Fatal Cerebral Phaeohyphomycosis in an Immunocompetent Individual due to *Thielavia subthermophila*: Case Report and Review of the Literature

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ABSTRACT

We report the first case of fatal brain infection in an Indian farmer caused by *Thielavia subthermophila*, a dematiaceous thermophilic fungus in the order *Sordariales* and present a review of previous infections from this order. The patient failed amphotericin B therapy combined with surgical excision despite low MICs *in vitro*. 
A 39-year-old male presented in the Emergency Department of Government Medical College Hospital (GMCH), Chandigarh with complaints of multiple episodes of generalized tonic clonic seizures for the past ten days. In addition to the seizures, he experienced uncontrolled movements of his limbs, incontinence of urine, rolling of eyes and production of foam from his mouth. There was a history of fever for two days, associated with an attack of seizures. He was a resident of Ambala (Haryana state, Northern India) and farmer by occupation with low socioeconomic status. He was first admitted to a local hospital in Ambala, and was subsequently transported to the GMCH. In the past he never had headache or any other significant complaints. There was no history of any trauma, roadside accidents, near-drowning, or similar predisposing factors.

On examination the patient was having an altered sensorium and was disoriented with a Glasgow Coma Score of E2V2M4. On the basis of generalized tonic clonic seizures, a presumptive diagnosis of meningioma was made. His chest X-ray and ECG were normal. A lumbar puncture was done and cerebral spinal fluid (CSF) was sent for cytological, biochemical and microbiological examination. Gram staining, Ziehl-Neelsen (ZN) staining and fungal smears of CSF were negative and there was no growth of either bacteria or fungi. The cytological and biochemical examination of CSF was non-contributory. The other laboratory investigations revealed haemogram, white blood cells, serum electrolytes, liver function, and glucose concentration within normal ranges.

The magnetic resonance image (MRI) of the brain showed a large supratentorial intracranial right frontotemporal (7.0×7.5×8.6 cm) space-occupying lesion and also small components devoid of frank edema alongside the frontal part of the falx cerebri, suggestive of meningioma in the right sphenoidal wing (Fig. 1A-B). Therefore, right frontotemporal craniotomy was done for excision of the intracranial mass, which intraoperatively showed a white, cheesy and gelatinous substance suggestive of infective pathology rather than of meningioma. The excised intracranial mass was sent for histopathological and microbiological examination. Direct microscopy revealed neither Mycobacterium nor other bacteria with ZN staining and Gram staining, respectively but KOH preparations showed septate, branched fungal hyphae (Fig. 2A). Histopathological examination with Periodic Acid-Schiff (PAS) staining
showed fungal granulomas in the brain parenchyma centered around blood vessels. Granulomas were made up of epithelioid cells with giant cells and collections of neutrophils. These granulomas and giant cells contained septate fungal hyphae and blood vessels showed evidence of vasculitis. There was also angioinvasion by the fungal hyphae however necrosis was not seen (Fig. 2B). Fungal culture was done with Sabouraud’s Dextrose Agar (SDA, HiMedia, Mumbai, India) with and without antibiotics and incubated at both 37°C and 22°C. The bacterial and mycobacterial cultures remained sterile. On the second day of incubation, SDA plates showed mycelial growth at both 37°C and 22°C. Lacto-phenol cotton blue (LCB) mounts of the black fungal growth showed sterile, dematiaceous hyphae which did not allow morphological identification.

The patient was treated with intravenous amphotericin B deoxycholate (1 mg/kg/day) but his condition deteriorated rapidly and he developed respiratory distress for which tracheostomy was necessary. Despite antifungal therapy the patient died two weeks later due to cardiac arrest and respiratory failure.

**MYCOLOGY**

The fungal culture was deposited in the reference collection of CBS-KNAW, Utrecht, The Netherlands under the accession number CBS 125981. Stock cultures were maintained on slants of 2% malt extract agar (MEA, Difco, Leeuwarden, The Netherlands) and oatmeal agar (OA) at 24°C (16). Colonies showed rapid growth, were flat, velvety to floccose with an olivaceous-black reverse on OA (Fig. 3A). 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 hyphae were observed. Supplementary cultures were prepared on MEA, potato carrot agar (PCA, Difco), potato dextrose agar (PDA) and oatmeal agar (OA) with or without Lupine stems and incubated at 25, 35, 42, 45 and 50°C for a period of 3 weeks under alternate near-ultraviolet light to suppress growth of aerial hyphae and induce adequate ascomata formation (16). After two weeks of incubation, scattered black ascomata were observed on all media tested (Fig. 3B). Walls of the ascomata consisted of *textura epidermoidea* (i.e. of jig-saw-shaped cells; 16) coated with dark hyphae. The brown, fusiform
ascospores were single-celled, 10−12 × 7.5−8.5 µm and had a characteristic, sub-apical germ-pore measuring 1 to 1.5 µm (Fig. 3C–E). Thermotolerance tests showed that the isolate grew rapidly at both 35°C and at higher temperatures of 42°C, 45°C and 50°C. The fungus was subsequently phenotypically identified as a *Thielavia* species. Sequencing was used to further identify it to the species level.

For molecular analyses, the fungus was grown on 2% MEA plates and DNA was extracted using an Ultra Clean Microbial DNA Isolation Kit (Mobio, Carlsbad, CA, U.S.A.) according to the manufacturer’s instructions. PCR amplification and sequencing were carried out according to Badali et al. (9). Briefly, the universal fungal primer pairs V9G (5-TTA CGT CCC TGC CCT TTG TA-3) / LS266 (5-GCA TTC CCA AAC AAC TCGA CTC-3) and LROR/LR7 were used for amplification of internal transcribed spacer ribosomal DNA (ITS) and 28S ribosomal rRNA (nucLSU), respectively. PCR reactions were performed on a Gene Amp PCR System 9700 (Applied Biosystems, Foster City, CA, U.S.A.) in 50 µL volumes containing 25 ng template DNA, 5 µL reaction buffer (0.1 M Tris-HCl, pH 8.0, 0.5 M KCl, 15 mM MgCl₂, 0.1% gelatin, 1% Triton X-100), 0.2 mM of each dNTP and 2.0 U Taq DNA polymerase (ITK Diagnostics, Leiden, The Netherlands). Amplification of ITS and nucLSU was performed with cycles of 2 min at 94°C for primary denaturation, followed by 35 cycles at 94°C (45 s), 52°C (30 s) and 72°C (120 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, U.K.). 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, U.S.A.). Sequence data obtained in this study were adjusted using the SeqMan of Lasergene software (DNASTar Inc., Madison, WI, U.S.A.). Sequences were compared with entries in GenBank and by using local blast searching in a molecular database maintained at the CBS and validated by ex-type strains. Isolate CBS 125981 was identified as *Thielavia subthermophila* by 98.98% and 100% identity at ITS rDNA and nucLSU regions, respectively, comparing GenBank submissions which included AJ271575.1 from the recent revision of *Thielavia* by Stchigel et al. (30), and by 99.6% identity with CBS 509.74, the ex-type strain of *T. subthermophila*. This led to the final diagnosis of the infection as a cerebral phaeohyphomycosis due to *Thielavia subthermophila*. 
In vitro antifungal susceptibility testing was performed using the M38-A2 reference method of the Clinical Laboratory Standards Institute (CLSI) by using a microbroth dilution format (15, 18). Briefly, ascospore suspensions were prepared from more than 3-week-old cultures on OA with Lupine stems (16) at 37°C by gently scraping the surface of mature colonies with a sterile moistened cotton swab. If large aggregates existed, they were allowed to settle for several minutes, the homogenous suspension of asci and ascospores were then transferred to sterile tubes and the supernatants were counted with a hemocytometer. Broth dilutions were done in RPMI 1640 medium with L-glutamine, without bicarbonate, and buffered with 0.165 M morpholinepropanesulfonic acid (MOPS). Paecilomyces variotii (ATCC 22319), Candida parapsilosis (ATCC 22019) and Candida krusei (ATCC 6258) were used as quality control organisms (15). The isolate showed the following antifungal susceptibility profile: amphotericin B, 0.03 µg/ml; fluconazole, 8 µg/ml; itraconazole, 0.016 µg/ml; voriconazole, 0.016 µg/ml; posaconazole, 0.016 µg/ml; isavuconazole, 0.016 µg/ml. The minimum effective concentrations of caspofungin and micafungin were 4 and 0.25 µg/ml, respectively.

**DISCUSSION**

During recent decades the diversity of fungal agents causing systemic disease has increased dramatically, especially in immunocompromised hosts, patients using broad-spectrum antibiotics, or patients with severe underlying diseases or undergoing solid organ transplantation. Melanized (dematiaceous) fungi are particularly significant because they not only infect debilitated hosts but also apparently healthy individuals giving infections which range from mild cutaneous to fatal brain disease (14, 17, 21-23, 29). Primary cerebral phaeohyphomycosis is a rare disorder characterized by black necrotic tissue and the production of pus, frequently occurring in humans without known predisposing factors or immunodeficiency (21, 23, 28). The infection is recognized as a disease associated with high mortality and ultimately a poor prognosis despite the application of surgery and antifungal therapy (14, 23). If untreated the infection may lead to death within weeks, months, or occasionally, years. The majority of etiological agents belong to a single order of known environmental fungi, the Chaetothyriales (e.g. Cladophialophora, Exophiala, Rhinocladiella and Fonsecaea). Occasionally species from
other orders are involved such as Sordariales (Chaetomium), Pleosporales (Bipolaris, Exserohilum), Xylariales (Nodulisporium), and Botryosphaeriales (Neoscytalidium) (23).

Thielavia is a common genus of environmental ascomycetes belonging to the family Chaetomiaceae in the order Sordariales. The genus is characterized by spherical, non-ostiolate ascomata with a thin peridium (ascoma wall), producing one-celled, darkly pigmented ascospores (7, 8, 16, 27). Taxonomy and phylogeny of Thielavia has been the subject of some confusion, because optimal markers for species distinction have not yet been established (30). A morphotaxonomic revision by von Arx et al. (7, 8) described 14 species, but developments in molecular techniques revolutionized species concepts. Thielavia subthermophila (not to be confused with T. thermophila Fergus & Sinden) was introduced by Mouchacca (26) for a species from desert soil which showed good growth at 45°C. The species is further characterized by the presence of pigmented mycelium and brownish-black, hairy ascomata. Thielavia gigaspora (27), T. arenaria, T. hyrcaniae and T. microspora (7) are distinguished by criteria of ascospore size and position of germ pores (30). Thielavia species have rarely been proven to be involved in human infections. Bourbeau et al. (13) described a disseminated infection due to Myceliophthora thermophila, the anamorph of Thielavia heterothallica and Theoulakis et al. (31) reported a keratitis due to T. subthermophila in a 10-year-old girl. Our patient is the first reported case of severe systemic infection due to T. subthermophila. Identification by a molecular approach like sequencing of ITS rDNA and sequence comparison to GenBank is the best and most simple approach for identification of these difficult sporulating fungi. This is an addition to the list of potential agents of primary brain abscesses in apparently healthy individuals, such as the black yeast-like relatives Cladophialophora bantiana, Exophiala dermatitidis, Fonsecaea monophora and Rhinocladiella mackenziei (9-11, 29). Primary or secondary brain infections are distinguished by their modes of infection, either by supposed hematogenous spread from an unrecognized pulmonary focus, or through direct extension from an adjacent focus, e.g. in paranasal sinuses or by a penetrating trauma to the head (14, 21, 24, 29).

Thielavia is closely related to Chaetomium (Sordariales, Chaetomiaceae), a large genus of saprobic ascomycetes that are widespread in soil, in plant debris and on wood. Both species have been recovered during air surveys in jute fields in India (32) suggesting the probable source in our patient, a farmer, has been airborne. Chaetomium globosum, C. atrobrunneum, C. funicola, and C. strumarium have occasionally been encountered in a wide variety of human
infections, such as onychomycosis, sinusitis, pneumonia and cerebral abscess. Fatal cerebral infection was repeatedly reported in intravenous drug users with a high mortality despite the administration of antifungal combination therapy (1). The disorder may be underdiagnosed because many ascomycetes in the Sordariales do not produce anamorphs and may appear as sterile mycelium. In addition, the correct identification of species described 20 years ago can be doubted, as these isolates have not been reexamined with molecular methods. We have the impression that members of Sordariales target relatively frequently the cerebrum (23). The optimal temperature for most Chaetomium species lies between 25 and 35°C (12, 25, 30), while those that have been reported to cause invasive infection grow very well above 35°C to 45°C.

The invasive infections due to Thielavia and Chaetomium species reported to date are summarized in Table 1. Cases of brain infection due to C. strumarium occurred in intravenous drug abusers, and in patients with hematologic malignancies and solid organ transplantation (1). Outcome was generally poor. Al-Aidaroos et al. (4) reported fatal invasive infections in two immunocompromised paediatric patients due to C. atrobrunneum. The present study adds another thermophilic member of the Sordariales (T. subthermophila) to the list of potential agents of phaeohyphomycotic brain infections.

The keratitis due to T. subthermophila reported by Theoulakis et al. (31) was successfully treated with topical amphotericin B and oral voriconazole. Treatment of cerebral phaeohyphomycosis generally includes surgical debridement, combined with antifungal therapy and/or immune enhancement. Although the etiologic agent in our case had a low in vitro MIC of amphotericin B, the patient died despite amphotericin B therapy. Murine studies showed no benefit from amphotericin B in cerebral phaeohyphomycosis due to poor penetration into the CNS (2) which may explain, in part, the treatment failure. Currently there is no accepted standard therapy for brain infections by melanized fungi, but in vitro data (9, 10, 11) and one clinical study (3) suggest that posaconazole may be a potential choice.

**Nucleotide sequence accession numbers:** The ITS rDNA and nucLSU sequences from isolate CBS 125981 determined in this study have been deposited in GenBank under accession numbers HM448441 and HM448442, respectively.
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Potential conflicts of interest

J.F. Meis received grants from Astellas, Merck, Basilea and Schering-Plough. He has been a consultant to Astellas, Basilea and Merck and received speakers fees from Merck, Pfizer, Schering-Plough and Janssen Pharmaceutica. All other authors report no conflicts of interest.
REFERENCES


Table 1. Overview of reported cases of systemic infections by members of the *Sordariales*.

<table>
<thead>
<tr>
<th>Agent</th>
<th>Age/sex</th>
<th>Host status</th>
<th>Site of infection</th>
<th>Therapy</th>
<th>Outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. subthermophila</em></td>
<td>39/M</td>
<td>Healthy</td>
<td>Brain</td>
<td>AmB</td>
<td>Died</td>
<td>present case</td>
</tr>
<tr>
<td><em>T. subthermophila</em></td>
<td>10/F</td>
<td>Healthy</td>
<td>Cornea</td>
<td>Ciprofloxacin, tobramycin, AmB, cefazolin</td>
<td>Cured</td>
<td>[30]</td>
</tr>
<tr>
<td><em>C. strumarium</em></td>
<td>20/M</td>
<td>IVDA</td>
<td>Brain</td>
<td>Ceftriaxone, penicillin, acyclovir</td>
<td>Died</td>
<td>[1]</td>
</tr>
<tr>
<td><em>C. strumarium</em></td>
<td>25/M</td>
<td>IVDA</td>
<td>Brain</td>
<td>Amoxicillin, acyclovir, AmB, rifampin, isoniazid</td>
<td>Died</td>
<td>[1]</td>
</tr>
<tr>
<td><em>C. strumarium</em></td>
<td>28/M</td>
<td>IVDA</td>
<td>Brain</td>
<td>Oxacillin, cefotaxime, metronidazole</td>
<td>Died</td>
<td>[1]</td>
</tr>
<tr>
<td><em>C. atrobrunneum</em></td>
<td>31/M</td>
<td>Multiple myeloma, allogeneic BMT</td>
<td>Brain, lung</td>
<td>AMB, ITC</td>
<td>Died</td>
<td>[19]</td>
</tr>
<tr>
<td><em>C. atrobrunneum</em></td>
<td>32/M</td>
<td>Renal transplant</td>
<td>Brain</td>
<td>Unknown</td>
<td>Died</td>
<td>[5]</td>
</tr>
<tr>
<td><em>C. globosum</em></td>
<td>19/F</td>
<td>Lymphoma / autologous BMT</td>
<td>Lungpleura</td>
<td>imipenem, vancomycin, amikacin, AmB</td>
<td>Died</td>
<td>[24]</td>
</tr>
<tr>
<td><em>C. globosum</em></td>
<td>24/M</td>
<td>ALL</td>
<td>Lung</td>
<td>AmB</td>
<td>Died</td>
<td>[20]</td>
</tr>
<tr>
<td><em>C. perlucidum</em></td>
<td>78/F</td>
<td>Asthma, chronic bronchiectasis</td>
<td>Lung</td>
<td>RML lobectomy</td>
<td>Cure</td>
<td>[12]</td>
</tr>
<tr>
<td><em>C. perlucidum</em></td>
<td>47/F</td>
<td>Leukemia, umbilical cord blood transplant</td>
<td>Multiple organs</td>
<td>L-AmB</td>
<td>Died</td>
<td>[12]</td>
</tr>
<tr>
<td>Chaetomium sp.</td>
<td>19/M</td>
<td>AML</td>
<td>Lung</td>
<td>L-AmB</td>
<td>Died</td>
<td>[33]</td>
</tr>
<tr>
<td>Chaetomium sp.</td>
<td>73/F</td>
<td>None</td>
<td>Left maxillary sinus</td>
<td>Infundibulectomy</td>
<td>Cured</td>
<td>[6]</td>
</tr>
</tbody>
</table>

Abbreviations used: BMT, bone marrow transplant; RML, right middle lobe; IVDA, intravenous drug abusers; AML, acute myelogenous leukemia; ALL, acute lymphocytic leukemia.
Legends to the Figures

Fig. 1. Coronal (A) and axial (B) MRI images of the brain demonstrating a large supratentorial intracranial right fronto-temporal space occupying lesion and also small, left frontal components devoid of frank edema on both sides of the falx cerebri.

Fig. 2. Septate hyphae observed in KOH wet mount (A) and (B) photomicrograph of brain parenchyma showing granuloma and septate hyphae. (PAS staining; 400x).

Fig. 3. *Thielavia subthermophila* (CBS 125981). (A) Culture on oatmeal agar at 42°C after 2 weeks in darkness with Lupine stem grew rapidly, was flat, velvety or floccose with olivaceous-black reverse. (B-C) Scattered black ascomata developing within hyphae in all. The brown, fusiform ascospores are single-celled (10-12×7.5-8.5 µm), and have a characteristic subapical germ pore measuring 1 to 1.5 µm (D-E). Scale bars, 10 µm.