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Detection of *Paracoccidioides brasiliensis* in Tissue Samples by a Nested PCR Assay

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A nested PCR assay for the detection of *Paracoccidioides brasiliensis* DNA was evaluated, using a sequence of the immunogenic *gp43* gene as a target. This gene encodes an outer membrane protein unique to this dimorphic fungus. DNA from six clinical isolates and the ATCC strain 60885 of *P. brasiliensis*, as well as DNA from closely related fungi, was examined to determine detection limits and cross-reactions. PCR was done on DNA extracts of lung homogenates from 23 experimentally *P. brasiliensis*-infected and two uninfected BALB/c mice and from 20 *Histoplasma capsulatum*-infected ICR mice. The results were compared to quantitative cultures. A detection limit of 0.5 fg of specific DNA was determined using cloned plasmid DNA. In all seven *P. brasiliensis* isolates, the expected 196-bp nested PCR product was found. Their sequences were 100% identical to the *gp43* gene sequence in GenBank. DNA extracts of all other, related fungi were negative. The PCR assay was positive in 21 out of 23 culture-positive lung homogenates with concentrations of 1×10^3 to 1.3×10^7 CFU of *P. brasiliensis* per g of lung. Uninfected BALB/c mice and *H. capsulatum*-infected mice samples gave negative results. The high sensitivity and specificity of this new nested PCR assay for the detection of *P. brasiliensis* in tissue samples were demonstrated. The assay may be useful for diagnosis in human tissue samples.

Paracoccidioidomycosis occurs sporadically in adults, predominantly in men aged 30 to 50 years, whereas the infection is acquired at an early age equally in both sexes. The infection is usually subclinical or limited to the lungs (13). Acute pulmonary and acute or subacute disseminated forms have been described as a juvenile form predominantly found in children and young adults. Chronic pulmonary or chronic disseminated forms are seen in adults, with a preponderance of men affected. The disease occurs rarely in patients with AIDS as an opportunistic infection. The diagnosis is based on culture, histopathology, and detection of antibodies. The latter may be problematic, as the antibodies can cross-react with *Histoplasma capsulatum* antigens. In nonendemic areas, diagnosis is hampered by lack of experience and the necessity of a high biosafety level to grow the fungus. In histological sections, the etiological agent might be missed or confused with other dimorphic fungi such as *Histoplasma* spp. or *Coccidioides immitis* (13). Because of reduced production of antibody, immunodiagnosis might be unusable in immunocompromised patients.

Recently, PCR techniques have been introduced for the detection of systemic fungal infections (6, 12). A *Paracoccidioides brasiliensis* PCR assay for diagnosis might be advantageous due to rapidity, sensitivity, and minimized health risk compared to the above-named methods.

(The presented data are part of the doctoral thesis of A. Ibricevic.)

MATERIALS AND METHODS

Microorganisms. Six isolates of *P. brasiliensis* (R-2878 to R-2883), originating from A. Restrepo in Colombia, and the ATCC 60885 strain were grown on potato flakes agar at 30°C for 2 weeks. Their identity as *P. brasiliensis* was confirmed in the Fungus Testing Lab, San Antonio, Texas. Mycelial colonies were scraped off the agar, dissolved in sterile water, frozen, and stored at −20°C. After thawing, 2×200 μl of each suspension was used for DNA extraction. One isolate of *Aspergillus fumigatus*, three of *Blastomyces dermatitidis*, one of *Candida krusei*, two of *Cryptococcus neoformans*, and three isolates of *H. capsulatum* were grown on blood or Sabouraud agar and identified by standard methods. Fungal suspensions were prepared as described above.

Tissue samples. BALB/c mice were infected by intranasal instillation of 3×10^6 conidia of *P. brasiliensis* (ATCC 60885) and sacrificed several days and weeks thereafter, as described in detail elsewhere (3). Lungs were removed from mice under aseptic conditions, weighed, and homogenized in 2 ml of saline; the lung tissue homogenate was serially diluted and plated in duplicate on Sabouraud agar. After 30 days of incubation at 18°C, the number of CFU g of tissue^{-1} was calculated (3). The remaining lung homogenates were stored frozen (−70°C) for up to 5 years before DNA extraction was done. Lung homogenates of 20 ICR mice intravenously infected with 8×10^3 CFU of *H. capsulatum* and sacrificed on days 1, 5, and 11 after infection were used as controls. All lung homogenates were positive by quantitative culture in a range of 1×10^3 to 7×10^6 CFU of *H. capsulatum* per g of lung (R. Bialek et al., unpublished data).

DNA extraction. To 200 μl of each fungal suspension or thawed lung homogenate, 180 μl of ATL buffer of the QIAamp tissue kit (Qiagen, Hilden, Germany) and proteinase K (Qiagen) to a final concentration of 1 mg/ml were added. After incubation at 55°C for at least 3 h or overnight, the samples were boiled for 5 min, then exposed to three cycles of freezing in liquid nitrogen for 1 min and boiling for 5 min afterwards to disrupt the fungal cells. After cooling to room temperature, proteinase K (Qiagen) was added again to a final concentration of 1 mg/ml. After incubation at 55°C for 1 h, DNA was extracted using the QIAamp tissue kit (Qiagen) following the manufacturer's instructions. This extraction is based on detergents and proteinase K for solubilization of the tissue, the addition of ethanol and chaotrophic salts to allow binding of DNA to a silica membrane in columns, washing steps to remove protein, and elution of DNA from the silica by an alkaline buffer (pH 9.0). The exact composition of the buffers is part of the manufacturer's patent and the information is unavailable.

Primer design. The sequence of *gp43* of *P. brasiliensis* (1) deposited in GenBank (U26160) was screened for primers. The outer primers were para I, 5′-AAC TAG AAT ATC TCA CTC CCA GTC C-3′, and para II, 5′-TGT AGA CGT TCT TGT ATG TCT TGG G-3′, being complementary to positions 846 to 870 and 1200 to 1176 of the GenBank sequence, respectively, defining a 355-nucleotide amplicon. The inner primer set consisted of para III, 5′-GAT CGC CAT CCA TAC TCT CGC AAT C-3′, and para IV, 5′-GGG CAG AGA AGC ATC CGA AAT TGC G-3′, which were complementary to the nucleotide positions 953 to 978 and 1148 to 1124 of the deposited sequence, respectively. They delimit a 196-nucleotide sequence.

PCR assay. The reaction mix of the first PCR consisted of 10 μl of DNA extract in a total volume of 50 μl, with final concentrations of 10 mM Tris-HCl

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TABLE 1. PCR results for lung homogenates[a]

Source of lung homogenate(s)	*P. brasiliensis* CFU/g of lung	*P. brasiliensis* nested PCR	Mouse actin PCR	Inhibitor control PCR
BALB/c mouse no.				
1	0.1×10^4	Pos	Pos	ND
2	1.1×10^4	Pos	Pos	ND
3	1.3×10^4	Pos	Pos	ND
4	1.3×10^4	Pos	Pos	ND
5	1.4×10^4	Neg	Pos	Neg
6	3.2×10^4	Pos	Pos	ND
7	3.4×10^4	Neg	Pos	Pos
8	3.7×10^4	Pos	Pos	ND
9	6.2×10^4	Pos	Pos	ND
10	7.8×10^4	Pos	Pos	ND
11	8.5×10^4	Pos	Pos	ND
12	9.4×10^5	Pos	Pos	ND
13	1.0×10^5	Pos	Pos	ND
14	1.7×10^5	Pos	Pos	ND
15	2.6×10^5	Pos	Pos	ND
16	2.8×10^5	Pos	Pos	ND
17	3.5×10^5	Pos	Pos	ND
18	3.8×10^5	Pos	Pos	ND
19	7.0×10^5	Pos	Pos	ND
20	7.3×10^5	Pos	Pos	ND
21	1.3×10^6	Pos	Pos	ND
22	3.6×10^6	Pos	Pos	ND
23	1.3×10^7	Pos	Pos	ND
24	0 (control)	Neg	Pos	Pos
25	0 (control)	Neg	Pos	Pos
20 *H. capsulatum*-infected ICR mice	20×0	$20 \times$ Neg	$20 \times$ Pos	$20 \times$ Pos

[a] ICR, Institute for Cancer Research; Pos, positive; Neg, negative; ND, not done.

(pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$ (10× Perkin-Elmer buffer II plus $MgCl_2$ solution [Roche Molecular Systems, Branchburg, N.J.]), a 1-μM concentration of each primer (Roth, Karlsruhe, Germany), 1.5 U of AmpliTaq DNA polymerase (Perkin-Elmer), and a 100-μM concentration of each deoxynucleotide triphosphate (Promega, Madison, Wis.). The reaction mix of the second PCR was identical, except that 1 μl of the first reaction, a 50-μM concentration of each deoxynucleotide triphosphate, and a 1-μM concentration of each second primer were used. Reactions with the outer primer set were thermal cycled once at 94°C for 5 min, 35 times at 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min, and then once at 72°C for 5 min. For the nested PCR product, reactions were thermal cycled once at 94°C for 5 min, 30 times at 94°C for 30 s and 72°C for 1 min, and then once at 72°C for 5 min. The high melting temperatures of the inner primers allowed a two-step nested PCR with high stringency. The PCR was run in a Primus PCR thermocycler Tc 9600 (MWG Biotech, Ebersberg, Germany). The PCR products were analyzed by electrophoresis on 1.5% agarose gels, stained with ethidium bromide, and visualized on an UV transilluminator.

Controls. A sample of 100 fg of plasmid DNA ($\sim 2 \times 10^4$ gene copies) containing the target sequence of the *P. brasiliensis gp43* gene was used in every PCR assay as a positive control. Sterile water was included in the DNA extraction and was used as a negative control after every fifth sample in the nested PCR assay to monitor for crossover contaminations. Reaction mix without DNA was run in the first and second cycling to try to detect contaminations. In order to screen for inhibitors, all negative samples were examined a second time after adding 2 μl ($\sim 4 \times 10^3$ gene copies) of the positive control.

DNA extraction was controlled by a PCR using the primer set actin 4 (5′-AGC CAT GTA CGT AGC CAT CCA GGC T-3′) and actin 5 (5′-GGA TGT CAA CGT CAC ACT TCA TGA TGG-3′), amplifying a 450-bp sequence of the mouse actin gene and thus indicating the presence of amplifiable murine DNA. The reaction mix was identical to the mix described above, except that 3 mM $MgCl_2$, a 1-μM concentration of each primer, and only 5 μl of the DNA extract were used. The reaction was thermal cycled according to the first PCR protocol above, except for an annealing temperature of 57°C.

Sequencing. The nested PCR products were purified by using the QIAquick PCR purification kit (Qiagen), based on DNA binding to a silica membrane. Automated sequencing was done with the BigDye terminator cycle sequencing kit and PCR primers in accordance with the recommendations of the manufacturer and was analyzed on the ABI 373 automated DNA sequencer (Applied Biosystems Division, Perkin-Elmer Biosystems, Foster City, Calif.). Sequences were generated from both strands, edited and aligned with the Sequence Navigator software (Applied Biosystems), and used for a BLAST search in GenBank (National Center for Biotechnology Information, Washington, D.C.).

Cloning. The PCR product of the DNA extracted from the ATCC 60885 *P. brasiliensis* strain after the first round of amplification was purified by Qiagen spin columns (Qiagen). The amplicon was inserted into the PCR II.1 cloning vector using the Original TA cloning kit following the manufacturer's instructions (Invitrogen, Groningen, The Netherlands). After the bacteria were cultured and harvested, the plasmid DNA was purified by a Qiagen plasmid maxi kit (Qiagen) consisting of alkaline lysis of bacteria, separation and binding of plasmid DNA to anion exchange resin, wash steps, and final elution. The DNA concentration was measured by absorption at 260 nm. Serial dilutions were used to determine the detection limit of the nested PCR assay. The amplified product was sequenced to prove homology to the sequence in GenBank.

RESULTS

In three independent assays on three different days done by two examiners, the detection of specific, cloned DNA covered a range from 0.5 fg, equivalent to ~100 gene copies, to at least 100 pg per sample. Whereas all seven *P. brasiliensis* isolates, including the ATCC strain, gave a positive result in the PCR, all other fungal isolates remained negative in several experiments, demonstrating the specificity of the primer pairs and the target sequence. As shown in Table 1, all except 2 of 23 *P. brasiliensis* culture-positive lung homogenates were positive by the nested PCR assay. The presence of DNA was demonstrated by the positive actin PCR in all samples. One lung homogenate (BALB/c mouse 5) remained negative after 2 μl of the positive control was added, demonstrating the presence of specific inhibitors of the *P. brasiliensis* nested PCR. A second DNA extraction could not be done due to a limited sample volume. The lung homogenates of two control BALB/c mice and 20 ICR mice infected with and culture-positive for *H.*

capsulatum were negative by the *P. brasiliensis* nested PCR assay.

The lungs with a mean weight of 100 mg were homogenized in 2 ml of saline, and a tenth of this solution was used for DNA extraction. The DNA was finally dissolved in 150 μl of buffer, of which 10 μl was used for the PCR. Calculating with the minimum CFU concentration of 1×10^3 per g of lung (BALB/c mouse 1), the DNA amount of 1 CFU of *P. brasiliensis* per sample gave a positive PCR signal. Thus, the detection limit of our nested PCR assay determined with cloned plasmid DNA was verified in tissue samples.

Sequencing of all nested PCR products from lung homogenates and from seven *P. brasiliensis* isolates demonstrated 100% identity of the amplicons to positions 953 to 1148 of the *gp43* sequence of *P. brasiliensis* in GenBank.

DISCUSSION

The nested PCR assay described herein enabled us to detect *P. brasiliensis* DNA in culture and tissue samples. The results show that physical destruction of fungal cells is sufficient to liberate the DNA. We previously accomplished this by incubation with zymolase but changed to the current protocol when contamination with DNA from *Saccharomyces cerevisiae* was found in commercially available enzyme preparations (7, 10).

A common target of diagnostic fungal PCR assays is the 18S rRNA gene because its frequency in the genome guarantees a high sensitivity. Screening the 18S rRNA genes of *P. brasiliensis*, a high homology to *B. dermatitidis* (99%) and *H. capsulatum* was found (R. Bialek, A. Ibricevic, A. Fothergill, and D. Begerow, submitted for publication), and therefore another target was searched. We examined the *gp43* gene because the corresponding outer membrane protein has been shown to be highly immunogenic, of diagnostic value in paracoccidioidomycosis, and unique to this dimorphic fungus (9). We could find neither homology between the chosen primer sequences and other sequences deposited in GenBank nor a cross-reaction in the nested PCR assay of closely related dimorphic fungi. Another target sequence of a diagnostic PCR, described by Goldani and coworkers, is a cloned 110-bp fragment of *P. brasiliensis* (4, 5). The authors detected specific DNA in blood and lung samples of infected mice and reported a detection limit of 10 pg. When we used specific, cloned plasmid DNA, the lowest amount to be detected by our nested PCR was 0.5 fg of *P. brasiliensis* DNA. This high sensitivity was verified in tissue samples. Specific DNA was amplified from lungs with a tissue burden between 1,000 and 13,000,000 CFU of *P. brasiliensis* per g of tissue determined by quantitative culture. The tissue burden corresponds to a single fungal cell up to several thousands in one histological section of *P. brasiliensis*-infected organs. Two out of 23 culture-positive lung homogenates were negative by the nested PCR assay. In one sample, specific inhibitors were demonstrated, whereas in the second with a low CFU count, either the target DNA was missed in the reaction mix of the PCR assay or specific DNA was lost during extraction. The negative results of lung homogenates from uninfected mice and *H. capsulatum*-infected mice and of DNA extracts from other fungal pathogens demonstrates the specificity of this highly sensitive nested PCR. However, Sano et al. (11) failed to demonstrate gp43 and its gene in two isolates from soil in an endemic area. It remains to be determined whether *P. brasiliensis* isolates from humans differ in their *gp43* gene content and its expression.

McEwen and coworkers described another potential target sequence of a diagnostic PCR assay. They showed that 91% of their *P. brasiliensis*-infected patients had antibodies against a recombinant 27-kDa protein (8). It was intended to use the gene for a PCR to screen for the natural habitat of the fungus, but it has not yet been used for diagnostic purposes in humans or animals. Da Silva and colleagues reported on the heat shock protein gene of *P. brasiliensis* (2). We did not use this gene as a target for a diagnostic PCR because the authors had demonstrated high homology of the gene to those corresponding to other members of the hsp70 family, and thus there was a risk of cross-reactions with other fungi.

In conclusion, we have developed a highly sensitive and specific nested PCR assay for the detection of *P. brasiliensis* in tissue samples. The assay awaits further evaluation in human specimens.

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