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Molecular Detection and Identification of *Paracoccidioides brasiliensis*

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Nearly 800 nucleotides from the 5′ terminus of the 28S ribosomal gene of *Paracoccidioides brasiliensis* were sequenced, and a 14-base DNA probe specific for this species was identified. Hybridization results showed that the probe identified *P. brasiliensis* ribosomal DNA in a panel of ribosomal DNAs representing a total of 48 species of fungi.

Paracoccidioidomycosis is a deep-seated systemic infection of humans caused by the thermal dimorphic fungus *Paracoccidioides brasiliensis* and is a major health problem in Central and South America. Diagnosis is based mainly upon the detection in clinical materials of large, budding yeast mother cells with distinctive pilot wheel morphology and the detection of a 43-kDa glycoprotein exoantigen or humoral antibody to this antigen (22).

Historical difficulties in identifying and classifying this organism are summarized in a monograph on paracoccidioidomycosis (9). The agent of paracoccidioidomycosis was first cultured by Lutz in 1908 (17). From this and subsequent work (1, 21), the agent was finally designated *P. brasiliensis* (1). Other members of the genus were recognized subsequently: *P. cerebriformis* (18), *P. tenuis* (19), *P. antarcticus* (11), and *P. loboi* (2). However, upon further study by classic mycological methods, *P. tenuis* (7) and *P. antarcticus* (10) were reclassified as *P. brasiliensis*. *P. loboi* remains a valid and distinct species (15). Efforts to culture *P. loboi* have not been successful, and the organism is recognized from an appropriate clinical presentation, biopsy material, and production of the gp43 exoantigen first recognized with *P. brasiliensis*. Artagaveytia-Allende and Montemayor (3) subsequently determined that the single isolate of *P. cerebriformis* reported by Moore (18) definitely is not *P. brasiliensis*. The identity of this single isolate remains enigmatic.

We have developed a uniform diagnostic protocol for fungal detection and identification (20) that has wide applicability with medically important fungi harbored in culture or clinical samples. The approach consists of using common cell lysis and DNA purification procedures combined with a common PCR amplification driven by primers specific for the 5′ terminus of fungal 28S ribosomal gene sequences. The protocol is completed by characterization of amplicons made in the PCR. Others have conceived of a similar approach (4, 14) or different approaches using probes for rRNA sequences (23). In this report, we present DNA sequencing results for several isolates of *P. brasiliensis* and identify a DNA probe specific for this organism that is compatible with our uniform diagnostic protocol.

Reagents, procedures, and PCR primers used in this paper are identical to those in our original work (20). PCR with primers P1 and P2 produces amplicons of about 800 bp encoding the 5′ terminus while primers U1 and U2 produce an amplicon of about 200 bp whose location begins about 400 bp from the 5′ terminus. This latter amplicon represents a hypervariable region containing species-specific sequences (20). Neither the P nor the U primer pairs amplify human ribosomal sequences.

Cultures of nine *P. brasiliensis* isolates (Table 1 and Fig. 1) were checked for purity by subculturing, and concurrently a small amount of culture was lysed for use in PCR. Subculturing results showed that isolates 135 (24) and 9919 (human) were contaminated with a fast-growing fungus. Amplicons made with the latter two cultures were suitable for hybridization but not for DNA sequencing. Amplicons from pure cultures were sequenced on both strands (Table 1). For each isolate, a total of about 800 bases was identified inclusive of the P1-P2 primer pair. We found 100% identity in all isolates with one exception. The exception, isolate 262 from dog food contaminated with soil (8), contained a single base difference; an A replaces the G (underlined) in the consensus sequence string ACTCCCCCCG TGGTCG GGC CAG.

To find a species-specific probe, the region from *P. brasiliensis* mapping between coordinates 400 and 600 starting from the 5′ terminus was aligned with the comparable regions from 47 other species of fungi representing 25 genera listed in Fig. 1, and a unique 14-mer of DNA, ACTCCCCCGT GGTC, was identified. Next, the 14-mer was compared to the full GenBank

TABLE 1. DNA sequencing of *P. brasiliensis* isolates[a]

Isolate	Source or organism	No. of bases sequenced	% Identity to 556	Accession no.
262	Dog food/soil	761	99.9[b]	U93304
265	Human	761	100	
556	Human[c]	761	100	U81263
BAT 2	Human	761	100	
IBIA 2	Soil	761	100	
927	Penguin feces[d]	761	100	
9894	Human	761	100	
	B. dermatitidis	760	98.0	U26882[e]
	H. capsulatum	760	96.2	U26909[e]

[a] All *P. brasiliensis* cultures are from the Laboratory of Medical Mycology, Instituto de Medicina Tropical de São Paulo, University of São Paulo, São Paulo, Brazil.
[b] Isolate 262 differs by a single base from 556 (see text).
[c] Originally *P. tenuis* (18) but reclassified (7).
[d] Originally *P. antarcticus* (11) but reclassified (10).
[e] Reference 20 and unpublished data.

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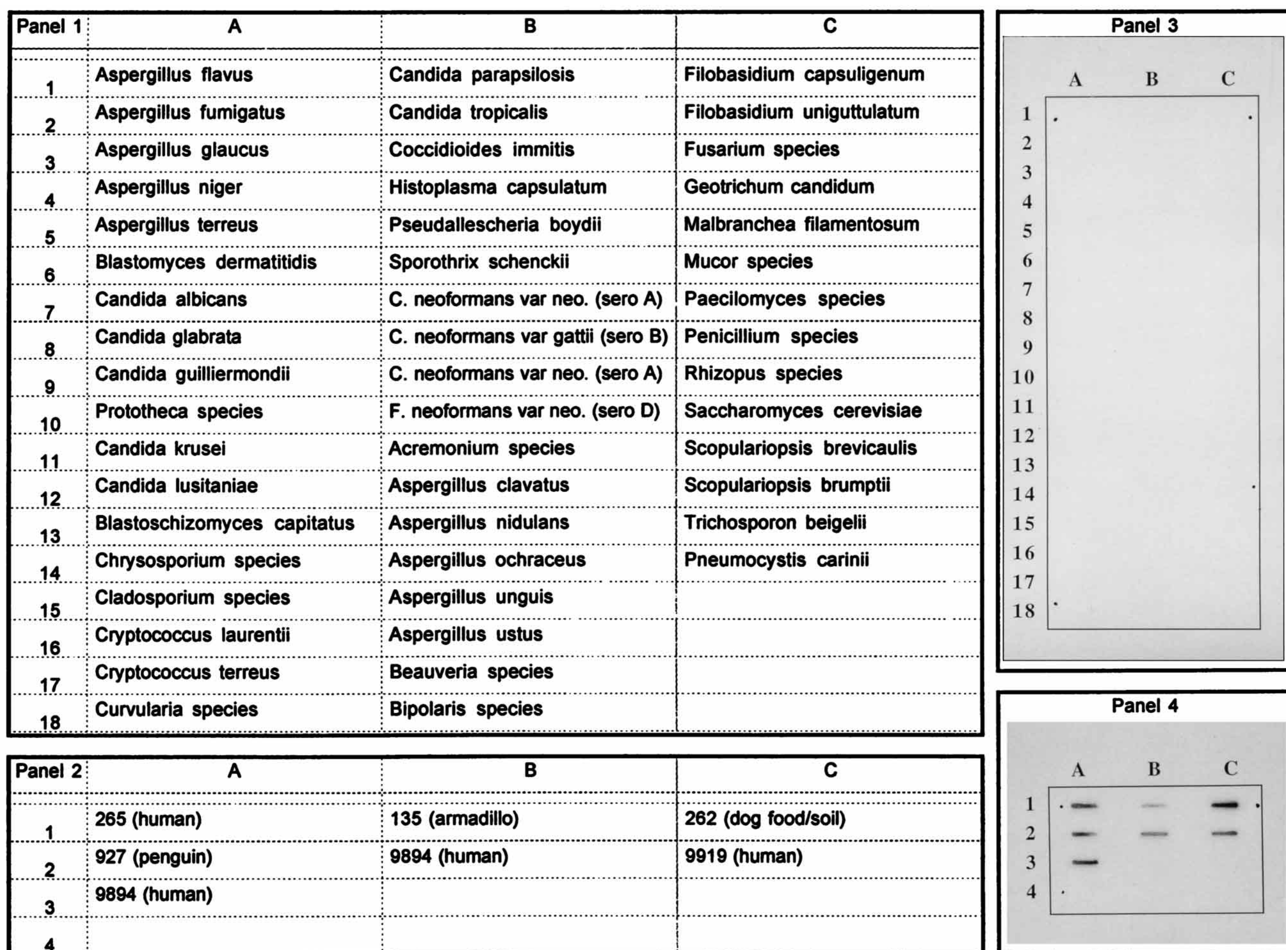

Panel 1	A	B	C
1	Aspergillus flavus	Candida parapsilosis	Filobasidium capsuligenum
2	Aspergillus fumigatus	Candida tropicalis	Filobasidium uniguttulatum
3	Aspergillus glaucus	Coccidioides immitis	Fusarium species
4	Aspergillus niger	Histoplasma capsulatum	Geotrichum candidum
5	Aspergillus terreus	Pseudallescheria boydii	Malbranchea filamentosum
6	Blastomyces dermatitidis	Sporothrix schenckii	Mucor species
7	Candida albicans	C. neoformans var neo. (sero A)	Paecilomyces species
8	Candida glabrata	C. neoformans var gattii (sero B)	Penicillium species
9	Candida guilliermondii	C. neoformans var neo. (sero A)	Rhizopus species
10	Prototheca species	F. neoformans var neo. (sero D)	Saccharomyces cerevisiae
11	Candida krusei	Acremonium species	Scopulariopsis brevicaulis
12	Candida lusitaniae	Aspergillus clavatus	Scopulariopsis brumptii
13	Blastoschizomyces capitatus	Aspergillus nidulans	Trichosporon beigelii
14	Chrysosporium species	Aspergillus ochraceus	Pneumocystis carinii
15	Cladosporium species	Aspergillus unguis	
16	Cryptococcus laurentii	Aspergillus ustus	
17	Cryptococcus terreus	Beauveria species	
18	Curvularia species	Bipolaris species	

Panel 2	A	B	C
1	265 (human)	135 (armadillo)	262 (dog food/soil)
2	927 (penguin)	9894 (human)	9919 (human)
3	9894 (human)		
4			

FIG. 1. Testing a *P. brasiliensis* probe for species specificity. The sequence of the probe is described in the text. The grid numbers in the upper and lower panels identify positions of denatured, 28S amplicons from the indicated species blotted onto a nylon membrane before probing. Absence of a listing in a block signifies no DNA target. The numbers in panel 2 correspond to the numbers of *P. brasiliensis* isolates listed in Table 1. C., *Cryptococcus*; neo., *neoformans*; F., *Filobasidiella.*

database which includes over 300 28S genes from fungal species. No fungal matches were found, but the BI-1 calcium channel gene from the European rabbit (accession no. X57476) showed complete homology. The possibility of this sequence complicating the diagnostic protocol results is remote because it would not be amplified in a PCR by our ribosomal gene primers.

The specificity of the 14-mer probe in slot blot hybridization is shown in Fig. 1. Panels 1 and 2 represent the positions on the two membranes of denatured, target amplicons made with the U1-U2 primer pair. Both membranes were exposed to the 14-mer probe simultaneously in a common solution. After washing, the membranes were exposed to film (panels 3 and 4). The results show that the probe hybridized specifically only with *P. brasiliensis* isolates. In contrast, primer U1 used as a probe hybridized to all the targets in panel 3 (data not shown).

Phylogenetic analysis of 18S (5) and 28S (16) (Table 1) sequences indicates that *Histoplasma capsulatum*, *Blastomyces dermatitidis*, and *P. brasiliensis* are close relatives. Subsequent to the results in Fig. 1, the 14-mer *P. brasiliensis* probe was tested by in situ hybridization, and we found that it reacted with *P. brasiliensis*, but not *H. capsulatum* or *P. loboi* (6a). Thus, the *P. brasiliensis*-specific probe also distinguishes between the two recognized *Paracoccidioides* species.

Goldani et al. (12) have identified a 110-bp probe sequence from a cryptic gene in *P. brasiliensis*. Also, Cisalpino et al. (6) have deposited in GenBank the nucleotide sequence for the *P. brasiliensis* gp43 exoantigen, which represents another potential probe. The existence of multiple probes for a species is useful in the event of laboratory contamination with amplicons or subsequent diminishment in probe specificity as more organisms are examined. The last possibility follows from the fact that only a handful of the estimated 69,000 to 1.5 million species of fungi which are thought to exist (13) have been sequenced.

In closing, we emphasize that utility of molecular probes ultimately rests on the accuracy of fungal identifications initially made by classical taxonomic means. Obviously, it also rests on a stringent level of sequence conservation within a species. Our data in Fig. 1 and Table 1 are consistent with both points.

Nucleotide sequence accession numbers. The sequences of isolates 556 and 262 have been deposited in GenBank (accession no. U81263 and U93304, respectively) as representative of the consensus sequence and a variant.

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