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Development and validation of a quantitative real-time PCR assay for the early diagnosis of coccidioidomycosis ☆

Sara Gago [a], María José Buitrago [a], Karl V. Clemons [b,c,d,*], Manuel Cuenca-Estrella [a], Laurence F. Mirels [b,c], David A. Stevens [b,c,d]

[a] Servicio de Micología, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Majadahonda, Madrid, Spain
[b] California Institute for Medical Research, San Jose, CA, USA
[c] Department of Medicine, Santa Clara Valley Medical Center, San Jose, CA, USA
[d] Department of Medicine, Stanford University, Stanford, CA, USA

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ABSTRACT

A new real-time polymerase chain reaction (RT-PCR) assay based on a *Coccidioides* genus–specific molecular beacon probe was developed for the detection of coccidioidomycosis and validated with tissues from animal models and clinical samples. The assay showed high analytic reproducibility ($r^2 > 0.99$) and specificity for cultured strains (100%); the lower limit of detection was 1 fg of genomic DNA/μL of reaction. Fungal burdens in the organs of mice infected with *Coccidioides posadasii* strain Silveira were more accurately quantified by RT-PCR compared to colony-forming unit for all tissues. The RT-PCR assay was positive for 97.7% of spleen and 100% of liver or lung. Progression of infection in all organs was similar by both methods ($P > 0.05$). The sensitivity of the assay also was 100% for paraffin-embedded samples and samples from patients with positive cultures. Our RT-PCR assay is effective for the diagnosis and monitoring of *Coccidioides* infection, and its use also avoids the biohazard and time delay of identifying cultures in the clinical setting.

1. Introduction

Coccidioidomycosis is endemic in the southwestern United States, as well as in Latin America (Laniado-Laborin, 2007; Pappagianis, 1993, 1994). This endemic mycosis is caused by 2 closely related species, *Coccidioides immitis* and *Coccidioides posadasii*, which are described as Californian and non-Californian species, respectively. Differences in the manifestation of disease or the diagnosis of the infection have not been reported for these species (Fisher et al., 2002). Infection usually occurs after inhalation of arthroconidia and initiation of a respiratory infection. Predisposing factors for life-threatening infection include racial ethnicity, immunosuppression, and pregnancy (Pappagianis, 1988, 1993). Despite this, most infections are asymptomatic, while symptomatic presentation can be similar to community-acquired pneumonia. A minority of patients develop disseminated infection, which can present in a variety of tissues, such as skin lesions, osteomyelitis, or meningitis (Ampel, 2010). The incidence of coccidioidomycosis has increased in the last 10 years, which could be related to climatologic factors, soil disturbance, or an influx of susceptible individuals to the endemic areas (CDC, 2013; Hector et al., 2011).

Although culture is the gold standard for definitive diagnosis of fungal infections, it often requires extended times of incubation (2–7 or more days) (Sutton, 2007). Moreover, for coccidioidomycosis sensitivity of culture is less than 50% (Saubolle, 2007) and even less than 1% for blood cultures (Saubolle et al., 2007). Although serological tests for the diagnosis of coccidioidomycosis are the most frequently used assays, serology can be falsely negative especially in immunocompromised hosts or early after infection. In addition, positive serological results may not always be indicative of active infection as antibodies can persist, even following appropriate therapy (Ampel, 2010; Crabtree et al., 2008; Durkin et al., 2009; Kuberski et al., 2010). Although histopathological studies can also be useful, fungal structures are often observed only when the infection is advanced (Cuenca-Estrella et al., 2011). *Coccidioides* has been included as a potential pathogen for bioterrorism, and handling this hazardous pathogen is limited to biosafety level 3 laboratories (Dixon, 2001). Thus, the development of new tools for early diagnosis and reducing the risk of working with cultures of *Coccidioides* are needed.

Molecular methods for the diagnosis of fungal infections may be useful, but validation of the assays is needed (Cuenca-Estrella et al., 2011; Hsu et al., 2011; Perfect, 2013). Real-time polymerase chain reaction (RT-PCR) protocols are useful tools for the diagnosis of fungal infections as they allow the detection of small amounts of fungal DNA in clinical samples. Conventional PCR and RT-PCR approaches for the

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* Corresponding author. Tel.: +1-408-998-4557; fax: +1-408-998-2723.
E-mail address: Clemons@cimr.org (K.V. Clemons).

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diagnosis and identification of *Coccidioides* spp. have been described previously; however, their sensitivity and specificity in clinical samples have varied (Bialek et al., 2004; Binnicker et al., 2007; Sheff et al., 2010; Vucicevic et al., 2010).

Animal models of coccidioidomycosis are useful, since their susceptibility to the infection is similar to that described in humans (Shubitz, 2007). Although rabbits have proven to be a good model of coccidioidal meningitis, mouse models are the most frequently used for pulmonary and systemic infections (Clemons et al., 2007). Furthermore, animal models have been used to demonstrate the usefulness of RT-PCR protocols compared to colony-forming unit (CFU) enumeration for monitoring fungal burdens of mycelial organisms during the course of the infection (Abad-Diaz-De-Cerio et al., 2013; Bowman et al., 2001; Buitrago et al., 2005; Clemons and Stevens, 2009; Morton et al., 2011; Singh et al., 2005).

In this report, we describe the development of an RT-PCR approach for the diagnosis of coccidioidomycosis. Validation of the technique was performed using unrelated clinical strains and tissues from a mouse model of systemic coccidioidomycosis. We analyzed the correlation of the RT-PCR and the CFU enumeration to determine the progression of the infection and the utility of the RT-PCR assay. The sensitivity of the RT-PCR assay was also tested in paraffin-embedded samples from animal models of coccidioidal meningitis and samples from patients.

2. Materials and methods

2.1. Design of the RT-PCR assay

2.1.1. Primers and probe design

PCR primers and a molecular beacon probe were designed on the basis of the consensus DNA sequence of the internal transcribed spacer (ITS) region from the rDNA of 16 strains of *C. immitis* and *C. posadasii* (Table 1). Three of these strains belonged to the Mould Collection from the Spanish National Center of Microbiology. Sequences for the remaining strains were obtained from GenBank. The complete ITS region sequences, aligned by using Mega 5.1 Software (Tamura et al., 2011), were used for primer and molecular beacon probe design using Beacon Designer 70 Software (Premier Biosoft, Palo Alto, CA, USA). The primers and molecular beacon probe selected were subjected to a BLASTn search in the GenBank database, and in the database of the Department of Mycology of the Spanish National Center for Microbiology, the latter of which contains more than 8000 mould sequences, to avoid cross-homology with other species, as well as be genus-specific for *Coccidioides*. The primers, CIP-1 (′5-CACCCGTGTTTACTGAAC-3′), CIP-2 (′5-CAAAGATTCGATGATTCAC-3′), and the molecular beacon probe MB-CIP (6FAM-CGCGATCTTATGTGAAGATTGTCAGTCTGAGCAGATCGCG-BHQ1) are located in a conserved region of the ITS1, which avoids differences between the 2 species of *Coccidioides*.

A previously described internal control, designed on the basis of a jellyfish-derived sequence cloned into a pICJF plasmid, with a specific molecular beacon end-labeled with CY5 and a pair of primers, was also included in each assay (Bernal-Martinez et al., 2013).

Table 1
Species identification and GenBank identification numbers from the strains used for primers and probe design in this study.

Strain	Species	GenBank ID*
Strain Silveira (ATCC28868)	*C. posadasii*	AB232886
CBS 113846	*C. posadasii*	AB186786
CBS 113843	*C. posadasii*	AB186785
CNM-CM-2912	*C. posadasii*	KF700275
CNM-CM-2911	*C. posadasii*	KF700274
RMSCC1036	*C. posadasii*	HQ219089
IFM 45815	*C. immitis*	AB232890
IFM 45816	*C. immitis*	AB232891
IFM 50992	*C. immitis*	AB232894
IFM 50995	*C. immitis*	AB232897
CBS 116.51	*C. immitis*	EF186783
CBS 113852	*C. immitis*	EF186787
CBS 113851	*C. immitis*	EF186788
CBS 113856	*C. immitis*	EF186789
RMSCC2099	*C. immitis*	EF186790
CNM-CM-7056	*C. immitis*	KF700276

* Sequence submission ID.

2.1.2. Fungal strains

Genomic DNA (gDNA) from *C. posadasii* strain Silveira (ATCC28898), *C. posadasii* (CNM-CM-2912), and *C. immitis* (CNM-CMM 7056) were used to standardize the RT-PCR assay. The specificity of this assay was assessed by including gDNA from other fungal pathogens from the mould and yeast culture collection of the Spanish National Center of Microbiology. These were: *Candida albicans* (CNM-CL-5719), *Candida parapsilosis* (CNM-CL 5683), *Candida tropicalis* (CNM-CL 5742), *Candida glabrata* (CNM-CL 5533), *Candida guilliermondii* (CNM-CL 7127), *Candida krusei* (CNM-CL 7057), *Histoplasma capsulatum* (CNM-CM 2721), *Paracoccidioides brasiliensis* (CNM-CL 2902), *Aspergillus fumigatus* (CNM-CM AF237, CNM-CM 7012, CNM-CM 7168), *Aspergillus ochraceus* (CNM-CM 7033), *Aspergillus alliaceus* (CNM-CM 7036), *Aspergillus terreus* (CNM-CM 7082, CNM-CM 3508), *Aspergillus lentulus* (CNM-CM 7152), *Aspergillus clavatus* (CNM-CM 7169), *Aspergillus flavus* (CNM-CM 7198. CNM-CM 3509), *Blastomyces dermatitidis* (CNM-CM 3113, CNM-CM 3114, CNM-CM 3115, CNM-CM 3192), *Fusarium oxysporum* (CNM-CM 2914), *Fusarium solani* (CNM-CM 3035), *Scedosporium prolificans* (CNM-CM 1627), *Scedosporium apiospermum* (CNM-CM 3169), *Rhizopus arrhizus* (CNM-CM 3020), *Rhizopus microsporus* (CNM-CM 4244), *Cryptococcus neoformans var. neoformans* (CNM-CL 3801), *Cryptococcus gattii* (CNM-CL 5007). Human and mouse DNA (Promega, Madrid, Spain) were also tested. All fungal strains had been identified previously by amplification and sequencing of the ITS region of their rDNA.

2.1.3. Fungal DNA extraction

DNA extractions from the mycelia of *H. capsulatum*, *C. immitis*, *C. posadasii*, and *P. brasiliensis* were performed in biosafety level III facilities using Wizard Genomic Purification Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions and in compliance with the Spanish laws (Real Decreto 664/1997), following the recommendations described by the CDC (2009). DNA extraction from the other filamentous fungi and yeast species was performed using a phenol-chloroform method (Tang et al., 1993).

2.1.4. RT-PCR assay

PCR reactions were performed in a Chromo 4 thermocycler real time detection system (Biorad, Hercules, CA, USA). A 2× SensiMix Probe II kit (Bioline, Taunton, MA, USA) was used following the recommendations of the manufacturer. The PCR reactions (20 μL final volume) contained 10 μL of the SensiMix mastermix (i.e., dNTPS, Taq, $MgCl_2$, etc.), 0.5 μmol/L of each CIP primer, 0.25 μmol/L of the internal control primers, 0.2 μmol/L CPI probe, 0.1 μmol/L of internal control probe, 2 fg of the internal control plasmid (pICJF), and 2 μL of the fungal DNA. The thermocycling conditions were as follows: an initial step of 95 °C for 10 min followed by 50 cycles of 95 °C for 25 s, 50 °C for 30 s, and 72 °C for 15 s. Results were considered positive when a significant fluorescent signal above the baseline was detected, as determined by second-derivate analysis, and were expressed as the crossing point (Cp).

Standard curves were constructed from PCR results from four repetitions of dilutions (from 2 ng to 2 fg of gDNA per reaction) of *C. posadasii* CNM-CM-7056, *C. posadasii* strain Silveira, and *C. immitis* CNM-CM-7056. A linear regression line ($y = mx + b$) was generated