First case of disseminated phaeohyphomycosis in an immunocompetent individual due to Alternaria malorum

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A 27-year-old Iranian, previously healthy male presented with sub-cutaneous necrotic lesions with a localized dermatosis affecting the anterior chest, neck and face. These lesions consisted of singular, well-defined verrucous plaques which gradually developed and disseminated over time. The dermatosis was followed by the development of necrotic swollen lesions localized on the hard palate. The patient did not recall any history of trauma or puncture at any of the sites of infection. While histopathological examination of periodic acid-Schiff (PAS) stained material revealed irregular, unbranched, septate hyphae, direct examination (KOH 10%) of lesion samples demonstrated the presence of septate indistinct brownish hyphae. Alternaria malorum was isolated (CBS 126589) and its identity was confirmed by sequencing of the internal transcribed spacer (ITS rDNA). Since the palatal lesion reoccurred after 10 years and the patient’s condition did not improve with amphotericin B combination therapy, the lesion was surgical excised and he underwent antifungal therapy with amphotericin B and itraconazole. There was no dehiscence or fistula formation or any evidence of relapse of fungal infection during a one year follow-up and the patient was successfully cured. In vitro antifungal susceptibility tests revealed that the MIC values for those antifungals employed in this case were amphotericin B (0.125 μg/ml), fluconazole (32 μg/ml), itraconazole (0.125 μg/ml), voriconazole (1 μg/ml), and posaconazole (0.063 μg/ml). The MECs for caspofungin and anidulafungin were 0.25 μg/ml and 0.016 μg/ml, respectively. However, treatment of A. malorum infections with the latter agents remains to be evaluated.

Keywords Alternaria malorum, Pleosporales, ITS rDNA, in vitro susceptibility

Introduction

Numerous fungi can cause infections in otherwise healthy individuals after their traumatic implantation [1]. Among these are many black yeast-like fungi, which are often treated as a single group but phylogenetically belong to different ascomycete orders. This is particularly the situation with those that have melanized cell walls that cause human and animal disorders [1], such as species of Cladophialophora, Exophiala, Fonsecaea which belong to the order Chaetothyriales [1,2], while hyphomycete Cladosporium spp. and coelomycetes (fungi producing asexual fruit-bodies) are members of the order Dothideales [2]. Cladosporium species and certain coelomycetes are widely distributed in soil, on wood, and plant debris, and are often encountered as plant pathogens [3]. Fast-growing dematiaceous fungi such as Bipolaris, Curvularia,
*Exserohilum*, and *Alternaria* belong to the order *Pleosporales*, which may cause allergic sinusitis, or cutaneous and sub-cutaneous phaeohyphomycoses in immunocompromised hosts [3–5]. The term ‘phaeohyphomycoses’ is used to cover a heterogeneous group of cutaneous, subcutaneous and systemic fungal infections in which melanized hyphae or yeast cells are noted in samples through histopathology. The only common factor in phaeohyphomycotic infections is the presence of brownish fungal elements in the tissue, which excludes *Aspergillus* spp., dermatophytes and yeasts [1,5]. Here we present a case of disseminated phaeohyphomycosis due to *Alternaria malorum* in a 27-year-old immunocompetent male.

**Case report**

A 27-year-old Iranian, otherwise healthy male was admitted to the department of reconstructive surgery at Hazrat Fatemeh Hospital, due to a complaint of sub-cutaneous lesions with a localized dermatosis affecting the anterior chest, neck and face. The lesions consisted of singular, well-defined verrucous plaques which gradually developed and disseminated and this was followed by the formation of necrotic swollen lesions localized on the hard palate. A brief clinical history revealed that 11 years prior to his present admission, the patient had subcutaneous lesions on the chest, neck and face preceding the appearance of necrotic lesions in the mouth as a cleft palate. Physical examination revealed subcutaneous purple nodules on the chest, neck and shoulder, which were not painful, had no signs of inflammation and did not discharge any granules at the site of infections (Fig. 1 A–B after treatments).

Although initial computerized tomography (CT) scans were normal without any sinus infections, a second CT scan revealed postoperative changes of the bilateral maxillary and ethmoidal air cells without orbital involvement and ethmoidal sinuses with chronic mucoperiosteal change, left greater than right. In addition, imaging studies of cleft palate revealed an extensive mass growth which affected the surrounding bone. The growth was resected en mass including adjoining bone and palatal defect was covered by local tissue (Fig. 1 C–D). The patient did not remember any history of trauma or puncture at the sites of the infections. Laboratory investigations including a full blood count, blood chemistry, glucose concentration, liver and renal functions were within normal ranges. The patient underwent antibacterial therapy for 5–6 days with intravenous ceftriaxone and clindamycin during hospitalization. Biopsy specimens were obtained from all lesions, smears were prepared from this material and stained by Gomori-Grocott methenamine silver (GMS) and Periodic Acid-Schiff (PAS) stains. Histopathological examination

![Image](image_url)
of PAS stained sections revealed fungal granulomas in the fibrotic tissues centered on blood vessels. Granulomas were made up of epithelial cells and central and peripheral giant cells surrounded by neutrophils and eosinophils. These granulomas and central or peripheral giant cells contained irregular, unbranched, septate hyphae surrounded by considerable exudate, and blood vessels showed evidence of vasculitis (Fig. 2 A–B). The remaining biopsy tissue was sent for mycological investigation. Direct examination (KOH 10%) of a portion of the material revealed indistinct, septate, brownish hyphae. In addition, the tissue samples were inoculated onto Sabouraud’s dextrose agar (SDA; Difco) and SDA supplemented with chloramphenicol (50 μg/ml), which were incubated at 30–37°C for up to one week. No bacteria were detected in cultures of the biopsy specimens and serological test for human immunodeficiency virus (HIV) were negative, but fungi were detected in culture after 4–5 days of incubation. Growth of a melanized fungus was recognizable and identified based on conventional mycological method as a *Cladosporium* species. For case management, the lesion of the palate was treated surgically and antifungal therapy was started with intravenous amphotericin B deoxycholate (5 mg/kg/day, for one month) combined with oral itraconazole (400 mg/day for 6 month). During a one-year follow-up clinical improvement was observed, but after 10 years, the patient was again admitted to the heart clinic because of chest pain associated with severe toothache.

His complaints on the latest admission included purulent nasal discharge, epistaxis, nasal stiffness and obstruction, fever, cough, sneezing, anosmia, halitosis, headache and toothache. His physician recommended the extraction of all teeth and the use of dentures. Subsequently, a necrotic lesion appeared in the cleft palate (palatoschisis) which created problems including difficulty in eating, hearing, speaking and socializing. Repeat CT imaging revealed persistent sinusitis, and due to concerns about a possible invasive infection, he was returned for surgery. Cultures inoculated with portions of the surgically extracted tissue and sections of the same material stained with GMS and PAS resulted in findings similar to those reported above. The patient again underwent the combination of surgical and antifungal therapy with deoxycholate (5 mg/kg/day, for one month) and itraconazole (400 mg/day for one year), which resulted in gradual improvement in his condition. Finally, the decision to employ free tissue transfer was made after several confirmations that the surgical area was disease-free. To cover this 38 × 42 mm three-dimensional defect in the palate, a well vascularized thin tissue providing viable skin was needed. Since the length of the pedicle was an issue to obtaining a successful microsurgical transplantation, a free radial forearm fasciocutaneous flap was chosen (Fig. 3 A–B). The patient underwent surgery once the necessary tests were conducted and the patency of the ulnar artery and deep palmar arch were confirmed. The flap was dissected on the non-dominant limb in a distal to proximal direction on its radial artery pedicle and venae comitantes. After preparation of the facial vessels as the recipient vessels, the flap was harvested and transplanted. The nasal lining was restored by split thickness skin graft. The patient received postoperative care and was discharged after the donor site dressing and immobilizing splint were removed (Fig. 3 C–D). There was no relapse during the one-year follow-up and he was successfully cured. This research was approved by the ethics committee of Tehran University of Medical Science and written informed consent was obtained from the patient.

**Mycological investigation**

The fungal culture was deposited in the reference culture collection of CBS-KNAW, Utrecht, Netherlands, under

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**Fig. 2** (A–B) Periodic Acid-Shiff (PAS) staining reveals necrosis with dense and diffuse mixed inflammatory infiltrates with granuloma formation with central and peripheral giant cells and the presence of numerous irregular, unbranched, septate hyphae (arrows) surrounded by a dense inflammatory response.
Disseminated phaeohyphomycosis caused by *A. malorum* accession number dH 21363 = CBS 126589. This culture was first grown on oatmeal agar (OA, Difco) at 24°C for a period of 4–6 days, then transferred to potato carrot agar (PCA, Difco) and grown under standardized conditions (one week incubation at 23°C under an alternating light/dark cycle consisting of 8 h of cool-white fluorescent daylight and 16 h darkness) to suppress the growth of aerial hyphae and induce adequate sporulation [2]. Colonies showed rapid growth and were flat, powdery, with an olivaceous-black reverse on OA (Difco). Smears from old cultures were prepared in lactic acid and in sterile water and examined with a Nikon Eclipse 80i microscope equipped with a Nikon digital-sight DS-Fi1 camera. Septate, branching, dark olivaceous, thinner and more thread-like hyphae were observed, as well as cylindrical didymo- and phragmoconidia produced in very long branching chains (Fig. 4 A–C). Optimal growth was achieved at 27°C, but was also observed to occur between 12 and 37°C, albeit no growth was seen at 40°C. The fungus was phenotypically identified as *Alternaria malorum*, with sequencing used to confirm this identification.

**Molecular identification**

Identification was accomplished by growing the isolate on plates of 2% MEA for 5 days at 27°C [2]. DNA was extracted from 7-day-old PCA cultures with Ultraclean Microbial DNA isolation kit (Mo Bio Laboratories, Solana Beach, CA, USA) according to manufacturer’s protocol and stored at −20°C. The ITS rDNA region was amplified using primers V9G (5-TCCTGAGGTGACCTGCGG-3) and LS266 (5-GCATTTCCAAAACACTCAGACTC-3) and subsequently sequenced with internal primers ITS1 (5-TCCGATGCGACCTCGGG-3) and ITS4 (5-TCCTCGCTTATGATGATGC-3). PCR amplification and sequencing were conducted as previously described [6]. Briefly, the amplification of ITS rDNA was performed with cycles of 5 min at 94°C for primary denaturation, followed by 40 cycles at 94°C (30 s), 48°C (30 s), and 72°C (80 s), with a final 7 min extension step at 72°C. PCR products were first run on 1.5% agarose gels and visualized with UV after ethidium bromide staining and then subsequently were purified using GFX PCR DNA (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 [6]. Sequence data obtained were adjusted using Lasergene SeqMan software (DNASTar, Inc., Madison, WI, USA) and compared with GenBank and through local blast with a molecular database maintained for research purposes at the CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands. The DNA sequence of the ITS region matched that of *Alternaria malorum* (CBS 126589 = GenBank JQ219160) by showing 99.8% similarity with the ex-type strain of the
species (CBS 112048 = GenBank FJ214883) which had originally been isolated from Vitis in USA. The molecular results confirmed the mycological and histopathological diagnosis of the disease as a sub-cutaneous to disseminated phaeohyphomycotic due to A. malorum.

In vitro antifungal susceptibility testing

The in vitro antifungal susceptibility tests of A. malorum utilized the microbroth dilution method of the Clinical and Laboratory Standard Institute (CLSI) [7], with the modification described by Badali et al. [8]. Briefly, the antifungal agents were dispensed into microdilution trays at final concentrations of 0.016–16 μg/ml for amphotericin B, itraconazole, voriconazole, posaconazole and caspofungin, 0.063–64 μg/ml for fluconazole, and 0.008–8 μg/ml for anidulafungin. Inoculum suspensions were prepared from one-week-old potato carrot agar by slightly scraping the surface of mature colonies with a sterile cotton swab wetted with sterile saline including Tween 40 (0.05%). The supernatants were adjusted spectrophotometrically at a wavelength of 530 nm to an optical density (OD) that ranged from 0.25–0.3 (2 × 10⁴ – 7 × 10⁴ cfu/ml). The inoculum suspension which included mostly conidia was diluted 1:25 in RPMI 1640 medium. Microdilution plates were inoculated with 100 μl of the diluted conidial inoculum suspensions, incubated at 30°C for 48 h and read visually after agitation. Paecilomyces variotii (ATCC 22319) and Candida parapsilosis (ATCC 22019) were used as quality control [7]. The following MICs were found; amphotericin B (0.125 μg/ml), fluconazole (32 μg/ml), itraconazole (0.125 μg/ml), voriconazole (1 μg/ml), and posaconazole (0.063 μg/ml). The MECs for caspofungin and anidulafungin were 0.25 μg/ml and 0.016 μg/ml, respectively.

Discussion

During recent decades the incidence of fungal infections, ranging from mild cutaneous to fatal brain encephalitis, due to opportunistic dematiaceous fungi has increased remarkably both in immunocompromised and immunocompetent individuals [1,4,8]. The pathways of these infections has not been clarified but they have been reported to originate through the inhalation, ingestion or traumatic implantation of fungal elements into the skin leading to superficial, cutaneous, subcutaneous and rarely disseminated phaeohyphomycosis [9–11]. Although over 150 species and 70 genera of melanized fungi have been implicated in human and animal disorders, these fungi are ubiquitous in soil, rotten wood, dead plant debris and plant pathogens [12]. Those of the order Pleosporales are commonly encountered as agents of allergic sinusitis, and are emerging in opportunistic agents of cutaneous infections.
in immunocompromised patients [1]. Among the most common etiologic agents of such skin disease are species of *Alternaria* [4,13,14], a large and taxonomically complex genus [6,15] mainly comprising plant pathogens, while a few members, particularly *A. infectoria*, are regularly involved in human infections [1,2]. Andersen et al. [6] analyzed this species and its relatives, and found that the *A. infectoria* species-group is phylogenetically distant from the more common *A. alternata* forming a separate group with environmental species like *A. malorum*, *A. phototistica* and *Chalastospora cetera* [6]. However, identification based on morphological criteria is extremely difficult due to the high degree of similarity among *Alternaria* species, as well as the latter’s resemblance with related fungi. Anderson et al. has shown three different and very distinct morphologies among *Alternaria* species (Table 1).

Human or animal phaeohyphomycosis due to plant pathogens have rarely been reported in the literature [16]. *Alternaria malorum* was first discovered in Washington State, USA, and originally described from stored apple fruits in 1930 but was soon discovered to be widespread and often dominant on market wheat in the state. In subsequent years, the fungus was documented on many different hosts, including seeds of various plants of agronomic importance [17].

While no standard therapies for fungal infections associated with melanized fungi are available, surgical excision has successfully been applied in a number of cases [18]. Most forms of disease caused by *Alternaria* spp. require both surgical and therapeutic treatment, except for allergic fungal sinusitis, where antifungal treatment is not usually dominant on market wheat in the state. In subsequent years, the fungus was documented on many different hosts, including seeds of various plants of agronomic importance [17].

Table 1 Morphological and physiological differences between *Alternaria malorum*, *A. infectoria* and *A. alternata*.

<table>
<thead>
<tr>
<th>Alternaria species</th>
<th>Colonies on PCA and type of conidia</th>
<th>Size of conidia (μm)</th>
<th>Growth at 37°C</th>
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<tr>
<td><em>A. infectoria</em> species complex</td>
<td>Expanding grey to olivaceous, powdery or felty Conidiophores mostly unbranched, flexuose, elongating considerably, ovoid, obpyriform or obclavate phragmoand dictyoconidia, strongly branched chains</td>
<td>80–90 × 3–6</td>
<td>+/−</td>
</tr>
<tr>
<td><em>A. alternata</em></td>
<td>Expanding, grey to olivaceous, powdery or felty Catenulate, obclavate to obpyriform dark conidia with short conical beaks, branched acropetal chainsand multicelled, Surface smooth to verruculose</td>
<td>23–56 × 8–17</td>
<td>+/−</td>
</tr>
<tr>
<td><em>A. malorum</em></td>
<td>Rapid growing, flat powdery, olivaceous, dark brown reverse, cylindrical, didymoconidia, phragmoconidia, long and branching</td>
<td>10–12 × 2–3</td>
<td>+</td>
</tr>
<tr>
<td><em>A. malorum</em> var. polymorpha</td>
<td>Dark brown and the mycelium thinner and more thread-like, cylindrical, didymoconidia, phragmoconidia, long and branching</td>
<td>10–12 × 2–3</td>
<td>+</td>
</tr>
</tbody>
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PCA, potato carrot agar.

**Nucleotide sequence accession numbers**

The sequence of the ITS rDNA region from isolate CBS 126589 determined in the present study have been deposited in GenBank with the accession numbers JQ219160.

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**References**


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