JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 2004, p. 778–783 Vol. 42, No. 2
0095-1137/04/$08.00+0 DOI: 10.1128/JCM.42.2.778–783.2004

PCR Assays for Identification of *Coccidioides posadasii* Based on the Nucleotide Sequence of the Antigen 2/Proline-Rich Antigen

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Received 25 July 2003/Returned for modification 23 October 2003/Accepted 8 November 2003

A conventional nested PCR and a real-time LightCycler PCR assay for detection of *Coccidioides posadasii* DNA were designed and tested in 120 clinical strains. These had been isolated from 114 patients within 10 years in Monterrey, Nuevo Leon, Mexico, known to be endemic for coccidioidomycosis. The gene encoding the specific antigen 2/proline-rich antigen (Ag2/PRA) was used as a target. All strains were correctly identified, whereas DNA from related members of the family *Onygenaceae* remained negative. Melting curve analysis by LightCycler and sequencing of the 526-bp product of the first PCR demonstrated either 100% identity to the GenBank sequence of the Silveira strain, now known to be *C. posadasii* (accession number AF013256), or a single silent mutation at position 1228. Length determination of two microsatellite-containing loci (GAC and 621) identified all 120 isolates as *C. posadasii*. Specific DNA was amplified by conventional nested PCR from three microscopically spherule-positive paraffin-embedded tissue samples, whereas 20 human tissue samples positive for other dimorphic fungi remained negative. Additionally, the safety of each step of a modified commercially available DNA extraction procedure was evaluated by using 10 strains. At least three steps of the protocol were demonstrated to sufficiently kill arthroconidia. This safe procedure is applicable to cultures and to clinical specimens.

The systemic fungal disease coccidioidomycosis is endemic exclusively in the Americas, in semiarid areas on both sides of the United States-Mexican border (20). In addition, small endemic foci are described in other parts of Mexico, Guatemala, Honduras, Venezuela, Colombia, Brazil, Paraguay, and Argentina, where it was described for the first time in the medical literature by Posadas and Wernicke in 1892 (26). The etiologic agent is the dimorphic fungus *Coccidioides immitis*, which lives as an arthroconidium-forming saprophyte in the earth and on the ground. After inhalation, the arthroconidia transform into enlarging spherules, producing endospores by invagination of the inner cell wall. Mature spherules will rupture and release up to 800 endospores, which continue asexual growth by turning into spherules. Based on single-nucleotide polymorphisms and the size of microsatellites, the former known Californian and non-Californian strains were recently divided into two species, *C. immitis* and *C. posadasii* (11).

An estimated 100,000 new infections occur annually in the United States, but two-thirds of coccidial infections go unrecognized and more than 90% of patients resolve their illness without therapy (9, 13). Pulmonary progression occurs in approximately 5% of infections, most represented by residual nodules or thin-walled cavities, which may pose diagnostic difficulties to distinguish tuberculosis, cancer, and other fungal infections. It is estimated that 0.5 to 1% of infected patients will develop a disseminated disease involving skin, joints, or basilar meninges, leading to diagnostic and therapeutic challenges requiring life-long therapy, especially in coccidial meningitis (9). Risk factors are immunosuppression, diabetes, male gender, and nonwhite ethnic group, but epidemiological data outside U.S. borders are limited.

Diagnosis is based on serology, histopathology, and culture. Although characterized by limited sensitivity, definitive diagnosis is based on growing the dimorphic fungus on media. *Coccidioides* spp. will grow rapidly within 2 to 5 days on most of the media used in mycology, but handling this hazardous pathogen is limited to biosafety level 3 laboratories. Definitive identification of the mycelial form requires conversion into the parasitic form in an animal or by culture which is restricted to some specialized laboratories. Identification is also possible by a specific DNA probe (24). Before mailing, inactivation of cultures by heat or formalin is required to minimize the risk of infection, but the latter is known to reduce the sensitivity of the commercial identification assay (14).

One aim of the present study was to screen the safety of a DNA extraction procedure based on a commercial kit which has been evaluated successfully with other potentially hazardous dimorphic fungi (2, 3, 5). The second aim was to establish PCR assays for specific identification of *Coccidioides* strains that might also have diagnostic potential for clinical specimens. Based on experience, the target sequence for the PCRs was chosen within a gene encoding a specific protein (2, 3, 4). The immunoreactive proline-rich antigen, or antigen 2, now named antigen 2/proline-rich antigen (Ag2/PRA), is known to be specific for *Coccidioides* spp. and it has been evaluated favorably as a vaccine candidate in mice (21, 23).

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TABLE 1. Oligonucleotide primers and LightCycler hybridization probes used in the PCR assays

Oligonucleotide	Sequence (5′-3′)[a]	Target gene	Nucleotide positions	GenBank accession no.	Reference
Cocci I	GTA CTA TTA GGG AGG ATA ATC GTT	Proline-rich antigen	841–864	AF013256	This study
Cocci II	GGT GTC AAC TGG TGG GAT GTC AAT	Proline-rich antigen	1366–1343	AF013256	This study
Cocci III	ATC CCA CCT TGC GCT GTA TGT TCG A	Proline-rich antigen	962–986	AF013256	This study
Cocci IV	GGA GAC GGC TGG ATT TTT TAA CAT G	Proline-rich antigen	1303–1279	AF013256	This study
Cocci-HP-1	CCA AAT TCT TGC ATC TCG CCC A-[FL]	Proline-rich antigen	1209–1230	AF013256	This study
Cocci-HP-2	[Red 640]-ATG GGA TAA GAT GAG AAG ATG GAA AG-Ph	Proline-rich antigen	1234–1259	AF013256	This study
GAC.1	ATG TCTCGC CTG GAG CAC	GAC microsatellite-containing locus			Fisher et al. (12)
GAC.2	GCT GTT CCA CGT TTC GAC				Fisher et al. (12)
621.1U	ACA ATG AAC GAG CAG CAA GG	621 microsatellite-containing locus			Fisher et al. (12)
621.2L	TGA AAG ATG TGT AGA CCC GA				Fisher et al. (12)

[a] FL, fluorescein; Red 640, LightCycler Red 640-*N*-hydroxysuccinimide ester; Ph, 3′-phosphate.

(The data presented here are part of the doctoral thesis of Jan Kern.)

MATERIALS AND METHODS

***Coccidioides* strains.** One hundred twenty strains had been isolated from clinical specimens sent to the Department of Microbiology in Monterrey, Nuevo Leon, Mexico, for routine diagnostic procedures between October 1991 and January 2002. The fungus was isolated from bronchoalveolar lavage and cerebrospinal fluid, organ biopsies, joint, pericardial, and pleural effusions, paravertebral abscesses, and lymph nodes of 114 patients. The cultures were identified by colony morphology and microscopy of typical arthroconidia. All strains had been maintained in water at room temperature. They were cultured on Sabouraud dextrose agar in slant tubes at 30°C for up to 3 weeks. Sterile water was added to the cultures; hyphae and arthroconidia were taken with a sterile wooden stick from colonies and dissolved in water. Two samples of 200 μl of suspension from each strain were used for DNA extraction. All procedures were done in a biosafety level 3 laboratory according to international recommendations.

Clinical specimens. One lung and two skin biopsies embedded in paraffin that were positive for spherules by microscopy were used for evaluation of the PCR assays as a diagnostic tool. Two 5-μm sections of each tissue sample were used for DNA extraction. In order to test specificity, DNA was used which had been extracted from 20 paraffin-embedded specimens that were positive by microscopy and by specific PCR assays for either *Histoplasma capsulatum*, *Paracoccidioides brasiliensis*, or *Blastomyces dermatitidis* in former studies (2, 4). Extracted DNA was stored frozen at −20°C until use.

Template DNA preparation. The QIAamp tissue kit (Qiagen, Hilden, Germany) was used following the manufacturer's instructions with additional cycles of freezing and boiling as described elsewhere (3). In brief, 180 μl of ATL buffer and proteinase K to a final concentration of 1 mg/ml were added to each sample of 200 μl of fungal suspension. After incubation at 55°C overnight, the samples were boiled for 5 min and then exposed to three cycles of freezing in liquid nitrogen for 1 min and boiling for 2 min to disrupt fungal cells. After cooling to room temperature, DNA was extracted with the QIAamp tissue kit based on binding of DNA to silica columns. For the paraffin-embedded material, deparaffinization by incubation with xylene and ethanol was done before adding ATL buffer and proteinase K as described in more detail elsewhere (4).

Safety of the DNA extraction procedure. Ten strains were chosen arbitrarily to control each step of the extraction procedure. Nine samples of 200 μl of suspension of each strain were prepared. One sample of each strain was plated on Sabouraud dextrose agar without further treatment as a viability control, whereas one sample of each strain was cultured (i) after adding 180 μl of ATL buffer, (ii) after adding ATL buffer and proteinase K (final concentration, 1 mg/ml), (iii) after adding ATL buffer and proteinase K followed by 3 h of incubation at 56°C in a water bath, (iv) after adding ATL buffer and proteinase K followed by overnight incubation at 56°C, (v) after solely incubation at 56°C overnight, (vi) after overnight incubation at 56°C followed by three cycles of freezing and boiling as described above, (vii) after adding ATL buffer and proteinase K and overnight incubation at 56°C followed by three cycles of freezing and boiling, and (viii) after three cycles of freezing and boiling. All samples were cultured on Sabouraud dextrose agar in slant tubes at 30°C up to 4 weeks before being recorded as negative.

Design of primers and hybridization probes. The outer primers Cocci I (5′-GTA CTA TTA GGG AGG ATA ATC GTT-3′) and Cocci II (5′-GGT GTC AAC TGG TGG GAT GTC AAT-3′) delimit a 526-nucleotide sequence of the gene coding for the cell wall immunoreactive protein that is unique to *C. immitis* Silveira strain, now regarded as *C. posadasii* (accession number AF013256, GenBank). The primers are complementary to nucleotide positions 841 to 864 and 1366 to 1343, respectively. As shown in Table 1, the inner primer set Cocci III (5′-ATC CCA CCT TGC GCT GTA TGT TCG A-3′) and Cocci IV (5′-GGA GAC GGC TGG ATT TTT TAA CAT G-3′) is complementary to positions 962 to 986 and 1303 to 1279, respectively, spanning a 342-bp-long sequence.

For real-time detection of *C. posadasii*-specific amplicons, a pair of LightCycler (Roche Diagnostics, Mannheim, Germany) hybridization probes were designed. The fluorescence-labeled oligonucleotides Cocci-HP-1 (CCA AAT TCT TGC ATC TCG CCC A-fluorescein) and Cocci-HP-2 (LightCycler Red 640-*N*-hydroxysuccinimide ester-ATG GGA TAA GAT GAG AAG ATG GAA AG-phosphate) were designed to hybridize adjacent to each other within the 342-bp nested PCR amplicon. They are complementary to nucleotide positions 1209 to 1230 and 1234 to 1259, respectively (Table 1).

***C. posadasii* nested PCR assay.** The reaction mixture of the primary PCR consisted of 10 μl of DNA extract in a total volume of 50 μl with final concentrations of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 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 outer primer (Roth, Karlsruhe, Germany); 1.5 U of AmpliTaq DNA polymerase (Roche); and a 50 μM concentration of each deoxynucleoside triphosphate (Promega, Madison, Wis.). The reaction mixture of the nested PCR was identical, except that 1 μl of the first reaction and a 1 μM concentration of each inner primer were used.

Reaction mixtures with the outer primer set were thermally 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, followed by a single terminal extension at 72°C for 5 min. For the nested PCR products, reaction mixtures were thermally cycled once at 94°C for 5 min, 30 times at 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, followed by a final elongation at 72°C for 5 min.

LightCycler PCR. Amplification mixtures contained 2 μl of 10× LightCycler FastStart DNA Master Hybridization Probes mix (Roche Diagnostics), 5 mM $MgCl_2$ (total concentration), 0.5 μM each Cocci III and IV or Cocci I and Cocci II primer oligonucleotide (Roth), 0.2 μM each Cocci-HP-1/-2 hybridization probe oligonucleotide (TIB Molbiol, Berlin, Germany), and 5 μl of template DNA in a final volume of 20 μl. Following an initial denaturation at 95°C for 10 min to activate the FastStart *Taq* DNA polymerase, the 50-cycle amplification profile consisted of heating at 20°C/s to 95°C with a 10-s hold, cooling at 20°C/s to 50°C with a 10-s hold, and heating at 20°C/s to 72°C with a 20-s hold. Fluorescence values of individual LightCycler reaction capillaries were measured and recorded at 640 nm at the end of each annealing step and plotted against PCR cycle numbers. To minimize variations in positioning or geometry of individual capillaries, the absolute signal of the reporter dye (LightCycler Red 640;

channel F2) divided by the signal of the donor dye (fluorescein; channel F1) is shown at the *y* axis of the LightCycler screen plots.

Following the amplification phase, a melting curve analysis was performed with a temperature transition rate of 0.2°C/s to determine the melting point values for the sequences targeted by the hybridization probes. For melting curve analysis, the first negative derivative of the fluorescence (-d*F*/d*T*) is plotted versus temperature and the T_m values were manually assigned from the turning point of this curve.

Microsatellite PCR. According to Fisher et al. (11), the PCR products of two microsatellite-containing loci named GAC and 621 are diagnostic for *C. posadasii* and *C. immitis*. These two loci were amplified from all 120 strains with primers without fluorescence labels according to the conditions described by Fisher et al. (12) (Table 1). PCR products were analyzed with an Agilent 2100 bioanalyzer (Agilent Technologies, Waldbronn, Germany), a commercially available chip-based nucleic acid separation technology. The LabChip (DNA 7500) separates nucleic acid fragments by capillary electrophoresis in a chip with microfabricated channels and automates the detection as well as on-line data evaluation as described in detail elsewhere (19). The products of two microsatellites of each strain were mixed and used for analyses following the instructions of the manufacturer. In addition, the lengths of the two microsatellites of 25 arbitrarily chosen strains were determined by electrophoresing PCR products gained by fluorescently (FAM) labeled primers through a 6.5% denaturing polyacrylamide gel with an ABI 377 (Applied Biosystems) automated sequencer according to the original method (12).

Cloning. An amplicon of the primary PCR with template DNA extracted from one strain was purified by Qiagen spin columns (Qiagen). The amplicon was inserted into the pCR 2.1-TOPO cloning vector with the Original TA Cloning Kit in accordance with the manufacturer's instructions (Invitrogen, Groningen, The Netherlands). After culturing and harvesting the bacteria, plasmid DNA was purified with the Qiagen Plasmid Maxi Kit 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. The amplified product was sequenced to prove homology to the sequence of the GenBank database.

Controls. Ten microliters containing 100 fg of purified plasmid DNA with an insert of *C. posadasii* cell wall immunoreactive protein partial gene sequence was used in every PCR assay as a positive control. In order to monitor crossover contamination, sterile water was included in the DNA extraction after every tenth sample and the extraction products were used in the PCR assays. Reaction mixtures without DNA were run in the first and nested PCR to monitor contaminations.

Sequencing. The 526-bp products amplified by the first PCR of template DNA from 35 arbitrarily chosen strains were purified with the QIAquick PCR purification kit (Qiagen). Automated sequencing was done with the BigDye terminator cycle sequencing kit and the outer primers of the nested PCR in accordance with the instructions of the manufacturer. PCR products were analyzed on an ABI 3100 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 the BLASTSearch program of the GenBank database (National Center for Biotechnology Information, Washington, D.C.).

Specificity and sensitivity testing of the nested PCR assay. In order to determine specificity of the nested PCR assay we tested DNA extracted from lab strains of closely related members of the family *Onygenaceae*, i.e., two strains of *Malbranchea* spp.; one strain each of *Auxarthron* sp., *Histoplasma capsulatum*, *Paracoccidioides brasiliensis*, and *Blastomyces dermatitidis*; and one strain of *Chrysosporium parvum*, because its colonies can resemble those of *Coccidioides* spp. Additionally, DNA from *Candida albicans*, *Candida dublinensis*, *Candida glabrata*, and *Candida tropicalis* was examined because it was reported that *Candida* surface antigen 1 has sequence similarity to motifs found in Ag2/PRA (16). Whereas colony morphology discriminates *Candida* and *Coccidioides*, the former could belong to the flora of clinical samples leading to PCR products if cross amplification is possible. To determine sensitivity, cloned plasmid DNA was measured photometrically at 260-280 nm, serially diluted 10-fold, and then used as template DNA.

RESULTS

Safety of the extraction procedure. Uncountable, i.e., >100, colonies of *Coccidioides* spp. were grown from all viability controls, i.e., from those samples cultivated without any treatment. Since 200 μl of suspension was cultured, the initial arthroconidium concentration must have been more than 5×10^2/ml. In contrast, all other cultures remained negative even after 4 weeks of incubation at 30°C. Therefore, addition of ATL buffer, which is included in the extraction kit, sufficiently kills *C. posadasii* arthroconidia and mycelia. In addition, two more steps of the extraction protocol, i.e., overnight incubation at 56°C and three cycles of freezing in liquid nitrogen and boiling, are each capable of securely inactivating cultures of this fungal pathogen. Thus, the extraction protocol with a commercial kit combines three steps to sufficiently kill cultures of *C. posadasii* and has to be considered safe.

Nested PCR and LightCycler PCR. A product of 526 bp was obtained from 116 of the 120 strains tested after the first PCR, whereas an expected 342-bp nested PCR product was detected by agarose electrophoresis from all 120 strains tested. All 120 strains were positive by real-time PCR.

Sequencing of the 526-bp product obtained from 35 strains demonstrated a complete identity to the sequence in GenBank database (accession number AF013256) except for an exchange of cytosine to guanosine at position 1228 in 28 of 35 strains. This was the only difference found. Since this mutation happens to be located within the binding site of the hybridization probe of the LightCycler PCR it was expected to detect this mutation by melting curve analysis. As shown in Fig. 1, the mutation shifts the melting point from 59°C to 57°C in the case of a mutation of C to G at position 1228. The results of melting curve analyses of the 35 strains were in accordance with the data obtained by sequencing. Thus, the melting curve analysis was regarded as being appropriate to detect the mutation at position 1228, which was found in 89 of 120 strains tested, whereas 31 strains demonstrated a melting point of 59°C. The mutation at position 1228, in comparison to the sequence deposited in the GenBank database, is found nearly three times more often in the 120 strains tested. Peng et al. described this mutation in two California strains and one strain each from Texas and Argentina (21). The authors found that nucleotide position 1228 belongs to an intron and therefore the nucleotide exchange has to be regarded as a silent mutation.

Microsatellites. With FAM-labeled primers, the ABI Sequencer, and the method described by Fisher et al. (12), the microsatellite-containing locus named GAC of 25 strains examined was exactly 206 bp long, whereas the length of the microsatellite-containing locus named 621 varied between 398 and 399 bp. These two markers identified the 25 strains as *C. posadasii* (11). The lengths of these microsatellite-containing loci were in the range of 195 to 198 bp and 388 to 395 bp, respectively, when examined by the LabChip-bioanalyzer method. Accordingly, the lengths of the two microsatellite-containing loci GAC and 621 of the remaining 95 strains, examined by bioanalyzer only, varied between 188 and 198 bp and 373 and 395 bp, respectively. Since the results were lower than or within the range of the 25 strains examined by both methods, all 120 strains were identified as *C. posadasii*.

Clinical specimens. A specific 342-bp product was obtained by nested PCR from three biopsies positive for spherules by microscopy, whereas no product was amplified from DNA extracted from samples positive for other dimorphic fungi. Attempts to identify the *Coccidioides* species by amplifying microsatellite-containing loci failed because the primers generated several products of different sizes (data not shown). No

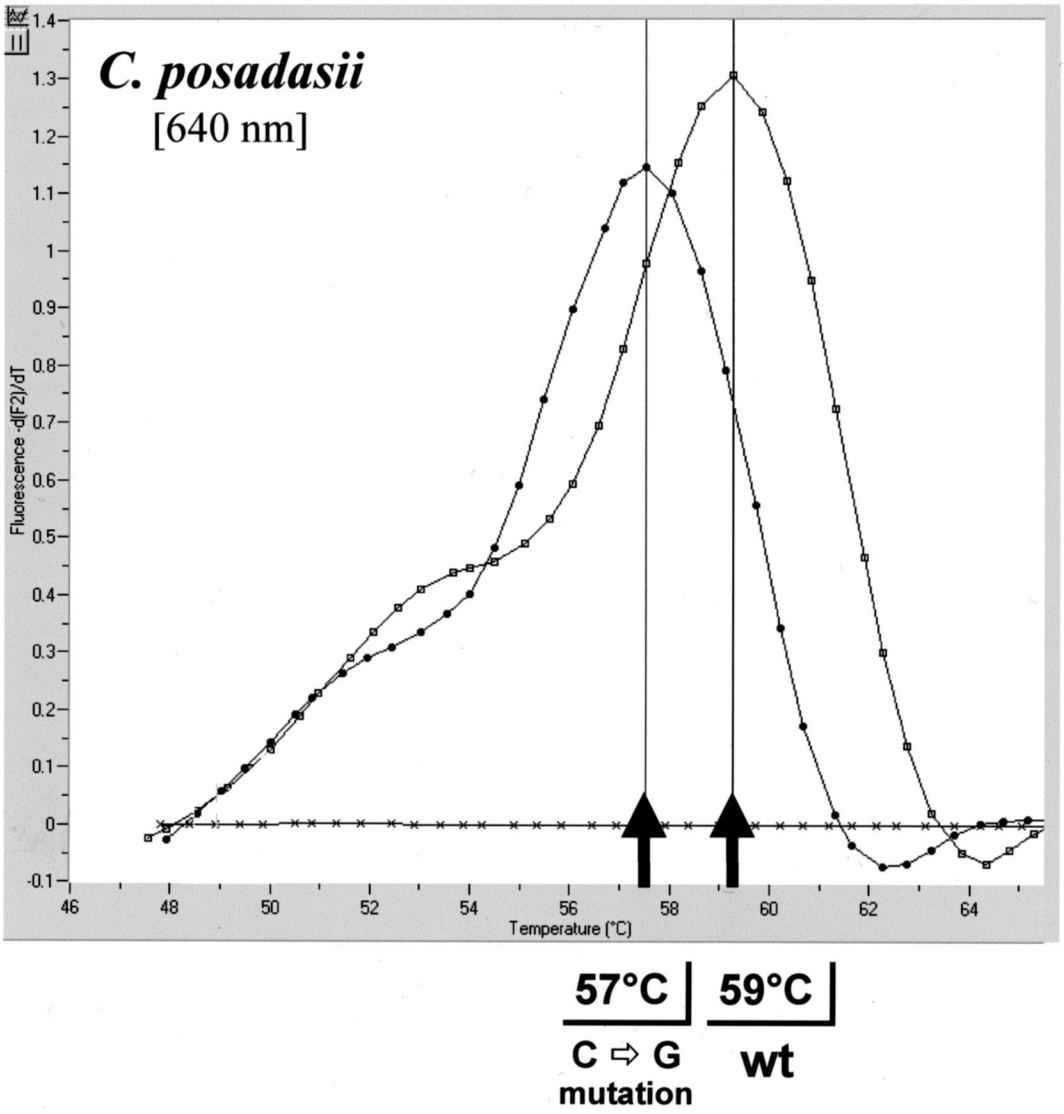

FIG. 1. Evaluation of the LightCycler PCR assay with two representative strains of *Coccidioides posadasii*. Results of the melting curve analysis are shown of a strain (wild type, wt) with cytosine at position 1228 in accordance with the GenBank sequence of antigen 2/proline-rich antigen (accession number AF013256) and a strain with a mutation to guanosine at this nucleotide position, shifting the melting point to 57°C.

PCR product was in the expected range. Whereas the newly developed nested PCR demonstrates specificity and diagnostic potential, identification of *Coccidioides* species by determination of microsatellite length from clinical human specimens seems to be impossible due to unspecific amplifications.

Specificity and sensitivity. Whereas all positive controls were positive by nested and LightCycler PCR, indicating appropriate amplification conditions, controls without specific *Coccidioides* DNA remained negative excluding crossover contaminations during DNA extraction and PCR preparations. A product was detected after the first PCR when a minimum of 100 fg of cloned plasmid DNA was used as a template. This detection limit was increased further a hundred times when an additional nested PCR was done. Thus, a detection limit of 1 fg of specific DNA in 10 μl of extracted DNA was determined for the conventional nested PCR assay, which is equivalent to 1 to 10 genome copies. DNA extracted from all other fungi used in this study, including *Candida* spp., was not amplified by the PCR assays.

DISCUSSION

A conventional nested PCR and a real-time PCR assay targeting the gene encoding the antigen 2/proline-rich antigen are described which specifically identified all 120 strains of *C. posadasii* examined in this study. The Ag2/PRA is conserved among non-Californian strains, now called *C. posadasii*, as well as among Californian strains now considered to be *C. immitis* (21). Peng et al. amplified and sequenced 673-bp-long segments of the Ag2/PRA gene of the Silveira strain and eight *Coccidioides* strains isolated from California, Arizona, Texas, and Argentina. Their target sequence includes 456 bp of our

526-bp PCR products, allowing us to consider that three of four primers and the two hybridization probes described in this study will bind to DNA from *Coccidioides* strains originating from other regions as well. However, it remains to be demonstrated that the PCR assays amplify DNA from *C. immitis* as well.

Since Ag2/PRA is regarded as specific for *Coccidioides* spp., these newly developed PCRs can be considered specific as well. Accordingly, neither DNA from related members of the family *Onygenaceae* nor DNA from *Chrysosporium parvum* and *Candida* spp. was amplified by these PCRs. Another target would have been the complement fixation antigen of *C. immitis*, known to be specific and immunoreactive as well (27). We did not use its coding gene for a diagnostic PCR because the antigen was identified as a chitinase with high homology to enzymes of other fungi. In addition, it was shown that antibodies from patients with other dimorphic fungal infections do cross-react with the cloned, i.e., nonglycosylated protein (27). Therefore, cross amplifications with DNA templates from other dimorphic fungi were expected.

Targeting a specific gene may decrease the sensitivity of diagnostic PCR assays in comparison to PCRs targeting multicopy rRNA genes (18). However, primers within conserved gene regions can amplify DNA of nonpathogenic related fungi as shown in former studies (6). Sandhu et al. developed specific probes within the large subunit rRNA genes from several fungi, including *C. immitis* (22). The authors stated that the use of radiolabeled probes in a routine laboratory is not the most desirable method. According to others the method is time-consuming and labor-intensive (17). In addition, a current BLASTSearch with the *C. immitis* probe reveals 100% identity with sequences of the large subunit of *Chrysosporium* spp. Lindsley et al. amplified the internal transcribed spacer (ITS) region and used an enzyme immunoassay-based hybridization procedure to identify fungal strains including *C. immitis* (17). Whereas this method might be applied to cultures, the use of universal primers faces problems when examining clinical specimens contaminated with spores of several fungi. The competition of binding regions might decrease sensitivity. In addition, the specificity of the probe has to be proven by sequencing ITS regions of all possibly contaminating fungi. In our experience, the identity of PCR product targeting fungal ribosomal DNA, i.e., conserved genes, can only be proven by sequencing, and even then, species identification might not be possible (2, 4, 6). Even if ITS sequences are variable, identification of PCR products from clinical specimens by oligonucleotide probes can only be relied on if ITS regions of all fungi have been sequenced and deposited in GenBank.

For diagnostic purposes. a highly specific and sensitive PCR has to be created. According to Peng et al. (21), only one copy of the Ag2/PRA gene per genome is expected. As demonstrated by cloned DNA, sensitivity can be increased 100-fold by running a conventional nested PCR assay. The detection limit of 1 fg of cloned plasmid DNA is within the range found in other diagnostic nested PCR assays targeting single-copy genes (2, 4). In addition, a higher number of *Coccidioides* genomes per sample can be expected in clinical specimens because spherules contain numerous endospores and thus several genomes. In the few paraffin-embedded samples examined in this study, the conventional nested PCR assay demonstrated sensitivity and specificity. PCR assays distinguishing pathogenic fungi are of clinical importance because *Coccidioides* spp. can resemble other dimorphic fungi in histological sections (15). Since therapy and prognosis differ considerably, methods to identify the different dimorphic fungi can be of high value, especially in nonendemic regions to which coccidioidomycosis is imported but rarely suspected and where experience in growing the pathogen is limited (1, 8). However, the diagnostic potentials of the conventional nested and LightCycler PCR assays remain to be determined.

The safety of the extraction procedure is essential when handling cultures of this hazardous fungal pathogen. The modified protocol of a commercial kit has to be regarded as very safe due to the combination of at least three steps, each one being able to sufficiently inactivate the mycelial form of *C. posadasii*. In order to liberate DNA, each sample is boiled for at least 11 min, interrupted by freezing in liquid nitrogen, which was effective to inhibit growth of *C. posadasii*. Boiling for 15 to 20 min has been reported as a safe procedure to inactivate suspensions of mycelia from *Coccidioides* spp. (14, 24). Adding ATL buffer from the kit as well as overnight incubation at 56°C can inactivate suspensions of mycelia from *C. posadasii* cultures. The additional effect of freezing in liquid nitrogen alone was not examined and has not been studied elsewhere so far.

For the safe extraction procedure described by Burt et al. (7) liquid cultures of *Coccidioides* spp. were frozen in liquid nitrogen and then lyophilized before DNA was extracted. Whereas this procedure can be applied to cultures, it has not been used for clinical specimens. In contrast, our protocol has been used successfully to extract fungal DNA from native, frozen, and formalin-fixed specimens obtained from humans and animals (2, 3, 4, 5). In tissue samples, spherules are usually found which are considered to be noninfectious, but reports of *Coccidioides* hypha-containing clinical specimens demand safe extraction procedures (25). Although not tested in this study, the extraction procedure appears appropriate for testing DNA of *Coccidioides* strains by AccuProbe, avoiding false-negative results due to formalin killing (14).

The distinction of two *Coccidioides* species is of tremendous interest in evolution (10). However, it seems to be of limited medical importance because there does not seem to be a significant difference in disease caused by them. In addition, determination of microsatellite size is limited to cultures because the primers used to amplify microsatellite-containing loci will not specifically amplify DNA from *Coccidioides* spp. in clinical samples according to our limited experience. Therefore, retrospective species identification in histopathological material from known coccidioidal infections does not seem to be possible.

As expected from excellent geographic studies (11) all 120 strains isolated in Monterrey, Nuevo Leon, i.e., North Mexico, were identified as *C. posadasii*. The sizes of microsatellites were exactly within the described range when examined by the original method with the ABI Sequencer and fluorescently labeled primers. In contrast, analysis by the time-saving LabChip 2100 Bioanalyzer system gave variable results. According to the manufacturer 5 to 10% variability in nucleotide size might occur with the LabChip. The length determinations of the microsatellites examined were within the lower deviation of

the expected size, allowing for species identification. However, it remains to be determined whether microsatellites of *C. immitis* which differ by minimal 12 and 19 nucleotides can be clearly distinguished from those of *C. posadasii* by the LabChip system. Due to repetitive elements direct sequencing of the microsatellites amplified from the strains in order to determine the exact size was not possible in our hands.

In conclusion, we have developed a conventional nested and a real-time PCR assay specific for the identification of *Coccidioides posadasii* DNA by targeting a gene coding for the unique Ag2/PRA. Limited examinations demonstrate the diagnostic potential of these assays to detect and identify specific coccidioidal DNA in tissue samples. In addition, a modified protocol of a commercially available extraction kit was shown to securely inactivate cultures of *C. posadasii*.

ACKNOWLEDGMENTS

We gratefully acknowledge the excellent technical assistance of Lidia Oviedo, Diana Rodriguez, and Maria-Bibiana Alberdi. We thank Qiagen GmbH, Hilden, Germany, for providing extraction kits and Agilent Technologies, Waldbronn, Germany, for providing the equipment of the 2100 Bioanalyzer.

We thank the reviewers for very constructive comments.

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