을 검출하기 위한 16S rDNA PCR법의 효율을 평가하였다.

방 법 : 배양 양성 99검체와 배양 음성 122검체로 구성된 총 221개의 혈액배양병을 대상으로 16S rDNA PCR을 시행하여 그 결과를 통상적인 배양법과 비교하였다. 또한 혈액배양병에서 DNA를 추출하기 위한 세 가지 방법 즉 proteinase K, triton X-100 및 benzyl alcohol-guanidine을 이용한 추출법들의 효율을 비교하였다.

결 과 : 16S rDNA PCR법은 배양 양성 99개 중 95개(*Staphylococcus aureus* 12주, coagulase 음성 포도구균 27주, 장구균 10주, 연쇄구균 5주, 그람음성간균 37주, corynebacteria 4주)에서 병원균을 검출하였다. *Corynebacteria* 2주와 *Escherichia coli* 1주, *S. aureus* 1주는 위음성 결과를 보였다. 배양 음성인 122개의 배양병에서 모두 16S rDNA PCR 검사에 음성 결과를 보였다. 배양결과를 기준으로 16S rDNA PCR의 민감도와 특이도, 양성 예측도와 음성 예측도를 산출해보면 각각 96.0%와 100%, 100% 및 96.8%이었다. 세 가지 DNA 추출법 중 benzyl alcohol-guanidine을 이용한 추출법이 가장 효율적이었다.

결 론 : 16S rDNA PCR이 혈액 배양병에서 다양한 병원균들을 검출할 수 있는 신속하고 효율적인 방법이므로 임상 미생물 검사실에서 사용될 잠재력이 크다.

INTRODUCTION

Bacterial ribosomal RNA (rRNA), such as 23S, 16S, 5S rRNA, are major constituents of the ribosome. Certain regions of rRNA have been highly conserved during evolution, and rRNA sequence homology is currently widely used to determine evolutionary relationships between organisms. The 16S rDNA coding for the 16S ribosomal gene component is only found in bacteria, and is thus not affected by contamination with mammalian DNA [1]. Eubacterial 16S rDNA amplification by PCR has been increasingly used to identify microbes in clinical samples. Eubacterial 16S rDNA PCR uses primers that are complementary to sequences conserved in all bacteria and allows detection even of non-culturable bacteria [2, 3].

We used the 16S rDNA PCR method to detect microbial DNA in blood culture broth and additionally assessed the efficiency of the 16S rDNA PCR assay. We compared three methods of DNA extraction of blood culture samples, including the benzyl alcohol-guanidine method, which has been advocated as an excellent method for the extraction of blood culture samples [3].

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MATERIALS AND METHODS

Bacterial strains : Four strains of bacteria, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 51-299, *Escherichia coli* ATCC 25922, and *Pseudomonas aeru-*

ginosa ATCC 27853, were used as positive controls.

A total of 221 culture-proven samples collected from BacT/Alert blood culture bottles (Organon Teknika Corporation, Durham, NC, USA) were obtained from Chonnam University Hospital. Aliquots (1 mL) from 99 culture positive- and 122 culture negative-bottles were removed and stored at -70℃ until used for PCR amplification.

Species identification of bacteria isolated from the blood cultures was performed based on biochemical reaction patterns using the VITEK II system (BioMerieux, Durham, NC, USA) according to the manufacturer's instructions.

Extraction of DNA

We compared the efficiency of DNA extraction from the blood culture broths using three DNA purification methods, the proteinase K, triton X-100, and benzyl alcohol-guanidine methods.

A. Proteinase K Method

DNA was extracted by the commonly used proteinase K method. Briefly, a total of 0.1 ml of the inoculated blood culture medium was added to 0.3 mL of 1 × TEN (1 M Tris [pH 8.0], 0.5 M EDTA, 5 M NaCl), followed by the addition of 20 μL of 10% SDS, and 20 μL of 20 g/L proteinase K. The samples were digested at 54℃ overnight.

B. Triton X-100 Method

DNA was extracted by the method of Shin et al [4]. Briefly, a washed pellet of bacteria was resuspended with 300 μL of triton X-100 buffer (1% triton X-100, 10 mM Tris, 1 mM EDTA [pH 8.0]) and boiled in a water bath for 20 min.

C. Benzyl alcohol-guanidine method

DNA was extracted by the method of Fredricks et al [3]. A total of 0.1 mL of the blood culture broth was added to 0.1 mL of lysis buffer consisting of 5 M guanidine hydrochloride and 100 mM Tris [pH 8.0] in sterile water and briefly mixed with a vortex mixer. A total of 0.4 mL of water was added, followed by the addition of 0.8 mL of 99% benzyl alcohol (Sigma Chemical Co. St. Louis, MO, USA). The sample was then mixed again by vortexing and centrifuged at 7,000 × g for 5 min. A total of 0.4 mL of the aqueous supernatant was removed and placed in a new centrifuge tube. A total of 40 μL of 3 M sodium acetate was added, followed by the addition of 0.44 mL of isopropanol, and the sample was centrifuged at 16,000 × g for 15 min. The precipitated DNA was washed with 70% ethanol and the pellet was air dried. The DNA was resuspended in 50 μL of distilled water.

PCR amplification of 16S rDNA

A 16S rDNA fragment was amplified by PCR using the universal primers 516F and 1541R. The primer 516F (5'-TGC CAG CAG CCG CGG TAA-3') is identical to positions 516 to 533 and 1541R (5'-AAG GAG GTG ATC CAA CCG-3') is complementary to positions 1541 to 1524 in the *E. coli* numbering system [3, 5].

A reaction mixture containing 1 × PCR buffer B (Promega, Madison, WI, USA), 2.0 mM $MgCl_2$, 0.2 μM of each PCR primer, 0.2 mM of each dNTP, and 1.25 U of *Taq* DNA polymerase (Promega) in a total volume of 50 μL was prepared. Each 50 μL PCR mixture contained 5 μL of target DNA. After a 5 min denaturation at 94℃, the reaction mixture was amplified using 35 cycles of denaturation for 30 sec at 94℃, annealing for 30 sec at 58℃ and extension for 1 min at 72℃, followed by a 7 min incubation at 72℃. Both reagent controls and positive controls were included in each PCR run. The reagent control consisted of all PCR components except for the template DNA. If the reagent control was positive, the entire set of PCR reactions was repeated.

Agarose gel electrophoresis

Gel electrophoresis was conducted in TBE buffer (0.1 M Tris, 0.09 M boric acid, 1 mM EDTA [pH 8.4]) at 100 V for 28 min using 2% (wt/vol) agarose gels. Gels were stained with ethidium bromide for 20 min and destained for 5 min with distilled water.

Lower limit of detection of the 16S rDNA PCR assay

To determine the lower limit of detection of the 16S rDNA, tubes of distilled water were artificially seeded with *S. aureus* ATCC 25923, *E. faecalis* ATCC 51299, *E. coli* 25922, and *P. aeruginosa* ATCC 27853 to final concentrations ranging from 10^1 to 10^{10} cells per mL. The exact colony counts were determined by plating aliquots of several dilutions of bacteria on blood agar plates concomitantly and counting the number of colonies after an overnight incubation.

The samples were treated to extract DNA by the benzyl alcohol-guanidine method and the isolated DNA was used for the 16S rDNA PCR reactions, as described above.

Table 1. Comparison of bacterial identification by 16S rDNA PCR and by blood culture tests

Blood culture	N	16S rDNA PCR	
		Positive	Negative
Positive	99	95	4
Negative	122	0	122
Total	221	95	126

Sensitivity 96.0%, specificity 100%, positive predictive value 100%, negative predictive value 96.8%.

RESULTS

Efficiency of the PCR assay

To assess the efficiency of the 16S rDNA PCR assay in clinical samples, we tested 221 culture-proven samples from BacT/Alert blood culture bottles. A comparison of the blood culture results and the 16S rDNA PCR results is shown in Table 1. Almost all of the samples, 217 of 221 (98.2%), showed equivalent results in each assay. Compared with culture results, the sensitivity, specificity, positive predictive value and negative predictive value of 16S rDNA PCR assay for detection of bacteria from blood culture broth were 96.0%, 100%, 100%, and 96.8 %, respectively.

Positive rate of 16S rDNA PCR by bacterial species

Of 99 strains from 22 different species obtained from positive blood culture bottles, 95 strains (96.0%) were detected correctly by the 16S rDNA PCR method. Four false-negative results were obtained: 1 *Corynebacterium glutamicum*, 1 *Corynebacterium* species, 1 *E. coli*, 1 *S. aureus*.

The percentage of correct positives using the 16S rDNA PCR method varied among different bacterial groups. The 16S rDNA PCR method correctly identified 98.2% of gram-positive cocci, 66.7% of gram-positive bacilli, and 97.4% of gram-negative bacilli (Table 2).

Comparison of DNA extraction methods

Aliquots of 10 blood culture-positive samples and a

Table 2. Percentage of 16S rDNA PCR positivity of bacterial species isolated from blood cultures

Group	Bacteria	16S rDNA PCR			
		N	Negative	Positive	(%)
G (+) C	Coagulase negative staphylococci	27	0	27	(100.0)
	Staphylococcus aureus	13	1	12	(92.3)
	Enterococcus spp.	10	0	10	(100.0)
	Strepococcus spp.	5	0	5	(100.0)
G (+) B	*Corynebacterium* spp.	6	2	4	(67.7)
G (-) B	*Escherichia coli*	14	1	13	(92.9)
	Klebsiella pneumoniae	4	0	4	(100.0)
	Pseudomonas spp.	3	0	3	(100.0)
	Serratia spp.	3	0	3	(100.0)
	Acinetobacter baumannii	3	0	3	(100.0)
	Burkholderia picketii	3	0	3	(100.0)
	Agrobacterium radiobacter	2	0	2	(100.0)
	Enterobacter cloacae	2	0	2	(100.0)
	Salmonella group D	1	0	1	(100.0)
	Citrobacter freundii	1	0	1	(100.0)
	Enterobacter aerogenes	1	0	1	(100.0)
	Haemophilus influenzae	1	0	1	(100.0)
Total		99	4	95	(96.0)

Abbreviations: G (+) C, gram positive cocci; G (+) B, gram positive bacilli; G (-) B, gram negative bacilli.

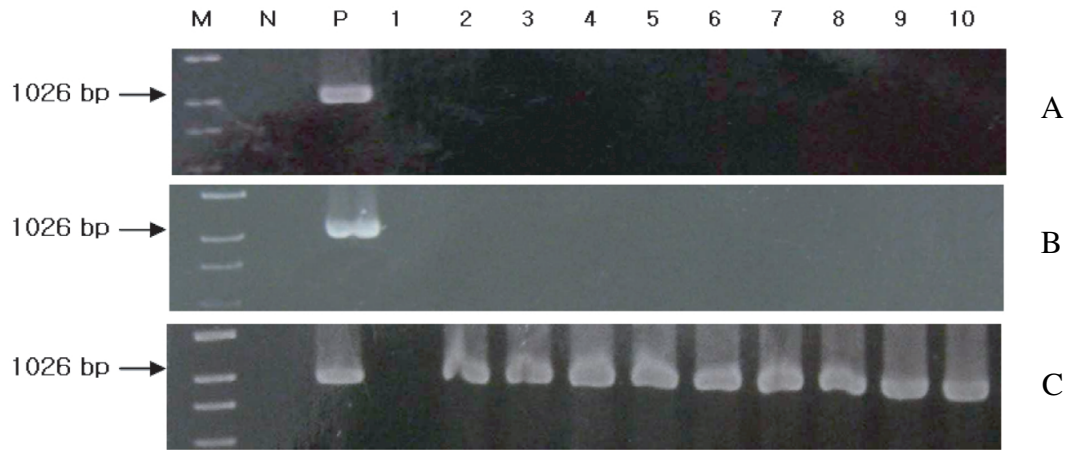

Fig. 1. Agarose gel electrophoresis of PCR-amplified 16S rDNA obtained by the different DNA extraction methods. A, Proteinase K Method; B, Triton X-100 method; C, benzyl alcohol-guanidine method. Lane M, molecular weight markers (100-bp ladder); Lane N, negative control; Lane P, positive control; Lanes 1 to 10, randomly selected blood culture-positive broth samples from 10 different patients.

loopful of bacterial colonies were subjected to each of three DNA extraction methods: proteinase K, triton X-100, and benzyl alcohol-guanidine. The extracted DNA was used as the target in the 16S rDNA PCR assay. The results of the 16S rDNA PCR amplification of DNA extracted by each of the methods is shown in Fig. 1. Among these three methods, the benzyl alcohol-guanidine method was the most effective for isolating DNA from blood culture broth samples. No 16S rDNA was detected by PCR of samples isolated using proteinase K or triton X-100 for blood culture broth (Fig. 1), although all of the bacterial colony samples tested showed positive results by each of the three DNA extraction methods (data not shown). Thus, we selected the benzyl alcohol-guanidine method for DNA extraction for assays to determine the detection limit of the PCR method.

Lower limit of detection of the 16S rDNA PCR

To determine the lower limit of detection of the 16S rDNA PCR, 10-fold serially diluted aliquots of four species of bacteria, *E. coli*, *S. aureus*, *E. faecalis*, and *P. aeruginosa*, were tested. The lower limit of detection was 3×10^7/mL for *S. aureus*, 5×10^7/mL for *E. faecalis*, 3×10^5/mL for *E. coli*, and 4×10^7/mL for *P. aeruginosa*.

DISCUSSION

Molecular techniques such as 16S rDNA PCR have been successfully used to identify a wide range of organisms including Chlamydia [6-10]. Bacterial 16S rDNA PCR can be used as a tool for rapid detection of bacteria in normally sterile clinical samples and would be useful to differentiate bacteria from viral infections. This would confirm the necessity for antibiotic treatment and would influence patient management.

However PCR-based detection systems for blood-borne pathogens face two challenges: (i) the low density of the pathogens and (ii) the inhibitory effects of certain blood components on PCR. A common technical problem with PCR is amplification failure due to the presence of PCR inhibitors. Known PCR inhibitors include the heme compounds found in blood, the aqueous and vitreous humors, heparin, EDTA, urine, polyamines, and plant polysaccharides [3, 11-13].

Fredricks and colleagues identified sodium polyanetholesulfonate (SPS) as a potent PCR inhibitor present in blood culture media. SPS is a common component in commercially available blood culture medium. They reported that several standard DNA purification methods failed to remove SPS, resulting in failed PCR amplification. However, an organic extraction procedure with a buffer containing benzyl alcohol and guanidine hydrochloride successfully removed SPS, yielding DNA that can be amplified by PCR without further processing or dilution [3]. Our study confirmed these

findings. When we compared the efficiency of three DNA purification methods, the benzyl alcohol-guanidine method, the proteinase K method, and the triton X-100 method, only the benzyl alcohol-guanidine method effectively extracted PCR-ready DNA from blood culture broth.

Jordan et al. reported that the sensitivity, specificity, and positive- and negative- predictive values of bacterial 16S rDNA PCR were 96.0%, 99.4%, 88.9%, and 99.8%, respectively [14], which is similar to our results in the current study. However Sleigh et al. reported that 16S rDNA PCR was about twice as sensitive as blood culture in detecting bacteremias in intensive care unit patients and might be especially useful in patients who have received prior antibiotics [1]. The lower limit of detection of the 16S rRNA PCR method in detecting *E. coli* in CSF or blood in a study by Backman and colleagues was 10^4 CFU/mL [15], which is better than the limit of detection we were able to obtain here. A possible cause of the relatively low sensitivity of PCR in the present study may be the difference in the method of DNA extraction, particularly the small volume (100 μL) of sample for DNA extraction and the influence of PCR inhibitors such as hemoglobin.

Backman et al. selected the Dynabeads DNA DIRECT Kit after comparing several DNA extraction methods and the diagnostic sensitivity of the resulting assay in detection of bacteria in CSF was high (97%) [15]. Thus, selection of an optimum method to isolate bacterial DNA from samples containing only a few bacteria seems to be an important factor in increasing the diagnostic sensitivity of the assay. Jordan et al. appreciated the benefits of pre-filtering the PCR master mix using the Centricon YM-100 centrifugal filter device (Millipore Corporation, Billerica, MA, USA) [14].

Jordan et al. reported that when they attempted to detect the bacterial 16S rRNA gene from whole blood samples with volumes of appreciably less than 200 μL, a dramatic loss in sensitivity was observed. They therefore established a minimum blood volume requirement of 200 μL for 16S rRNA gene PCR testing [14]. Sleigh et al. used 400 μL of EDTA chelated blood. In their study, the RBCs were lysed by NH_4Cl and the WBCs were removed and heme reduced with 15 μL of H_2O_2 [1]. When we adopted this method for efficient removal of hemoglobin prior to DNA extraction in several PCR-negative and blood culture-positive samples, the PCR-negative result converted to positive in several samples (data not shown).

For samples containing PCR inhibitory substances such as blood culture broth, pilot studies adopting a new sample preparation method and using an inhibitor binding protein such as bovine serum albumin (BSA) in the assay may be useful [12]. Factors such as the number of copies of the 16S rDNA present in different bacterial species or the efficiency of DNA extraction from different bacteria may also influence positive PCR results in blood culture samples.

The prerequisites for successful amplification of bacterial 16S rDNA seem to be optimization of PCR conditions, selection of proper primers, and implementation of efficient DNA extraction methods. Using the best method to extract bacterial DNA efficiently and to remove PCR inhibitors completely from samples is highly recommended. To increase the number of bacteria in samples it may be helpful to use a larger sample volume, or to culture the bacteria prior to DNA extraction.

Almost all species of bacteria commonly isolated from blood cultures were detected by 16S rDNA PCR. However, the positive rate of detection of Corynebacteria was relatively lower than those of the other microorganisms.

Lu et al. used PCR with universal primers for 16S rDNA PCR coupled with restriction endonuclease digestions, in order to detect and identify common bacterial pathogens in cerebrospinal fluid [6]. We tried the methods of restriction endonuclease digestion to identify some bacterial pathogens (data not shown). However, the restriction endonuclease digestion patterns of the bacteria that we tested were not reproducible and the band intensity was not strong enough to recognize specific patterns easily. Because Lu et al. developed the restriction patterns only for common bacterial pathogens, unusual pathogens can not be identified by their method. For those bacteria showing nonspecific patterns by *Hae III* digestion, another digestion step is required. Therefore, we prefer DNA sequencing of amplified bacterial DNA for species identification, despite the fact that DNA sequencing is expensive, time consuming, and requires cloning.

In conclusion, these data show that the 16S rDNA PCR assay is a rapid and sensitive method to detect bacterial pathogens in blood culture broth samples and will be useful to differentiate bacterial from viral infections. This assay would confirm the necessity for antibiotic treatment and would influence patient management. Our method is capable of detecting almost all species of bacteria isolated from blood cultures. Therefore, it has great potential for use in clinical microbiology laboratories.

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Evaluation of a 16S rDNA PCR Assay for Detection of Bacterial Pathogens in Blood Culture Broth

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Background: Rapid detection of pathogens in blood is important in patient management, because the mortality rate associated with bloodstream infections is very high. We evaluated the efficiency of a 16S rDNA PCR assay for the detection of various pathogens in blood culture broth in a clinical laboratory.

Methods: 16S rDNA PCR was performed on 221 blood culture bottles consisting of 99 culture-positive and 122 culture-negative samples. The results were compared with conventional culture methods. We also compared the efficiency of three DNA extraction and purification methods using proteinase K, triton X-100, and benzyl alcohol-guanidine DNA extraction of blood culture broths.

Results: The 16S rDNA PCR method detected 95 (12 *Staphylococcus aureus*, 27 coagulase negative staphylococci, 10 enterococci, 5 streptococci, 37 gram negative bacilli, 4 corynebacteria) of 99 positive culture bottles. Four false-negative results were obtained for bottles containing 2 *Corynebacterium*, 1 *Escherichia coli*, and 1 *S. aureus* species. All 122 bottles that showed no blood culture growth were negative by 16S rDNA PCR. Overall, the sensitivity, specificity, positive predictive values and negative predictive values of 16S rDNA PCR relative to the culture results were 96.0%, 100%, 100%, and 96.8%, respectively. Among the three DNA extraction methods, the benzyl alcohol-guanidine method was most effective.

Conclusion: The 16S rDNA PCR assay is a rapid and efficient means of detecting various pathogens in the blood and has great potential for use in the clinical microbiology laboratory.

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Keywords: 16S rDNA, Polymerase chain reaction, Blood culture, Sepsis

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