Case Report

Pyrenochaeta romeroi: a causative agent of phaeohyphomycotic cyst

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A subcutaneous phaeohyphomycotic cyst caused by *Pyrenochaeta romeroi* in a 47-year-old Indian female is described. The organism was isolated repeatedly from the aspirated material from the cyst. It was identified by colony and microscopic characteristics, and sequencing of internal transcribed spacer regions of the rDNA. Although the patient recovered without antifungal therapy, the isolate appeared resistant to commonly used antifungal agents. To the best of our knowledge, this is only the second report of subcutaneous phaeohyphomycotic cyst caused by *Pyrenochaeta romeroi*.

Introduction

The members of the genus *Pyrenochaeta* are widely distributed in the environment in soil or in association with wood and plant debris (de Hoog *et al.*, 2000; Badali *et al.*, 2010). So far, four species, namely *Pyrenochaeta romeroi*, *Pyrenochaeta mackinnonii*, *Pyrenochaeta unguis-hominis* and *Pyrenochaeta keratinophila*, have been implicated in human infections (de Hoog *et al.*, 2000; Ferrer *et al.*, 2009; Badali *et al.*, 2010; Verkley *et al.*, 2010). Previously, *Pyrenochaeta unguis-hominis* has been isolated from cases of onychomycosis (English, 1980). However, the aetiological role of this species in nail infection has not been established unequivocally (Punithalingam, 1979). Most of the isolates of *Pyrenochaeta romeroi* and *Pyrenochaeta mackinnonii* have originated from clinical material obtained from patients with mycetoma (André *et al.*, 1968; Baylet *et al.*, 1968; David-Chaussé *et al.*, 1968; Young *et al.*, 1973; Thammayya *et al.*, 1979; Cerar *et al.*, 2009). The lesions usually develop slowly following trauma and remain localized to cutaneous and subcutaneous tissues (Borelli, 1979; Serrano *et al.*, 1998; Girard *et al.*, 2004). Apart from the aetiological role of *Pyrenochaeta romeroi* in black grain mycetoma, the species has recently been isolated from a case of subcutaneous phaeohyphomycotic cyst in an Indian female, thus increasing the spectrum of its clinical manifestations (Badali *et al.*, 2010). Here, we present a second case of subcutaneous

phaeohyphomycotic cyst caused by *Pyrenochaeta romeroi* in an Indian female with acute lymphoblastic leukaemia (ALL).

Case report

A 47-year-old Indian female with ALL, a resident of Kuwait since September 2006, was diagnosed with ALL in September 2006 and completed a hyper-CVAD (cyclophosphamide, vincristine, Adriamycin, dexamethasone, a high dose of methotrexate and a high dose of cytosine arabinoside) regimen in May 2007. In June 2007, she was started on maintenance therapy comprising prednisone, vincristine, methotrexate and 6-mercaptopurine. During her regular follow-up, she noticed a small round swelling over the proximal interphalangeal joint of the right index finger in July 2009. This lesion developed gradually over a 6-month period and remained localized with no discharge or sinus formation (Fig. 1). It was painless and attained a diameter of about 10 mm with central necrosis. The patient gave no history of trauma at the site of infection. She had not visited her home country since 2006. The pus from the cystic lesion was aspirated. It was non-sanguineous and free from any discrete granules. Microscopic examination of the aspirated material with 10% KOH–calcofluor and of smears stained with Gomori methenamine–silver revealed septate hyphal elements with irregular swellings (Fig. 2a, b). Haematoxylin and eosin-stained smears also showed fungal elements along with an acute inflammatory response containing a few epithelioid granulomas and multinucleated giant cells. No antifungal therapy was administered. Following repeated drainage of the exudates, the swelling subsided gradually over a 2-month period.

Abbreviation: ALL, acute lymphoblastic leukaemia.

The GenBank/EMBL/DDBJ accession number for the sequence of the ITS region of the *Pyrenochaeta romeroi* isolate from this study is FN826905.

Fig. 1. Phaeohyphomycotic cyst at the intraphalangeal joint of the right index finger.

Mycological investigations and antifungal susceptibility

A portion of the specimen was cultured on Sabouraud dextrose agar (SDA; Difco) supplemented with chloramphenicol (50 mg l^{-1}). The plates were incubated at

Fig. 2. Presence of branched, septate hyphae with swellings in the aspirated pus from the cystic lesion. (a) KOH mount with calcofluor; (b) KOH mount. Original magnification $\times 60$.

Fig. 3. Colony morphology (dark-olive-grey in colour) of *Pyrenochaeta romeroi* on potato dextrose agar after 2 weeks of growth at 25°C .

30°C and a dematiaceous mould grew at the site of inoculation after 4 days of incubation. The colonies were initially brownish but became olivaceous grey on further incubation. Microscopic examination of the culture showed brown-coloured septate hyphae without conidia. In order to identify the isolate, subcultures were made on potato dextrose agar (PDA), oat meal agar (OMA) and malt extract agar (MEA) and the plates were incubated at 25°C and 37°C . In addition, slide cultures were also prepared on these media and plates were incubated at 25°C and 37°C for up to 6 weeks and observed periodically for the formation of conidia, pycnidia or other characteristic morphological structures (de Hoog *et al.*, 2000). On MEA and PDA, at 25°C , the colonies were floccose, olivaceous grey and attained a diameter of 35 mm and 43 mm, respectively, after 2 weeks of incubation (Fig. 3). Pycnidia were formed on SDA as well as on PDA and OMA after 6 weeks of incubation at 25°C , mostly observed in the submerged growth on the periphery of the colony. They appeared pyriform in shape, measuring $85\text{--}120 \mu\text{m}$ in size, covered with dark-brown-coloured hyphae (Fig. 4a). Setae were also observed. A large number of hyaline, single-celled ellipsoidal to bacilliform conidia, measuring $2.2\text{--}4 \times 1.5\text{--}1.8 \mu\text{m}$ in size, were produced within pycnidia (Fig. 4b). No conidia were seen on aerial hyphae. The isolate grew well at 37°C but its growth was inhibited at 40°C .

In vitro susceptibility was determined by Etest (AB Biodisk) on RPMI 1640 medium supplemented with 2% glucose and buffered to pH 7.0 with 0.165 M MOPS (CLSI, 2002). The test was performed according to the manufacturer's instructions. The hyphal growth from 1-week-old culture was gently scraped from the surface and suspended in 2 ml normal saline and mixed with a vortex mixer. The larger growth particles were allowed to settle. The upper portion of the suspension was removed into another tube and an inoculum with an OD_{600} of 0.15 was used to inoculate the plates using a sterile cotton swab. The MIC was read after 72 h incubation at 35°C at a point where the border of the

Fig. 4. (a) Pycnidium (dark-brown) of *Pyrenochaeta romeroi* produced on oat meal agar after 6 weeks of incubation at 25 °C (bar, 40 µm); (b) ellipsoidal to bacilliform conidia discharged in large numbers from the pycnidium (bar, 5 µm).

elliptical inhibition zone intersected the scale on the antifungal strip. The MICs for the isolate were as follows: voriconazole, 0.008 µg ml⁻¹; posaconazole, 0.064 µg ml⁻¹; amphotericin B, 8 µg ml⁻¹; itraconazole, 3 µg ml⁻¹; fluconazole, >256 µg ml⁻¹; caspofungin, 6 µg ml⁻¹; and anidulafungin, 0.5 µg ml⁻¹ (Table 1).

Molecular identification

The DNA from the cultured isolate was prepared as described in detail previously (Ahmad *et al.*, 2007). The entire ITS region (containing the ITS-1, 5.8S rRNA and ITS-2) of rDNA was amplified by PCR by using extracted DNA and panfungal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCTTTTCCTCCGCTTATTGATATGC-3') according to the protocol described previously

(Ahmad *et al.*, 2005). The amplicons were purified by using a PCR product purification kit according to the instructions supplied by the kit manufacturer (Qiagen). Both strands of amplified DNA fragments were sequenced. The sequencing reactions were carried out by using the cycle DNA sequencing kit (DTCS CEQ2000; Beckman Coulter) as described in detail previously except that ITS1, ITS4, ITS1FS (5'-ACCTGCGGAAGGATCATT-3'), ITS2 (5'-TCGCTGCGTTCTTCATCGATGC-3'), ITS3 (5'-TCGCATCGATGAAGAACGCAGC-3') or ITS4RS (5'-GATATGCTTAAGTTCAGCG-3') (Khan *et al.*, 2008, 2010) were used as sequencing primers. GenBank basic local alignment search tool (BLAST) searches (<http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi>) were performed for species identification. The sequence of the entire ITS region (561 nt) of our isolate (EMBL accession no. FN826905) exhibited 100 % identity with the corresponding sequences from two strains (IP888.65 and B36) of *Pyrenochaeta romeroi* (GenBank accession nos DQ836803 and EF488398, respectively). The complete ITS region sequences from the type strain (IP 862.63/CBS 252.60) as well as two other strains (CBS 122784 and IP571.61) of *Pyrenochaeta romeroi* (GenBank accession nos DQ836802, FJ528674 and DQ836801, respectively) also exhibited only three nucleotide differences to the sequence of the entire ITS region of our isolate. In contrast, the ITS region sequence (GenBank accession no. EU885415) of a recently described species, *Pyrenochaeta keratinophila*, exhibited ~80 % identity only. Based on previous observations that strains belonging to same species exhibit >99 % nucleotide identity in the ITS-1 and ITS-2 regions of rDNA (Rakeman *et al.*, 2005; Desnos-Ollivier *et al.*, 2006), the molecular identity of our isolate was determined as *Pyrenochaeta romeroi*.

Discussion

The members of the anamorphic genus *Pyrenochaeta* are widely distributed in the environment and have been occasionally implicated in the aetiology of chronic cutaneous and subcutaneous infections (Badali *et al.*, 2010). The identification of *Pyrenochaeta* species is difficult because of the inability of some of the strains to readily produce characteristic diagnostic structures in culture and also due to the limited expertise available in most of the diagnostic microbiology laboratories. The classification of species in the genus *Pyrenochaeta* and allied genera is still under revision. Desnos-Ollivier *et al.* (2006) compared the interspecies sequence similarities of six *Pyrenochaeta* species by sequencing the entire ITS region of rDNA of several strains that were available in different culture collection centres. The results indicated that isolates identified as *Pyrenochaeta romeroi* were heterogeneous with intraspecies similarities ranging from 40 to 100 %, suggesting that several of these isolates were erroneously identified. In fact, only three strains were identified as *Pyrenochaeta romeroi* and clustered with the type strain IP 862.63 (Pasteur Institute Collection of Fungi, Paris, France). Recently, de

Table 1. Antifungal susceptibility profile of *Pyrenochaeta romeroi* isolates

Reference	Patient nationality	Site of infection	No. of isolates	Methods	Antifungal susceptibility ($\mu\text{g ml}^{-1}$)*						
					AP	FL	IT	VO	POS	CAS	AND
Cerar <i>et al.</i> (2009)	British Pakistani	Eumycetoma of right foot	1	EUCAST	—	—	—	>8	0.25	—	—
				Microbroth	—	—	>32	0.25	—	—	—
				EUCAST Macrobroth	—	—	>32	0.25	—	—	—
Badali <i>et al.</i> (2010)	Indian	Phaeohyphomycotic cyst	1	CLSI	4	>64	0.5	4	0.5	8	1
Present study	Indian	Phaeohyphomycotic cyst	1	Etest	8	>256	3	0.008	0.064	6	0.5

*AP, Amphotericin B; FL, fluconazole; IT, itraconazole; VO, voriconazole; POS, posaconazole; CAS, caspofungin; AND, anidulafungin.

Gruyter *et al.* (2009) also sequenced the 18S and 28S nrDNA gene regions of 18 *Phoma* strains and compared the sequences with representative strains of 39 allied anamorph genera including strains of *Pyrenochaeta* species of human and plant origin. While both the strains of *Pyrenochaeta romeroi* tested in this study formed a distinct subclade, they were only distantly related to strains of *Pyrenochaeta nobilis*. More recently, a new genus, *Pyrenochaetopsis*, has been created to accommodate the type species *Pyrenochaetopsis leptospora* comb. nov. along with several other strains that were previously classified in the genera *Phoma* and *Pyrenochaeta* (de Gruyter *et al.*, 2010). The above studies elucidate the confusion that exists in defining the taxonomic status of species that belong to the genera *Pyrenochaeta*, *Phoma* and *Pleurophoma*.

In 1975, Ajello (1975) proposed the term phaeohyphomycosis to include cutaneous, subcutaneous and systemic infections caused by a heterogeneous group of fungi that form dark-walled, dematiaceous, septate mycelial elements in tissues. The subcutaneous form of the disease usually remains localized to the deep dermis and underlying tissues and it has often been referred to as 'phaeohyphomycotic cyst' (Ajello, 1975; Sutton *et al.*, 2009). The centre of the cyst may undergo necrosis and eventually disappear. Histopathological examination of the aspirated material from the lesion may reveal dematiaceous yeast forms, pseudohyphae-like elements or septate hyphae or any combination of these morphological forms (Fader & McGinnis, 1988). Although the infection usually arises as a result of traumatic implantation of the fungus, it is not always recalled by the patients due to the protracted nature of the disease (Sutton *et al.*, 2009). We believe that this apparently was also the case with our patient. Subcutaneous phaeohyphomycosis is more commonly seen in countries of tropical and subtropical regions with a warm climate. Since *Pyrenochaeta* species are widely distributed in the soil, particularly in association with plants or decaying vegetable debris, agricultural/plantation workers could be at increased risk of acquiring infection due to accidental trauma. Most of the clinical isolates of *Pyrenochaeta romeroi* have originated from mycetoma patients with a history of residence in India, Pakistan and

Venezuela (Badali *et al.*, 2010). Phaeohyphomycotic cyst is a relatively new clinical condition caused by this species. Recently, a case of keratitis due to a novel species, *Pyrenochaeta keratinophila*, was described from Spain (Ferrer *et al.*, 2009). This species formed phialoconidia from the aerial hyphae, a feature that has not been observed in any of the other species of the genus *Pyrenochaeta* (Verkley *et al.*, 2010).

Information on the antifungal susceptibility of *Pyrenochaeta* species remains scanty due to non-availability of a large number of clinical isolates (Cerar *et al.*, 2009; Badali *et al.*, 2010). No antifungal susceptibility breakpoints are available for this species. The two isolates that have been tested by broth dilution methods showed reduced susceptibility to amphotericin B, fluconazole, itraconazole, voriconazole and caspofungin. Consistent with the above studies, our isolate appeared resistant to amphotericin B, fluconazole, itraconazole and caspofungin, but susceptible to voriconazole and posaconazole, by Etest. The lower MICs for our isolate with respect to voriconazole and posaconazole may be attributed to different methodologies used in susceptibility testing (Table 1). Likewise, little information is available about the management of subcutaneous infections caused by *Pyrenochaeta romeroi*. Based on the therapeutic experience with mycetoma cases, itraconazole and possibly other newer triazoles may be preferred over amphotericin B in the treatment of this mycosis (Cerar *et al.*, 2009; Badali *et al.*, 2010). Surgical drainage or debridement of the infected lesion along with prolonged use of one of the triazoles (such as itraconazole or posaconazole) appears to be the best available option. However, our patient did not require antifungal treatment and the lesion subsided with drainage alone, which is consistent with several other reports of subcutaneous mycoses caused by melanized fungi (Revankar & Sutton, 2010).

In conclusion, a case of phaeohyphomycotic cyst caused by *Pyrenochaeta romeroi* is described in a 47-year-old Indian female with ALL. The infection was probably acquired in Kuwait as the patient had not visited her native country for the previous 6 years. Despite her immunosuppressed state

due to anticancer therapy, the infection remained localized to subcutaneous tissue and required no antifungal treatment. The report highlights the role of *Pyrenochaeta romeroi* as an agent of phaeohyphomycotic cyst.

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