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A Case of Relapsed Chromoblastomycosis Due to *Fonsecaea monophora*: Antifungal Susceptibility and Phylogenetic Analysis

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Abstract Chromoblastomycosis is a chronic cutaneous and subcutaneous mycosis. The management of this infection continues to be challenging because there is no consensus on the therapeutic regimen. We report here a case of a 69-year-old male patient with cauliflower-like lesions on his left leg and foot. He had already been treated with itraconazole at a dose of 200 mg/day for 5 months, with mycological cure for all the affected areas. However, the lesions relapsed at both sites, and treatment with itraconazole was resumed at the dose previously used. Initially, direct mycological examination, cultural, and microculture slide observation were performed. Afterward, sequencing of the ITS1-5.8S rDNA-ITS2 region of the fungal DNA and evaluation of its susceptibility to antifungal agents alone and in combination were performed. In direct mycological examination, the presence of sclerotic cells was verified, and the fungus was identified as *Fonsecaea* based on cultural and microscopic examinations. Identification as *Fonsecaea monophora* was confirmed after sequencing of the ITS region and phylogenetic analysis. The isolate was susceptible to itraconazole and terbinafine. The combinations of amphotericin B and terbinafine and terbinafine and voriconazole were synergistic. The use of drugs for which the causative agent is susceptible to singly or in combination may be an alternative for the treatment of mycosis. Furthermore, the identification of the agent by molecular techniques is important for epidemiological purposes. To the best of our knowledge, this is the first case of relapsed chromoblastomycosis caused by *F. monophora* in Brazil.

Keywords Checkerboard technique · Chromoblastomycosis · *Fonsecaea monophora* · In vitro antifungal susceptibility · ITS rDNA · Recurrence

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Introduction

Chromoblastomycosis is a chronic mycosis of polymorphic appearance, slowly progressing, that affects

the skin and subcutaneous tissue. It is caused by several species of dematiaceous fungi, such as *Fonsecaea spp.* [1–3]. This mycosis has a cosmopolitan distribution, being mostly prevalent in tropical and subtropical regions [4]. Recently, different species within the *Fonsecaea* genus were described [5–7]. *Fonsecaea monophora*, one of the species, has a distinctive clinical aspect in relation to other species, appearing as a general opportunist fungus causing, besides cutaneous and subcutaneous chromoblastomycosis, infections in internal organs, such as the brain [8, 9]. Furthermore, it is reported that this agent has a better response to treatment compared to *F. pedrosoi* [9–11], although recently resistance was verified in a case of chromoblastomycosis caused by *F. monophora* [12]. Relapse of the infection caused by this fungus is rarely reported in the literature [13], and to the best of our knowledge, this is the first case of relapsing chromoblastomycosis by *F. monophora* in Brazil.

Therefore, we report a case of chromoblastomycosis caused by *F. monophora* in Brazil where mycological cure was initially achieved with itraconazole, but the lesions relapsed later. The clinical isolate after the relapse was identified molecularly and evaluated for its susceptibility to drugs currently used in the clinic, both singly and in combination.

Case Report

A male 69-year-old patient, Brazilian, farmer, presenting lesions in his left leg and foot with a diagnosis of chromoblastomycosis was sent from a Health Unit of the city of São Leopoldo, Rio Grande do Sul, Brazil, to the Dermatology Department at Santa Casa de Misericórdia de Porto Alegre Hospital Complex, in the same State, in January 2011. Prior to referral to the Hospital Complex, the patient had already been treated with 200 mg/day itraconazole for 5 months, showing mycological cure of all the affected sites. In 2010, there was a recurrence of the lesions, and the treatment with 200 mg/day itraconazole was retaken. At the Dermatology Service, the patient denied comorbidities, except for the deformation in the foot. He presented edema in the left foot and leg, verrucous lesions, and papules on the dorsum of the foot and heel (Fig. 1a, b). For confirmation of the diagnosis, a mycological direct exam was carried out, with 20 % potassium hydroxide, and the presence of sclerotic cells was verified (Fig. 1c). After confirmation of the presence of fungal material, a sample was inoculated on Sabouraud agar and incubated for 10 days at 25 °C. Following incubation, the presence of dark-olivaceous filamentous colonies were observed (Fig. 2b). The culture was observed microscopically with lactophenol cotton blue staining. Dematiaceous septate hyphae and conidiogenesis were characteristic of the *Fonsecaea* species (brown conidiophores and ovoid conidia located either at the end or at the side of conidiophores) (Fig. 2a). The treatment with itraconazole in the dosage mentioned above was maintained, with significant regression of lesions after 1 month, and the patient was lost to follow-up.

The molecular identification of the isolate was performed from the culture grown aerobically in Sabouraud broth at 28 °C, with shaking at 180 rpm for 4 days. Total genomic DNA was extracted and purified from 100 mL cultures using the UltraClean® Soil DNA Isolation Kit (Mobio, USA). The Internal Transcribed Spacer (ITS) region was PCR amplified as described previously [14]. The PCR product was purified by the UltraClean® PCR Clean-Up Kit (Mobio,USA). Cycle sequencing employed standard protocols with the following primers: ITS1 (5′-TCC

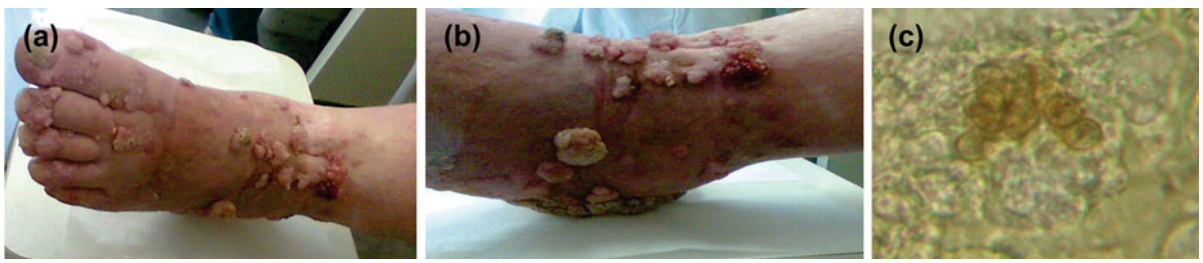

Fig. 1 **a** and **b** Lesions caused by *Fonsecaea pedrosoi* (69,704) in the pacient's left foot and leg. **c** Direct microscopic examination (KOH 20 %) revealed sclerotic cells (X 400)

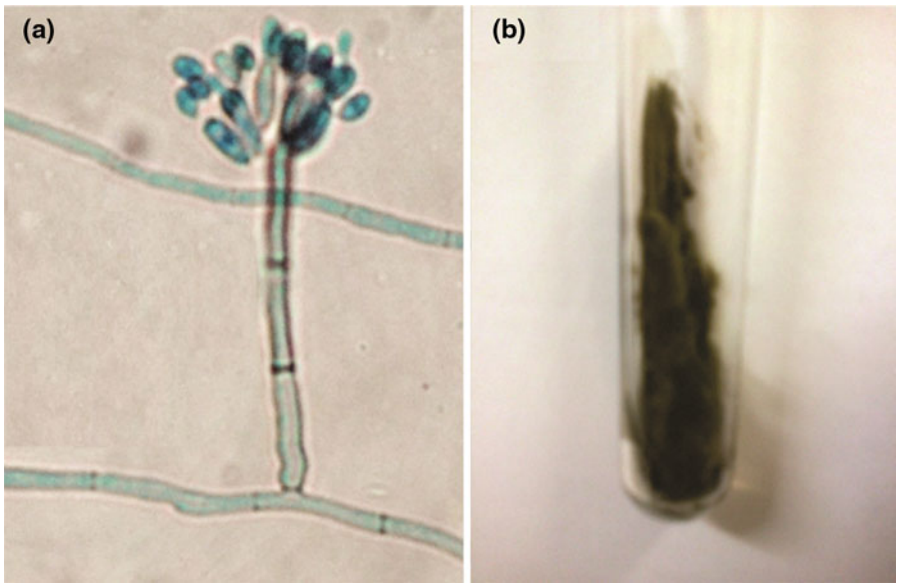

Fig. 2 **a** Lactophenol cotton blue-stained microculture of the fungus, showing septate hyphae with erected conidiophores and ovoid conidia located either at the end or at the side of conidiophores, characteristic of the genus *Fonsecaea*. (X 400) **b** Presence of dark-olivaceous filamentous colonies in Sabouraud dextrose agar cultured for 10 days, at 25 °C

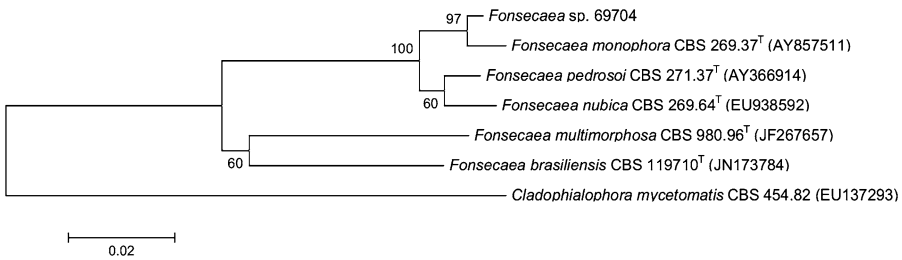

Fig. 3 ITS tree showing the phylogenetic relationship among the genus *Fonsecaea*, obtained by neighbor-joining analysis using Mega 5.0. The numbers given on the branches are the frequencies with which a given branch appeared in 1,000 bootstrap replications. *Cladophialophora mycetomatis* CBS 454.82 was used as outgroup. Strain 69,704 and *Fonsecaea monophora* CBS 269.37^T are typed in boldface

GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′). Sequences were obtained with an ABI-PRISM 3100 Genetic Analyzer (Applied Biosystems), according to the manufacturer's instructions. Alignments and phylogenetic trees were constructed with MEGA 5 [15], using the neighbor-joining method with bootstrap analysis based on 1,000 random samplings. Gaps were treated as missing data. Additional sequences were retrieved from GenBank (accession numbers are indicated on the phylogenetic tree). The isolate was identified as *F. monophora* after the phylogenetic analysis (Fig. 3). This strain was added into the culture collection of Pathogenic Fungi Laboratory of the Federal University of Rio Grande do Sul as 69,704.

As there has been a relapse after the first treatment with itraconazole, we made a proper assessment of the susceptibility profile of this isolate to the antifungal agents employed either individually or in combination.

The evaluation of antifungal susceptibility was performed according to the M38-A2 document of the *Clinical and Laboratory Standards Institute* [16], and, in parallel, an evaluation of combinations of the antifungal agents through the checkerboard technique [17] was carried out. The interaction coefficient among drugs was quantitatively evaluated by means of the fractional inhibitory concentration index (FICI), which was calculated by the following formula: FICI = (MIC A in combination/MIC A) + (MIC B in combination/MIC B). The interaction was defined as synergistic if the FIC index was ≤ 0.5, no interaction if $0.5 > \text{FICI} \leq 4.0$, and antagonistic if FICI was > 4.0, as applied in recent studies [18–20]. The antifungals itraconazole (ITZ), voriconazole (VRZ),

terbinafine (TRB) and amphotericin B (AMB) were used in the concentration ranges from 0.015 to 8 μg/mL for the first two, 0.001 to 2 μg/mL for TRB, and 0.03 to 16 μg/mL for AMB. The isolate was susceptible to ITZ and to TRB, with minimum inhibitory concentration (MIC) of 0.125 μg/mL, followed by VRZ with MIC of 1 μg/mL and by AMB, with a MIC of 2 μg/mL. In relation to the combinations, the associations of TRB and AMB (FICI = 0.27) and of TRB and VRZ (FICI = 0.37) proved to be synergistic, and the first combination showed greater synergism, with a decrease in the antifungal MICs in combination when compared to the MICs of the drugs alone. The other associations were indifferent.

Discussion

Fonsecaea spp. are the major etiologic chromoblastomycosis agents in Brazil and worldwide [3]. Among the most commonly used methodologies for molecular identification of agents of chromoblastomycosis are the techniques of sequencing and the analysis of sequence data of the ribosomal DNA (rDNA) Internal Transcribed Spacer (ITS) regions [15, 21, 22]. With these techniques, there was a significant increase in knowledge concerning the genus *Fonsecaea.*

There was a recent taxonomic revision of this genus [5–7, 23], and from data obtained by sequencing, five species are currently recognized: *F. pedrosoi*, *F. monophora*, *F. nubica, F. multimorphosa* and *F. brasiliensis*. The first three species were found to cause fungal infections in humans. These species are morphologically identical, but appear distinctly in the clinic. Whereas *F. pedrosoi* and *F. nubica* seem to be associated exclusively with chromoblastomycosis, *F. monophora* was also isolated from brain abscesses, cervical lymph nodes, and bile [8, 13, 24].

The phylogenetic analysis of the ITS sequences demonstrates that *F. pedrosoi* and *F. monophora* form a sister cluster to *F. nubica* and are more distantly related to *F. multimorphosa* and *F. brasiliensis* (Fig. 3). As several sequences deposited in GenBank as *F. pedrosoi* belong in fact to *F. monophora*, the identification of strain 69,704 could only be attained after the phylogenetic analysis. Strain 69,704 grouped with the type strain of *F. monophora* (CBS 269.37^{T}).

Usually individuals affected by chromoblastomycosis are farmers, male, with 30 years or more [25], characteristics which are consistent with our patient. Probably, he has been contaminated by contact of an injured foot with soil or organic matter where fungal propagules were present, due to his occupation.

Many strategies are used in chromoblastomycosis because the treatment of this disease is extremely difficult. Often patients are refractory to various therapeutic options and are prone to relapses. In general, the management of this mycosis should be guided according to clinical, mycological, and histopathological criteria [1, 3].

Among the methods used are the physical therapies, chemotherapy, and combination of both. Concerning the first category, surgical excision, cryotherapy, local therapy with heat, and electrodessication can be highlighted [1, 2]. Regarding chemotherapy, various drugs have been used, such as ketoconazole, posaconazole, voriconazole, amphotericin B, 5-flucytosine, terbinafine, and itraconazole [26–31]. Amphotericin B and 5-flucytosine are used in combination, as well as terbinafine and itraconazole [3, 32]. Itraconazole and terbinafine seem to be the drugs with better results for the treatment of chromoblastomycosis [2, 3, 32]. This was confirmed in our in vitro susceptibility assays, where these two drugs used alone showed the lowest MICs.

The combination of terbinafine and itraconazole was evaluated in vitro on 18 strains of *F. monophora*, and for 12 isolates, this combination showed a synergistic effect and did not show an antagonistic effect [33]. In our study, we found that only the combination of amphotericin B and terbinafine, and terbinafine and voriconazole were synergistic in vitro. It is interesting that both voriconazole and amphotericin B, which had the highest MICs when used alone, have shown synergism when combined with terbinafine. In this sense, the use of combinations of antifungal agents in situations where there is no response to drug therapy when used alone or in cases with high severity, which usually occur in this mycosis, may be an option to enhance the efficacy of each antifungal and obtain high efficiency using low doses [34].

We know that our patient was previously treated with itraconazole and had mycological cure, but suffered relapse. Hypothesis for this recidive are as follows:

1. The use for 6 months or less in antifungal therapy can result in good clinical results; however, relapses during therapy or after are common [3].

2. Itraconazole needs a low pH of the stomach to be properly absorbed [35]. The elderly may have a decreased production of gastric juice, and this results in higher stomach pH, thus reducing the bioavailability of this drug and hence its activity [36].
3. In elderly, cells of the immune system decrease in number and defense capability, and there is a decline in the immune response [37].

In the light of the large number of recurrences and the fact that there is no treatment of choice for this neglected mycosis, the alternating use of antifungal agents which present satisfactory activity or even the use of antifungal agents that present synergistic activity in combined form are possible ways to avoid the relapse problem. In the present study, the combination of amphotericin B and terbinafine, for example, which showed the best results in vitro, would be an eligible choice therapy. Furthermore, the use of molecular tools for proper identification is of utmost importance in order to generate accurate epidemiological information about the causative agents of chromoblastomycosis.

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Conflict of interest The authors declare there are no conflicts of interest.

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