Case Report

Coccidioidal pericarditis: a rapid presumptive diagnosis by an in-house antigen confirmed by mycological and molecular methods

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Coccidioidal pericarditis is a condition found in approximately 1–5 % of patients infected by *Coccidioides* species. It is associated with widely diverse clinical symptoms. This paper reports a case of coccidioidal pericarditis diagnosed by an in-house *Coccidioides posadasii* antigen and confirmed with mycological and molecular methods. From February to September 2005, the patient suffered from fever, weight loss, a non-productive cough, thoracic pain and tachycardia. He received a positive diagnosis of coccidioidal pericarditis only in October 2005. The macromorphological examination of the culture showed a whitish felt-like colony, which became brownish with age. Preparations in lactophenol cotton blue stain showed hyaline septate hyphae with fragmentation and thin arthroconidia-like structures. Pericardial fluid and sera samples were positive for *Coccidioides* antibodies by immunodiffusion and ELISA with a *C. posadasii* in-house antigen preparation. The *C. posadasii* identification was confirmed by nested PCR of the antigen 2/proline-rich antigen (Ag2/PRA) encoding gene.

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Introduction

Coccidioidomycosis is an emerging fungal infection caused by *Coccidioides immitis* and *Coccidioides posadasii* (Saubolle *et al.*, 2007). Although infections by *Coccidioides* species are usually asymptomatic or result in a mild flu-like illness, disseminated disease may occur in 1 % of cases (Saubolle *et al.*, 2007). Approximately 60 % of primary infections by *Coccidioides* spp. are asymptomatic and are only detected by positive skin tests. However, 5 % of patients with primary coccidioidomycosis may develop persistent pulmonary disease, manifested by chronic progressive pneumonia, miliary disease, pulmonary nodules or cavitation (Galgiani *et al.*, 2005).

Coccidioides spp. are dimorphic fungi that have a saprophytic phase, characterized by mycelia that produce enterothallic arthroconidia, and a parasitic phase with endosporulating *in vivo* spherules (De Hoog *et al.*, 2000).

Since 2002, based on genetic analysis of single-nucleotide polymorphisms and the size of microsatellites, *C. immitis* was recognized as two separate species, *C. immitis* and *C. posadasii*. Both species are now recognized as aetiological agents of coccidioidomycosis (Fisher *et al.*, 2002).

Coccidioidomycosis occurs only in the Americas, within a geographically delineated area of the USA, known as the Lower Sonoran Life Zone, and in semi-arid areas of Mexico, Central America and South America (Hector & Laniado-Laborin, 2005). In Brazil, coccidioidomycosis is an endemic disease in the semi-arid part of the north-east region. Since 1978, human cases have been documented in the states of Maranhão, Bahia, Piauí and Ceará (Sidrim *et al.*, 1997; Wanke *et al.*, 1999; Cordeiro *et al.*, 2006c).

Although the epidemiology of coccidioidomycosis is poorly understood in the endemic countries, it is estimated that in the USA, 150 000 new infections occur annually,

which have a substantial effect on medical costs due to the management of critically ill patients (Galgiani *et al.*, 2005). Based on the importance of coccidioidomycosis, this study reports a case of coccidioidal pericarditis diagnosed by an in-house *C. posadasii* antigen and confirmed with mycological and molecular methods.

Case report

The present case occurred in a patient from Ibiapina (3° 55' 24' S, 40° 53' 22' W), Ceará State, North-East Brazil. In February 2005, a 35-year-old man sought medical assistance at a local tertiary hospital, as he was suffering from fever (38 °C), chest pain, chills, a persistent productive cough and weight loss. Admission exams showed altered reactive C protein and haemosedimentation velocity. After clinical examination, the patient was diagnosed by echocardiogram as showing pericardial effusion, and he was treated with antibiotics and anti-inflammatory drugs for 8 days. After a month, the patient returned to the hospital with persistent clinical symptoms. At this point he underwent echocardiogram examination, which revealed a moderate dilatation of the left ventricle, signs of diastolic restriction and mild mitral regurgitation, the last of which spontaneously ceased after 8 days. At that time, the clinical diagnosis was myopericarditis. After a few days, the symptoms worsened and he was readmitted to the hospital, with fever (38 °C), chills, a persistent cough and weight loss. The patient was monitored for the following 8 months by the hospital's cardiology and internal medicine staff. From February to September 2005, the patient suffered from fever, weight loss, a non-productive cough, thoracic pain and tachycardia. In this period, the main diagnosis hypothesis was non-specific pericarditis. In October 2005, pericardial fluid was collected for pathological and microbiological tests. The analysis showed a low cell count for red cells, leukocytes and typical mesothelium cells. The exam was negative for neoplastic cells. Only at this time was the diagnosis positive for *Coccidioides* sp. Therefore, the patient was treating with 200 mg fluconazole twice a day for 6 months and his condition improved.

Methods

Microbiological procedures. A search for *Mycobacterium* spp. in pericardial fluid was performed with cultures in Löwenstein–Jensen medium and by the standard Ziehl–Neelsen method, but all the results during February to October 2005 were negative. Mycological tests of the pericardial fluid were conducted by direct microscopic examination in 30 % KOH, and culture on Sabouraud glucose agar (SGA), SGA with 0.01 % chloramphenicol and Mycosel agar slants, as previously described (Cordeiro *et al.*, 2006a). Slides were prepared with lactophenol cotton blue stain and then microscopically examined. All procedures were carried out within a class II biological safety cabinet in a biosafety level 3 laboratory, at the Specialized Medical Mycology Center (CEMM).

In-house antigen preparation. The antigenic extract was prepared with a *C. posadasii* strain (CEMM 01-6-085) isolated in Ceará State

(North-East Brazil) from a clinical source. The isolate belongs to the CEMM culture collection and was identified by mycological analysis, as well as by reactivation spherules in an animal model (Sidrim *et al.*, 1997). In brief, cultures in the mycelia phase were grown in a 2 % glucose/1 % yeast extract broth for approximately 30 days at 30 °C. Each culture was killed with 0.2 g thimerosal l⁻¹ (ethylmercuritho-salicylic acid sodium salt; Synth) and the supernatant was collected by paper filtration. Protein was precipitated with solid ammonium sulfate (Sigma) until the filtrate reached 90 % saturation. The mixture was kept at 4 °C for 24 h, and then the precipitated proteins were recovered by centrifugation and dialysed exhaustively against a 10× volume of distilled water in a dialysis membrane with a 10 kDa molecular mass cut-off. The dialysate was stored at –20 °C, and used for immunodiffusion and ELISA tests.

Serological assay. A presumptive diagnosis of coccidioidomycosis was made on the basis of the serology of pericardial fluid and two sera samples from the patient – the first one collected 1 week before the antifungal treatment and the second at 1 month after the start of the fluconazole treatment. Serology tests consisted of immunodiffusion assays with commercial *C. immitis* IDCF antigen (Immy Immunodiagnostics), carried out according to the manufacturer's instructions, and *C. posadasii* antigen prepared in our laboratory carried out according to the standard protocol described elsewhere (Camargo *et al.*, 2003), as well as indirect ELISA with our antigenic preparation. All reactions employed *Coccidioides* serum (anti-IDCF; Immy Immunodiagnostics) as a positive control. Serological analyses were also conducted by indirect ELISA with our *C. posadasii* crude antigen preparation. Microtitre plates (Corning Costar) were coated with 50 µl in-house *C. posadasii* antigen (0.5 µg per well) diluted in PBS, and incubated overnight at 4 °C. The plates were blocked with 1 % BSA (Calbiochem)/PBS, 100 µl per well, for 1 h 30 min at 37 °C. After incubation, the plates were washed four times with PBS/Tween 20 (0.05 %), and 50 µl pericardial fluid and sera samples (1:400 to 1:51 200 dilution) were added to each well. Then the plates were incubated at 37 °C for 1 h. A pool of serum samples from negative and positive controls was included in the tests for comparison. The plates were then washed again, 100 µl peroxidase-labelled goat anti-human IgG (1:2000 dilution; Sigma) was added to each well, and the plates were incubated for 1 h at 37 °C. After four washes, the reaction was developed by adding 100 µl orthophenylenediamine solution (0.4 mg ml⁻¹; Sigma) and 0.01 % (v/v) H₂O₂ in 0.1 M citrate/phosphate buffer, pH 5.0. After incubation for 30 min in the dark, the reaction was stopped by adding 25 µl 1.25 M H₂SO₄. The absorbance was measured at 492 nm by using an ELISA microplate reader (Titertek). Positive and negative serum samples were included as controls and an absorbance value twice that of the negative control (mean) value was considered as a positive result. The results of the indirect ELISA for IgG detection revealed that both serum samples from the patient gave a negative result, but the pericardial fluid presented a positive result.

Molecular identification. *C. posadasii* identification was confirmed by nested PCR of the antigen 2/proline-rich antigen (Ag2/PRA) encoding gene, as suggested by Bialek *et al.* (2004). For DNA extraction, the suspicious culture of *C. posadasii* isolated from the pericardial fluid was grown in saprophytic phase at 37 °C in 100 ml brain heart infusion medium (Difco) for 10 days. After this period, the whole culture flask was transferred to an autoclave and steamed at 1 atm (100 °C) for 15 min, as recommended by Burt *et al.* (1995). After this procedure, DNA extraction was performed with CTAB (N-cetyl-N,N,N-trimethylammonium bromide) buffer and chloroform/isoamyl alcohol solution (24:1, v/v), as suggested by Talbot (2001) for DNA extraction of filamentous fungi. Genomic DNA from a *C. posadasii* strain (CEMM 01-6-085), extracted as described above, was added as a positive control. A negative control of molecular biology-grade water was included in each reaction. Electrophoresis in 6 % polyacrilamide gels with 1× TBE buffer [89 mM Tris-borate, 2 mM

EDTA (pH 8.0)] and silver staining (Sanguinetti *et al.*, 1994) were also carried out.

Results

Mycological diagnosis

Direct microscopic examination of the clinical specimen was negative. However, after 2 weeks, a mycelial growth was detected on the SGA with chloramphenicol and Mycosel agar tubes. Slides were prepared with lactophenol cotton blue stain and then microscopically examined. We observed hyaline arthroconidia (3.5–5.0 µm), cylindrical to barrel shaped, alternating with empty disjunct cells, suggesting *Coccidioides* spp. Macromorphological examination of the fungal growth revealed a whitish felt-like colony, which became brownish with age.

Presumptive diagnosis by an in-house antigen

A positive reaction in the immunodiffusion assays was observed only with the in-house coccidioidal antigen and the pericardial fluid sample. Additionally, the serological analyses by indirect ELISA for IgG detection revealed that both serum samples from the patient were negative, but the pericardial fluid gave a positive result. Commercial positive control serum displayed positive results in commercial and experimental immunodiffusion tests, and in the experimental ELISA (Table 1).

Molecular diagnosis of *C. posadasii*

Finally, the *C. posadasii* identification was confirmed by nested PCR of Ag2/PRA antigen encoding gene, which evidenced a PCR product of approximately 340 bp. The *C. posadasii* amplicon was seen for both cultures, the one obtained from the pericardial fluid and the one from the *C. posadasii* positive control (CEMM 01-6-085 strain), as displayed in Table 1.

Discussion

In endemic sites coccidioidal arthroconidia may be aerosolized and carried over long distances or survive in appropriate soil for a long and indefinite period (Kolivras

et al., 2001). In this paper, we report a case of coccidioidomycosis in a patient who travelled to towns situated in the semi-arid region of Brazil that are considered endemic areas for coccidioidomycosis. Despite the association between armadillo hunting and coccidioidomycosis in Brazil (Cordeiro *et al.*, 2006a), the patient in this study had never hunted these burrowing animals. He stated that until coming down with the symptoms described above his health had always been excellent. He had never used immunosuppressive drugs, had never presented any base illness, had never smoked nor had tuberculosis. As tuberculosis is a very prevalent disease in Brazil, the patient was initially suspected of having a mycobacterial infection, but the searches for *Mycobacterium* spp. in pericardial fluid cultures in Löwenstein–Jensen medium and by the standard Ziehl–Neelsen method were negative from February 2005 to October 2005. In fact, coccidioidomycosis has often been misdiagnosed as pulmonary tuberculosis in Brazil (Cordeiro *et al.*, 2006b).

Only a few cases of pericarditis caused by coccidioidomycosis have been reported (Schwartz *et al.*, 1976; Chowdhury & Habibzadeh (1977); Faul *et al.*, 1999; Aquilar *et al.*, 2001; Visbal *et al.*, 2003; Arsura *et al.*, 2005; Crum-Cianflone *et al.*, 2006). This case report describes a patient with pericardial effusion and chronic fibrosing pericarditis, who later presented constrictive pericarditis. It is worth noting that pericardial coccidioidomycosis is a disease with no pathognomonic symptoms, which may hamper clinical diagnosis. Therefore, simultaneous laboratory approaches such as culture, PCR amplification and serology were necessary to confirm the diagnosis.

Although *Coccidioides* spp. colonies show rapid growth on conventional mycological media (Sutton, 2007), the *C. posadasii* strain isolated in this study showed visible growth only after 2 weeks of incubation. We presume that the isolated strain may be a mutant displaying slow growth in synthetic substrate. Further studies will be conducted in our laboratory in order to test this hypothesis.

Immunodiffusion tests against in-house antigen preparations and commercial *Coccidioides* spp. antigens revealed discrepant results. A positive reaction was detected only between the pericardial liquid sample and our crude antigen preparation. According to Pappagianis (2001),

Table 1. Laboratory test results for the patient with coccidioidal pericarditis

Sample	Commercial immunodiffusion (antigen from <i>C. immitis</i>)	Experimental immunodiffusion (antigen from <i>C. posadasii</i>)	Experimental ELISA (IgG) (antigen from <i>C. posadasii</i>)
Pericardial liquid	Negative	Positive	Positive
Serum 1 (before treatment)	Negative	Negative	Negative
Serum 2 (during treatment)	Negative	Negative	Negative
Commercial positive control serum (against <i>C. immitis</i>)	Positive	Positive	Positive
Negative control serum	Negative	Negative	Negative

negative serological results in patients with fungal infections are relatively infrequent and are associated with immunocompromised states, and in these cases negative results do not rule out the presence of the disease. Since the patient in this study was not immunocompromised, we believe that our antigen may contain molecules with different epitopes that are recognized by antibodies produced during infections with local *C. posadasii* strains. A remarkable aspect to this case is the negative *Coccidioides* serology results of serum samples with both commercial and experimental antigens. Based on the ELISA results, we presume this patient had a localized infection rather than a disseminated disease, which could explain the positive immune reactions only in the pericardial fluid. Negative serology among patients with disseminated coccidioidomycosis has been previously described (Assi *et al.*, 2006).

Although *in vivo* conversion is still regarded as an important tool to confirm identification of suspected isolates of *Coccidioides* spp. (Clemons *et al.*, 2007), in our laboratory we decided to perform *C. posadasii* identification based on three independent steps: culture, serology and specific PCR reaction. In our opinion, the use of these approaches simultaneously can substitute for animal experimentation.

After laboratory diagnosis, the patient was treated with 200 mg fluconazole twice a day for 6 months. The symptoms regressed and he did not have any clinical relapse after finishing the treatment. Arsura *et al.* (2005) reported that pericardial coccidioidomycosis can be treated with fluconazole or amphotericin B, but there is high morbidity and mortality (23 % of the patients die). Pericardiectomy for pericardial coccidioidomycosis has been achieving good results in young patients, as was observed in our patient.

Coccidioidal pericarditis has a relatively unfavourable prognosis. In this study, we used different laboratory approaches to achieve the correct diagnosis. Culture of clinical specimens and molecular identification of *C. posadasii* by PCR may become an important approach for a precise diagnosis of non-pulmonary coccidioidal infections. The results here show the importance of producing antigens from local strains in order to improve coccidioidomycosis diagnosis, mainly in extra-pulmonary infections. To the best of our knowledge, so far, there have been no reports of Brazilian strains being used to prepare antigens for serological diagnosis of coccidioidomycosis.

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