

Rhinochadiella aquaspersa, proven agent of verrucous skin infection and a novel type of chromoblastomycosis

H. BADALI *†‡, A. BONIFAZ§, T. BARRÓN-TAPIA#, D. VÁZQUEZ-GONZÁLEZ§, L. ESTRADA-AGUILAR#, N. M. CAVALCANTE OLIVEIRA+, J. F. SOBRAL FILHO\$, J. GUARRO^, J. F. G. M. MEIS@ & G. S. DE HOOG*†~

*CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands, †Institute of Biodiversity and Ecosystem Dynamics, University of Amsterdam, Amsterdam, The Netherlands, ‡Department of Medical Mycology and Parasitology, School of Medicine/Molecular and Cell Biology Research Center, Mazandaran University of Medical Sciences, Sari, Iran, §Mycology Department, Dermatology Service, General Hospital of Mexico, #Dermatology Service, Regional Hospital Lic. Adolfo López Mateos, Mexico, +Laboratório de Micologia, Departamento de Ciências Farmacêuticas, \$Serviço de Dermatologia, Hospital Universitário Lauro Wanderley, Universidade Federal da Paraíba, Joao Pessoa, Brazil, ^Mycology Unit, Medical School, IISPV, Rovira i Virgili University, Reus, Spain, @Department of Medical Microbiology and Infectious Diseases, Canisius Wilhelmina Hospital, Nijmegen, The Netherlands, and ~Peking University Health Science Center, Research Center for Medical Mycology, Beijing, China

We report a case of chromoblastomycosis which resembled sporotrichosis due to the presence of warty nodules and lymphatic distribution on the forearm in a 56-year-old male. Mycological and histopathological investigation of exudates and biopsy tissue samples revealed a granulomatous lesion with muriform cells, the hallmark of chromoblastomycosis. The infection showed only localized expansion with verrucous plaques suggesting a new clinical type of the disease. The causative agent was identified as *Rhinochadiella aquaspersa*. This case prompted a study of the clinical spectrum of *R. aquaspersa*, through which we identified a second case caused by this fungus in a 62-year-old Brazilian female. The case was unusual in that *R. aquaspersa* exhibited hyphae rather than muriform cells in tissue. Given the difficulties treating chromoblastomycosis and other infections caused by melanized fungi, we evaluated the *in vitro* activities of extended-spectrum triazoles, amphotericin B, and echinocandins against these clinical isolates of *R. aquaspersa*. Itraconazole (MIC; 0.063 mg/l) and posaconazole (MIC; 0.125 mg/l) had the highest *in vitro* activities, while voriconazole and isavuconazole had somewhat lower activities (MICs; 2 mg/l) against the isolates. Amphotericin B and anidulafungin each had an MIC of 1 mg/l, whereas the MIC of caspofungin was 8 mg/l.

Keywords chromoblastomycosis, *Rhinochadiella aquaspersa*, *Rhinochadiella phaeophora*, ITS rDNA, black yeasts

Introduction

Chromoblastomycosis is a chronic, progressive cutaneous and subcutaneous disorder histologically characterized by nodular and verrucous skin lesions. Tissue proliferation usually occurs around the area of traumatic inoculation of the etiologic agent, producing crusted, verrucose,

wart-like lesions eventually leading to emerging, cauliflower-like forms [1]. Infections occur primarily in immunocompetent individuals and are supposed to originate by traumatic implantation of fungal elements into the skin. The fungus multiplies in the tissue producing muriform cells, which represent the invasive form of fungi causing chromoblastomycosis [1]. The most common etiological agents of chromoblastomycosis are the melanized fungi namely *Fonsecaea pedrosoi*, *F. monophora*, *Cladophialophora carrionii* and *Phialophora verrucosa*, all members of the ascomycete order *Chaetothyriales* in the family *Herpotrichiellaceae* [2–3]. There are occasional reports

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Correspondence: G. S. de Hoog, CBS-KNAW Fungal Biodiversity Centre, PO Box 85167, NL-3508 AD, Utrecht, The Netherlands. Tel: +31 30 2122663; fax: +31 30 2512097; E-mail: de.hoog@cbs.knaw.nl

of chromoblastomycosis involving other members of the order such as *Exophiala jeanselmei*, *E. spinifera*, *E. dermatitidis* [4–6] and *Cladophialophora samoënsis* [3]. A few species outside the order *Chaetothyriales* have been implicated in chromoblastomycosis-like infections, such as *Chaetomium funicola* (order *Sordariales*, family *Chaetomiaceae*) [7], *Catenulostroma chromoblastomycosum* (order *Capnodiales*, family *Teratosphaeriaceae*) [8], and an unidentified capnodialean fungus [9]. However, the latter were primarily concerned with infections with hyphae and chlamyospore-like cells in tissue which does not fit with the concept of chromoblastomycosis.

Chromoblastomycosis is currently classified into five clinical types [1]; nodular, plaque, tumoral, cicatricial and verrucous, based on the description of Carrión in 1950 [10]. Queiroz-Telles *et al.* [1] graded lesions according to their expansion, viz. mild, moderate and severe, and noted that, chronic lymphedema and ankylosis developed in the most severe cases. However, some atypical cases of chromoblastomycosis show lymphatic dissemination, a trait usually associated with sporotrichosis [11,12]. For example, Muhammed *et al.* [13] reported a case of lymphangitic chromoblastomycosis due to *F. pedrosoi* in a 40-year-old male who presented with a non-healing ulcer on the right big toe and multiple nodules over the anterior aspect of the right leg and foot. Interestingly, Ogawa *et al.* 2003 [11] used lymphoscintigraphy as an objective method to show the morphology of the lymph vessels and to assess lymphedema secondary to chromoblastomycosis.

Lymphatic dissemination leads to a different appearance of chromoblastomycosis, as illustrated in the present case reports of a Mexican male and a Brazilian female infected by *Rhinocladiella aquaspersa* [14–16]. The chaetothyrialean fungus *R. aquaspersa* is a rare cause of chromoblastomycosis with cases generally confined to Latin America. The infections caused by species of the genus *Rhinocladiella* are clinically diverse. *R. aquaspersa* is associated with skin infections, while *Rhinocladiella mackenziei* causes brain infections in otherwise healthy individuals which are associated with high mortality [17,18]. The aforementioned cases of *R. aquaspersa* chromoblastomycosis prompted a study on the clinical spectrum of *R. aquaspersa*, based on strains verified by sequencing and an analysis of infections caused by related species. This led us to evaluate the *in vitro* activities of amphotericin B, fluconazole, itraconazole, voriconazole, posaconazole, isavuconazole, caspofungin, and anidulafungin against this fungal pathogen.

Case report 1

A 56-year-old male rural professional plumber of drainage systems (residual water handling), who is a native and resident of Alpuyecá, Morelos (120 km south of Mexico City),

presented with a localized dermatosis affecting the dorsum of the left hand and the ipsilateral forearm. The lesions consisted of multiple, well-defined verrucous plaques with the same color as the skin or slightly grayish. They followed the path of lymphatic vessels of the arm, with mild pruritus (Fig. 1A,B). The dermatosis reportedly had begun 15 years prior to his presentation. The patient referred to several work-related traumata. Prior treatment included topical ketoconazole. The entire biopsied tissue was subjected to mycological and histopathological investigation. Direct examination of scales of the verrucous plaques in KOH (20%) demonstrated multiple dark brown muriform cells, approximately 6 µm in diameter. Section of the biopsied material stained with hematoxylin and eosin (H&E) revealed pseudoepitheliomatous hyperplasia and a granulomatous inflammatory infiltrate with lymphocytes, multinucleated giant (Langhan's type) and muriform cells (Fig. 1D). Clinical specimens were inoculated onto Sabouraud's glucose agar (SGA; Difco) and on SGA supplemented with chloramphenicol (0.5 mg/l) and were incubated at 27–30°C for two weeks. Growth of a melanized fungus was observed repeatedly and the mold was provisionally identified as a *Rhinocladiella* sp. on the basis of morphological criteria, and a voucher strain was deposited in the CBS-KNAW culture collection (accession number CBS 122635). The patient was treated successfully with oral terbinafine (500 mg/day), with good clinical response (significant reduction of the affected area) after six months of therapy. He was then put on maintenance therapy with oral itraconazole solution (200 mg/day) for 3 months. The patient also had bimonthly cryosurgery sessions for one year. Follow-up showed improvement of the dermatosis, with residual hypochromic areas and recovery of terminal hair in the previously verrucous plaques, though a 5-mm lesion remained in the distal third of the forearm (Fig. 1C).

Case report 2

A 62-year-old black female agricultural worker from Solânea in the state of Paraíba in the north east of Brazil consulted the hospital in 2001 because of the presence of multiple verrucose lesions measuring 2–8 cm in diameter, forming plaques on her left foot (Fig. 2A). The patient had a history of puncture injury due to an unidentified cactus approximately 20 years prior to her examination. Mycological and histopathological investigations of the biopsied verrucous lesion by direct examination in KOH (20%) and KOH supplemented with lactophenol revealed dark brown septate hyphae (Fig. 2B). Examination of a section of the biopsied material stained with H&E showed no muriform cells. Clinical specimens were cultured on SGA with and without chloramphenicol (0.5 mg/l) and incubated at 27–30°C for two weeks which resulted in the repeated

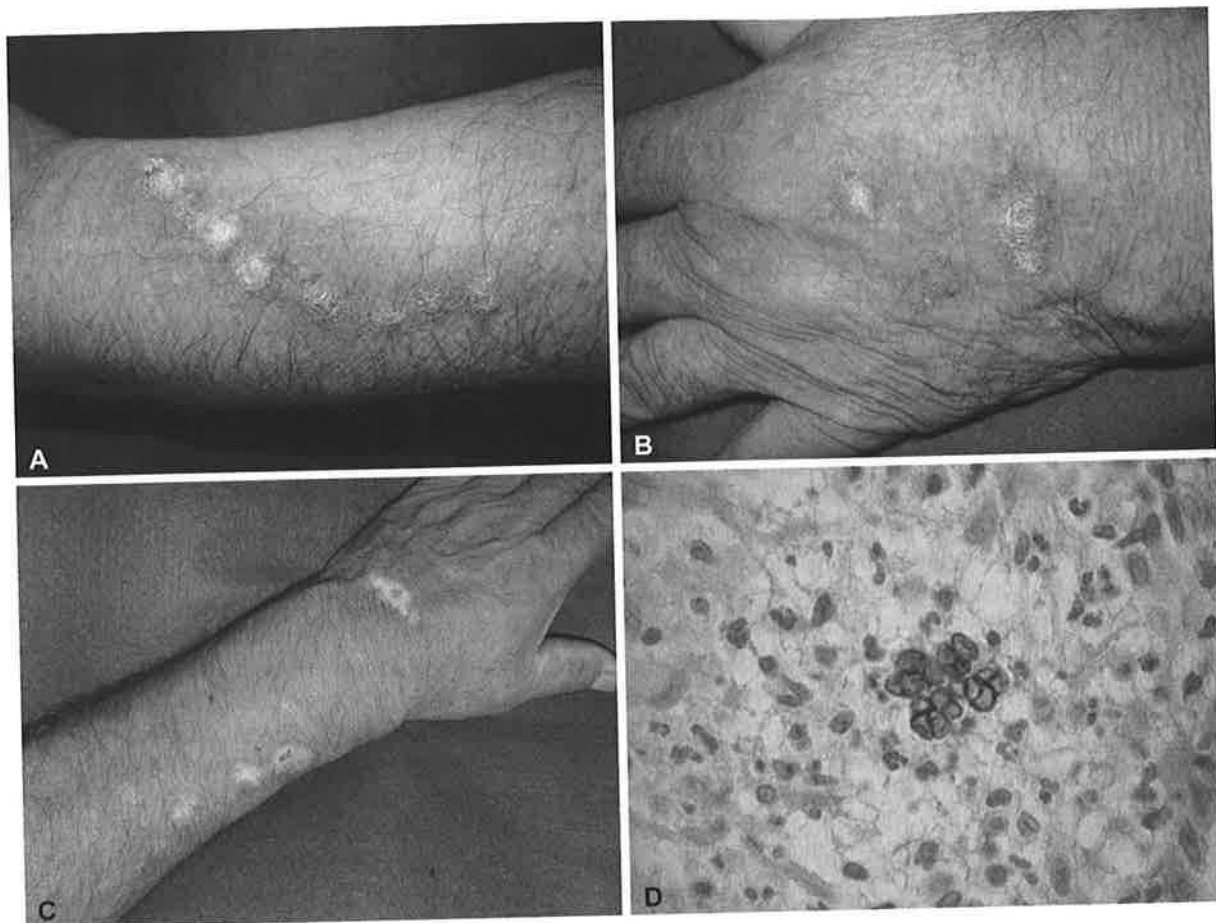


Fig. 1 Case 1: (A, B) Multiple and well-defined verrucous plaques due to *Rhinocladiella aquaspersa* concolorous with the skin or slightly greyish, and following the trajectory of lymphatic vessels of the arm. (C) Improvement of the dermatosis, residual hypo-chromic areas with recovering of the terminal hair in the previously verrucous plaques. (D) Histopathological examination showing muriform cells stained with hematoxylin & eosin (H&E $\times 400$).

growth of a melanized fungus. The mold was provisionally identified as a *Rhinocladiella* sp. by morphological criteria, and a voucher strain was deposited in the culture collection of the Universitat Rovira i Virgili School of Medicine in Reus, Spain (accession number FMR 7699). The patient was treated with antiseptics, antibiotics, and corticoids without improvement; followed by electrocauterization and oral itraconazole (200 mg/day). The lesions improved progressively, and the patient successfully cured.

Materials and methods

Mycology

Stock cultures of both representative strains (Table 1) were maintained on slants of 2% malt extract agar (MEA; Difco) and oatmeal agar (OA; Difco) at 24°C [19]. Microscopic studies were accomplished through the use of the slide culture technique using potato dextrose agar (PDA; Difco)

or OA to readily induce sporulation and suppress aerial hyphal growth [20]. Each slide culture was prepared in culture plates containing sterile water into which a U-shaped glass rod was placed, extending above the water surface. A block of freshly-grown fungal colony (ca. 1cm²) was placed onto a sterile microscope slide, covered with a somewhat larger, sterile glass cover slip, and incubated in this moist chamber. After 2 weeks slides were stained with both lactic acid and lactophenol cotton blue and light micrographs recorded using a Nikon Eclipse 80i microscope fitted with a Nikon digital sight DS-Fi1 camera.

For DNA extraction, mycelia were grown for 2 weeks at 24°C on plates of 2% MEA. DNA was extracted using an Ultra Clean Microbial DNA Isolation Kit (Mobio, Carlsbad, CA, USA) according to the manufacturer's instructions. Methods for PCR amplification and sequencing were those of Badali *et al.* [4]. DNA from both strains (strains from case-1 and case-2) including the ex-type strains of *R. aquaspersa* (CBS 313.73) and *R. phaeophora*

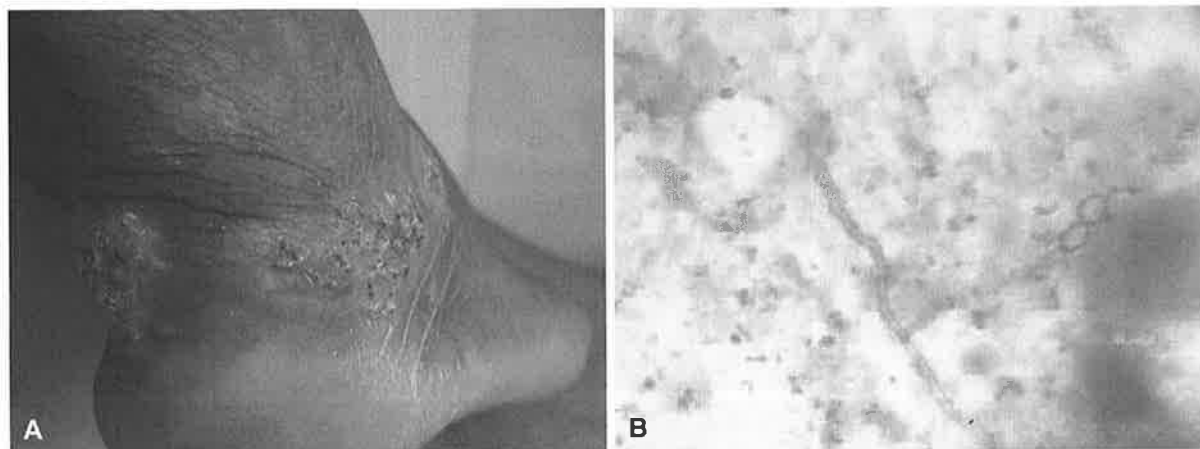


Fig. 2 Case 2: (A) Verrucose lesions due to *Rhinocladiella aquaspersa*. (B) Direct examination of verrucous lesion in KOH (20%) supplemented with lactophenol demonstrating dark brown septate hyphae.

(CBS 496.78) were subjected to DNA sequencing of internal transcribed spacer region (ITS rDNA), the partial β -tubulin gene (*TUB*) and the partial gene and introns of actin (*ACT1*). In addition, the nuclear ribosomal small subunit (*nucSSU*) gene of the ex-type strains was also sequenced (Table 1). Obtained sequences were compared with selected sequences in the GenBank (<http://www.ncbi.nlm.nih.gov>) and using a black yeast database maintained for research purposes at the CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands.

Antifungal susceptibility testing

The *in vitro* minimal inhibitory concentrations (MICs) and minimum effective concentrations (MECs) of the three available clinical isolates of *R. aquaspersa* and environmental isolate of *R. phaeophora* were determined for eight antifungal agents according to Clinical and Laboratory Standards Institute guidelines [21]. Amphotericin B (Bristol-Myers-Squib, Woerden, The Netherlands), fluconazole (Pfizer Central Research, Sandwich, UK), itraconazole (Janssen Research Foundation, Beerse, Belgium), voriconazole (Pfizer), posaconazole (Schering-Plough, Kenilworth, USA), isavuconazole (Basilea Pharmaceutica International AG, Basel, Switzerland), caspofungin (Merck Sharp & Dohme, Haarlem, The Netherlands) and anidulafungin (Pfizer) were obtained as reagent-grade powders from their respective manufacturers. Antifungal agents were dissolved as prescribed by the CLSI [21], i.e., stock solutions of the experimental triazole isavuconazole were prepared in DMSO. Antimycotics were diluted in RPMI 1640 medium (GIBCO BRL, Life Technologies, Woerden, The Netherlands) buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) (Sigma-Aldrich Chemie

GmbH, Steinheim, Germany) and dispensed into 96-well microdilution trays at the following concentration ranges: (1) amphotericin B, itraconazole, voriconazole, posaconazole, caspofungin from 0.016–16 mg/l; (2) fluconazole from 0.063–64 mg/l; and (3) isavuconazole, anidulafungin from 0.008–8 mg/l. Microdilution trays were stored at -70°C prior to use. Conidial suspensions in physiological saline containing Tween 40 (0.05%) were adjusted spectrophotometrically (530 nm) to optical densities in the range 0.15–0.17, then diluted 1:50 in buffered RPMI 1640 medium to prepare final inoculum suspensions containing 0.5×10^4 – 4×10^4 CFU/ml, as verified by colony counts on SGA [21]. Microtiter plates inoculated with *R. aquaspersa* were incubated at 35°C , whereas microtiter plates inoculated with *R. phaeophora* were incubated at 30°C . After 96 h the plates were examined visually and MICs and MECs determined. *Candida parapsilosis* (ATCC 22019) [21], *Candida kruzei* (ATCC 6258) [21], and *Paecilomyces variotii* (ATCC 22319) [21] were used for quality control.

Results

Colonies of *R. aquaspersa* were moderately expanding with velvety, elevated, olivaceous-black upper surfaces and reverses that were dark olivaceous in color (Fig. 3A,B). Hyphae were pale olivaceous, smooth- or slightly rough-walled. Conidiophores were straight, upright, unbranched, thick-walled and dark brown. Conidiogenous cells were usually terminal, cylindrical, 9–23 μm long, with crowded, slightly prominent denticles with dark scars and hyaline centres (Fig. 3C–E). Conidia were subhyaline, smooth- and thin-walled, one- or occasionally two-celled, ellipsoidal to clavate, 4.5–7.5 \times 1.8–2.4 μm (Fig. 2F). Presence of annellides are occasionally described for this fungus but were

Table 1 Isolation data of examined strains.

Name	Reference number	Gene Bank	Source	Origin
		ITS, TUB, ACT, SSU		
<i>Rhinochadiella aquaspersa</i>	CBS 313.73 (T)	GU017733, GU079660, GU079656, RAU20512	Chromoblastomycosis, male	Mexico
<i>Rhinochadiella aquaspersa</i>	CBS 122635	GU017732, GU079659, GU079658, GU079655	Chromoblastomycosis, male	Mexico
<i>Rhinochadiella aquaspersa</i>	FMR 7699	GU053606, -, -, -	Phaeohyphomycosis, male	Brazil
<i>Rhinochadiella phaeophora</i>	CBS 496.78 (T)	EU041811, GU079661, GU079657, EF137366	Maize-field soil	Colombia

CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; ITS rDNA, Internal Transcribed Spacer ribosomal DNA; TUB, the partial β -tubulin gene; ACT1, the partial gene and introns of actin; nucSSU, the nuclear ribosomal small subunit; T, ex-type culture.

not noticed in our isolates. The teleomorph is unknown. Growth occurred between 9 and 37°C (optimum, 27°C) and no growth was noted at 40°C.

The strains isolated from chromoblastomycosis (case-1, CBS 122635) and phaeohyphomycosis (case-2, FMR 7699) were identified as *R. aquaspersa* by 100% identity with multilocus sequence typing to ex-type strain of *R. aquaspersa* (CBS 313.73; originated from human chromoblastomycosis, Mexico). The related species *Rhinochadiella phaeophora* (ex-type strain CBS 496.78) was unambiguously distinguishable from *R. aquaspersa* by all genes sequenced (97%, 95% and 97.8% similarity in ITS, TUB and ACT1 genes, respectively) forming a robust branch with 100% bootstrap support. Morphologically *R. phaeophora* had sympodial, brown conidiophores which were arranged in a more profusely branched conidial apparatus than *R. aquaspersa*.

MICs for the three clinical isolates of *R. aquaspersa* towards eight antifungal drugs surveyed were amphotericin B, 1–2 mg/l; fluconazole, 32–64 mg/l; itraconazole and posaconazole, 0.063–0.125 mg/l, isavuconazole and voriconazole, 2 mg/l; caspofungin, 8 mg/l; anidulafungin, 1 mg/l. For the environmental isolate of *R. phaeophora* examined, itraconazole (MIC, 0.5 mg/l) and posaconazole (MIC, 0.25 mg/l) were the most active drugs, followed by amphotericin B, voriconazole, and isavuconazole (2 mg/l). MICs for caspofungin and anidulafungin towards *R. phaeophora* were each 8 mg/l, whereas fluconazole had an MIC > 64 mg/l.

Discussion

Our previous study [3], based on multigene phylogeny analysis, showed that *Rhinochadiella* is a genus of black yeast-like fungi closely related to other members of the order *Chaetothyriales* that have sympodial conidiogenesis [22] and that are consistently associated with opportunistic infections, i.e., primarily species belonging to the family *Herpotrichiellaceae* (e.g., *Cladophialophora*, *Exophiala* and *Fonsecaea*). The initial *R. aquaspersa* isolate (strain CBS 313.73) was obtained by Borelli [14] as '*Acrotheca*

aquaspersa Borelli' in 1972 from a chromoblastomycosis patient in Mexico. The species was later renamed '*R. aquaspersa*' by Schell *et al.* [23] following the isolation of the organism from a patient in Brazil. Padhye *et al.* [6] re-described these two earlier cases and presented other reports of chromoblastomycosis from Colombia, Brazil, and Mexico ascribed to this fungus. Arango *et al.* [15] reported a further case in a 60-year-old male with auricular localization and an infiltrative, dark-colored encrusted, scaly lesion. This patient had had the infection for 5 years, but it was successfully treated with itraconazole. Marques *et al.* [16] reported an unusual case involving *R. aquaspersa* chromoblastomycosis in a 52-year-old male farm worker from Brazil, who had extensive lesions in scaly plaques erupting in three different body sites over two years. Jae *et al.* [24] published a further case of chromoblastomycosis due to this fungus in a 52-year-old female from Korea. In this case the lesion, an erythematous, verrucous abdominal plaque 2 cm \times 1.3 cm, was successfully treated with oral itraconazole solution (200 mg/day administered over 4 months) together with surgical excision. All cases of chromoblastomycosis due to *R. aquaspersa* were diagnosed on the basis of clinical and pathological data and confirmed by conventional microscopic examinations of samples from lesion biopsies which showed numerous muriform cells. Misidentification of *R. aquaspersa* is likely using conventional morphological methods, especially as the literature is replete with incorrectly-named organisms. It may be noted that the species has dark conidial denticles with hyaline scars, resembling the poroconidia of dematiaceous fungi [20].

Over the past two decades chromoblastomycosis and phaeohyphomycosis have been attributed principally to *C. carrionii*, *F. pedrosoi*, *F. monophora* and *P. verrucosa* [1–3]. Our data confirm that *R. aquaspersa* also can cause chromoblastomycosis. Our case report from Mexico clinically resembled sporotrichosis. The dry, encrusted, verrucous, violaceous lesions and chronic lymphedema are characteristic of chromoblastomycosis, but the infection usually shows only localized expansion with verrucous plaques [1,20]. Therefore, our cases represent a new

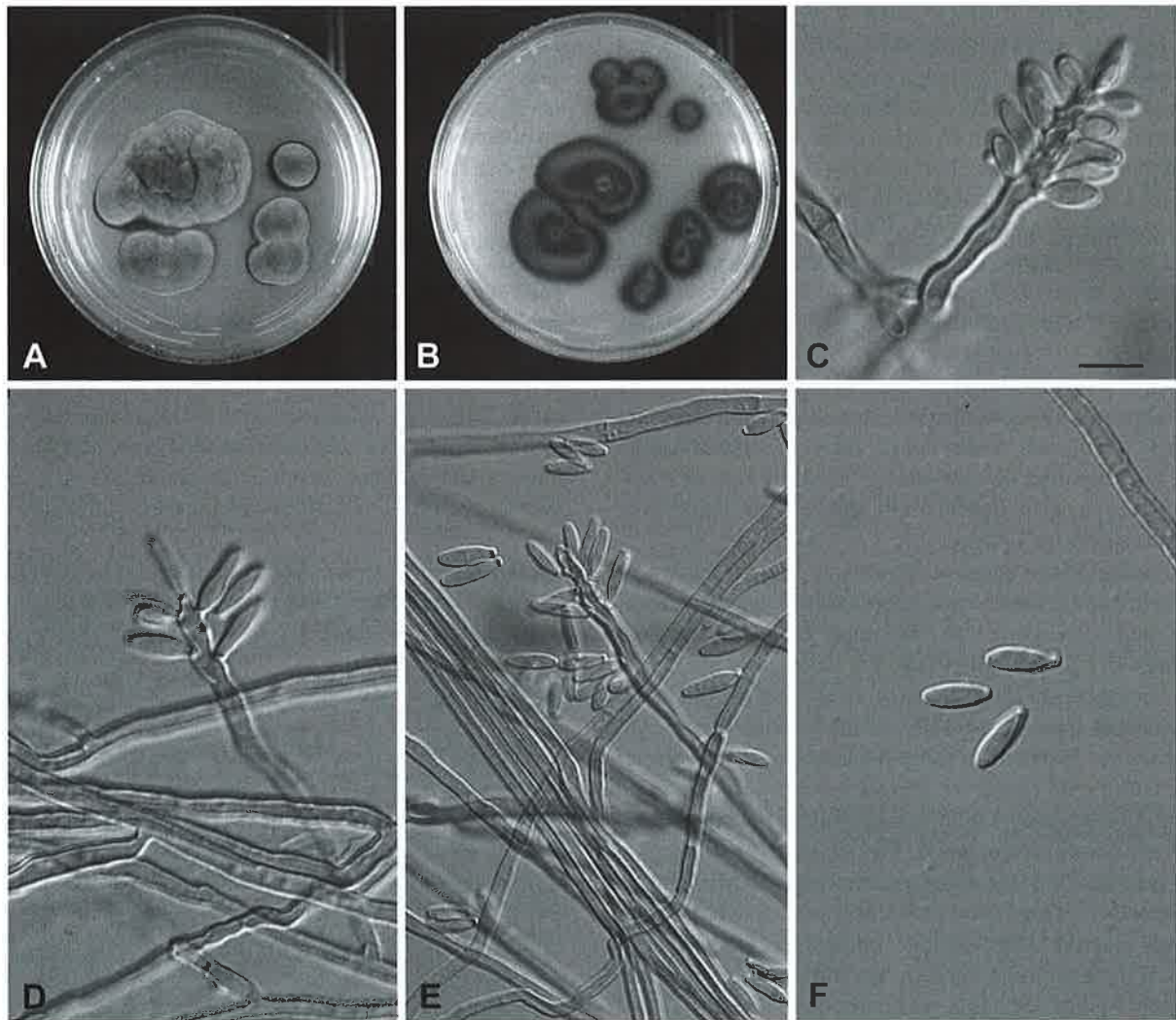


Fig. 3 (A, B) Colonies of *Rhinocladiella aquaspersa* on malt extract agar (MEA, Difco) and potato dextrose agar (PDA, Difco) moderately expanding, velvety, elevated, and olivaceous-black. (C–E) Conidiophores straight, upright, unbranched, thick-walled, and dark brown. (F) Conidia subhyaline, smooth- and thin-walled, one- or occasionally two-celled, ellipsoidal to clavate. Scale bar = 10 μ m.

clinical presentation of the disease that also may occur in patients infected with *F. pedrosoi* [11–13]. Lymphatic distribution in chromoblastomycosis may appear after cryosurgical treatment (R. Isa, Dominican Republic, personal communication).

D'Ávila *et al.* [25] characterized cell-mediated tissue reactions in chromoblastomycotic skin lesions and showed that distinct immunohistopathological alterations correlated with clinical aspects of the lesions. Patients with verrucous plaques had a type Th2 immunological response, whereas patients with erythematous atrophic plaques had a type Th1 response. Corbellini *et al.* [26] studied delayed-type hypersensitivity responses to crude and fractionated antigens from *F. pedrosoi* cultivated in different media, and suggested that a delayed-type skin

test using antigens produced in synthetic media may be useful for assessing primary exposure to chromoblastomycosis. Iwatsu *et al.* [27] observed cutaneous delayed hypersensitivity in animal experiments, and Ahrens *et al.* [28] reported enlargement and metastasis of lesions caused by *F. pedrosoi* in immunosuppressed but not in immunocompetent mice [28]. Esterre *et al.* [29] suggested that, relative to cell-mediated immunity, humoral immunity does not seem to offer much protection against chromoblastomycosis. Cardona-Castro and Agudelo-Flórez [30] detected chronic infection after healthy mice were inoculated intraperitoneally with *F. pedrosoi*. An unambiguous connection between host defense mechanisms and clinical aspects of chromoblastomycosis remains to be proven.

Most countries in which cases of *R. aquaspersa* have been reported have subtropical climates with alternating well-defined dry and humid seasons. The related fungus *R. phaeophora* originally was recovered from maize field soil from Colombia, but *R. aquaspersa* thus far has been found only in humans. For infections caused by black yeast-like fungi, the mammalian host seems to select a small number of opportunistic species. De Hoog *et al.* [31] found that a *C. carrionii*-positive patient was repeatedly pricked by cacti which carried *Cladophialophora yegresii* rather than *C. carrionii* on their spines. The patient in this case possibly was infected by *C. carrionii* present at sub-detectable levels on the cactus spines, or repeated exposure to *C. yegresii* sensitized the patient to subsequent exposure through a different venue to *C. carrionii*. Harutyunyan *et al.* [32] reported the presence of distantly related *Rhino-cladiella* species in lichens from arid habitats which were not pathogenic for humans.

Treatment of chromoblastomycosis is difficult, since prolonged therapy is needed and relapses are frequent. Itraconazole and terbinafine are the most frequently applied antifungals [1,33,34], but no standard therapy has been identified, and there are limited data on *in vitro* antifungal activity against *R. aquaspersa*. MICs of most non-dermatophyte opportunistic filamentous fungi for amphotericin B cluster in the 0.5–2 mg/l range, but correlations between MIC and outcome of treatments with this antifungal for filamentous fungi are poorly documented. Vanden Bossche *et al.* [35] reported that for black fungi amphotericin B MIC values were mostly > 4 mg/l. In contrast, Marques *et al.* [16], using a broth macrodilution method, obtained amphotericin B MICs of 0.8 mg/l for *R. aquaspersa*. Our results corroborate previous studies [36] that itraconazole (0.063 mg/l) and posaconazole (0.125 mg/l) have potent activity against *R. aquaspersa*, whereas voriconazole and isavuconazole have lower activities. Cuenca-Estrella *et al.* [37] likewise noted that posaconazole was a superior drug against *Rhino-cladiella* strains while caspofungin had poor activity. However, we also found that *R. aquaspersa* was more sensitive to anidulafungin (MIC, 1 mg/l) than was *R. phaeophora* (MIC, 8 mg/l). Odabasi *et al.* [38] showed that anidulafungin is a potent drug against *P. verrucosa*, and Badali *et al.* [39] demonstrated that, although anidulafungin had potent *in vitro* activity against *Alternaria* clinical isolates (*Alternaria infectoria*, *A. alternata*), it had poor activity against *Alternaria* environmental isolates (*A. malorum*). This is concordant with the finding presented here, that anidulafungin had higher *in vitro* activity against clinical isolates of *Rhino-cladiella* (*R. aquaspersa*) than against environmental isolates of this genus (*R. phaeophora*).

We conclude that itraconazole and posaconazole may become therapeutic options and alternatives to amphotericin B for treating infections due to the rare etiologic agent

R. aquaspersa, although *in vitro* studies using considerably larger numbers of strains, as well as animal models of infection and relevant clinical trials will be required before these azoles receive an official imprimatur from the medical community for treatment of *R. aquaspersa* chromoblastomycosis.

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