Molecular diagnosis of bacterial and fungal infections in blood and body fluids using 16sRNA and 18sRNA direct amplification

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Introduction

Identification of bacterial and fungal pathogens is crucial for effective antimicrobial therapy. Cultures have been the mainstay of diagnosis until now, but definitive identification of the causative organisms is usually not available until 24 to 48 hours. A more rapid diagnostic method is needed for clinicians in certain medical emergencies. In this respect, molecular diagnostic methods are now inevitable in clinical application, and with the development of the PCR technique[1], microbial identification using rRNA gene sequences has been made possible[2].

PCR primers that are targeted at highly conserved rDNA gene sequences make amplification of the genes of virtually any bacterial or fungal species possible[3]. The selected eubacterial gene in this study is the 16sRNA, and the eufungal gene 18sRNA. The DNA sequence of the amplification product enables identification of the causative microorganism in clinical specimens[3]. During recent years, this approach has proven its usefulness for the identification of bacterial isolates that are difficult to be cultured in clinical situations where antibiotics have already been administered[4]. More important is the application of this method to the identification of rare pathogens with unusual growth requirements[5, 6]. Earlier studies have involved a limited number of specimens and have focused on one defined infectious diseases only, such as endocarditis[7], meningitis[4], bacteremia[8], and intra-amniotic infection[9].

We report here the usefulness of the PCR technique in the amplification of the 16sDNA genes of bacteria and 18sDNA genes of fungus and DNA direct sequencing of the PCR products in the diagnosis of a wide spectrum of bacterial and fungal infections.

Materials and Methods

1. Samples

A total of 291 clinical specimens from 260 patients were analyzed at Department of Internal Medicine, Yonsei University College of Medicine from March, 1999 to May, 2001. The clinical specimens consisted 206 specimens of blood, 76 specimens of body fluids, 5 specimens of pus from various abscesses, and 4 biopsy specimens from tissues. The specimens were collected upon request of the attending doctors of the involved departments.

2. Conventional microbiological cultures

Cultures of blood and body fluids were performed by conventional methods in the department of clinical pathology. The laboratory had no knowledge of the PCR results. Cultures for most samples included aerobic culture on blood agar or blood Mackonkey agar plates and when necessary, anaerobic culture on a fastidious anaerobic agar plate. Body fluids were concentrated by centrifugation and biopsy specimens were homogenized in brain heart infusion broth prior

to cultivation. On the basis of the Gram stain, appropriate subculturing is done for definitive microbiological identification. Identification of the colonies isolated was based on routine microbiological methods.

3. DNA purification

Blood was centrifuged at 1,500 rpm for 15 minutes and the supernatant was collected for centrifuge at 12,000 rpm for 10 minutes. The pellet was digested with lysozyme (Sigma Chemical Co., St. Louis, MO, USA) and proteinase K (Sigma Chemical Co.) 0.1 mg/ml at 55℃ for 2 hours. DNA was extracted with two phenol-chloroform-isoamyl alcohol extractions followed by one ether wash. Pleural fluid, peritoneal fluid and pus was centrifuged at 3,500g for 10 minutes and the pellet in 100 l distilled water was treated with proteinase K 0.1 mg/ml at 55℃ for 2 hours and after heat inactivation DNA was extracted with two phenol-chloroform-isoamyl alcohol extractions followed by two ether washes. Cerebrospinal fluid and pericardial fluid was boiled at 94℃ for 10 minutes. Proteinase K 0.1 mg/ml was treated at 55℃ for overnight. After heat inactivation DNA was extracted with two phenol-chloroform-isoamyl alcohol extractions followed by two ether washes.

4. PCR and direct sequencing

The primers used in the 16sDNA PCR of bacteria was as follows: forward- 5′-AGT TTG ATC ATG GCT CAG-3′, reverse- 5′-GGA CTA CCA GGG TAT CTA AT-3′. The primers used in the 18sDNA PCR of fungus was as follows: forward-5′-ATT GGA GGG CAA GTC TGG TG-3′, reverse-5′-CCG ATC CCT AGT CGG CAT AG-3′. PCR reactions were performed in a total volume of 50 l. The reaction mixture contained taq polymerase (HT Biotechnology, UK), buffer containing 10mM Tris-HCl (pH9.0), 1.5mM MgCl2, 50mM KCl, 0.1%(v/v) Triton X-100, 20 m of each dNTP (Pharmacia Biotech, Sweden), and 0.1 M of each primers, and 10 l of template DNA. Model 2400 PE Thermal Cycler (Applied Biosystems, San Hose, CA, USA) was used for bacteria amplification carried out at 94℃ for 5 minutes followed by 30 cycles of 94℃ for 1 minute, 56℃ for 30 seconds, and 72℃ for 1 minute. Fungus amplification was carried out at 95℃ for 10 minutes followed by 35 cycles of 95℃ for 30 seconds, 67℃ for 1 minute, and 70℃ for 1 minute. A band at 840 bp was detected for positive bacterial PCR, and a band at 520 bp for positive fungal PCR. To identify the microorganism, direct sequencing was performed with the identical set of primers used for amplification. The 16sDNA and 18sDNA sequences that were obtained were analysed using the BLAST (Basic Local Alignment Search Tool) program to detect relationships among sequences which share only isolated regions of similarity[10]. The best matches and sequence homologies are reported according to the original results, but not reanalyzed with the sequences in the expanded database.

Results

1. Culture and PCR positivity

Culture of either bacteria and fungus was positive in 53 of 291 specimens (18.2%). Blood culture was positive in 17.0%, body fluid 19.7%, and pus 60%. Among the body fluids, peritoneal fluid showed the highest positivity of 36.4%. PCR of bacterial specimens was 43.8% and fungal specimens was 36.5%. Bacterial PCR of blood was positive in 42.1%, of body fluid 43.6%, of pus 75%, and of tissue 66.7%. Fungal PCR of blood was 37.0%, of body fluid 33.3%, and of pus 100% (Table 1).

2. Bacterial PCR and sequencing

Of all 291 specimens analysed, 176 specimens were run for bacterial PCR and 115 specimens for fungal PCR. Among 176 specimens, bacterial culture was positive in 36 specimens (21%), of which PCR correlated as positive in 31 specimens. There were 5

Table 1. Positive results of specimens analyzed by the 16sRNA/18sRNA PCR and culture

Specimen	No. of (+) specimens / No. of analyzed specimens (%)		
	16sDNA PCR	18sDNA PCR	Culture
Blood	48/114 (42.1%)	34/92 (37.0%)	35/206 (17.0%)
Body fluids	24/55 (43.6%)	7/21 (33.3%)	15/76 (19.7%)
Cerebrospinal fluid	6/20 (30.0%)	2/10 (20.0%)	5/30 (16.7%)
Pleural fluid	9/19 (47.4%)	1/2 (50.0%)	3/21 (14.3%)
Peritoneal fluid	6/7 (85.7%)	2/4 (50.0%)	4/11 (36.4%)
Pericardial fluid	2/6 (33.3%)	1/3 (33.3%)	2/9 (22.2%)
Bone marrow	1/2 (50.0%)	1/2 (50.0%)	1/4 (25.0%)
Synovial fluid	0/1 (0 %)	0/0 (0 %)	0/1 (0 %)
Pus	3/4 (75.0%)	1/1 (100%)	3/5 (60.0%)
Tissue	2/3 (66.7%)	0/1 (0 %)	0/4 (0 %)
Total	77/176 (43.8%)	42/115 (36.5%)	53/291 (18.2%)

specimens which had positive bacterial cultures with negative PCR results, and 1 specimen was highly suspected of culture contamination. The decision of culture contamination was made when scarce growth of coagulase-negative staphylococci and/or gram-positive rods such as Corynebacterium sp., Propionibacterium sp., or Bacillus sp., in the primary culture plate, or when detection of one or more of the aforementioned species or other species known to have low levels of pathogenecity, such as an Enterococcus sp., or pseudomonas-like rods, in one of three enrichment cultures, often after a prolonged culture period. In all patients, the clinical presentation of the disease and the immunologic status of the patient were verified before classification of the culture finding as contamination.

Among 140 bacterial culture negative specimens, 94 specimens also showed negative PCR results. However, 46 specimens of the 140 bacterial specimens had positive PCR results despite negative cultures. Among the 31 bacterial cases of positive blood culture and PCR results, the sequencing results revealed identical bacteria in 29 cases, sequencing failed in 1 case, and discordant bacteria was isolated in 1 case (Table 2). The case which showed discordant results on PCR and culture identified different bacterial species in the blood sample. *Bacteroides fragilis* was identified on the basis of sequencing, and *Staphylococcus aureus* by culture. No definite conclusions about the contribution of these microbes to the clinical disease in this 72-year-old aspiration pneumonia patient can be made, however, because Bacteroides spp. is a fastidious microorganism, sequencing may be a better tool in determining the organism in this case.

The importance of PCR as a useful diagnostic tool is emphasized in the cases where bacterial culture showed no growth, but PCR showed positive results with sequencing detecting the causative microorganism. Among these 46 cases, only 4 cases failed in sequencing, and 42 cases detected a microorganism (Table 3), in which 33 cases supplied useful clinical information in guiding the infectious diseases specialist to decide the proper antibiotics, and 9 cases were difficult to clinically consider as the related pathogen, which could possibly be PCR contamination. Among the 33 microbes, 25 had normal growth requirements and 8 were fastidious (Figure 1). In 6 cases, gram stain correlated with the sequencing results. Effective antibiotics were being administered at the time of sampling in 44 cases (96%), in the cases with positive PCR and negative culture results.

Table 2. Description of clinical specimens with positive 16sDNA PCR and positive cultures

Specimen	Culture	Sequencing %	Homology	Sequence length	Final diagnosis
Cerebrospinal fluid	*S. pneumoniae*	*S. pneumoniae*	95.5	820bp	bacterial meningitis
Peritoneal fluid	*S. aureus*	*S. aureus*	100	813bp	CAPD peritonitis
Peritoneal fluid	*A. baumanii*	*A. baumanii*	99.2	855bp	post-surgical peritonitis
Brain abscess	*S. milleri*	*S. millleri*	100	690bp	brain abscess
Empyema pus	*S. aureus*	*S. aureus*	98.3	810bp	pneumonia
Pericardial fluid	*S. aureus*	*S. aureus*	99.3	820bp	bacterial pericarditis
Pleural fluid	*S. aureus*	*S. aureus*	94.4	810bp	pneumonia
Pleural fluid	*P. aeruginosa*	*P. aeruginosa*	99.9	840bp	VAP
Blood	*S. aureus*	*B. fragilis*	95.5	720bp	pneumonia, sepsis
Blood	*C. freuindii*	failed	-	-	pneumonia
Blood	*E. coli*	*E. coli*	98.9	840bp	UTI, bacteremia
Blood	*E. coli*	*E. coli*	99.9	810bp	UTI, sepsis
Blood	*S. aureus*	*S. aureus*	99.0	820bp	VAP
Blood	*S. aureus*	*S. aureus*	96.9	840bp	catheter infection
Blood	*S. aureus*	*S. aureus*	99.5	840bp	otitis media, sepsis
Blood	*S. aureus*	*S. aureus*	93.9	780bp	endocarditis
Blood	*S. aureus*	*S. aureus*	99.2	840bp	bacteremia
Blood	*P. aeruginosa*	*P. aeruginosa*	95.5	730bp	catheter infection
Blood	*P. aeruginosa*	*P. aeruginosa*	97.0	830bp	cellulitis
Blood	*P. aeruginosa*	*P. aeruginosa*	99.9	840bp	pneumonia
Blood	*S. epidermidis*	*S. epidermidis*	94.2	810bp	bed sore, sepsis
Blood	*S. epidermidis*	*S. epidermidis*	99.0	800bp	catheter infection
Blood	*S. epidermidis*	*S. epidermidis*	99.5	800bp	CAPD peritonitis
Blood	*A. baumanii*	*A. baumanii*	99.1	840bp	VAP
Blood	*A. baumanii*	*A. baumanii*	95.5	800bp	sepsis
Blood	Bacillus spp.	Bacillus spp.	99.9	830bp	bacteremia
Blood	*K. pneumoniae*	*K. pneumoniae*	95.8	820bp	pneumonia
Blood	*S. mutans*	*S. mutans*	100	840bp	endocarditis
Blood	*E. cloacae*	*E. cloacae*	100	800bp	typhlitis
Blood	*E. faecium*	*E. faecium*	99.3	840bp	UTI, sepsis
Blood	*S. marcescens*	*S. marcescens*	98.8	780bp	pneumonia

CAPD: continuous ambulatory peritoneal dialysis, VAP: ventilator-associated pneumonia, UTI: urinary tract infection

3. Fungal PCR and sequencing

Among 115 specimens tested for fungal PCR, fungus culture was positive in 17 specimens. There were only two specimens which had positive fungus cultures with negative PCR results. Both of these specimens were thought to be clinically true infections, since these specimens were blood and catheter cultures of the identical patients which revealed *candida albicans* and *candida tropicalis* respectively.

Among the 15 cases of positive fungal culture and PCR results, the sequencing results revealed identical fungus in 12 cases, sequencing failed in 2 cases, and discordant fungus was isolated in 1 case (Table 4). The case which showed discordant results on PCR and culture identified different fungal species in the blood sample. *Candida parapsilosis* was identified on

Table 3. Microorganisms identified by PCR / sequencing in culture negative specimens (n=42)

Staphylococcus aureus	6
Bacteroides fragilis	6
Escherichia coli	5
Staphylococcus epidemidis	4
Pseudomonas aeruginosa	4
Enterobacter cloacae	2
Streptococcus bovis	1
Streptococcus constellatus	1
Streptococcus mutans	1
Enterococcus faecium	1
Citrobacter freundii	1
Klebsiella oxytoca	1
Acinetobacter baumanii	1
Proteus mirabilis	1
Proteus vulgaris	1
Stenotrophomonas maltophilia	1
Salmonella paratyphi	1
Alcaligenes xylosoxidans	1
Actinomyces	1
Clostridium perfringens	1
Prevotella melaninogenica	1

Figure 1. Analysis of 16sRNA PCR / sequencing and cultures of bacterial specimens

	Culture (+)		Culture (-)		Total
PCR (+)	Concordant results	29	Routinely cultured organism	25	
	Discordant results	1	Fastidious organism	8	
	Sequence failure	1	PCR contamination (possible)	9	
			Sequence failure	4	
	Total	31	Total	46	77
PCR (-)	Clinically significant	4			
	Culture contamination	1			
	Total	5	Total	94	99
Total		36		140	176

Figure 2. Analysis of 18sRNA PCR / sequencing and cultures of fungal specimens

	Culture (+)		Culture (-)		Total
PCR (+)	Concordant results	12	Routinely cultured fungus	21	
	Discordant results	1	PCR contamination (possible)	3	
	Sequence failure	2	Sequence failure	3	
	Total	15	Total	27	42
PCR (-)	Clinically significant	2			
	Culture contamination	0			
	Total	2	Total	71	73
Total		17		98	115

the basis of sequencing, and *Candida cruzei* by culture. No definite conclusions about the contribution of these microbes to the clinical disease in this immunocompromised patient who had been on broad spectrum antibiotics can be made, however, because the patient was on fluconazole and was successfully treated, the probable causative organism was *Candida*

Table 4. Description of clinical specimens with positive 18sDNA PCR and positive cultures

Specimen	Culture	Sequencing %	Homology	Sequence length	Final diagnosis
Cerebrospinal fluid	*C. albicans*	*C. albicans*	99.9	520bp	fungal meningitis, SLE
Cerebrospinal fluid	*C. albicans*	*C. albicans*	98.1	520bp	fungal meningitis
Peritoneal fluid	*C. glabrata*	*C. glabrata*	95.5	500bp	CAPD peritonitis
Peritoneal fluid	*C. albicans*	*C. albicans*	99.9	490bp	CAPD peritonitis
Pleural fluid	*A. fumigatus*	*A. fumigatus*	100	510bp	fungal pneumonia
Pus from catheter	*P. marnefeii*	*P. marnefeii*	99.7	550bp	CAPD peritonitis
Blood	*C. cruzei*	*C. parapsilosis*	99.5	520bp	fungemia, leukemia
Blood	*C. tropicalis*	*C. tropicalis*	98.5	520bp	fungemia
Blood	*C. albicans*	*C. albicans*	99.9	610bp	fungemia, endocarditis
Blood	*C. albicans*	*C. albicans6*	95.5	530bp	CRI
Blood	*C. albicans*	*C. albicans*	99.6	520bp	fungemia
Blood	*C. albicans*	failed	-	-	fungemia, CRI
Blood	*C. cruzei*	*C. cruzei*	100	510bp	pneumonia
Blood	*C. glabrata*	*C. glabrata*	100	510bp	pneumonia, fungemia
Blood	*C. glabrata*	failed	-	-	fungemia

SLE: systemic lupus erythematosus, CAPD: continuous ambulatory peritoneal dialysis, CRI: catheter related infection

parapsilosis, since *Candida cruzei* is naturally resistant to azoles.

Among 98 fungus culture negative specimens, 71 specimens also showed negative PCR results. However, 27 specimens of the 98 fungus specimens had positive PCR results despite negative cultures. In these cases, sequencing detected the causative fungus in all but 3 cases, and in 21 cases identification of the fungus supplied useful clinical information in guiding the infectious diseases specialist to decide the proper antifungals (Figure 2). In these cases with positive PCR and negative culture results, effective antifungals were being administered at the time of sampling in 25 cases (93%).

4. Performance of bacterial and fungal PCR compared to cultures

The sensitivity and specificity of bacterial PCR was 86.1% and 67.1%, respectively. The sensitivity and specificity of fungal PCR was 88.2% and 72.4%, respectively. Positive and negative predictive value of bacterial PCR was 40.3% and 94.9%, and of fungal PCR was 35.7% and 97.3%, respectively.

Discussion

Usefulness of PCR of the conserved sequences of microorganisms and direct sequencing in the diagnosis and identification of a wide spectrum of bacterial and fungal infections has been examined in this study. Molecular methods correlated well with conventional microbiological methods. The results were identical for 73% of the specimens. In bacterial specimens, among 140 culture negative cases, 46 cases (33%) revealed PCR positive, and in fungal specimens, among 98 culture negative cases, 27 cases (28%) revealed PCR positive. Most of these cases were culture negative because antibiotics or antifungals have already been administered, and detecting the causative pathogens in these cases comes to be important in certain clinical situations, such as when unresponsive empirical treatment needs regimen modification or when drug adverse effects brings clinicians to change drugs. Another reason why some of these cases were culture negative was because the pathogens were fastidious organisms or rare pathogens not grown on routine culture media. This is a matter of improving the detection rate of causative organisms as well as shortening the time to identification of pathogens. In a patient with peritonitis on continuous ambulatory peritoneal dialysis, fungus culture revealed an unidentifiable organism, perhaps because it was an unfamiliar fungus to the microbiologists. In 2 days, *Penicillium marnefeii* was identified by sequencing, and in 26 days, was microbiologically proven to be the first case of *P. marnefeii* peritonitis in Korea, where until then, *P. marnefeii* infections had been unknown in our country.

In analyzing the usefulness of a novel diagnostic tool, a currently available conventional diagnostic method is usually the golden standard in evaluating the sensitivity and specificity[11]. However, a pitfall exists in such evaluation especially when the golden standard does not perfectly reflect the disease status. In diagnosing an infectious disease, culture rate is not sufficiently high in most specimens, and a problem exists in assessing culture contamination as a positive result. Also, when the culture is negative, it is difficult to interpret it as infection not existing. In this study, when excluding the cases of culture contamination, sequencing failures, discordant cases, cases which the PCR results does not clinically correlate with the therapeutic responses, the sensitivity and specificity of bacterial PCR rises enormously to 93.9% and 88.0% respectively, and of fungal PCR to 94.3% and 92.2% respectively. Although evaluating the performance of PCR may seem more accurate this way, there is a limitation in precisely defining contamination of both culture and PCR, and there is also difficulty in categorizing sequence failures to PCR positivity.

Several limitations of the conserved sequence

rDNA PCR exists. Bacterial or fungal resistance and sensitivity to drugs remains unknown. Polymicrobial infections in the same specimen may identify only one organism, although cloning may identify more than one organism, this may be an over-expensive procedure. In some cases, only genus can be determined, while the species remains undefined. This is a problem when the treatment differs in infections caused by two organisms of identical genus and different species.

Since conserved sequence rDNA PCR is a broad-range PCR, the risk of contamination is higher than in specific assays. Traces of environmental bacterial and fungal fragments may be amplified, and therefore, we sterilized or filtered reagents, enzymes, buffers and almost every material that is involved in the procedure. In our PCR laboratory, contamination was rarely experienced, and only 12 samples among 291 samples were thought to be probable contaminations in comparison to culture results.

There may be difficulty in applying molecular diagnostic techniques in clinical microbiology due to its expensiveness and requirements of highly specialized laboratories with well trained technicians. This is where novel DNA microarray techiniques should come into place, for this may spare unnecessary skills and labor in the diagnostic procedures. In the near future, when microarray becomes more popular and inexpensive, the conserved sequence PCR method may become even more useful.

We conclude that although not currently competitive with conventional microbiological methods, conserved sequence PCR and direct sequencing appears to be a valuable alternative technique for the diagnosis of infectious diseases in certain clinical situations. The molecular methods that we have described may be applied as a basic knowledge for the development of less time consuming and less laborious molecular methods for the identification of causative microorganisms in a wide range of infectious diseases.

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