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Detection of *Coccidioides* Species in Clinical Specimens by Real-Time PCR▽

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***Coccidioides* spp. are dimorphic fungal pathogens endemic to the semiarid regions of North, Central, and South America. Currently, direct smear and culture are the most common means of identifying *Coccidioides* spp. While these methods offer relatively sensitive and specific means of detecting *Coccidioides* spp., growth in culture may take up to 3 weeks, potentially delaying the diagnosis and initiation of appropriate antifungal therapy. In addition, growth of the organism represents a significant safety risk to laboratory personnel. The need for a rapid and safe means of diagnosing coccidioidomycosis prompted us to develop a real-time PCR assay to detect *Coccidioides* spp. directly from clinical specimens. Primers and fluorescent resonance energy transfer (FRET) probes were designed to target the internal transcribed spacer 2 region of *Coccidioides*. The assay's limit of detection is below 50 targets per reaction. An analysis of 40 *Coccidioides* sp. clinical isolates grown in culture demonstrated 100% sensitivity of the assay. A cross-reactivity panel containing fungi, bacteria, mycobacteria, and viruses was tested and demonstrated 100% specificity for *Coccidioides* spp. An analysis of 266 respiratory specimens by LightCycler PCR demonstrated 100% sensitivity and 98.4% specificity for *Coccidioides* spp. compared with culture. Analysis of 66 fresh tissue specimens yielded 92.9% sensitivity and 98.1% specificity versus those of the culture method. The sensitivity of the assay testing 148 paraffin-embedded tissue samples is 73.4%. A rapid method for the detection of *Coccidioides* spp. directly from clinical material will greatly assist in the timely diagnosis and treatment of patients, while at the same time decreasing the risk of accidental exposure to laboratory personnel.**

Coccidioidomycosis is caused by the dimorphic fungi *Coccidioides immitis* and *Coccidioides posadasii*. These organisms are endemic to the southwestern regions of the United States, northern Mexico, and areas of Central and South America. The illness commonly manifests as a self-limited, upper respiratory tract infection, but it can also result in disseminated disease that may be refractory to treatment (5). Clinical onset generally occurs 10 to 16 days following inhalation of coccidioidal spores (arthroconidia) (9), and disease progression may be rapid in previously healthy or immunosuppressed individuals. It has been estimated that the inhalation of as few as 10 arthroconidia spores may be sufficient to cause disease (5), and therefore, *Coccidioides* spp. are considered a potential bioweapon (6, 7) and are currently included on the select agent list issued by the Centers for Disease Control (http://www.cdc.gov/od/sap/).

The identification of *Coccidioides* spp. by clinical laboratories plays a critical role in the management of infected patients, as the clinical presentation of coccidioidomycosis may closely resemble that of other infectious or noninfectious etiologies (12, 30). Currently, the laboratory methods most frequently used to diagnose coccidioidomycosis include serology, direct smear, histopathology, and culture with confirmation by DNA probe analysis (15, 22, 24, 28). Various serologic assays, including tube precipitin, complement fixation, and immunodiffusion, have been used for many years in the diagnosis of coccidioidomycosis (17, 19, 24). These assays have demonstrated reliable sensitivity and specificity, especially when used in combination (e.g., complement fixation and immunodiffusion). However, serology may take 1 to 2 weeks following the onset of symptoms to become positive and may remain negative despite infection in immunosuppressed patients (1, 2, 23). Direct smear and histopathology have also been used in the diagnosis of *Coccidioides* disease, but these methods lack sensitivity and specificity (15, 20, 25). At present, the gold standard for the diagnosis of coccidioidomycosis is culture of the organism from clinical specimens. Culture is highly sensitive, and the implementation of DNA probe assays for confirmatory testing of culture isolates has yielded excellent specificity (22, 28). However, growth in culture may take several days to several weeks. This often delays the diagnosis and initiation of appropriate treatment in infected individuals. In addition, the propagation of *Coccidioides* spp. in the clinical laboratory represents a significant safety hazard to laboratory personnel and may serve as an important cause of laboratory-acquired infections (26) if not quickly identified and handled appropriately in a biosafety level 3 facility.

Due to the limitations of current laboratory methods available for the diagnosis of coccidioidomycosis, we set out to develop a method that would allow for the rapid and reliable detection of *Coccidioides* spp. directly from clinical specimens. In this report, we describe a real-time PCR assay based on the LightCycler (Roche Applied Sciences, Indianapolis, IN) plat-

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form that can be used to detect *Coccidioides* spp. directly from a variety of clinical specimens, including respiratory specimens, fresh tissue, and formalin-fixed, paraffin-embedded tissue.

MATERIALS AND METHODS

Clinical specimens. A total of 266 respiratory specimens (bronchoalveolar lavage [BAL] fluid, bronchial washings, pleural fluid, and sputum) were obtained from patients seen at either the Mayo Clinic Rochester or the Mayo Clinic Scottsdale between September 2005 and August 2006. The specimens included in this study were processed in parallel for general fungal culture, as described below, to allow for comparison to the LightCycler PCR results. Of the 266 total respiratory specimens, 261 were obtained from the Mayo Clinic in Scottsdale, Arizona, an area where *Coccidioides* spp. are endemic. These specimens were shipped via overnight courier to Rochester, MN, and processed for LightCycler PCR analysis within 7 days of collection. In addition to respiratory specimens, fresh tissue and formalin-fixed, paraffin-embedded tissue samples were also sent from Mayo Clinic Scottsdale for testing. An institutional review board of the Mayo Foundation approved the use of all specimens included in this study.

Culture of clinical specimens. Respiratory specimens were inoculated onto inhibitory mold agar supplemented with chloramphenicol (125 μg/ml) (Becton Dickinson Diagnostic Systems [BDDS]), brain heart infusion (BHI) blood agar supplemented with chloramphenicol (50 μg/ml) and gentamicin (40 μg/ml) (BHI-3; BDDS), and brain heart infusion blood agar supplemented with chloramphenicol (50 μg/ml), gentamicin (40 μg/ml), and cycloheximide (0.5 mg/ml) (BHI-4; BDDS). Plates were incubated at 30°C and held for up to 24 days. Colonies exhibiting morphologies consistent with those of *Coccidioides* spp. were examined microscopically for characteristic patterns of alternating arthroconidia and then tested at Mayo Clinic Rochester via a *Coccidioides* DNA probe hybridization assay (Gen-Probe, San Diego, CA) for confirmation.

Generation of positive control plasmid. A patient isolate confirmed by microscopy and DNA probe hybridization analysis to be *Coccidioides* spp. was used to construct a positive control plasmid. In brief, a portion of the patient isolate was suspended in 200 μl of PrepMan Ultra reagent (Applied Biosystems) and the inoculated tube was then heated at 95°C for 10 min. The lysate was allowed to cool to 4°C and was then diluted 1:10 in S.T.A.R. buffer (Roche Applied Sciences). The diluted lysate was mixed, and 200 μl was extracted on the MagNA Pure Compact (Roche Applied Sciences) as described below. The isolated nucleic acid was then subjected to the LightCycler PCR assay described below, utilizing primers designed to specifically amplify a portion of the internal transcribed spacer 2 (ITS2) region of *Coccidioides* spp. The ITS2 region is present in a conserved ribosomal gene cluster in the *Coccidioides* genome. The PCR product was then ligated into the pCR2.1 vector by using the pCR2.1-TOPO TA cloning kit (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. The plasmid construct was then purified using the High Pure plasmid isolation kit (Roche Applied Sciences) according to the manufacturer's instructions. Restriction enzyme analysis demonstrated the presence of a single insert of the appropriate size, and subsequent DNA sequencing confirmed the insert to be the ITS2 region derived from *Coccidioides* spp.

Specimen processing and nucleic acid extraction. All *Coccidioides* culture isolates were lysed in PrepMan Ultra reagent (Applied Biosystems) as described above. Respiratory specimens (BAL fluid, bronchial washings, pleural fluid, and sputum) were processed by pipetting 500 μl of raw specimen and 100 μl proteinase K (Roche Applied Sciences) into a 1.5-ml tube containing 0.1-mm silica glass beads and 2.4-mm zirconia beads (BioSpec Products). Samples were then incubated at 55°C for 15 min on a thermomixer (Eppendorf) at 1,400 rpm and subsequently placed on a 95°C heat block for 5 min. To facilitate complete lysis and nucleic acid liberation, samples were placed on a Disruptor Genie (Scientific Industries, Bohemia, NY) for 2 min and then centrifuged briefly at 1,000 × *g* to 5,000 × *g* to collect the sample at the bottom of the tube. Five hundred microliters of the prepared lysate was pipetted into a sample tube for MagNA Pure Compact extraction (Roche Applied Sciences) utilizing the nucleic acid isolation kit (large volume), the total NA plasma program, and a final elution volume of 100 μl.

Fresh tissue specimens were processed for LightCycler PCR analysis by first placing a small piece of tissue, approximately 0.5 cm^2, into a sterile 1.5-ml tube containing 400 μl of 1× Tris-EDTA (Sigma Aldrich), 100 μl proteinase K (Roche Applied Sciences), and 50 μl 10% sodium dodecyl sulfate (Sigma Aldrich). The samples were vortexed briefly and then placed on a thermomixer (Eppendorf) overnight at 55°C with a mixing speed of 500 rpm. The following day, 200 μl of the digested tissue was transferred into a MagNA Pure Compact tube and extracted utilizing the nucleic acid isolation kit (small volume), the total NA plasma program, and a final elution volume of 100 μl.

Formalin-fixed, paraffin-embedded tissue sections were processed by placing one 50-μm section (from large tissue sections) or two 50-μm sections (from punch biopsies) into a 1.5-ml tube containing 500 μl xylene (Sigma Aldrich). The sections were then incubated in xylene for 5 min at room temperature. The tubes were then centrifuged for 30 s at 20,800 × *g*, and the xylene was removed with a fine-tip disposable pipette. A second incubation in 500 μl xylene at room temperature was performed, followed by centrifugation and removal of xylene. Subsequently, 500 μl of 95% ethanol was added to the tubes, and the samples were gently vortexed and then incubated for 5 min at room temperature. The samples were then centrifuged for 3 min at 20,800 × *g*, and the alcohol was removed using a fine-tip disposable pipette. Following removal of the alcohol, 400 μl of 1× Tris-EDTA, 100 μl proteinase K, and 50 μl 10% sodium dodecyl sulfate were added to the tube. The samples were then vortexed and placed on

TABLE 1. Cross-reactivity panel tested with the *Coccidioides* LightCycler PCR assay

Organism	Organism
Bacteria	*Propionibacterium acnes*
Acinetobacter baumannii	*Propionibacterium granulosum*
Acinetobacter lwoffii	*Proteus mirabilis*
Actinomyces odontolyticus	*Proteus vulgaris*
Aeromonas hydrophila	*Pseudomonas aeruginosa*
Bartonella quintana	*Pseudomonas cepacia*
Bartonella henselae	*Pseudomonas fluorescens*
Bordetella bronchiseptica	*Staphylococcus aureus*
Bordetella parapertussis	*Staphylococcus epidermidis*
Bordetella pertussis	*Stenotrophomonas maltophilia*
Borrelia burgdorferi	*Streptococcus pneumoniae*
Campylobacter jejuni	*Streptococcus pyogenes*
Chlamydia trachomatis	*Tropheryma whipplei*
Corynebacterium hemolyticum	*Ureaplasma urealyticum*
Corynebacterium diphtheriae	
Corynebacterium pseudodiptheriae	Fungi
Escherichia coli	*Arthroderma gloriae*
Haemophilus influenzae	*Aspergillus fumigatus*
Klebsiella oxytoca	*Aspergillus flavus*
Klebsiella pneumoniae	*Aspergillus versicolor*
Legionella jordanis	*Aspergillus nidulans*
Legionella pneumophila	*Aspergillus glaucus*
Legionella longbeachae	*Auxathron zuffianum*
Legionella dumoffii	*Blastomyces dermatitidis*
Legionella bozemanii	*Candida albicans*
Legionella micdadei	*Candida glabrata*
Listeria monocytogenes	*Candida parapsilosis*
Moraxella catarrhalis	*Candida tropicalis*
Morganella morganii	*Candida krusei*
Mycobacterium africanum	*Chrysosporium* spp.
Mycobacterium bovis	*Cryptococcus neoformans*
Mycobacterium bovis BCG	*Fusarium* spp.
Mycobacterium microti	*Gymnoascus longitrichus*
Mycobacterium tuberculosis	*Histoplasma capsulatum*
Mycobacterium avium	*Malbranchea albolutea*
Mycobacterium gordonae	*Paracoccidioides* spp.
Mycobacterium kansasii	*Penicillium* spp.
Mycobacterium intracellulare	*Rhizopus* spp.
Mycobacterium smegmatis	*Uncinocarpus reesii*
Mycoplasma pneumoniae	
Mycoplasma fermentans	Viruses
Mycoplasma salivarium	Adenovirus
Mycoplasma hominis	Parainfluenza 1 and 3
Mycoplasma buccale	Influenza A/B
Mycoplasma orale	Respiratory syncytial virus A2/B
Mycoplasma faucium	Cytomegalovirus
Mycoplasma genitalium	Epstein-Barr virus
Neisseria gonorrhoeae	Enterovirus
Neisseria meningitidis	Herpes simplex virus 1/2
Nocardia brasiliensis	Coronavirus
Nocardia brevicatena	Measles
Nocardia carnea	Mumps
Nocardia dassonvillei	Human herpesvirus 6, 7, and 8
Nocardia farcinica	Varicella-zoster virus
Nocardia otitidis	BK
Nocardia transvalensis	
Prevotella melaninogenica	Human DNA

TABLE 2. Detection of *Coccidioides* spp. from culture by LightCycler PCR

LightCycler PCR result	No. of specimens with indicated result by culture		
	Positive	Negative	Total
Positive	40	0	40
Negative	0	24	24
Total	40	24	64

a thermomixer (Eppendorf) overnight at 55°C with a mixing speed of 500 rpm. The following day, 200 μl of the digested specimen was transferred into a MagNA Pure Compact sample tube and extracted utilizing the nucleic acid isolation kit (small volume), the total NA plasma program, and a final elution volume of 100 μl.

LightCycler PCR. The ITS2 region of *Coccidioides* was selected as the target for the PCR assay. Primers and FRET probes were designed to amplify and detect a 170-bp region within the ITS2. The designed primers were of the following sequences: 5′-CGA GGT CAA ACC GGA TA-3′ (forward) and 5′-CCT TCA AGC ACG GCT T-3′ (reverse). The FRET hybridization probes were of the following sequences: 5′-GAG CGA TGA AGT GAT TTC CC-3′ (anchor probe [3′ fluorescein labeled]) and 5′-TAC ACT CAG ACA CCA GGA ACT CG-3′ (donor probe [5′ LC RED-640 labeled]).

The PCR assay was performed using the LightCycler FastStart DNA Master hybridization probes kit (Roche Applied Sciences), with each reaction consisting of 3 mM $MgCl_2$, 1× LightCycler FastStart mix, 0.5 μM of each primer, 0.2 μM fluorescein-labeled probe, and 0.4 μM LC-Red 640-labeled probe. The total volume per reaction was 20 μl (15 μl master mix plus 5 μl extracted nucleic acid). PCR amplification with real-time detection was performed on the LightCycler instrument (Roche Applied Sciences). Cycling parameters consisted of 1 cycle at 95°C for 10 min, followed by 45 cycles of 95°C for 10 s, 60°C for 15 s, and 72°C for 20 s. Following amplification, a melting curve analysis was performed by measuring the fluorescent signal during the following cycling profile: 95°C for 0 s, 59°C for 20 s, 45°C for 20 s with a 0.2°C/s transition, and 85°C for 0 s with a 0.2°C/s transition.

Analytical sensitivity and specificity. The analytical sensitivity of the assay was determined by testing a dilution series of the positive control plasmid. Tenfold dilutions of the positive control plasmid (10^4 copies/μl down to 10^0 copies/μl) were made in PCR-grade water and tested in triplicate.

The analytical specificity of the assay was determined by performing a BLAST search of the primer and probe sequences at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov). In addition, an extensive panel of nucleic acid extracted from 114 potentially cross-reacting organisms, including fungi, bacteria, mycobacteria, viruses, and human DNA, was tested (Table 1). Amplification and sequencing of either 16S (bacteria) or D2 LSU (fungi) was utilized to confirm the presence of amplifiable nucleic acid in the specificity panel. When available, specific viral PCR assays were used to demonstrate the integrity of viral nucleic acid.

RESULTS

Analytical sensitivity and specificity. The limit of detection of the assay was determined to be less than 10 copies of target per microliter (less than 50 copies of target per reaction) (data not shown). Furthermore, the assay did not demonstrate cross-reactivity with any of the organisms included in the specificity panel (Table 1).

Clinical sensitivity and specificity. Forty clinical isolates determined to be *Coccidioides* spp. by microscopic examination and DNA probe hybridization analysis were lysed, extracted to purify the nucleic acid, and tested using the LightCycler PCR assay. All 40 isolates were positive by the real-time PCR assay (100% sensitivity) (Table 2), demonstrating an average melting curve of 60.4°C (standard deviation, ±0.07°C) (Fig. 1). All amplification products generated from the clinical isolates

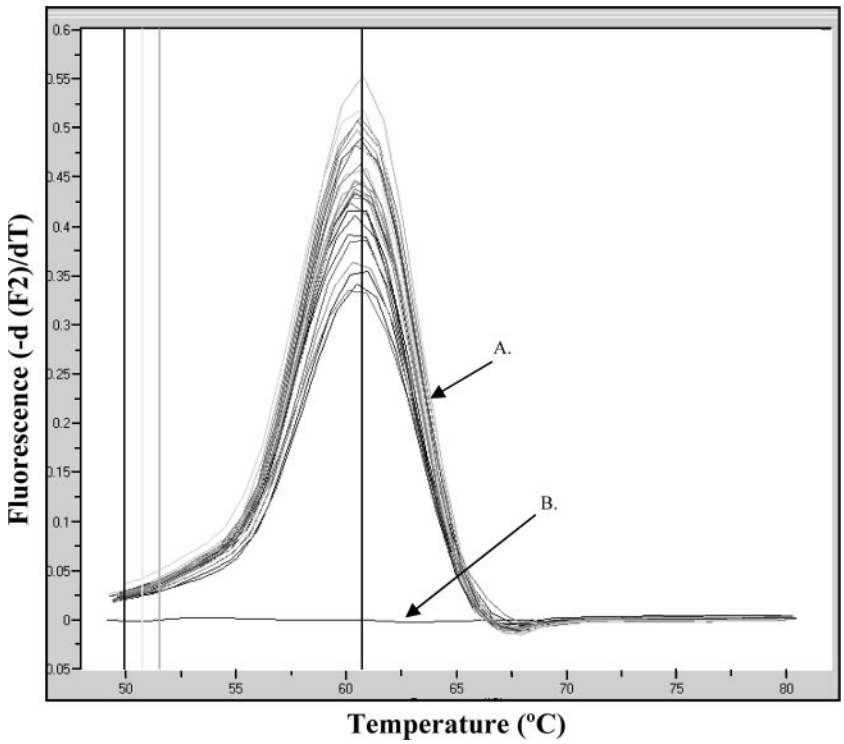

FIG. 1. Melting curve analysis of *Coccidioides* sp. clinical isolates. Isolates grown in culture were processed as described in the text and analyzed by LightCycler PCR. Postamplification melting curve analysis demonstrated an average melting temperature of 60.4°C ± 0.07°C (mean ± standard deviation). (A) Positive clinical isolates. (B) Negative control.

TABLE 3. Comparison of LightCycler PCR and culture for the detection of *Coccidioides* spp. in respiratory specimens

LightCycler PCR result	No. of specimens with indicated result by culture		
	Positive	Negative	Total
Positive	16	4	20
Negative	0	246	246
Total	16	250	266

were sent for DNA sequencing, and the results confirmed the identity of each as *Coccidioides* spp. (data not shown). In addition, 24 clinical isolates determined to be fungi other than *Coccidioides* were processed in an identical fashion and tested by the PCR assay. None of the non-*Coccidioides* clinical isolates yielded positive PCR results (Table 2).

A total of 266 respiratory specimens, including bronchial washings (119), BAL fluid (92), pleural fluid (32), sputum (21), and lung abscess fluid (2), were coexamined by general fungal culture and the LightCycler PCR assay (Table 3). Sixteen specimens were positive for *Coccidioides* spp. by both LightCycler PCR and fungal culture, while 246 specimens were negative by both methods. Interestingly, there were no culture-positive, PCR-negative specimens. However, four specimens were positive by the LightCycler PCR method but negative by culture (Table 3). It should be noted that the 20 PCR-positive specimens were derived from 17 different patients (14 patients from Scottsdale and 3 patients from Rochester). Overall, the clinical sensitivity and specificity values of the PCR assay from respiratory specimens were 100 and 98.4%, respectively.

In addition to respiratory specimens, fresh and paraffin-embedded tissues were also examined for the presence of *Coccidioides* spp. Fresh tissue specimens were examined by culture as a means of comparison to LightCycler PCR results. Thirteen fresh tissues were positive for *Coccidioides* spp. by both culture and PCR, while 51 specimens were negative by both methods (Table 4). One specimen did yield a positive culture result (one colony) with a negative PCR result. In addition, one specimen was positive by PCR in the absence of growth in culture (Table 4). The PCR assay exhibited clinical sensitivity and specificity values from fresh tissue of 92.9 and 98.1%, respectively.

Although paraffin-embedded tissue has historically been viewed as an insensitive source for PCR analysis, we examined 148 paraffin-embedded specimens by both pathological examination and PCR for the presence of *Coccidioides* spp. (Table 5). Positive sections were identified by retrospective review of archived material from cases in which coccidioidomycosis was either specifically diagnosed or suggested in the final diagnosis.

TABLE 4. Comparison of LightCycler PCR and culture for the detection of *Coccidioides* spp. in fresh tissue

LightCycler PCR result	No. of specimens with indicated result by culture		
	Positive	Negative	Total
Positive	13	1	14
Negative	1	51	52
Total	14	52	66

TABLE 5. Comparison of LightCycler PCR and histopathology for the detection of *Coccidioides* spp. in paraffin-embedded tissue

LightCycler PCR result	No. of specimens with indicated result by histopathology		
	Positive	Negative	Total
Positive	47	0	47
Negative	17	84	101
Total	64	84	148

Paraffin sections were identified as positive when necrotizing granulomas with either intact or degenerated spherules were observed. Of the 148 total sections examined, 47 were positive by LightCycler PCR with concordant histopathologic findings. Eighty-four specimens were negative for *Coccidioides* by both methods. While none of the paraffin-embedded specimens were found to be PCR positive, pathology negative, there were 17 specimens that displayed features suggestive of *Coccidioides* spp. by histopathology, but were negative by PCR (Table 5). These results demonstrated overall clinical sensitivity and specificity values from paraffin-embedded tissue of 73.4 and 100%, respectively.

Whenever enough amplification product was generated from a positive clinical sample (typically when a positive signaled at less than 33 cycles), the amplicon was sent for DNA sequencing. All sequences obtained from positive specimens (respiratory, fresh tissue, or paraffin-embedded tissue) were confirmed to be of *Coccidioides* origin by BLAST analysis or alignment with the sequence derived from the positive control plasmid (data not shown).

DISCUSSION

Several previous reports have described the identification of *Coccidioides* spp. by PCR, either from environmental sources (14), clinical isolates (3, 29), or clinical specimens (18). However, most of the *Coccidioides* PCR assays described in the literature have utilized conventional PCR technology (14, 18, 21, 29), a method which possesses limited utility as a diagnostic tool due to it being an open system and therefore having an increased potential for contamination events. In addition, to date, no report has included a thorough clinical verification in which large numbers of clinical specimens have been tested.

In this report, we have described the development of a real-time PCR assay based on the LightCycler platform for the rapid detection of *Coccidioides* spp. directly from clinical specimens. A total of 480 clinical specimens (266 respiratory specimens, 148 paraffin-embedded tissue samples, and 66 fresh tissue samples), most of which were collected in an area where *Coccidioides* spp. are endemic, were tested by the PCR assay, and the results were compared to those derived from a conventional "gold standard" method. These studies determined the PCR assay to be highly sensitive and specific, with clinical sensitivities and specificities of 100 and 98.4% (from respiratory specimens), 73.4 and 100% (from paraffin-embedded tissue), and 92.9 and 98.1% (from fresh tissue), respectively. It should be stressed that the majority of specimens tested in this study were acquired from an area where *Coccidioides* spp. are

endemic, and therefore, further studies will need to be carried out to accurately determine the performance characteristics of this assay in low-incidence, nonendemic regions. Furthermore, the number of positive respiratory specimens included in this study is limited. An ongoing clinical validation will be performed to discern whether the sensitivity of the assay is affected as more positive specimens are obtained.

Based on our findings, the PCR assay does not differentiate between the two known species of *Coccidioides*, *C. immitis* and *C. posadasii*. The two species differ in single-nucleotide polymorphisms, microsatellite size, and geographical distribution, with *C. immitis* and *C. posadasii* being historically known as the Californian and non-Californian strains, respectively (10, 11). We performed PCR and melting curve analyses on known, previously typed *C. immitis* and *C. posadasii* strains supplied to us by the Arizona Department of Public Health, and these studies demonstrated that the melting curves were indistinguishable between the two species (data not shown). While differentiation of the two species is important in evolutionary and epidemiologic studies, the clinical manifestations and therapies for *C. immitis* and *C. posadasii* are identical. Therefore, there is no utility for speciation of *Coccidioides* in the clinical laboratory.

It is often reported that PCR displays greater sensitivity over conventional detection methods, such as culture (8, 27). Therefore, it was of interest to us that four respiratory specimens were positive by PCR but were negative by culture. These discordant results may indicate potential PCR cross-reactivity or may provide further evidence of the enhanced sensitivity of PCR over culture for certain pathogens. In order to explain these discordant results, a thorough examination of the clinical histories and supplemental laboratory results for the four patients was conducted. The first patient had growth of *Coccidioides* spp. in a second specimen (the PCR was positive from this second specimen as well), and therefore, the initial discordant result is likely due to a greater sensitivity of the PCR assay. The second patient demonstrating discordant results presented with community-acquired pneumonia, and initial *Coccidioides* serologies were negative. However, the patient's follow-up *Coccidioides* enzyme immunoassay was positive for both immunoglobulin M and immunoglobulin G. The patient was treated for presumed coccidioidomycosis and recovered on antifungal therapy. The third patient presented with a right upper lung nodule, and the clinical impression was coccidioidomycosis versus adenocarcinoma. Although cultures and serologies for *Coccidioides* remained negative, the patient was treated empirically with fluconazole for possible pulmonary coccidioidomycosis and was lost to follow-up. The fourth patient with discordant results was a long-term hemodialysis patient who presented with multilobar bronchopneumonia. All serologies and cultures for *Coccidioides* remained negative. However, following the initial positive PCR result using the ITS2 primer/probe set, a second PCR was performed using a primer/probe set specific for a separate *Coccidioides* target (*ura5*; NCBI accession no. AF022892). The *ura5* target was initially included in our study as a potential target for the real-time assay, but it demonstrated less sensitivity than did the ITS2 primer/probe set despite exhibiting specificity for *Coccidioides* spp. (unpublished data). The *ura5* PCR was also positive (data not shown), providing further evidence that *Coccidioides* sp. nucleic acid was present in the specimen.

Taken together, these results suggest that the *Coccidioides* PCR assay may be more sensitive than conventional laboratory methods, such as culture. However, these findings do not rule out the potential for rare cross-reactivity with organisms not included in our specificity panel. In addition, further studies may need to be carried out to discern the assay's true specificity for disease. As with other PCR assays, it is sometimes difficult to interpret whether a positive PCR result indicates the presence of viable, disease-related organisms or simply the detection of transient colonizers or nucleic acid that is persisting from old disease. An additional limitation of this study is that the specimens were tested in a nonconsecutive fashion due to the relatively low prevalence of the disease and the shear volume of specimens that would have been necessary to obtain enough positives using a consecutive approach. As a result, defined positive and negative predictive values cannot be calculated and further studies will need to be performed to provide these data for regions of both endemicity and nonendemicity.

In summary, we have described the development of a real-time PCR assay for the detection of *Coccidioides* spp. directly from clinical specimens. The implementation of this method into the clinical laboratory will allow for the identification of *Coccidioides* spp. in respiratory specimens in less than 4 h. This turnaround time includes the time for specimen processing and nucleic acid extraction, using a protocol which is safe for laboratory personnel, and obviates the need to work with the organism in culture for identification purposes. In addition, this method will allow for the detection of *Coccidioides* spp. in fixed tissue. While long-term formalin fixation has been demonstrated to decrease the sensitivity of PCR (4, 13, 16), the ability to analyze these specimens may prove extremely beneficial in cases where no cultures are ordered because a noninfectious etiology is initially suspected, but the possibility of infection is raised on histopathology. A rapid and safe means of detecting *Coccidioides* spp. directly from patient specimens will have a major impact in the clinical arena, allowing for the timely diagnosis of coccidioidomycosis and the appropriate initiation of antifungal therapy.

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