Medical Mycology, March 2010, **48**, 318–327

informa
healthcare

The clinical spectrum of *Exophiala jeanselmei*, with a case report and *in vitro* antifungal susceptibility of the species

H. BADALI*†‡, M. J. NAJAFZADEH*†, M. VAN ESBROECK§, E. VAN DEN ENDEN§, B. TARAZOOIE*, 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, Mazandaran University of Medical Sciences, Sari, Iran, §Institute of Tropical Medicine, Antwerp, Belgium, and ^Department of Medical Microbiology and Infectious Diseases, Canisius Wilhelmina Hospital, Nijmegen, The Netherlands*

Exophiala jeanselmei is clinically redefined as a rare agent of subcutaneous lesions of traumatic origin, eventually causing eumycetoma. Mycetoma is a localized, chronic, suppurative subcutaneous infection of tissue and contiguous bone after a traumatic inoculation of the causative organism. In advanced stages of the infection, one finds tumefaction, abscess formation and draining sinuses. The species has been described as being common in the environment, but molecular methods have only confirmed its occurrence in clinical samples. Current diagnostics of *E. jeanselmei* is based on sequence data of the Internal Transcribed Spacer (ITS) region of ribosomal DNA (rDNA), which sufficiently reflects the taxonomy of this group. The first purpose of this study was the re-identification of all clinical ($n = 11$) and environmental strains ($n = 6$) maintained under the name *E. jeanselmei*, and to establish clinical preference of the species in its restricted sense. Given the high incidence of eumycetoma in endemic areas, the second goal of this investigation was the evaluation of *in vitro* susceptibility of *E. jeanselmei* to eight conventional and new generations of antifungal drugs to improve antifungal therapy in patients. As an example, we describe a case of black grain mycetoma in a 43-year-old Thai male with several draining sinuses involving the left foot. The disease required extensive surgical excision coupled with intense antifungal chemotherapy to achieve cure. *In vitro* studies demonstrated that posaconazole and itraconazole had the highest antifungal activity against *E. jeanselmei* and *E. oligosperma* for which high MICs were found for caspofungin. However, their clinical effectiveness in the treatment of *Exophiala* infections remains to be determined.

Keywords *Exophiala jeanselmei*, black yeasts, ITS rDNA, mycetoma, antifungal susceptibility testing

Introduction

Mycetoma is defined as a localized, chronic, granulomatous, suppurative and progressive inflammatory disease of subcutaneous tissue and contiguous bone after a traumatic inoculation of the causative organism [1]. Etiologic agents are recovered from humans as well as domestic animals [2]. In advanced stages of the infection tumefaction, abscess formation and draining sinuses arise. The hallmark of a mycetoma is the presence of the fungus in the form of grains. In Africa the infection is particularly common from Sudan to Senegal, an area known as the 'mycetoma belt'. The main etiologic agents in that area are *Madurella mycetomatis* and *Leptosphaeria senegalensis*. Other recurrent fungal agents are *Madurella grisea* and several species of coelomycetes.

Received 9 March 2009; Received in final revised form 6 May 2009; Accepted 28 June 2009

Correspondence: G.S. de Hoog, Uppsalalaan 8, 3584 CT, Utrecht, The Netherlands. Tel: +31 30 2122 663; E-mail: de.hoog@cbs.knaw.nl

DOI: 10.3109/13693780903148353

Classically, the black yeast *Exophiala jeanselmei* has been reported to be involved in subcutaneous lesions of traumatic origin, eventually causing eumycetoma [3,4]. Members of the ascomycete order *Chaetothyriales*, are remarkably frequently encountered and reported as agents of disease. Many of these species are oligotrophic [5] and are therefore found in uncommon habitats. This property also seems to be a predisposing factor for human infection, since a large number of species are found as etiologic agents of disease. Several members of *Chaetothyriales* have been reported involved in eumycotic mycetoma in humans and animals. Werlinger *et al*. [6] and Bonifaz *et al*. [7] described cases of mycetoma caused by *Cladophialophora bantiana*. Guillot *et al*. [8] reported on a case in a dog caused by the same species. *Cladophialophora mycetomatis* is a novel species recently described by Badali *et al*. [9] as an agent of eumycetoma in Mexico.

Black yeasts identified as *E. jeanselmei* have also been reported from many other types of infection, such as chromoblastomycosis or mild cutaneous disease [10], disseminated infections, endocarditis and arthritis [11]. However, recent molecular studies have shown that the species is very heterogeneous. The original morphological varieties have now been raised to species level [12], and additional, morphologically nearly indistinguishable species have been described [13]. Hence, the clinical predilection of *E. jeanselmei* has to be re-evaluated on the basis of correctly identified material. With obsolete mycological procedures, identification of black yeasts remains difficult [3]. Since the original isolates no longer are available for most cases published in older literature, their identification down to the species level cannot be performed by molecular methods. It is possible that the etiological agent may often have been misdiagnosed. In this article, a case of eumycetoma caused by *E. jeanselmei* matching the original clinical concept of the species is presented.

Current diagnostics of *E. jeanselmei* is achieved through the use of sequence data of the Internal Transcribed Spacer (ITS) region of ribosomal DNA (rDNA), which reflects the taxonomy of this group [14]. Therefore, the first purpose of this study was the re-identification of all clinical (n = 11) and environmental strains (n = 6) preserved under the name *E. jeanselmei* in the collection of Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands, and to establish the species clinical manifestations. Given the high incidence of eumycetoma in endemic areas, the second goal of this study was the evaluation of the *in vitro* susceptibility of *E. jeanselmei* to eight conventional and new generations of antifungal drugs to improve the antifungal therapy in patients. New antifungal agents with a better activity may help to improve the management of eumycetoma as sufficient knowledge is gained as to the *in vitro* activity of new antifungal agents.

Materials and methods

Fungal strains

Strains used in this study were obtained from the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands (Table 1), deposited and phenotypically identified *E. jeanselmei.* Stock cultures were maintained on slants of

Table 1 Summary of source and identification of strains tested.

CBS number	Other collection	Source	Country	Reference	Final name
CBS 122239	dH 18920	Man, mycetoma	Thailand	Current paper	*Exophiala jeanselmei*
CBS 119095	dH 13453	Man, foot skin	USA, Dallas	None	*Exophiala jeanselmei*
CBS 109635	dH 12305	Man, arm lesion	USA, San Antonio	[13]	*Exophiala jeanselmei*
CBS 148.97	dH 10808	Man, subcutaneous cyst	Japan, Ibaraki	[22]	*Exophiala jeanselmei*
CBS 507.90	dH 15933 (T)	Man, mycetoma	France	[20]	*Exophiala jeanselmei*
CBS 116.86	dH 15309	Man, chromomycosis	Japan	[10]	*Exophiala jeanselmei*
CBS 677.76	dH 16163	Man, mycetoma	Pakistan	[21]	*Exophiala jeanselmei*
CBS 664.76	ATCC 34123	Man, skin infection	None	[23]	*Exophiala jeanselmei*
CBS 528.76	ATCC10224	Man, hand skin lesion	None	None	*Exophiala jeanselmei*
CBS 814.95	dH 16266	Soil biofilter	Netherlands, Delft	[13]	*Exophiala oligosperma*
CBS 634.69	dH 16108	Wood, ship resting	Baltic Sea	None	*Exophiala oligosperma*
CBS 538.76	dH 15984	Man, bronchus	None	None	*Exophiala oligosperma*
CBS 537.76	dH 15981	Man, eye infection	Italy	[24]	*Exophiala oligosperma*
CBS 642.82	dH 16117	Soft rot of preservative-treated wood	Australia, Lucia	None	*Exophiala oligosperma*
CBS 527.76	dH 15966	Culture contaminant of *Hyphodontia breviseta*	Sweden, Bohuslan	None	*Exophiala xenobiotica*
CBS 102241	dH 11605	Soil under coffee plant	Brazil, Paraná	None	*Exophiala bergeri*
CBS 663.76	dH 16149	Wood	None	[23]	*Capronia pilosella*

Abbrevations used: ATCC = American Type Culture Collection, Manassas, U.S.A.; CBS = Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; DH = G.S. de Hoog working collection; IFM = Research Institute for Pathogenic Fungi, Chiba, Japan; IHM = Laboratory of Mycology, Faculty of Medicine, Montevideo Institute of Epidemiology and Hygiene, Montevideo, Uruguay; BMU = Beijing Medical University. *T* = *ex-type culture.*

2% Malt Extract Agar (MEA; Difco) and Oatmeal Agar (OA; Difco) and incubated at 24°C for two weeks [15]. The identification of 17 strains was verified with sequence data of the internal transcriber spacer regions (ITS) of the rDNA. An isolate from a recent case of mycetoma was added (CBS 122339).

DNA extraction and sequencing

Mycelia were grown on 2% MEA plates for 2 weeks at 24°C [15] and a sterile blade was used to scrape off the mycelium from the surface of the plate. DNA was extracted using an Ultra Clean Microbial DNA Isolation Kit (Mobio, Carlsbad, CA 92010, USA) according to the manufacturer's instructions. DNA extracts were stored at −20°C until used [9]. Internal Transcribed Spacers rDNA (ITS) were amplified using primers V9G and LS266 and sequenced with the internal primers ITS1 and ITS4 [16]. PCR reactions were performed on a Gene Amp PCR System 9700 (Applied Biosystems, Foster City, USA) in 50 µl volumes containing 25 ng of template DNA, 5 µl reaction buffer (0.1 M Tris-HCl, pH 8.0, 0.5 M KCl, 15 mM $MgCl_2$, 0.1% gelatine, 1% Triton X-100), 0.2 mM of each dNTP and 2.0 U Taq DNA polymerase (ITK Diagnostics, Leiden, The Netherlands). Amplification was performed with cycles of 5 min at 94°C for primary denaturation, followed by 35 cycles at 94°C (45 s), 52°C (30 s) and 72°C (90 s), with a final 7 min extension step at 72°C. Amplicons were purified using GFX PCR DNA and gel band purification kit (GE Healthcare, Ltd., Buckinghamshire UK). Sequencing was performed as follows: 95°C for 1 min, followed by 30 cycles consisting of 95°C for 10 s, 50°C for 5 s and 60°C for 2 min. Reactions were purified with Sephadex G-50 fine (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and sequencing was done on an ABI 3730XL automatic sequencer (Applied Biosystems, Foster City, CA, USA).

Alignment and phylogenetic reconstruction

Sequence data obtained in this study were adjusted using the SeqMan of Lasergene software (DNAStar Inc., Madison, Wisconsin, USA). ITS sequences were aligned manually using BioNumerics version 4.61 (Applied Maths, Kortrijk, Belgium). The program RAxML-VI-HPC v.7.0.0 [17], as implemented on the Cipres portal v.1.10, was used for the tree search and the bootstrap analysis (GTRMIX model of molecular evolution and 500 bootstrap replicates). Bootstrap values equal or greater than 70% were considered significant [18]. Newly generated sequences were subjected to a BLAST search of the NCBI databases, sequences with high similarity (≥98%) were downloaded from GenBank and sequences were compared based on the alignment using a black yeast molecular database maintained at the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands. If the similarity of sequences of the ITS region was more than 99% between a studied strain and its nearest neighbor, the strain concerned was considered to be the same species as its nearest neighbor.

In vitro susceptibility

MICs (minimum inhibitory concentration) and MECs (minimum effective concentration, echinocandins only) were determined as described in the Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) document M38-A2 [19]. The eight antifungal agents evaluated: amphotericin B (AMB, Bristol-Myers Squib, Woerden, The Netherlands); fluconazole (FLU, Pfizer Central Research Sandwich, U.K.); itraconazole (ITC, Janssen Research Foundation, Beerse, Belgium); voriconazole (VOR, Pfizer); posaconazole (POS, Schering-Plough, Kenilworth, USA); isavuconazole (ISA, Basilea, Basel, Switzerland); caspofungin (CAS, Merck Sharp & Dohme, Haarlem, The Netherlands) and anidulafungin (ANI, Pfizer) were provided by the manufacturers as reagent-grade powders. As per the CLSI guidelines, stock solutions of the drugs were prepared in the appropriate solvent [19]. The drugs were diluted in the standard RPMI-1640 medium (Sigma Chemical Co.) buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) buffer (Sigma) with L-glutamine without bicarbonate to yield two times their concentrations were as follows: amphotericin B, itraconazole, voriconazole, posaconazole and caspofungin 0.016–16 µg/ml; fluconazole 0.063–64 µg/ml; isavuconazole 0.002–2 µg/ml and anidulafungin 0.008–8 µg/ml. Plates were stored at −70°C until they were used. Briefly, inoculum suspensions were prepared from 7 to 14 days potato dextrose agar (PDA; Difco) cultures by adding sterile saline solution with Tween 40 (0.05%) and slightly scarping the surface of mature colonies with sterile cotton swab. If large aggregates existed, they were allowed to settle for several minutes, the homogenous conidial suspensions were then transferred to sterile tubes and the supernatants were performed spectrophotometrically at 530 nm wavelength to optical density (OD) that ranged from 0.17–0.15 (68 to 71 T%). Therefore, the final size of the stock inoculum suspensions of the isolates tested ranged from 0.4×10^4–3.1×10^4 CFU/ml as performed by quantitative colony count on sabouraud glucose agar (SGA; Difco) to determine the viable number of colony forming units per milliliter [19]. The inoculum suspensions which consisted primarily of non-germinated conidia were diluted 1:50 in RPMI 1640 medium. Microdilultion plates were incubated at 35°C and examined optically and spectrophotometrically at 420 nm after 72 h (if insufficient growth was found, plates were incubated longer, until 96 h) for MICs and MECs determinations. *Paecilomyces variotii* (ATCC

22319), *Candida parapsilosis* (ATCC 22019) and *Candida krusei* (ATCC 6258) were used for quality control. The MICs endpoints for amphotericin B, itraconazole, voriconazole, posaconazole and isavuconazole were determined with the aid of a reading mirror as the lowest concentration of the drug that prevented any recognizable growth (100% inhibition) and for fluconazole as a prominent reduction of growth, i.e., ≥50% inhibition compared to drug-free growth control. The MEC of the echinocandins (caspofungin and anidulafungin) was defined microscopically as the lowest concentration of the drug that led to the growth of small, rounded, compact hyphal forms as compared with the long, unbranched hyphal clusters that were seen in the growth control [19].

Case report

A 43-year-old Thai man was admitted in 2004 to the Institute of Tropical Medicine, Antwerp, Belgium, due to a complaint of a tumefaction that localized and developed on the left leg at the dorsum of the foot affecting the fourth and fifth toe (Fig. 1A,B). The patient did not remember any history of trauma or puncture at the site of the lesion. First surgical resection biopsy was performed in 2005 and initial clinical diagnosis was that of chromoblastomycosis. Histopathologically there was no evidence of presence of muriform cells. Subsequently, the patient underwent surgical excision of the medial mass with skin graft closure and no antifungal drugs were prescribed. Two years later, he was referred again to the Institute of Tropical Medicine, presenting with a relapse of the lesion which consisted of a deformed tumorous area, with nodules and multiple draining sinuses. Sinuses discharged small amounts of sanguineous fluid without visible grains. The patient limped due to discomfort at the site of infection. Radiography of the foot was normal with no visible bone destruction or osteomyelitis, and no significant regional lymphadenopathy was noted. Results of bacteriological (syphilis) and parasitological (leishmaniosis) evaluations, and serological test for human immunodeficiency virus (HIV) were all negative. Laboratory investigations including full blood count, blood chemistry and renal tests were within normal limits, whereas liver functions were slightly disturbed, i.e., SGOT 46 (normal <59), SGPT 82 (normal <72), GGT 166 (normal <73). Therapy with oral itraconazole at 400 mg/day for 6 months yielded good clinical response.

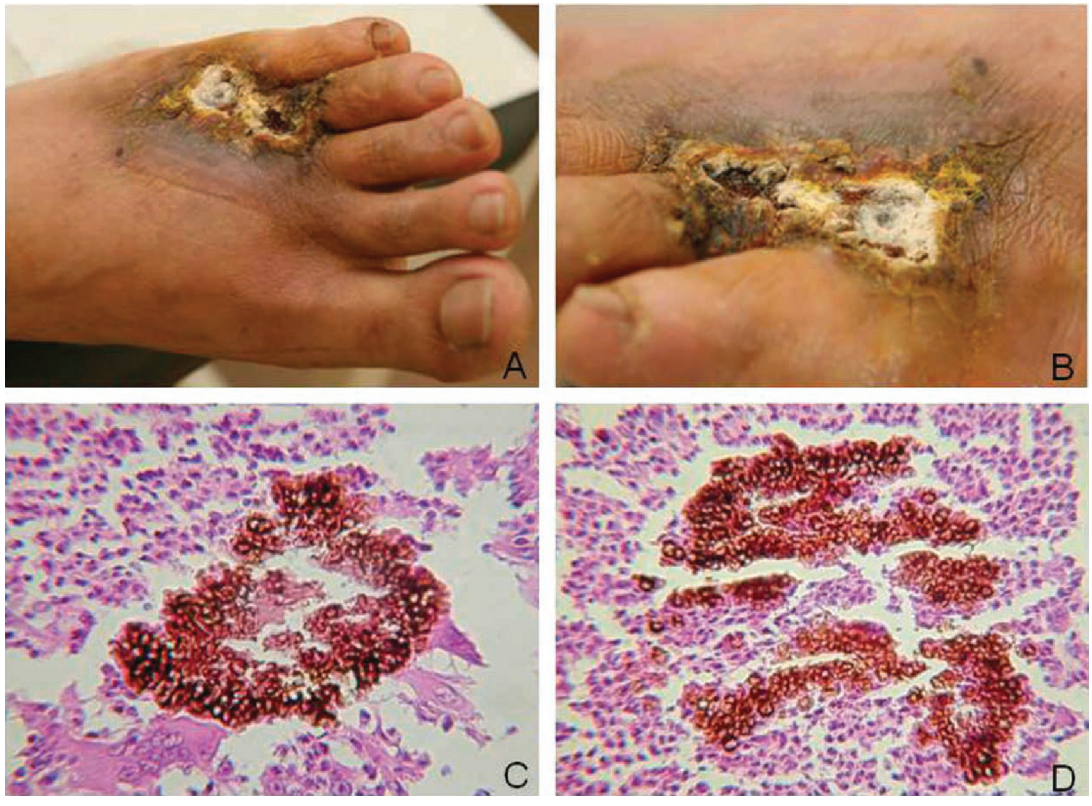

Fig. 1 (A,B) Clinical signs of mycetoma caused by *Exophiala jeanselmei*, deformed tumorous area of the foot, with nodules, draining sinuses with discharging and ulcers. (C,D) Section of the biopsy material stained with hematoxylin and eosin (H&E) exhibiting pigmented granules; some were sickle-shaped, and surrounded by a dense inflammatory infiltrate consisting of numerous neutrophils (H&E stain × 400).

The slight liver tests abnormalities were considered no contraindication for itraconazole treatment, but the patient was requested to avoid alcoholic beverages. Additional local heat therapy (hot footbath, local heated dry pillow, heat bag and/or infrared lamp) was suggested, but it remained unclear how far this was implemented, since verbal communication was rather difficult. On January 2008, there was a clear improvement of the wound, but no complete healing. Liver tests had normalized since he stopped alcoholic drinking. Afterwards, the patient did not return for follow up.

Mycological identification

The entire biopsied tissue was used for mycological and histopathological investigations. Direct examination with KOH (10%) revealed black granules and hyphal elements but were not obviously clear. Microscopic examination of biopsy sections stained with hematoxylin and eosin (H&E) revealed pigmented granules, some sickle-shaped, and surrounded by a dense inflammatory infiltrate consisting of numerous neutrophils and to the periphery chronic inflammation with fibrosis (Fig. 1C,D). The histopathological observations led to the diagnosis of a eumycotic mycetoma caused by an *Exophiala* species.

Clinical specimens were cultured on Sabouraud glucose agar (SGA: Difco) and SGA supplemented with chloramphenicol (0.5 µg/ml) and incubated at 27–30°C for 7 days. Growth of dematiaceous fungi was observed and these were morphologically classified as *Exophiala* species. Stock cultures were maintained on slants of 2% MEA and OA at 24°C [15], and a voucher strain was deposited in the CBS culture collection as CBS 122339. Microscopic studies using the slide culture techniques with PDA or OA were conducted. These media were selected because they readily induce sporulation and suppress growth of aerial hyphae [3]. After two weeks, slides were prepared from these cultures in lactic acid or lactophenol cotton blue and light micrographs were taken using Nikon Eclipse 80*i* microscope with a Nikon digital sight DS-Fi1 camera. Colonies were moderately expanding and initially moist (yeast-like), forming velvety, olivaceous-green aerial hyphae; colony reverse was olivaceous-black (Fig. 2A). Numerous sub-spherical to ellipsoidal budding cells were present in young cultures, giving rise to short torulose hyphae that gradually changed into un-swollen hyphae. Conidia were often cohering in long chains and converted into hyphae. Conidiogenous cells were intercalary or lateral, then rocket-shaped, brown, with inconspicuous, slightly tapering annellated zones, producing smooth, narrow, thin-walled, broadly ellipsoidal conidia 2.6–5.9 × 1.2–2.5 µm (Fig. 2B,C). Cardinal growth temperatures of strain CBS 122339 were growth between 9–37°C, optimum at 27°C, no growth was observed at 40°C.

Results

Seventeen strains from different geographical locations maintained as *Exophiala jeanselmei* in the CBS culture collection were sequenced using the ITS region (542 characters) and compared with alignable members of genus *Exophiala*. The ex-type strains of *E. jeanselmei* (AY156963), *E. spinifera* (AY156976), *E. exophialae* (AY156973), *E. dermatitidis* (FJ974060), *E. oligosperma* (AY163551), *E. xenobiotica* (DQ182587), *E. bergeri* (EF551462) and *E. lecanii-corni* (FJ974061) were used. The species

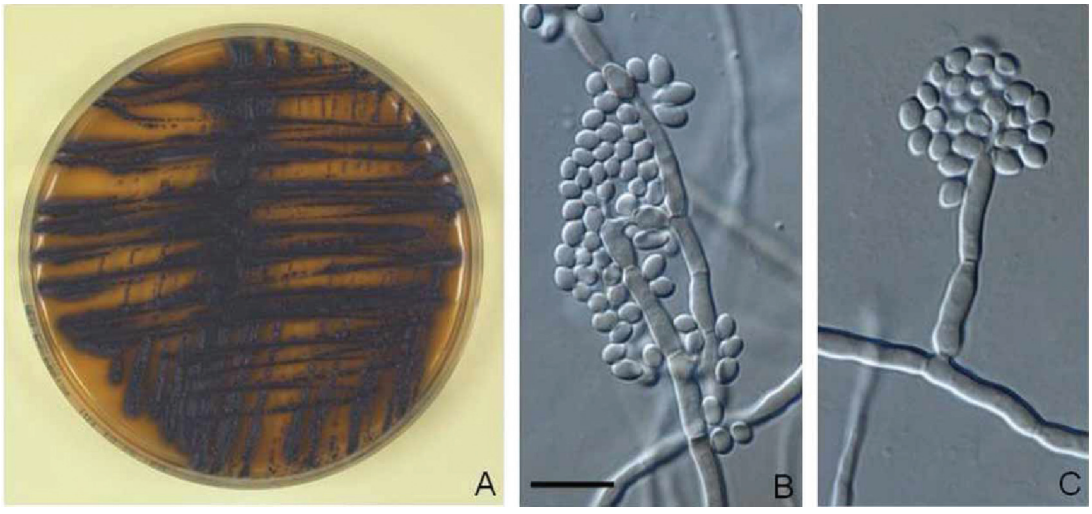

Fig. 2 (A) Culture on Malt extract agar (MEA, Difco) produced dark, moist, olivaceous-black yeast-like colony at room temperature. (B,C) Conidia clustered at the apices of the tapered annellides and long, thick-walled, septate conidiophores. Scale bar = 10 µm.

E. jeanselmei was unambiguously distinguished in a robust branch with >98% bootstrap support (Fig. 3). Nine out of 17 strains showed 98% identity with the *E. jeanselmei* ex-type strain (CBS 507.90) which had originally been isolated from a true mycetoma-like infection. Five strains were re-identified as *E. oligosperma*, and one strain each was confirmed as *E. bergeri* (CBS 102241), *E. xenobiotica* (CBS 527.76) and *Capronia pilosella* (CBS 663.76) (Table 1). Strains confirmed to be *E. jeanselmei* all originated from subcutaneous infections in human patients [current case,10,13,20-23], whereas strains which originated from environmental samples like soil, wood and plant or from clinical relevant eye or disseminated infections were considered to belong to *E. oligosperma* and *E. xenobiotica* [24].

Table 2 summarizes the results of *in vitro* antifungal susceptibility testing of eight antifungal drugs against *E. jeanselmei* ($n = 9$) and *E. oligosperma* ($n = 5$) employing the methods described in the CLSI guideline (M38-A2). The MIC_{90} was not determined due to insufficient numbers of isolates. Amphotericin B MICs for most non-dermatophyte opportunistic filamentous fungi isolates are clustered between 0.5–2 μg/ml. However, there is very little data available regarding the correlation between MICs and outcome of treatments with AmB for filamentous fungi. Results have shown that amphotericin B

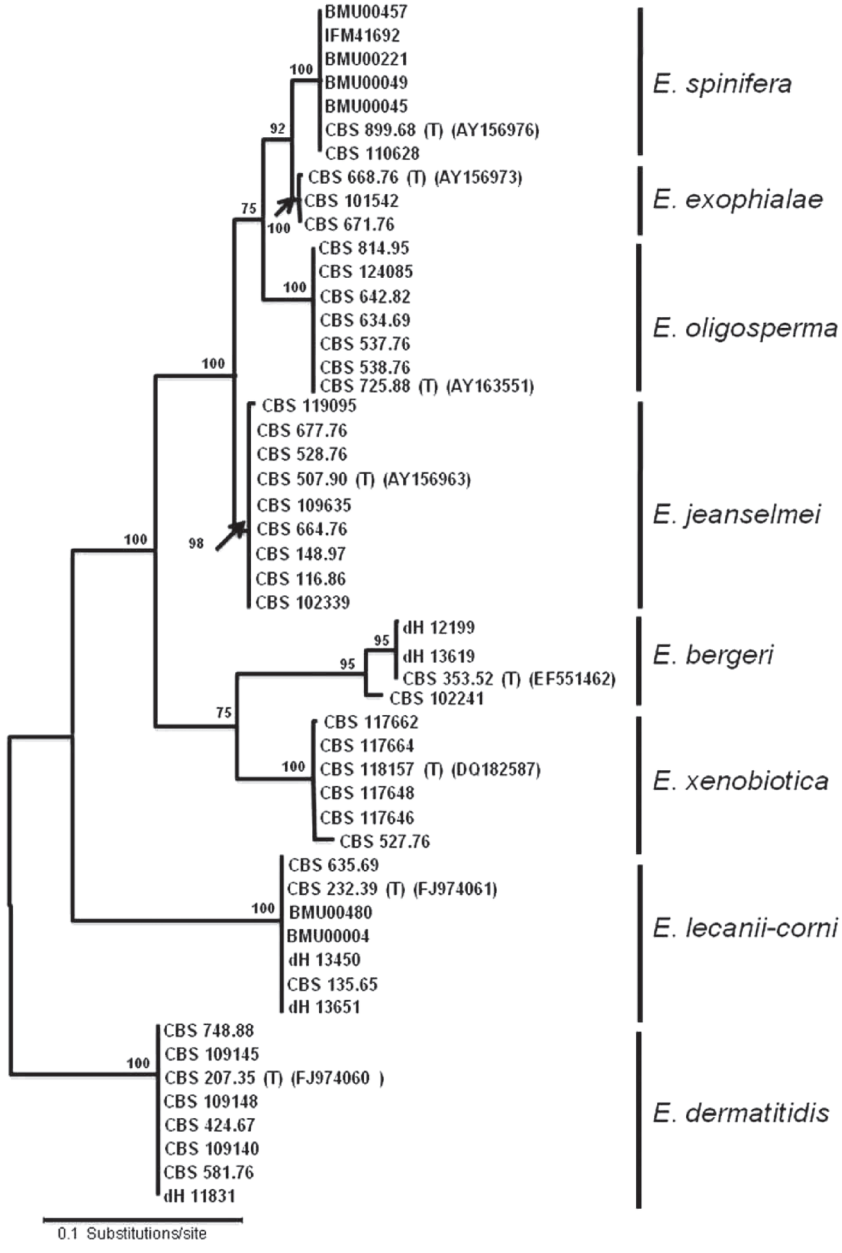

Fig. 3 Consensus tree of ITS rDNA obtained from a ML analysis using RAxML. Bootstrap support values were estimated based on 500 replicates, and are shown above the branches. The *Exophiala dermatitidis* Clade was taken as outgroup.

Table 2 *In vitro* susceptibility of *Exophiala jeanselmei* and *Exophiala oligosperma* for eight antifungal drugs expressed in μg/ml.

Source (no. of strains) and drugs	MIC range	MIC_{50}
Exophiala jeanselmei (9)		
Amphotericin B	0.25–2	1
Fluconazole	8–32	16
Itraconazole	0.031–0.25	0.125
Voriconazole	0.25–2	1
Posaconazole	0.016–0.063	0.031
Isavuconazole	0.25– > 2	2
Caspofungin	2–8	4
Anidulafungin	0.063–4	0.5
Exophiala oligosperma (5)		
Amphotericin B	1–2	1
Fluconazole	16–32	32
Itraconazole	0.031–0.25	0.063
Voriconazole	1–4	1
Posaconazole	0.016–0.125	0.031
Isavuconazole	0.5–2	1
Caspofungin	0.25–4	4
Anidulafungin	0.016–2	0.5

Note. MIC_{90} not determined due to insufficient numbers of isolate tested.

MICs ranged from 0.25–2.0 and 1–2 μg/ml for *E. jeanselmei* and *E. oligosperma*, respectively. Itraconazole and posaconazole showed potent activity against all *E. jeanselmei* and *E. oligosperma* isolates. Interestingly in terms of MIC_{50} (0.031 μg/ml) posaconazole was more active against *E. jeanselmei* than itraconazole (MIC_{50} 0.125). Filamentous fungi are usually not susceptible to fluconazole and most MICs are >64 μg/ml for these isolates. In our study, we found that fluconazole had the widest range and the highest MICs against *E. jeanselmei* and *E. oligosperma*, ranging between 8–32 and 16–32 μg/ml, respectively. Voriconazole and isavuconazole had highest MICs with complete inhibition end points with MIC_{50} (1 μg/ml and 2 μg/ml) against *E. jeanselmei* and MIC_{50} (both 1 μg/ml) against *E. oligosperma*. Posaconazole demonstrated the lowest MIC_{50} (0.031 μg/ml) of all azoles. Concerning the echinocandin drugs caspofungin and anidulafungin, both exhibited low *in vitro* activity against *E. jeanselmei* and *E. oligosperma. Exophiala jeanselmei* showed higher MEC_{50} (4 μg/ml) with caspofungin than to anidulafungin (MEC_{50}=0.5 μg/ml). Overall, in terms of MEC_{50} caspofungin had no activity against *E. jeanselmei* and *E. oligosperma.* There was no statistically significant difference when comparing the susceptibilities of *E. jeanselmei* and *E. oligosperma* isolates for all antifungal drugs ($P<0.05$).

Discussion

In our previous studies, phylogenetic analysis of nucSSU, nucLSU and *RPB1* genes have shown that the ex-type strain of *E. jeanselmei* (CBS 507.96) is a member of the order *Chaetothyriales*, within the family *Herpotrichiellaceae* [9,25]. Identification of this and related fungi with standard mycological procedures is difficult because of their high numbers of budding cells and poorly differentiated conidial structures. Nishimura *et al.* [26] studied the conidiogenesis of *E. dermatitidis*, *E. jeanselmei*, and *E. spinifera* and reported that the conidial ontogenesis of these three species is annellidic and may sometimes be difficult to differentiate. Masuda *et al*. [27] and Dixon *et al*. [28] showed that the morphological variants of *E. jeanselmei* distinguished earlier by de Hoog [29] are quite distinct on the molecular level and subsequently segregated into a number of individual species. Masuda *et al*. used DNA-DNA hybridization to classify *E. jeanselmei* into six groups. They suggested that DNA similarities between one group and another seem too low for these groups to comprise a single species [27]. Kawasaki *et al*. classified strains of *E. jeanselmei* into 18 types based on mitochondrial restriction profiles and believed that the organism constitutes a complex [30]. Wang *et al.* [31] used the DNA and amino acid types of the mitochondrial cytochrome *b*. They proposed that on the basis of mtDNA RFLP typing and similarities of nucleotide and amino acid sequences, that some *E. jeanselmei* strains should be re-identified. Moreover, Haase *et al*. suggested *E. jeanselmei* var. *lecanii-cornii* should be a distinct species, *Exophiala lecanii-corni*, based on investigation of ribosomal DNA [32].

In the present study, considerable sequence differences were found in nine out of 17 strains in the CBS culture collection. The isolate from the present case report exhibited ≥98% ITS similarity to the ex-type strain of *E. jeanselmei* (CBS 507.90) and formed a single cluster of *E. jeanselmei* and consequently should be regarded as identical to this species. The intraspecific variability within two strains ITS1 region was 2 bp (in the nucleotide position of 82 and 107) and 4 bp in ITS2 region (in the nucleotide position of 492, 519, 528 and 531), positions of ITS1 and 2 are counted after ATCATT. The ex-type strain originated from a case of mycetoma in a patient in France who was an immigrant from Martinique [20]. Strain CBS 116.86 represents the etiologic agent of chromoblastomycosis-like infection [10] with muriform cells in tissue. However, the clinical manifestation and the tissue form are different from that of the grains described by Langeron [20]. CBS 677.76 originated from a black-grain mycetoma in a patient from Pakistan [21] and formed grains *in vivo* that were morphologically identical to those formed by the ex-type strain that also originated from mycetoma. Sequence data of CBS 677.76 proved it to be close to *E. jeanselmei*. CBS 148.97 caused phaeohyphomycotic cysts in a 59-year-old Japanese male with enlarging nodules on his finger and forearm associated with wooden splinters [22]. It was

identified as *E. jeanselmei* by morphological and molecular analysis. In contrast, the remaining strains (CBS 119095, CBS 109635, CBS 528.76 and CBS 664.76) from unpublished cases were maintained as originating from cutaneous infections (arm, hand or foot). Environmental strains formerly identified as *E. jeanselmei* were considered to be distinct from *E. jeanselmei s. str*. They were within 1% differences with ex-type strains of *E. oligosperma* (CBS 725.88), *E. xenobiotica* (CBS 118157) and *E. bergeri* (CBS 353.52) (Fig. 3). Although *E. oligosperma* is phenotypically similar to *E. jeanselmei* and has mostly been confused with that taxon, genetically the species are clearly distinct.

In our case report on CBS 122339, granules, the hallmark of mycetoma, were seen histopathologically in tissue (Fig. 1C,D). Parts of the grains were reniform, and clinical features of mycetoma, such as tumefaction, abscess formation, and fistulae were observed.

In summary, nine strains confirmed as *E. jeanselmei* originated from mycetoma or subcutaneous phaeohyphomycosis, as well as in one instance from chromoblastomycosis according to histopathological examination. Strains with identity (100%) or near-identity (approximately 99%) with the ex-type strain of *E. jeanselmei* appeared to be very consistent in clinical behavior. The case of chromoblastomycosis is remarkable. The switch between grain and muriform cell as a response to host factors needs to be studied.

Over the past two decades, more serious black yeast infections have been recognized. Different antifungal agents have been used in the treatment of mycetoma, such as amphotericin B, fluorocytosine, itraconazole, fluconazole, and voriconazole [33]. In the past, amphotericin B was the most potent antifungal drug for severe invasive fungal infections, but its use is associated with severe side effects, particularly nephrotoxicity and low efficacy and its use has been replaced by newer azoles and echinocandins. Unfortunately, little data are available on the *in vitro* antifungal susceptibility of conventional and new antifungal agents against confirmed strains of *E. jeanselmei*. In a comparative overview of *in vitro* activities of posaconazole, itraconazole, voriconazole, and amphotericin B against over 19,000 clinically yeasts and moulds, just 14 *Exophiala* species were included, without molecular identification [34]. Data have shown that posaconazole has good activity against agents that cause chromoblastomycosis, mycetoma, and phaeohyphomycosis, including *Exophiala* species and posaconazole was generally more active than itraconazole and amphotericin B against these organisms [34,35].

Two recent studies reported *in vitro* antifungal data on amphotericin B, itraconazole, posaconazole and voriconazole against molecularly confirmed *Exophiala* species. In the first, 188 clinical strains were analyzed and most appeared to be susceptible to the four widely used antifungal agents, except *Exophiala attenuata* which was resistant to amphotericin B. MIC_{50}s of all *E. jeanselmei* isolates relative to amphotericin B, itraconazole, voriconazole and posaconazole were 0.5, 0.03, 0.125 and $\leq$0.015 μg/ml, respectively, whereas the MIC_{50}s for *E. oligosperma* were 0.25, 0.06, 0.25, and 0.03 μg/ml respectively [35]. Fothergill *et al*. [36] studied the *in vitro* susceptibility to amphotericin B, itraconazole, posaconazole and voriconazole of 160 *Exophiala* strains including *E. jeanselmei* (n=8, originating from subcutaneous and deep infections) and *E. oligosperma* (n=40, from cutaneous, subcutaneous and deep infection). Concerning *E. jeanselmei*, the MIC_{50}s were determined for amphotericin B (0.5 μg/ml), itraconazole (0.03 μg/ml), posaconazole ($<$0.015 μg/ml) and voriconazole (0.125 μg/ml) but posaconazole was more active than others antifungals [36]. Similar results were found in our series, posaconazole showed high activity against *E. jeanselmei* and *E. oligosperma* in terms of MIC_{50}. *In vitro* testing of isavuconazole and voriconazole exhibited broad-spectrum activity against the majority of the opportunistic and pathogenic fungi [37]. In the present study, low *in vitro* activities isavuconazole (MIC_{50} 2 μg/ml) and caspofungin (MIC_{50} 4 μg/ml) were found with *E. jeanselmei* suggesting that these drugs might not be the optimal choice for treatment. In contrast, posaconazole demonstrated potent *in vitro* activity (MIC_{50} 0.031 μg/ml) against *E. jeanselmei* that cause mycetoma. Itraconazole has been used clinically in cases of mycetoma due to *E. jeanselmei*, with successful outcome [38]. Voriconazole has only been employed in some cases of cutaneous infections [39]. Isavuconazole until now has not been used clinically for *Exophiala* infections, but itraconazole was the only clinically active antifungal drug in the treatment of mycetoma due to *E. jeanselmei*. Infections by *Exophiala* species may require a combination of surgical and medical treatment. Although amphotericin B and itraconazole, with or without additional flucytosine, are currently regarded to be efficacious against cutaneous and subcutaneous lesions, the newer triazole agents, itraconazole and posaconazole, expand the therapeutic options for these mycoses. In conclusion, posaconazole and itraconazole demonstrated the highest *in vitro* antifungal activity against *E. jeanselmei* and *E. oligosperma* that had high MICs for caspofungin. Therefore posaconazole and itraconazole seem to be the most active drugs for treating *Exophiala* infections. We did not investigate the relation between MIC and clinical response to treatment; their clinical effectiveness in the treatment of *Exophiala* infections remains to be determined.

Acknowledgements

This study and the work of Hamid Badali were funded by a grant (No. 13081) from the Ministry of Health and Medical Education of Islamic Republic of Iran and the School of Medicine, Mazandaran University of Medical Sciences, Sari, Iran. In addition, a part of this study was supported by a grant from Basilea Pharmaceutica, Basel, Switzerland. We thank I. Curfs-Breuker for help in building up part of the antifungal susceptibility test.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

1 McGinnis MR. Mycetoma. *Dermatol Clin* 1996; **14**: 97–104.
2 Hay RJ. Agents of eumycotic mycetomas. In: Ajello L, Hay RJ (eds). *Topley and Wilson's Microbiology and Microbial Infections*, vol. 4. *Mycology*. London: Arnold, 2000: 487–496.
3 De Hoog GS, Guarro J, Gene J, Figueras MJ. *Atlas of Clinical Fungi*, 2nd ed. Utrecht/Reus: Centraalbureau voor Schimmelcul-tures/Universitat Rovira i Virgili, 2000.
4 Bittencourt AL, Londero AT. Tropical mycotic diseases. In: Doerr BW, Uehlinger E (eds). *Tropical Pathology*, 2nd edn. Vol. 8. Berlin: Springer Verlag, 1995: 707–798.
5 Satow MM, Attili-Angelis D, De Hoog GS, *et al*. Selective factors involved in oil flotation isolation of black yeasts from the environment. *Stud Mycol* 2008; **61**: 157–163.
6 Werlinger KD, Yen Moore A. Eumycotic mycetoma caused by *Cladophialophora bantiana* in a patient with systemic lupus erythematous. *J Am Acad Dermatology* 2005; **52**: 114–117.
7 Bonifaz A, De Hoog GS, McGinnis MR, *et al*. Eumycetoma caused by *Cladophialophora bantiana* successfully treated with itraconazole. *Med Mycol* 2009; **47**: 111–114.
8 Guillot J, Garcia-Hermoso D, Degorce F, *et al*. Eumycetoma caused by *Cladophialophora bantiana* in a dog. *J Clin Microbiol* 2004; **42**: 4901–4903.
9 Badali H, Gueidan C, Najafzadeh MJ, *et al*. Biodiversity of the genus *Cladophialophora*. *Stud Mycol* 2008; **61**: 175–191.
10 Naka W, Harada T, Nishikawa T., Fukushiro R. A case of chromoblastomycosis with special reference to the mycology of the isolated *Exophiala jeanselmei*. *Mykosen* 1986; **29**: 445–452.
11 Nucci M, Akiti T, Barreiros G, *et al*. Nosocomial fungemia due to *Exophiala jeanselmei* var. *jeanselmei* and a *Rhinocladiella* species: newly described causes of bloodstream infection. *J Clin Microbiol* 2001; **39**: 514–518.
12 De Hoog GS, Takeo K, Yoshida S, *et al*. Pleoanamorphic life cycle of *Exophiala (Wangiella) dermatitidis*. *Antonie van Leeuwenhoek* 1994; **65**:143–153.
13 De Hoog GS, Vicente V, Caligiorne RB, *et al*. Species diversity and polymorphism in the *Exophiala spinifera* clade containing opportunistic black yeast-like fungi. *J Clin Microbiol* 2003; **41**: 4767–4778.
14 Desnos-Ollivier M, Bretagne S, Dromer F, *et al*. Molecular identification of black-grain mycetoma agents. *J Clin Microbiol* 2006; **44**: 3517–3523.
15 Gams W, Verkley GJM, Crous PW. *CBS Course of Mycology,* 5th ed. Utrecht: Centraalbureau voor Schimmelcultures, 2007.
16 White TJ, Bruns T, Lee S, Taylor J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: InnisMA, Gelfand DA, Sninsky JJ, White TJ (eds). *PCR Protocols: A Guide to Methods and Applications.* SanDiego: Academic Press, 1990; 315–322.
17 Stamatakis A, Hoover P, Rougemont J. A rapid bootstrap algorithm for the RAxML Web-Servers. *System Biol* 2008; **57**: 758–771.
18 Hillis DM, Bull JJ. An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. *System Biol* 1993; **42**: 182–192.
19 Clinical and Laboratory Standards Institute (CLSI). *Reference method for broth dilution antifungal susceptibility testing of filamentous fungi*. *Approved Standard M38-A2*; 2nd edn: CLSI, Wayne, PA, USA, 2008.
20 Langeron M. Mycétome à *Torula jeanselmei* Langeron, 1928. Nouveau type de mycétome à grains noirs. *Ann Parasitol Hum Comp* 1928; **6**: 385–403.
21 Murray IG, Dunkerley GE, Hughes KEA. A case of Madura foot caused by *Phialophora jeanselmei*. *Sabouraudia* 1963; **3**:175–177.
22 Kawachi Y, Tateishi T, Shojima K, *et al*. Subcutaneous phaeomycotic cyst of the finger cased by *E. jeanselmei*: association with a wood splinter. *Cutis* 1995; **56**: 41–43.
23 Yamada Y, sugihara K, Van Eijk GW, *et al*. Coenzyme Q system in ascomycetous black yeasts. *Antonie van Leeuwenhoek* 1989; **56**: 349–356.
24 Bossler AD, Richter SS, Chavez AJ, *et al*. *Exophiala oligosperma* causing olecranon bursitis. *J Clin Microbiol* 2003; **41**: 4779–4782.
25 Badali H, Carvalho VO, Vicente V, *et al*. *Cladophialophora saturnica* sp. nov., a new opportunistic species of *Chaetothyriales* revealed using molecular data. *Med Mycol* 2009; **47**: 51–62.
26 Nishimura K, Miyaji M. Studies on the phylogenesis of pathogenic 'black yeasts. *Mycopathologia* 1983; **81**: 135–144.
27 Masuda M, Naka W, Tajima S, *et al*. Deoxyribonucleic acid hybridization studies of *Exophiala dermatitidis* and *Exophiala jeanselmei*. *Microbiol Immunol* 1989; **33**: 631–639.
28 Dixon DM, Polak-Wyss A. The medically important dematiaceous fungi and their identification. *Mycoses* 1991; **34**:1–18.
29 De Hoog GS. *Rhinocladiella* and allied genera. *Stud Mycol* 1977; **15**: 1–144.
30 Kawasaki M, Ishizaki H, Matsumoto T, *et al*. Mitochondrial DNA analysis of *Exophiala jeanselmei* var. *lecanii-corni* and *Exophiala castellanii*. *Mycopathologia* 1999; **146**: 75–77.
31 Wang L, Yokoyama K, Miyaji M, Nishimura K. Identification, classification, and phylogeny of the pathogenic Species *Exophiala jeanselmei* and related species by mitochondrial cytochrome *b* gene analysis. *J Clin Microbiol* 2001; **39**: 4462–4467.
32 Haase G, Sonntag L, Melzer-Krick B, de Hoog GS. Phylogenetic inference by SSU-gene analysis of members of the *Herpotrichiellaceae* with special reference to human pathogenic species. *Stud Mycol* 1999; **43**: 80–97.
33 Ameen M, Arenas R. Emerging therapeutic regimes for the management of mycetomas. *Expert Opin Pharmacother* 2008; **9**: 2077–2085.
34 Sabatelli F, Patel R, Mann PA, *et al*. *In vitro* activities of posaconazole, fluconazole, itraconazole, voriconazole, and amphotericin B against a large collection of clinically important molds and yeasts. *Antimicrob Agents Chemother* 2006; **50**: 2009–2015.
35 Zeng JS, Sutton DA, Fothergill AW, *et al*. Spectrum of clinically relevant *Exophiala* species in the U.S.A. *J Clin Microbiol* 2007; **45**: 3713–3720.
36 Fothergill AW, Rinaldi MG, Sutton DA. Antifungal susceptibility testing of *Exophiala* spp: a head to head comparion of amphotericin B,

itraconazole, posaconazole and voriconazole. *Med Mycol* 2009; **47**: 41–43.
37 Guinea J, Peláez T, Recio S. *In vitro* antifungal activities of isavuconazole (BAL4815), voriconazole, and fluconazole against 1,007 isolates of Zygomycetes, *Candida*, *Aspergillus*, *Fusarium*, and *Scedosporium* species. *Antimicrob Agents Chemother* 2008; **52**: 1396–1400.
38 Jaffar A, Tawfiq AL, Amr SS, Madura leg due to *Exophiala jeanselmei* successfully treated with surgery and itraconazole therapy. *Med Mycol* 2009; 1-5 iFirst. DOI: 10.1080/13693780802669194.
39 Loulergue P, Hot A, Dannaoui E, *et al*. Successful treatment of black grain mycetoma with voriconazole. *Am J Trop Med Hyge* 2006; **75**: 1106–1107.

This paper was first published online on Early Online on 01 February 2010.