RESEARCH NOTE

Rapid diagnosis of coccidioidomycosis by nested PCR assay of sputum

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ABSTRACT

Coccidioidomycosis is a deep infection caused by two dimorphic fungi, *Coccidioides immitis* and *Coccidioides posadasii*. Diagnosis of the disease requires culture of suspicious clinical samples on mycological media. However, as these species are virulent pathogens, handling of their cultures is a high-risk activity, and is limited to Biosafety Level 3 laboratories. This study describes the direct detection of *C. posadasii* DNA in an inappropriate sputum sample by PCR amplification of the highly specific Ag2/PRA antigen gene. The results obtained suggest that direct detection of the Ag2/PRA sequence in sputum is an excellent method for rapid and specific diagnosis of coccidioidomycosis.

Keywords Ag2/PRA gene, coccidioidomycosis, DNA extraction, molecular diagnosis, PCR, sputum

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Coccidioidomycosis is a systemic infection caused by two dimorphic fungi, *Coccidioides immitis* and *Coccidioides posadasii* [1]. Depending on the host's immune response, *Coccidioides* spp. may cause asymptomatic disease, primary or chronic pneumonia, or disseminated disease [2]. Definitive diagnosis is based on viewing spherules in clinical specimens and growing *Coccidioides* spp. in culture media [3]. However, handling of *Coccidioides* cultures is a high-risk activity, and is limited to Biosafety Level 3 laboratories [1]. The aim of the present study was to detect *C. posadasii* directly in a sputum sample by PCR detection of the Ag2/PRA gene.

Sputum smears were examined by microscopy after 30% KOH mounts and Grocott's staining [4]. Specimens were also inoculated on Sabouraud glucose agar (SGA; Difco, Detroit, MI, USA), SGA with chloramphenicol, and Mycosel agar slants (Sanofi, Marnes-La-Coquette, France) and incubated at 28°C for 2 weeks. Slides of suggestive cultures of *Coccidioides* spp. were examined by microscopy. For DNA extraction, a 1-mL aliquot of sputum was mixed with 200 µL of NaOH 5% v/v, incubated at 37°C for 1 h and centrifuged at 14 000 *g* for 5 min. The resulting pellet was washed twice with phosphate-buffered saline and the cells were then incubated with 100 µL of lyticase (Sigma Chemical Co., St Louis, USA) 3 mg/mL at 37°C for 2 h; this was followed by incubation at 56°C for 1 h with lysis buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, SDS 2% w/v) containing proteinase K (Promega, Madison, USA) 150 mg/L. Lysed cells were centrifuged at 8000 *g* for 15 s and the supernatant was then used for PCRs.

A control *C. posadasii* strain (CEMM 01-6-085) was grown on SGA at 25°C for 10 days. After this period, 5 mL of saline was added to the agar slant, and the culture was resuspended with a cotton swab. The suspension was autoclaved at 100°C for 15 min. Fragments of mycelia and arthroconidia were collected by centrifugation at 10 000 *g* for 10 min and washed twice with phosphate-buffered saline. The pellet was incubated with 1 mL of lyticase 3 mg/mL at 37°C for 2 h, and then washed with phosphate-buffered saline. Cellular debris was resuspended in lysis buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, Triton X-100 2% v/v), and then exposed to three cycles of heating at 100°C for 10 min and freezing at −80°C for 10 min. Lysed cells were centrifuged

at 8000 *g* for 15 s and the supernatant was then used in PCRs. Aliquots (0.1 mL) of each lysed sputum and culture were incubated in brain–heart infusion broth at 37°C for 20 days before being recorded as negative. The absence of any growth assured the safety of both DNA extraction methods.

PCRs were performed according to Bialek *et al*. [1] with minor modifications. The first reaction was performed in a final volume of 25 μL containing 10 μL of DNA sample, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 1.25 mM dNTPs, 2 pmol of each of the outer primers (Cocci I and Cocci II) and 1 U of *Taq* polymerase (Promega). PCR comprised 5 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 57°C and 1 min at 72°C, with a final hold for 5 min at 72°C. For the nested PCR, 1 μL of the first reaction was added to a mixture containing the same reagents described above, but with 2 pmol each of the inner primers (Cocci III and Cocci IV). PCR amplification comprised 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 1 min, with a final hold at 72°C for 5 min. PCR products were analysed by electrophoresis on polyacrylamide 6% w/v gels followed by silver staining as described previously [5].

Microscopic analysis of the sputum sample showed scanty alveolar macrophages and polymorphonuclear leukocytes, but numerous squamous epithelial cells. A few thick-walled spherules suggestive of *Coccidioides* spp. were seen (Fig. 1). Sputum cultures were found to be heavily contaminated with commensal bacteria from the mouth and pharynx. The diagnosis of coccidioidomycosis was further confirmed by an immunodiffusion test (IDCF antigen; Immy Immunodiagnostics, Norman, OK, USA) and culture. PCRs yielded amplification products of *c*. 340 bp from both sputum and filamentous culture (Fig. 2). Negative controls using molecular biology grade water instead of DNA preparations did not yield any amplification products.

The present study used a PCR assay to detect a specific DNA sequence directly in sputum, which is the most common clinical sample examined in routine mycological laboratories. Cultures of inappropriate sputum are often difficult to interpret because of heavy bacterial contamination, which may affect the analytical potential of mycological diagnosis and lead to invalid results. Specific PCR amplification may overcome these difficulties. Identification of *C. posadasii*, based on amplification of the Ag2/PRA gene, was first reported by Bialek *et al*. [1], who detected the pathogen in biopsies. Although lung biopsies are widely recognised as a valuable tool for the

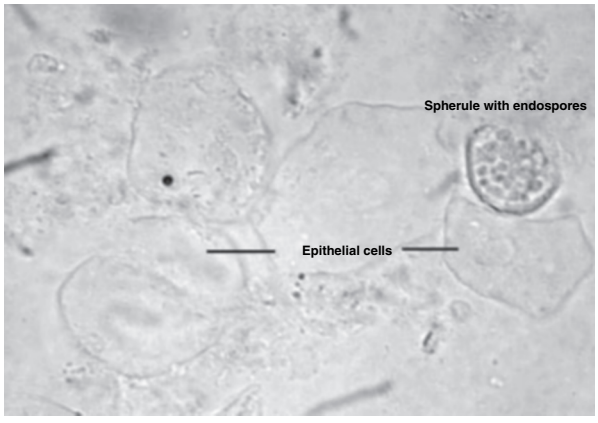

Fig. 1. Direct KOH microscopic examination of an inappropriate sputum sample showing epithelial cells and coccidioidal spherules filled with endospores.

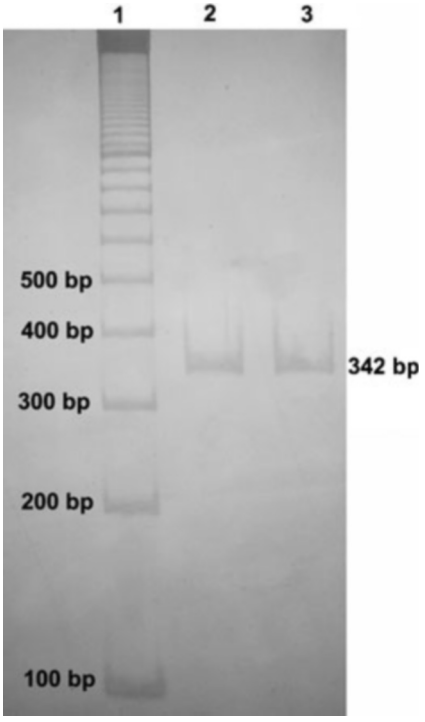

Fig. 2. PAGE of nested PCR products produced from *Coccidioides posadasii*. Lanes: 1, 100-bp ladder marker; 2, sputum sample; 3, filamentous culture (strain number CEMM 01-6-085). The 342-bp PCR amplification product specific to *C. posadasii* is indicated.

diagnosis of several pulmonary disorders [6], they should be performed only after other diagnostic procedures, e.g., bronchoalveolar lavage. In our experience, lung biopsies are rarely performed as the first-choice procedure for diagnosis of deep mycosis.

PCR amplification of DNA from both mycelia and spherules showed a single amplification product of *c*. 340 bp. A BLAST search showed homology only for *Coccidioides* spp. sequences, which suggests that amplicon sequencing is not necessary for routine diagnostic purposes. However, further studies with more patients are required to evaluate the sensitivity of the present protocol. In the protocol described by Burt *et al*. [7], filamentous cultures of *Coccidioides* spp. were grown in broth medium and then inactivated by heating, followed by freezing in liquid nitrogen and lyophilisation before extraction of DNA. The present study used an alternative method for safe DNA extraction from filamentous cultures growing on solid media. This protocol may be performed directly on suspicious cultures grown from clinical material, thereby reducing the time required to obtain definitive diagnostic results.

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RESEARCH NOTE

The use of antibiotic-containing agars for the isolation of extended-spectrum β-lactamase-producing organisms in intensive care units

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ABSTRACT

MacConkey agar containing either cefotaxime 1.0 mg/L or ceftazidime 1.0 mg/L was evaluated for use in screening for extended-spectrum β-lactamase (ESBL)-producing organisms. The media were evaluated using known ESBL-positive and -negative strains and 630 clinical specimens over a 6-month period. All Enterobacteriaceae isolated were identified and screened for ESBL production by phenotypic methods. In total, 14 ESBL-producing organisms were detected in the clinical samples. All known ESBL-positive strains were also detected. The use of both screening plates was required to detect all ESBLs.

Keywords Detection, Enterobacteriaceae, extended-spectrum β-lactamases, MacConkey selective agars, screening media, surveillance

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Detection of organisms producing an extended-spectrum β-lactamase (ESBL) is not always easy with routine susceptibility testing [1–3], and delays in recognition, resulting in unsuitable treatment with cephalosporins of severe infections caused by ESBL producers, has been linked with a rise in the mortality rate [4]. There is a need

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