In vitro inhibitory effect of antituberculosis drugs on clinical and environmental strains of *Coccidioides posadasii*

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Received 26 February 2006; returned 12 April 2006; revised 22 May 2006; accepted 23 June 2006

**Objectives:** The aim of the present study was to evaluate the *in vitro* effect of the first-line antimicrobial drugs for pulmonary tuberculosis against the fungal pathogen *Coccidioides posadasii*.

**Methods:** The *in vitro* activities of rifampicin, isoniazid, pyrazinamide and ethambutol against clinical and environmental strains of *C. posadasii* were determined in accordance with the CSLI M38-A macrodilution method. The antimicrobials were tested alone or in combinations of two or more drugs.

**Results:** With the exception of pyrazinamide, all of the tested drugs interfered with the *in vitro* growth of *C. posadasii*. The 2 day MIC ranges of the tested drugs were as follows: rifampicin 1060–4250 mg/L; isoniazid <250 mg/L; ethambutol <620 mg/L. Pronounced *in vitro* synergism was demonstrated for combined antituberculosis drugs. The combination of rifampicin plus pyrazinamide was the only one that did not inhibit fungal growth.

**Conclusions:** The present study showed that the first-line antituberculosis drugs, alone or in combinations, interfered with the vegetative growth of *C. posadasii* *in vitro*. Further studies in a murine model will need to be conducted in order to evaluate the *in vivo* effect of antituberculosis drugs on *Coccidioides* spp.

**Keywords:** susceptibility testing, coccidioidomycosis, pulmonary tuberculosis, Brazil

**Introduction**

Tuberculosis and coccidioidomycosis are pulmonary infectious diseases that share clinical, radiographic and histopathological characteristics.¹ The diagnosis of both diseases includes direct microscopy examination, culture and thorax radiography, as well as common clinical criteria.¹,²

Tuberculosis is one of the main public health problems in Brazil, where nearly 50 million people are infected by *Mycobacterium tuberculosis*, and it is believed that more than 100,000 new cases occur annually.³ More than 70% of all cases of the disease occur in northeast and southeast regions and this situation may be influenced by heterogeneous underlying factors such as social inequality, high AIDS prevalence in urban areas and the increasing multidrug resistance displayed by the bacilli.³

*Coccidioidomycosis* is a deep fungal infection caused by the dimorphic species *Coccidioides immitis* and *Coccidioides posadasii*.⁴ The disease is endemic in the semi-arid northeast region of Brazil and, since 1997, outbreaks have been documented in the states of Maranhão, Piauí, Ceará and Bahia.³,⁵ Unfortunately, the epidemiology of coccidioidomycosis in Brazil is poorly understood, as it is not a notifiable disease. Consequently, few cases of the disease have been diagnosed in the endemic areas, despite the suitable environmental conditions for coccidioidomycosis development, i.e. high temperatures, xerophytic vegetation, recurrent droughts and friable soils.³,⁷ In the State of Ceará, a previous epidemiological survey with spherulin revealed positivity of 11.5%³ but, since 1997, only 10 cases of coccidioidomycosis have been registered in this state.⁷ Despite the importance of the microbiological diagnosis, it is estimated that, in Brazil, ~26.7% of adult patients suspected of...
Materials and methods

Fungal cultures

A total of 10 strains of C. posadasii isolated in the State of Ceará from clinical (n = 7) and environmental (n = 3) sources were included in the study. The strains belong to the Medical Mycology Specialized Center Fungal Collection (Department of Pathology and Legal Medicine, Federal University of Ceará, Brazil). The identification procedures for each strain included mycological analysis, arthroconidia and hyphae was diluted 1:10 with RPMI 1640 medium and adjusted to 95% transmittance. The suspension containing arthroconidia and hyphae was diluted 1:10 with RPMI 1640 medium (Sigma Chemical Co., St Louis, MO, USA) containing L-glutamine and without sodium bicarbonate and buffered to pH 7.0 with 0.165 M MOPS. Final concentrations of each antimicrobial solution were prepared with RPMI 1640 medium buffered with 0.165 M MOPS (Sigma Chemical Co., St Louis, MO, USA) to obtain an inoculum of 1 x 10^3 to 5 x 10^3 cfu/mL.

Inoculum preparation for the antifungal susceptibility test

The strains of C. posadasii were obtained from storage in 0.9% saline at 4°C, subcultured onto Sabouraud glucose agar (Difco, Detroit, USA) and incubated at 25°C for 10 days. Prior to antimicrobial testing, the viability and purity of each isolate was evaluated by microscopic examinations. For the inoculum preparation, sterile normal saline was added to the agar slant, and the cultures were gently scraped with cotton swabs. The suspension was transferred to a sterile tube, allowed to settle for 5 min, and the upper homogeneous supernatant was read at 530 nm and adjusted to 95% transmittance. The suspension containing arthroconidia and hyphae was diluted 1:10 with RPMI 1640 medium (Sigma Chemical Co., St Louis, MO, USA) containing L-glutamine and without sodium bicarbonate and buffered to pH 7.0 with 0.165 M MOPS (Sigma Chemical Co., St Louis, MO, USA) to obtain an inoculum of 1 x 10^3 to 5 x 10^3 cfu/mL.

Antimicrobial drugs

Stock solutions of rifampicin, isoniazid, pyrazinamide and ethambutol (Ministério da Saúde, Brazil) were prepared in DMSO and stored at –20°C until use. Combinations of two or more antituberculosis drugs were prepared in DMSO at the time of use. Serial 2-fold dilutions of each antifungal solution were prepared with RPMI 1640 medium buffered with 0.165 M MOPS. Final concentrations of each antifungal drug or drug combinations ranged as displayed in Table 1.

In vitro susceptibility tests

The susceptibility pattern of C. posadasii strains to antituberculosis drugs was determined by a broth macrodilution assay. The analyses were performed according to CLSI (formerly NCCLS) guidelines, by way of the M38-A protocol, which was developed for in vitro antifungal susceptibility testing.10 Sterile plastic screw-cap tubes containing 0.1 mL of the antifungal drug or drug combinations described in Table 1 were inoculated with 0.9 mL of suspension of each isolate. The procedures were repeated at least twice and each fungal strain was tested in duplicate. The results were read visually, as recommended by CLSI M38-A method for antifungal drugs. MIC endpoints were determined after intervals of 2 days of incubation at 35°C. Additional readings were performed after 4 and 7 days of incubation. The MIC of each drug or drug combination was defined as the lowest drug concentration that caused 80% inhibition of visible fungal growth.10 The results were evaluated by means of qualitative analysis.

Results

With the exception of pyrazinamide, all the tested drugs interfered in the in vitro growth of C. posadasii (Table 2). The 2 day MIC ranges of the tested drugs were as follows: rifampicin 1060–4250 mg/L (mean of 2270 mg/L); isoniazid ≤250 mg/L; ethambutol ≤620 mg/L. The MIC values for each duplicate isolate were identical. MIC endpoints determined after 2, 4 and 7 days showed an increasing trend in the values for all the tested drugs. Better results were obtained with isoniazid, which presented smaller MICs than ethambutol and rifampicin.

Compared with the results obtained with single drugs, increased inhibitory effects were seen for the antituberculosis drugs used in combination (Figure 1). The combination formed by rifampicin with pyrazinamide was the only one that did not inhibit fungal growth even at the highest concentrations tested (rifampicin, 1500 mg/L; pyrazinamide, 12,500 mg/L). On the second day of the experiment, all C. posadasii strains were inhibited by the following drug combinations: ethambutol [70 mg/L] plus isoniazid [150 mg/L]; rifampicin [260 mg/L] plus isoniazid [70 mg/L] plus ethambutol [310 mg/L]; rifampicin [310 mg/L] plus isoniazid [460 mg/L] plus pyrazinamide [780 mg/L]; rifampicin [280 mg/L] plus isoniazid...
In vitro inhibitory effect of antituberculosis drugs

Table 2. In vitro susceptibility pattern of antituberculosis drugs against Brazilian C. posadasii isolates

<table>
<thead>
<tr>
<th>Strain source</th>
<th>MIC (mg/L)</th>
<th>rifampicin</th>
<th>isoniazid</th>
<th>ethambutol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 days</td>
<td>4 days</td>
<td>7 days</td>
<td>2 days</td>
</tr>
<tr>
<td>Environmental</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2120</td>
<td>8500</td>
<td>≥8500</td>
<td>&lt;250</td>
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<tr>
<td>2</td>
<td>4250</td>
<td>4250</td>
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<td>&lt;250</td>
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<td>3</td>
<td>1060</td>
<td>4250</td>
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<td>&lt;250</td>
</tr>
<tr>
<td>Human</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>2120</td>
<td>4250</td>
<td>≥8500</td>
<td>&lt;250</td>
</tr>
<tr>
<td>5</td>
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<td>4250</td>
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<td>&lt;250</td>
</tr>
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<td>7</td>
<td>4250</td>
<td>8500</td>
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<td>&lt;250</td>
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<td>9</td>
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<td>10</td>
<td>1060</td>
<td>4250</td>
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<td>&lt;250</td>
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<tr>
<td>Geometric range</td>
<td>2273</td>
<td>5607</td>
<td>8500</td>
<td>250</td>
</tr>
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</table>

Discussion

Coccidioidomycosis is a potentially fatal disease, but it is estimated that 60% of exposures to the fungi conidia do not present any symptoms, or that they are so mild that patients do not seek medical care.2,11 The most common form of the disease is acute or subacute pneumonic illness,12 and in these cases some patients may present a gradual resolution of symptoms, over 2–6 weeks, even without antifungal treatment. However, some patients seek medical attention because of the long duration of the disease or aggravation of symptoms.2,11 Coccidioidomycosis and tuberculosis share clinical features and several cases of both diseases occurring simultaneously have been reported since the early 1950s.13,14 In northeast Brazil, many people with the acute pulmonary form of coccidioidomycosis undergo inappropriate treatment for pulmonary tuberculosis. As primary coccidioidomycosis may be a self-limited and benign infection, it is believed that most patients recover spontaneously,11 even without antifungal therapy. Another possible hypothesis is that the drugs used to treat pulmonary tuberculosis may, somehow, promote the recovery of these patients. Both ideas may be considered in order to explain the reduced number of coccidioidomycosis cases in this region, in spite of the environmental conditions, previously mentioned.5,7 In northeast Brazil, tuberculosis and coccidioidomycosis seem to occur in a common geographic area.

In the present study, the in vitro effect of antituberculosis drugs on C. posadasii strains was evaluated by way of a macrodilution assay, in accordance with the document M38-A, published by CLSI, for in vitro antifungal testing of conidium-forming filamentous fungi. This protocol standardized important

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**Figure 1.** In vitro susceptibility pattern of antituberculosis drugs tested in combination against Brazilian isolates of Coccidioides posadasii, by way of macrodilution test broth. INH, isoniazid; EMB, ethambutol; RIF, rifampicin; PZA: pyrazinamide. A1: INH [0.15 mg/mL] plus EMB [0.07 mg/mL]; A2: INH [0.31 mg/mL] plus EMB [0.15 mg/mL]; A3: INH [0.62 mg/mL] plus EMB [0.31 mg/mL]; B1: RIF [0.26 mg/mL] plus INH [0.07 mg/mL] plus EMB [0.31 mg/mL]; B2: RIF [0.52 mg/mL] plus INH [0.15 mg/mL] plus EMB [0.62 mg/mL]; B3: RIF [2.08 mg/mL] plus INH [0.6 mg/mL] plus EMB [2.48 mg/mL]; B4: RIF [4.16 mg/mL] plus INH [1.2 mg/mL] plus EMB [4.96 mg/mL]; C1: RIF [0.31 mg/mL] plus INH [0.46 mg/mL] plus PZA [0.78 mg/mL]; C2: RIF [0.62 mg/mL] plus INH [0.93 mg/mL] plus PZA [1.56 mg/mL]; D1: RIF [0.28 mg/mL] plus INH [0.40 mg/mL] plus PZA [0.78 mg/mL] plus EMB [0.53 mg/mL]; D2: RIF [0.56 mg/mL] plus INH [0.81 mg/mL] plus PZA [1.56 mg/mL] plus EMB [1.06 mg/mL]. [400 mg/L] plus pyrazinamide [780 mg/L] plus ethambutol [530 mg/L]. There was an increasing trend seen in the MIC values on the seventh day of the experiment for all the tested drug combinations.
variables such as pH and medium composition, temperature and period of incubation, inoculum size, and MIC endpoints. It is a reference for the development of more effective tools for in vitro antifungal susceptibility testing. Although it deals specifically with antifungal drugs, M38-A was chosen as the experimental method in this study of antibacterial drugs because it allows reproducible results.

The results of the present study demonstrated that, with the exception of PZA, all the tested drugs interfered with the in vitro growth of C. posadasii strains. It is well known that, in vitro, pyrazinamide is active only in acid pH of 5.5 or less, similar to that found in early tubercular inflammatory lesions. The susceptibility assays in the study were performed at pH 7.0, which may have interfered with the drug action.

Previous studies revealed that the antituberculosis agents tested in the present study target several enzymes, causing damage to the bacterial metabolism. It has been proved that rifampicin inhibits bacterial RNA biosynthesis, by binding itself to the beta subunit of DNA-dependent RNA polymerase. Isoniazid and ethambutol interfere with the synthesis of the mycobacterial cell wall, by inhibiting fatty acid desaturase or arabinosyl transferases, respectively. Pyrazinamide may cause a collapse in the membrane potential, thus affecting the membrane transport function of mycobacterium in acid pH. Analogous binding sites to these drugs may be found in the fungal mitochondria, resulting in a mild inhibitory effect. In the present study, better results were obtained when the drugs were tested in combination, suggesting a synergistic effect on fungi structures.

During pulmonary coccidioidal infection, arthroconidia found in the mycelial form are inhaled and converted into yeast-like structures filled with endospores, which are easily seen in sputum. Although it is reasonable to suppose that the results of susceptibility tests against the parasitic form of the fungus are reliable, even with a possible therapeutic correlation, few studies have tested the susceptibility pattern of the yeast-like form of the microorganism. On the other hand, susceptibility tests with cells at the saprophytic stage are easier to perform and the standardized M38-A protocol can be applied, generating comparable results. The main advantage in performing susceptibility tests with the yeast-like form of the microorganism is biosafety, as the yeast-like structures are less virulent than the mycelial form. If the target site of the tested drugs is in the mitochondrion, both mycelial and yeast-like forms may have the same susceptibility pattern. Further investigations are required to evaluate this hypothesis.

Differences among the susceptibilities of C. posadasii strains isolated from clinical or soil samples were not detected. According to Cordeiro et al., clinical and environmental C. posadasii strains also displayed similar susceptibilities against antifungal drugs. The source of strain isolation may not be connected to in vitro antimicrobial susceptibility results. More extensive studies with a higher number of strains are required to test this hypothesis.

Other studies have indicated that amphotericin B and rifampicin act synergistically inhibiting in vitro C. immitis mycelial growth. Based on the results obtained in the present study, it would seem appropriate to assess the effect of antituberculosis drugs combined with antifungals on Coccidioides spp. growth.

In this study, it was seen that the first-line antituberculosis drugs alone or in combinations interfered with the vegetative growth of C. posadasii strains in vitro. Further studies with a murine model will need to be conducted in order to evaluate the in vivo effect of these drugs, together with epidemiological surveys of patients who have received antituberculosis therapy in northeast Brazil.

Acknowledgements
This work was supported by CNPq Conselho Nacional de Desenvolvimento Científico e Tecnológico (Process: 620053/2004-6) and FAPESP Fundação de Amparo à Pesquisa do Estado de São Paulo (Process: 4/14270-0).

Transparency declarations
None to declare.

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