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Detection of *Cryptococcus neoformans* Gene in Patients with Pulmonary Cryptococcosis

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Pulmonary cryptococcosis was diagnosed by nested PCR. Extraction of DNA was performed by mechanical destruction of the capsules of *Cryptococcus neoformans* by the glass bead technique. Nested PCR was positive for 4 of 5 culture-positive specimens but negative for 1 culture-positive specimen, 10 culture-negative specimens, and 1 specimen with undetermined culture results.

The clinical diagnosis of pulmonary cryptococcosis in patients without underlying diseases is generally difficult. Since the diagnosis is often established only by examination of tissue obtained from lung biopsy specimens, other more sensitive and specific methods are needed for the simple and fast detection of the fungus. One such approach involves the detection of fungal antigens in serum. We have previously evaluated different serological assays for detecting cryptococcal antigen in patients with pulmonary cryptococcosis (4, 12). However, sera from patients infected with *Trichosporon beigelii* showed false-positive reactions in these serological assays (4, 5).

We have recently reported the ability of the nested PCR method to detect as little as one cell of *Bacteroides fragilis* (14) and 0.1 cell of *Mycobacterium tuberculosis* (8) in clinical samples. The utility of the DNA probe and PCR technique in the identification of *Cryptococcus neoformans* has also been reported (2, 6, 7, 11, 13, 16). Polacheck et al. (9) used DNA probes for the diagnosis of and epidemiological screening for cryptococcosis in AIDS patients. However, the detection of *C. neoformans* by the PCR technique in clinical samples is not well documented. In the present study, we investigated the use of nested PCR for the genetic detection of *C. neoformans* in clinical specimens from patients with non-human immunodeficiency virus infection-related pulmonary cryptococcosis.

The following different categories of microorganisms were used (the two-letter codes refer to yeast isolates, *Cryptococcus* spp. [YC]; yeast isolates, fungi [YF]; and mold isolates, fungi [MF] [13]): (i) 5 strains of *C. neoformans* var. *neoformans* serotype A (strains YC-11, YC-18, YC-19, YC-22, and YC-27) (13); (ii) 6 *Candida* species (*Candida albicans* 7N, *Candida tropicalis* YF-587, *Candida* [*Torulopsis*] *glabrata* YF-557, *Candida krusei* YF-863, *Candida parapsilosis* YF-703, and *Candida guilliermondii* YF-708), 1 strain of *Saccharomyces cerevisiae* (strain YF-719), two strains of *Trichosporon beigelii* (strains YF-947 and YF-1019), and 5 *Aspergillus* species (*Aspergillus terreus* MF-6), *Aspergillus nidulans* MF-8, *Aspergillus fumigatus* MF-13, *Aspergillus niger* MF-16, and *Aspergillus flavus* MF-45); and (iii) 10 species of bacteria (*Staphylococcus epidermidis*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Serratia marcescens*, *Klebsiella pneumoniae*, *Haemophilus influenzae*, *Escherichia coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, and *Acinetobacter calcoaceticus*). *C. albicans* 7N was a kind gift from T. Arai, Chiba University. Other organisms were isolated from clinical specimens at Nagasaki University Hospital. We identified *C. neoformans* by the API 20C AUX system (Bio Merieux S.A., Marcy-l'Etoile, France). The serotype was confirmed by the Crypto Check system (Iatron, Tokyo, Japan).

C. neoformans and the other fungi were cultured in YEPD medium (1% yeast extract, 2% peptone, 2% dextrose) in a shaking incubator at 30°C for 48 h. After harvesting by centrifugation, the cells were washed twice with a washing solution consisting of 0.5% sodium dodecyl sulfate (SDS) and 50 mM EDTA (pH 8.5). The cell pellets were then suspended in 5 ml of the same washing solution containing glass beads (10 g, 0.4 mm in diameter). The mixture was first heated at 100°C for 10 min, then cooled to room temperature, and then stirred for 90 s with a vortex mixer. After centrifugation at 1,500 × *g* for 5 min, the supernatant was divided into aliquots of 600 μl, and each aliquot was incubated with 5 μl of RNase (10 mg/ml) at 37°C for 30 min. Genomic DNA was extracted by the phenol-chloroform method. Extraction of DNA from the bacteria was performed as described previously (14).

We evaluated 16 clinical specimens obtained from five patients with pulmonary cryptococcosis, including 2 specimens obtained by transbronchial lung biopsy (TBLB), 5 specimens collected from samples of bronchoalveolar lavage fluid (BALF), 3 bronchial aspirates collected by bronchoscopy, and 6 sputum samples. All patients were negative for human immunodeficiency virus infection. Culture of sputum, bronchial washing, or BALF from two patients was positive for *C. neoformans*. For the other three patients, *C. neoformans* was detected on histopathological examination of TBLB specimens. Other disseminated lesions, including disseminated meningitis lesions, were not recognized in any of the patients.

Each specimen was divided into two equal samples. The first sample was used for the microbiologic study, while the other was used for the PCR assay. Liquid specimens (BALF, bronchial aspirate, and sputum) were washed twice with a washing solution containing 0.5% SDS and 50 mM EDTA (pH 8.5). Solid specimens obtained by TBLB were crushed with a preparation glass and rinsed with the washing solution. After harvesting by centrifugation, the yeast cells were washed twice with the washing solution. The capsules of *C. neoformans* were

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TABLE 1. Amount of DNA extracted from 5.0×10^7 CFU of *C. neoformans*

Strain	Amt of DNA (μg) by the following DNA extraction method:		
	Glass beads	Enzyme[a]	Enzyme[a] + glass beads
YC-11	0.534	0.431	0.339
YC-27	0.677	0.443	0.226
YC-38	0.823	0.464	0.337
YC-39	0.697	0.486	0.238
Mean ± SD	0.683 ± 0.118	0.456 ± 0.024[b]	0.285 ± 0.061[c]

[a] *C. neoformans* was incubated with the lysing enzymes from *T. harzianum* for 2 h at 30°C.
[b] $P = 0.009$ compared with the glass bead method.
[c] $P = 0.001$ compared with the glass bead method.

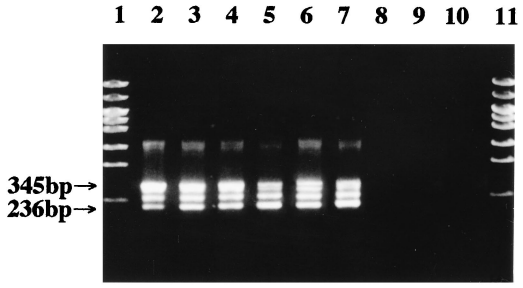

FIG. 1. Electrophoretic analysis of PCR products amplified from DNA extracted from six strains of *C. neoformans*. Lanes 1 and 11, DNA ladder marker; lanes 2 to 7, *C. neoformans* YC-11, YC-18, YC-19, YC-22, YC-25, and YC-27, respectively; lane 8, *C. albicans* 7N; lane 9, *A. fumigatus* MF-13; lane 10, *T. beigelii* YF947. The nested PCR was positive for all strains of *C. neoformans.*

mechanically destroyed with glass beads, and DNA was extracted by the phenol-chloroform method as described above.

Two primer pairs were selected to amplify the *C. neoformans URA5* gene (1). The sequences of primers used in the present study, designated A, B, C, and D, were as follows: A, 5′-ACGGTGAGGGCGGTACTATG-3′; B, 5′-AAGACCTCTGAACACCGTAC-3′; C, 5′-TCGTCATCATCGACGATGTT-3′; and D, 5′-GCCGGAGCTTTCATTGTAC-3′. The PCR conditions consisted of 36 cycles of consecutive denaturation, annealing, and DNA chain extension (60 s at 94°C, 90 s at 63°C, and 60 s at 72°C, respectively) in a Perkin-Elmer Cetus PJ2000 thermal cycler. Amplification with the first primer pair (primer A and primer B) produced a DNA fragment of 345 bp, while amplification with the second primer pair (primer C and primer D) produced a DNA fragment of 236 bp. Southern hybridization was performed with the internal DNA probe (5′-GTCGGAATTGTCCAGCTTGT-3′) to confirm that the DNA products were amplified from the rDNA region and that any unexpected products were the result of nonspecific amplification.

In preliminary studies of DNA extraction from *C. neoformans*, we used enzyme treatment by lysing enzymes from *Trichoderma harzianum* (Sigma, St. Louis, Mo.) for 2 h at 30°C to produce spheroplasts, according to the method described previously by Varma and Kwon-Chung (15). We also evaluated the method using mechanical destruction of the *C. neoformans* capsule by glass beads alone or following enzymatic treatment by the lysing enzyme for 2 h at 30°C. The rates of recovery of DNA by these three methods are summarized in Table 1. The results indicated that the mean rate of DNA extraction by the glass bead method was significantly higher than that by enzymatic treatment alone or when the enzymatic method was combined with the glass bead method ($P = 0.009$ and 0.001, respectively; Student's t test). Thus, the glass bead DNA extraction method was used in the remainder of experiments.

Nested PCR was positive for all five strains of *C. neoformans* (Fig. 1). No amplification product was observed when specimens of six *Candida* species, one strain of *S. cerevisiae*, two strains of *T. beigelii*, and five *Aspergillus* species were subjected to PCR. Furthermore, no genetic amplification was observed when specimens of *S. epidermidis*, *S. aureus*, *S. pneumoniae*, *S. marcescens*, *K. pneumoniae*, *H. influenzae*, *E. coli*, *P. mirabilis*, *P. aeruginosa*, and *A. calcoaceticus* were subjected to PCR (data not shown).

The detection limit of the nested PCR technique was 10 pg of the diluted template DNA (Fig. 2A). Nested PCR was also performed to amplify DNA from each template DNA extracted from different amounts of *C. neoformans*. A total of 1.0×10^3 CFU of *C. neoformans* was required to yield the equivalent amount of DNA from the DNA extraction (Fig. 2B).

The *C. neoformans* gene was detected by nested PCR in one BALF specimen, one specimen of bronchial aspirate, and two sputum specimens. Culture of these four specimens revealed 7.0×10^4 CFU in BALF specimens, 1.7×10^5 CFU in the bronchial aspirate specimen, and 6.0×10^4 CFU and 2.0×10^3 CFU, in the first and second sputum samples, respectively (Table 2). The nested PCR was negative for one sputum specimen containing 1.0×10^4 CFU of *C. neoformans* (specimen 8 from patient 1; Table 2). Nested PCR was also negative for the other 10 specimens which were culture negative and 1 specimen with undetermined culture results.

Until recently, the isolation of *C. neoformans* genomic DNA was based on enzymatic treatment of the fungus to produce spheroplasts (9, 13). However, the effective extraction of DNA

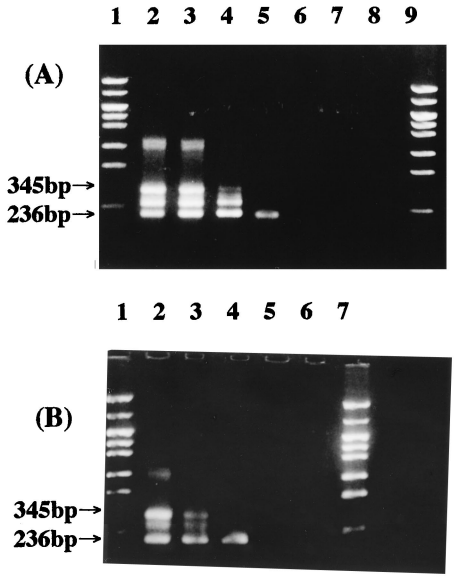

FIG. 2. Sensitivity of the nested PCR. (A) Electrophoretic analysis of PCR products amplified from different amounts of the *C. neoformans* DNA template. Lanes 1 and 9, DNA ladder marker; lanes 2 to 8, 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, and 10 fg of template DNA, respectively. The PCR was sensitive enough to detect 10 pg of the diluted template DNA. (B) Electrophoretic analysis of PCR products amplified from DNA extracted from different amounts of *C. neoformans*. Lanes 1 and 7, DNA ladder marker; lanes 2 to 6, DNA extracted from 10^5, 10^4, 10^3, 10^2, and 10 CFU of *C. neoformans*, respectively. A total of 1.0×10^3 CFU of *C. neoformans* was required to yield the equivalent amount of extracted DNA.

TABLE 2. PCR and culture of clinical specimens from patients with cryptococcosis[a]

Patient no.	Underlying disease	Specimen(s)[b]	Culture result (CFU)	PCR result
1.	Sjögren syndrome	1. TBLB		−
		2. BS	1.7×10^5	+
		3. BALF	7.0×10^4	+
		4. TBLB		−
		5. BS		−
		6. BALF		−
		7. SP	6.0×10^4	+
		8. SP	1.0×10^4	−
		9. SP		−
2.		BALF	NP[c]	−
3.		BALF		−
4.	Bronchiectasia	1. BS		−
		2. SP	2.0×10^3	+
5.	Acromegaly	1. BALF		−
		2. SP		−
		3. SP		−

[a] The diagnosis of pulmonary cryptococcosis was pathologically confirmed in patients 2, 3, and 5.
[b] BS, bronchial aspirate; SP, sputum.
[c] NP, not performed.

from *C. neoformans* is difficult since the thick capsule is resistant to enzymatic treatment. Restrepo and Barbour (10) used NovoZym 234 (lysing enzymes from *T. harzianum*) instead of zymolyase to digest the thick capsule of *C. neoformans*. They reported that the nonencapsulated strain (strain 309) yielded more DNA per cell than the encapsulated isolate (10). In addition, the susceptibility of encapsulated *C. neoformans* to the digestive activities of such enzymes varies from one strain to another (10). Almost all strains isolated clinically in Nagasaki Prefecture, Japan, had relatively high levels of resistance to enzymatic treatment compared with isolates from California (unpublished data).

Varma and Kwon-Chung (15) reported that the low DNA yield obtained by the enzymatic method was due to DNase contamination in multienzyme preparations, such as NovoZym 234 or zymolyase preparations (15). DNases are also produced by *C. neoformans* and are potent in reducing the DNA yield, since the enzymatic treatment necessary to produce protoplasts requires a longer incubation period than the glass bead technique. In the present study, DNA extraction was performed by the method described by Hazen and Cutler (3), with a slight modification. By this method, the capsule of *C. neoformans* is destroyed immediately with glass beads, and at the same time the reactivity of DNase is neutralized by the addition of 50 mM EDTA (15) and heat treatment. We believe that our glass bead method is suitable for the extraction of DNA from encapsulated strains of *C. neoformans* that are resistant to enzymatic treatment.

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