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Rapid, Sensitive and Simple Detection of Candida Deep Mycosis by Amplification of 18S Ribosomal RNA Gene; Comparison with Assay of Serum β-D-Glucan Level in Clinical Samples

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SAKAI, T., IKEGAMI, K., YOSHINAGA, E., UESUGI-HAYAKAWA, R. and WAKIZAKA, A. *Rapid, Sensitive and Simple Detection of Candida Deep Mycosis by Amplification of 18S Ribosomal RNA Gene; Comparison with Assay of Serum β-D-Glucan Level in Clinical Samples.* Tohoku J. Exp. Med., 2000, **190** (2), 119-128 —— We evaluated the clinical usefulness of a polymerase chain reaction (PCR) assay amplifying the 18S ribosomal RNA gene of fungi for the diagnosis of deep candidiasis, compared with that of the β-glucan test or Cand-Tec test. Thirty critically ill patients who had received prolonged care with intravenous hyperalimentation and endotracheal intubation in the intensive care unit and were suspected of having deep fungal infections were examined. Twenty-one were fungi positive in the PCR assay (70%). Among 24 samples in which the PCR assay, β-glucan test and Cand-Tec test were performed simultaneously, 75% of the samples (18/24) were fungi positive in the PCR assay, whereas only 54% (13/24) had positive reactions in the β-glucan test and 21% (5/24) in the Cand-Tec test. The results of the Cand-Tec test showed no relationship with those of the PCR or β-glucan test. The lower limit of detection in the PCR assay was 4-5 CFU/ml of *C. albicans* in blood. No fungal organism was amplified from the serum of 20 healthy individuals. The results of the PCR assay and β-glucan test showed a significant correlation in this study, but the PCR assay proved to be more sensitive than the β-glucan test ($p<0.05$), and to be more useful for the clinical diagnosis and monitoring of deep Candidiasis. ———— polymerase chain reaction; 18S ribosomal RNA; β-glucan; deep mycosis; *Candidiasis* © 2000 Tohoku University Medical Press

Disseminated candidiasis and nosocomial candidemia have emerged as common complications of immunocompromised hosts, surgically treated patients,

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trauma, burn injuries and babies in neonatal intensive care units (Horn et al. 1985; Morrison et al. 1986; Komshian et al. 1989; Banerjee et al. 1991; Walsh et al. 1995). However, conventional laboratory diagnostic culturing of fungal infections is sometimes difficult and time consuming. Although serodiagnostic kits have been developed for rapid diagnosis of deep mycoses (Buckley et al. 1992; Kohno et al. 1993), these have some problems of sensitivity or specificity (Ikegami et al. 1999). Molecular biological techniques have been developed in order to improve the sensitivity and specificity of the detection of pathogenic fungi (Buchman et al. 1990; Spreadbury et al. 1993; Makimura et al. 1994, 1996; Milwald et al. 1994; Walsh et al. 1995; Yamakami et al. 1996; Einsele et al. 1997; Baek et al. 1998; Kappe et al. 1998). We examined a polymerase chain reaction (PCR) assay which amplified a well conserved sequence of the 18S ribosomal RNA (18SrRNA) gene of fungi, analyzed its sensitivity and specificity, and assessed its usefulness for clinical application, compared with the serum β-glucan test.

Materials and Methods

Selection of DNA for amplification

In this study PCR primers amplifying conserved DNA sequences of 18SrRNA genes shared by most medically important fungi were employed. Primers B2F (5′-ACTTTCGATGGTAGGATAG-3′) and B4R (5′-TGATCA[G]TCTTCGATCCCCTA-3′) amplify a specific 687-bp fragment of the gene coding for 18SrRNA of clinically important fungi (Makimura et al. 1996), including variable species-specific regions. Primers to amplify a 16S ribosomal RNA sequence (331 bp) for bacteria and exon 20 of the tyrosine kinase proto-oncogene (243 bp) for humans were also used as internal controls.

Laboratory specimens

Serial 10 fold dilutions of *Candida albicans* (Strain TIMM 1623) were made in saline or fresh whole blood supplemented with EDTA. A hundred-μl aliquots were reserved for quantitative culture on Sabouraud's agar and for PCR. Non-albicans *Candida spp.* included *Candida glabrata* (Strain TIMM 1064), *Candida guilliermondii* (Strain TIMM 0260), *Candida kefyr* (Strain TIMM 0302), *Candida krusei* (Strain TIMM 0269), *Candida parapsilosis* (Strain TIMM 0292) and *Candida tropicalis* (Strain TIMM 0313). These fungal strains were provided by M. Okazaki, Department of Clinical Pathology, Kyorin University School of Medicine. Other genomic DNA specimens tested were human, methicillin resistant *Staphylococcus aureus* (MRSA) (Strain 0928-53), *Escherichia coli.* (*E. coli*) (Strain 51826) provided by H. Taguchi, Department of Microbiology, Kyorin University School of Medicine and *Mycobacterium leprae* (Strain Thai 53) provided by M. Matsuoka, Leprosy Research Center, National Institute of Infectious Diseases.

Clinical samples

Blood was obtained from thirty patients in the intensive care unit with suspicion of a deep mycotic infection. All of the patients included in this study were critically ill and had undergone prolonged intensive care, intravenous hyperalimentation and endotracheal intubation. Patient profiles are shown in Table 1. Deep mycosis was suspected due to prolonged fever over several days despite antibacterial therapy. The blood samples were supplemented with EDTA, and 500 μl aliquots of the samples were stored at -80°C until they were

TABLE 1. *A profile of 24 critically ill patients included in this study with prolonged intensive care, intravenous hyperalimentation and endotracheal intubation are shown*

Case (No.)	β-glucan (pg/ml)	Cand-Tec	Blood culture	SIRS	Antifungal agent
PCR positive group					
1	<5	N	N	No	No
2	<5	N	N	No	No
3	<5	P	N	Yes	No
4	6.3	N	N	Yes	No
5	7.9	N	N	No	No
6	10.5	N	N	No	No
7	10.6	N	GNR	Yes	No
8	12.9	P	N	No	No
9	14.8	N	GPC	Yes	No
10	16.3	P	N	Yes	No
11	33	N	GNR	Yes	FLCZ
12	33.2	N	GNR	Yes	AMPH
13	58.5	N	GPC	Yes	No
14	73	N	N	Yes	No
15	91.3	N	GPC	Yes	FLCZ
16	165	N	GPC	No	No
17	292	N	N	Yes	No
18	300<	N	GPC	Yes	No
PCR negative group					
19	<5	N	N	No	No
20	<5	P	GPC	Yes	AMPH
21	<5	N	N	No	FLCZ
22	<5	N	GNR	No	No
23	<5	N	N	Yes	No
24	8.4	P	N	Yes	No

N, negative; P, positive; GNR, gram negative rod; GPC, gram positive cocci; FLCZ, fluconazole; AMPH, amphotericin B.

examined for the presence of fungi by PCR, and other aliquots of the same blood samples were analyzed by bacterial and fungal cultures simultaneously. Twenty-four out of the 30 patients were also examined by the FUNGITEC G test MK (Seikagaku Kogyo, Co., Ltd., Tokyo) for the serum level of β-glucan and by the Cand-Tec test (Ramco Laboratories, Inc., Houston, TX, USA). (1→3)-β-D-glucan is a cell wall component in fungi and it reacts with coagulation factor G of the horseshoe crab (Morita et al. 1981). The Cand-Tec test measures the immunoreaction of *Candida* with rabbit anti-*Candida* antibody (Fung et al. 1986). The PCR assay and the β-glucan test were performed separately in different laboratories. Twenty blood samples from healthy individuals were also examined by PCR in the same manner.

Specimen processing

DNA was extracted using a modification of the method of Buchman et al. (1990). Five hundred-μl blood samples were initially lysed with detergent (1% Triton X-100, 1% Tween-20 and 1% NP-40 dissolved in 0.05 mol/liter Tris-HCl, pH 7.5). After microcentrifugation, the pellets were resuspended in half-strength detergent and again microcentrifuged. The pellets were then washed twice in 0.05 mol/liter Tris-HCl (pH 7.5) and 0.01 mol/liter magnesium chloride and resuspended in the same solution containing deoxyribonuclease I (Sigma Chemical Co., St. Louis, MO, USA) at a final concentration of 10 μg/ml to destroy human and bacterial DNA. After incubation for 15 minutes at 37°C, DNase was inactivated by the addition of EDTA to a final concentration of 0.001 mol/liter and heating to 85°C for 30 minutes. The fungi were pelleted and resuspended in a solution containing 0.3 mg/ml Zymolyase (Seikagaku Co., Tokyo), 0.05 mol/liter Tris-HCl (pH 7.5), 0.01 mol/liter EDTA, and 0.028 mol/liter β-mercaptoethanol to digest the fungal cell wall. After incubation for 30 minutes at 37°C, fungal DNA was extracted with SepaGene (Sanko Junyaku Co., Ltd., Tokyo).

Polymerase chain reaction

A TaKaRa PCR kit (TaKaRa Shuzo Co., Ltd., Shiga) was used according to the manifacturer's recommendations. The amplification was performed using the following temperature cycling conditions; initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 2 minutes and extension at 72°C for 3 minutes using a Takara PCR 4800 apparatus. Five-μl aliquots of the PCR products were electrophoresed in 1.5% agarose gels, and visualized by staining with ethidium bromide, and judged by one of us blinded to clinical information about the patients.

Statistical analysis

The chi-square test for independent variables and Fisher's exact probability test were used.

Results

Specificity of primers

The specificity of the primers mentioned above has been well documented (Makimura et al. 1994, 1996). DNA from non-fungal pathogens (*E. coli*, MRSA, *M. leprae*) and human DNA did not produce a PCR product band when amplified with the fungus-specific primers (Fig. 1).

Sensitivity of primers

We used DNA from *C. albicans* (strain TIMM 1623), extracted as described above, to evaluate the PCR sensitivity. The lower limit of detection of PCR assay in this study was 4–5 CFU/ml of *C. albicans* in saline or blood (data not shown).

Detection of fungus-specific DNA in blood samples

Among 30 clinical samples from patinets with suspected fungal infection, 21 samples were PCR-positive (21/30; 70%) (Fig. 2). The comparison of the results of PCR, β-glucan and Cand-Tec tests in 24 clinical samples is shown in Table 2. In the 24 specimens analyzed by both the PCR and β-glucan tests, positivity was 75% (18/24) in PCR, 54% in the β-glucan test and 21% in the Cand-Tec test.

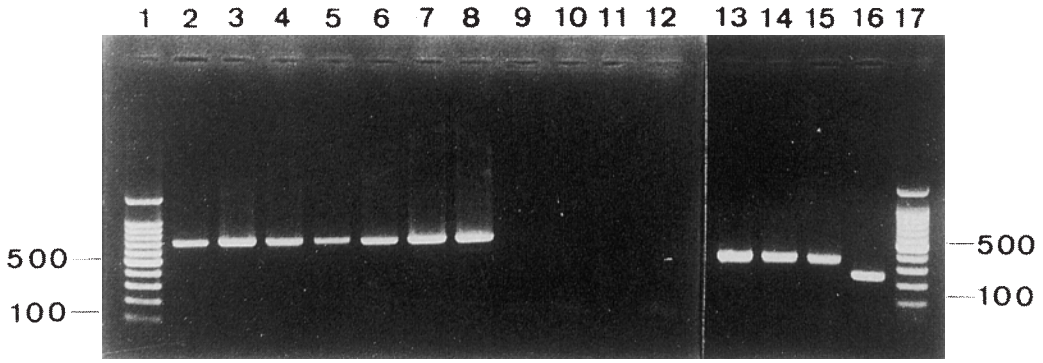

Fig. 1. Agarose gel electrophoresis of PCR products of *Candida* spp., bacteria and human DNA. Lanes 1 and 17 contaied 100-bp ladder molecular marker. Lanes 2 to 17 contaied DNA amplified with fungal specific primers B2F and B4R, and lanes 2 to 8 showed a 687-bp PCR product band which was specific for the fungal 18SrRNA gene (DNA in lane 2, *Candida albicans*, Strain TIMN 1623; lane 3, *Candida glabrata*, Strain TIMN 1064; lane 4, *Candida guilliermondii*, Strain TIMN 0260; lane 5, *Candida kefyr*, Strain TIMN 0302; lane 6, *Candida krusei*, Strain TIMN 0269; lane 7, *Candida paraplilosis*, Strain TIMN 0292; lane 8, *Candida tropicalis*, Strain TIMN 0313) but lanes 9 to 12 did not contaied a PCR product band (DNA in lanes 9 and 13, methicillin resistant *Staphylococcus aureus*, Strain 0928–53; lanes 10 and 14, *E. coli*, Strain 51826; lanes 11 and 15, *Mycobacterium leprae*, Strain Thai 53; lanes 12 and 16, Human DNA). Lanes 13 to 15 were amplified with bacteria-specific primers which amplified the 16SrRNA gene (331-bp product). Lane 16, human DNA, was amplified with primers of exon 20 of the receptor tyrosine kinase proto-oncogene (243-bp product).

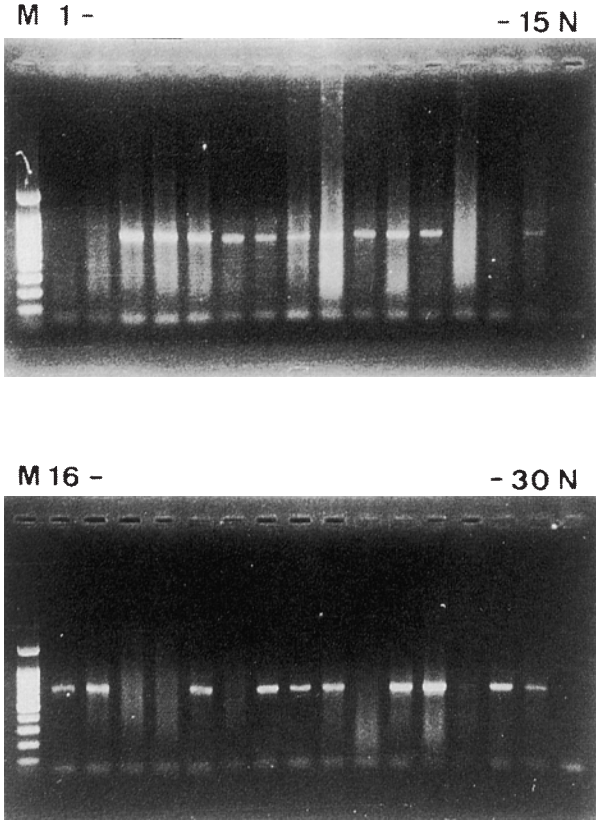

Fig. 2. Agarose gel electrophoresis of PCR products of clinical blood samples ($n=30$), using the the primers B2F and B4R. Seventy percent (24/30) revealed to be PCR positive (687 bp band). M, 100 bp ladder molecular marker. Lanes 1 to 15, and 16 to 30 were products from patients' blood samples. N, not amplified without templates (negative controls).

TABLE 2. *Patient profile (n = 24)*

Diagnosis	Number (male/female)	SIRS (+)
Trauma	14 (13/1)	8
H.E.	3 (0/3)	2
M.O.D.S.	1 (1/0)	0
C.V.A.	6 (4/2)	5

The relationship between the PCR results, serum β-glucan level, Cand-Tec test, blood culture and the clinical backgrounds of patients.

H.E., hypoxic encephalopathy; M.O.D.S., multiple organ dysfunction syndrome; C.V.A., cerebral vascular accident; SIRS, systemic inflammatory response syndrome (ACCP/SCCM Consensus Conference Committee 1992).

The sensitivity of PCR was significantly higher than that of the β-glucan test ($p<0.05$). The results of the PCR and β-glucan tests showed a significant correlation ($p<0.05$); however, one sample was PCR negative but had a β-glucan level of 8.3 pg/ml, and three samples were β-glucan negative but were PCR

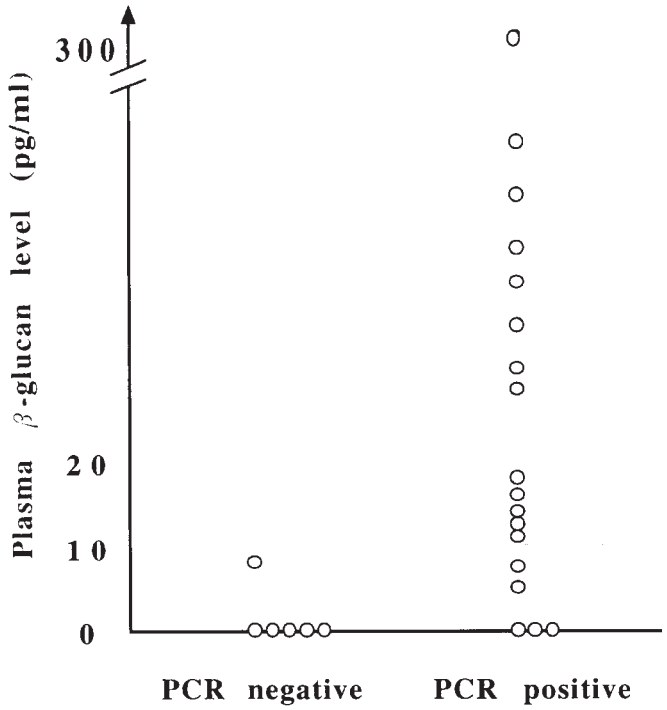

Fig. 3. Reaction specificity of PCR for the detection of fungi in patients' sera compared with that of the β-glucan test ($n=24$).

positive, as shown in Fig. 3. If we consider the optimal cutoff value for the β-glucan test to be 10 pg/ml based on receiver operating characteristic curves and the reports by the other investigators (Hanley and McNeil 1982; McClish 1987; Hiyoshi et al. 1999), the probability of correlation between the β-glucan test and PCR is more significant ($p<0.005$, $C=0.3939$, $\phi=0.6275$, $Q=1.0$). No specimens were β-glucan positive but PCR negative with this cutoff value, while 5 samples were PCR positive but β-glucan negative (Fig. 3). The results of the Cand-Tec test showed no relationship with those of the PCR assay or β-glucan test.

No fungal organism was detected in blood cultures in 24 patients examined. The 20 blood samples from healthy individuals were negative by PCR with fungus-specific primers in this study.

Discussion

Highly sensitive assays are essential for the detection of fungi in the blood of patients, since their blood samples are rarely culture positive. Culture and microbiological determination of the species of fungi from clinical material usually requires several days. Using the direct sequencing technique, DNA extraction and amplification of the PCR products and determination of the fungal species can be performed within 24 hours.

A method for the diagnosis and identification of clinically relevant fungi has been developed on the basis of the evaluation by PCR of the gene coding for the small ribosomal subunit 18SrRNA, which contains sequences that are universally conserved as well as sequences that are highly variable among individual fungal

species (Buchman et al. 1990; Lipschik et al. 1992; Miyakawa et al. 1992; Spreadbury et al. 1993). The identification of the causative fungal species can easily be determined by directly sequencing the PCR products of 18SrRNA. The incidence of the detection of *C. albicans* and non-albicans *Candida spp.* from fungicemic patients has been reported to be 81.7%-99.1% (Makimura et al. 1996; Chen et al. 1997). The primers used in this study could amplify the DNA of clinically important fungi such as *C. albicans*, non-albicans *Candida spp.*, *Aspergillus spp.*, *Pneumocystis carinii*, *Penicillium notatum*, *Coccidiodes immitis*, *Blastomyces dermatitidis*, *Histoplasma capsulatum and Trichopyton rubrum*. We could detect most deep fungal infections in clinical situations with these primers, and could identify the species of the amplified fungi by direct sequencing of the variable regions of 18SrRNA within the PCR products.

The results of the PCR assay and β-glucan test showed a significant correlation. Although measurement of the plasma level of β-glucan is also a useful method for the diagnosis of deep mycosis (Miyazaki et al. 1995; Obayashi et al. 1995; Hiyoshi et al. 1999) and has been commercially developed, in the present study 5 samples were PCR positive without elevation of the serum β-glucan level, that is, 21% of samples showed false negative results in the plasma β-glucan test compared with the PCR assay. Negative controls in the PCR assay showed that there was no artifactual contamination of fungal DNA with the laboratory techniques employed in this study. The primers used here were strictly specific for fungi, and no bacterial or human DNA was amplified with these primers. Moreover, in blood samples of 20 healthy individuals, no fungal DNA was detected by the same procedure. There were no false positives using our techniques.

The PCR method was significantly more sensitive than the β-glucan test by Fisher's exact probability test ($p<0.05$) in our study. The results of the Cand-Tec test showed no correlation with those of the PCR assay or β-glucan test. Our previous study also showed that the detection of Candidal antigen was not correlated with the results of PCR assays or measurements of the plasma level of β-glucan (Ikegami et al. 1999). Although these are preliminary data from the screening of a selected group of patients, the results showed that PCR is useful for the early diagnosis of antifungal therapy. The PCR assay shows high sensitivity and specificity for the diagnosis of Candida deep mycosis, and is clinically applicable. The PCR assay requires little blood, and the results are obtained quickly; therefore, this technique might be useful for the detection of fungi even in neonates.

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