A case of primary cutaneous mucormycosis produced by the new species Apophysomyces mexicanus.

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ABSTRACT

A case of fungal necrotizing fasciitis that appeared after a car accident is described in an immunocompetent Mexican woman. The patient did not respond to antifungal treatment and died four days later. The fungus was molecularly identified as a new species of Apophysomyces, namely Apophysomyces mexicanus.

CASE REPORT

A 46 year-old woman was admitted to the emergency department six days after suffering a rollover car accident, which occurred on a highway in a semi-arid region with 75% relative humidity; the patient was ejected 10 metres from the car during
the rollover, with consequent T12 vertebral fracture, Frankel grade B spinal cord injury and superficial abrasions and lacerations over the neck. The patient was immobilized with a Philadelphia collar straight after the accident and her medical history showed type-2 diabetes mellitus, controlled with metformin/glibenclamide and an allergy to non-steroidal anti-inflammatory drugs. She received initial treatment in a regional hospital, and was transferred to our hospital Emergency department six days later. After the collar was removed, a 3 cm fixed, irregular, red, swollen area was observed with a central necrosis of about 1 cm over the right posterior cervical triangle (Fig. 1A), along with multiple abrasions over the neck. Initial laboratory-test results showed: leukocytosis of 17 x 10⁹/L; glucose 10.77 mmol/L; haemoglobin, 121 g/L; hematocrit 0.352 L/L; urea nitrogen 3.92 mmol/L; urea 3.92 mmol/L; creatinine, 53.04 mmol/L. A CT scan of the neck after administering contrast material, revealed isodense homogeneous soft-tissue bulking posterior to the sternocleidomastoid muscle, with no evidence of foreign bodies. An escharectomy was carried out and ceftriaxone (2 g/day) and clindamycin (1.2 g/day) were administered. On the second day, the cellulitis and necrosis areas expanded to 10 cm (Fig. 1B), affecting the posterior, muscular and submandibular cervical triangles. The patient underwent surgical exploration of the neck and debridement, which showed necrotic tissue as far as the muscle. Material was collected and sent to the laboratory for histopathological examination and microbial culture. Direct mountings on KOH 10% w/v showed dichotomously branched, broad coenocytic hyphae (Fig. 1C), similar to those observed on hematoxylin-eosin histopathology preparations (Fig. 1D). Culturing on Sabouraud dextrose agar (Difco®, Becton Dikinson, México) produced white, cottony fungal
colonies made of pauci septated hyphae evocative of a fungus belonging to Mucorales. Consequently, primary cutaneous mucormycosis (PCM) was diagnosed, therefore amphotericin B deoxycholate 0.5 mg/kg/d and fluconazole 400 mg/d were administered. A second debridement removed all necrotic tissue, reaching the free edges of the lesion and leaving a surgical mark of 10 x 15 cm (Fig. 1E). Despite antifungal treatment, the patient showed significant overall deterioration and haemodynamic instability that required management with amines and antiarrhythmics. Two days later, the ulcer enlarged with more necrotic edges; a further surgical debridement was carried out and the dose of amphotericin B was increased to 0.75 mg/kg/d. After the third debridement, a surgical 20 cm diameter ulcer with clean edges was left. The borders reached the jaw angle, temporal and occipital scalp regions, the posterior cervical line, and the supraclavicular edge. The renal and hepatic function remained within normal ranges, but the patient developed metabolic acidosis, insulin therapy being necessary to control glucose levels. The cumulative dose of Amphotericin B was 380 mg. Although the patient's wound was clean and did not grow after the last debridement, and glucose levels were controlled, she was haemodynamically unstable, developed ventricular fibrillation and died four days after admission.

**Mycology.** The fungus was identified as *Apophysomyces* sp., characterized by culturing on potato dextrose agar (PDA Pronadisa, Spain), Czapek-Dox agar (CZA; Bifco, Becton Dickinson, France), and malt extract agar (MEA; 10 g of malt extract, 20 g of agar-agar, and 1000 mL of distilled water), for 4 d at 15, 25, 35, 37, 42 and 45°C incubation temperatures. The sporangiophores were mounted on
water and on lactic acid from CZA plates and incubated for six days at 37°C. They showed similar characteristics to the other species of *Apophysomyces*, with the exception of *A. elegans* (which produces both vase-shaped and funnel-shaped apophysises). The sporangiophore wall of our isolate was verrucose at maturity (including the apophysis) (Fig. 1F), remaining smooth-walled in the other species of the genus. The shape and the size of the sporangiospores of our isolate (Fig. 1G) were among those of *A. ossiformis* and *A. trapeziformis*. A carbon source assimilation profile was determined using the API 50 CH commercial kit (bioMérieux, Marcy, l’Etoile, France). The fungus assimilated glycerol, D-ribose, D-xylose, D-adonitol, D-glucose, D-fructose, D-mannose, D-mannitol, D-sorbitol, N-acetylglucosamine, D-maltose, D-trehalose, starch, glycogen, xylitol, D-lyxose, D-arabitol, L-arabitol, and potassium gluconate. Nitrogen source assimilation, growth in the presence of NaCl (2%, 5%, 7%, and 10%), MgCl$_2$ 2%, cycloheximide 0.1%, carried out as in Alvarez *et al.* (1), showed the ability of the fungus to assimilate arginine, cadaverine, creatine, creatinine, L-cysteine, L-leucine, L-lysine, L-ornithine, L-proline, L-tryptophan, and nitrate, as well as to grow on 2% NaCl and on 2% MgCl$_2$, like all the species of the genus. Esculin splitting, tested after inoculation on bile esculin agar (BEA; Panreac Quimica SA, Castellar del Vallés, Spain) in small Petri dishes (5 cm diam) and incubation at 37°C for up to one week, was negative despite the fungus being able to grow on this medium. We followed the previously described protocols in the DNA extraction, amplification and sequencing of the ITS region, D1/D2 domains of the 28S nrRNA gene and a fragment of the Histone H3 gene (H3) (1). The phylogenetic analysis of the combined data set (ITS, D1/D2 and H3; 1429 bp) encompassed the sequences of
our isolate and others corresponding to type or reference strains of the *Apophysomyces* spp. that we had used in a previous study (1). *Saksenaea vasiformis* (FMR 10131) was used as out-group. The alignments and phylogenetic analyses were carried out using MEGA v. 5.05 with Clustal W and maximum-likelihood (ML) algorithm, with the Kimura two-parameters as the substitution model. The robustness of the branches was assessed by bootstrap analysis of 1,000 replicates. In the ML tree (Fig. 2) we observed two main clades, one with a high bootstrap support (94% bs) and another one lower (65% bs). In the first one, we were able to distinguish two sub-clades: the *A. variabilis* sub-clade (77% bs) and another corresponding to the type species of the genus, *A. elegans* (100% bs). The second main clade split in two well-supported sub-clades: *A. ossiformis* (79% bs) and *A. trapeziformis* (99% bs), and an intermediate branch between them with our isolate (CBS 136361).

The combination of morphological, physiological and molecular results demonstrates that our isolate has enough differential characters from the other *Apophysomyces* species for us to propose the new species *A. mexicanus* to accommodate it.

*Apophysomyces mexicanus* Bonifaz, Cano, Stchigel et Guarro sp. nov. MycoBank MB 1122344. Fig. 1F-G.

Colonies on CZA attaining a diameter of 90 mm after 4 days of incubation at 37 °C, whitish, with scarce aerial mycelium, hyphae branched, hyaline, smooth-walled, 3–5.5 μm diam; reverse concolorous. Sporangiophores erect, arising singly, at first
hyaline but soon becoming light greyish-brown, straight to slightly sinuous, tapered towards the apex, unbranched, 100–700 μm long, 3.5 μm wide below the apophyses and 5–7 μm wide at the base, at first smooth-walled but finally strongly verrucose, thin- to slightly thick-walled, bearing hyaline rhizoids at the base and 1–2 lateral stolons. Sporangia apophysate, terminal, obpyriform, multisспорed, white at first, becoming light greyish-brown when mature, and 25–30 μm diam. Columella lens-shaped to subspherical, 8–15 x 12–20 μm. Apophyses short, cup- to funnel-shaped. Sporangiospores slightly trapezoid-shaped in side view, cylindrical in front view, with flattened to slightly concave lateral walls, hyaline to light brown in mass, smooth- and thin-walled, and 5–6.5(–10) x 3–4 μm. Colonies on PDA, and MEA floccose, whitish, and with less sporulation than on CZA. Minimum and maximum temperatures of growth 15 and 45 °C, respectively.

**Holotype.** CBS H-21410, isolated from biopsy material from a necrotic lesion in the neck of a patient (woman, diabetic), México D.F., México, 05-XI-2009, collected by L. Pintos and E. Guevara, isolated by A. Bonifaz. Living cultures: CBS 136361, FMR 12552.

*In vitro* antifungal susceptibility results as measured according to the Clinical and Laboratory Standards Institute M38-A2 guidelines for filamentous fungi (2) were as follows: 4 μg/mL amphotericin B, and 0.5 μg/mL posaconazole.

Cutaneous mucormycosis can be divided into two types: primary and secondary. The latter is the most frequent clinical presentation and, in most cases
derives from rhino-cerebral form. In our experience, approximately 70% have presented a cutaneous expansion related to uncontrolled diabetes mellitus and immunosuppression (neutropenia) (3). PCM is usually acquired by inoculation of material contaminated with spores, as in sites of venipuncture and application of adhesive tapes in severely immunocompromised hosts (3, 4). However, PCM can also occur in immunocompetent individuals, where the only predisposing factors identified are serious injuries (burns, car crashes) (3, 4). There have been recent reports and small outbreaks caused by lacerations and injuries due to tornadoes (5, 6) and other natural disasters, such as tsunamis and volcanic eruptions (4, 7).

The present case, from a clinical perspective, is similar to others reported (3, 8, 9). We believe the infection was acquired through a skin laceration and inoculation of a foreign body of small diameter during the accident and encouraged by the location of the Philadelphia collar, which probably created an optimal micro-environment for the fungus. A microscopic examination of KOH mountings of biopsy material from surgical debridement allowed the diagnosis of mucormycosis to be confirmed. We need to draw attention to the rapid progress of the infection, which spread and deepened easily, typically characteristic of necrotizing fasciitis (4, 10, 11). Although the patient would have been considered immunocompetent, the history of controlled type 2 diabetes mellitus should be emphasized, which is a high risk factor for infection by Mucorales (3, 12). Roden et al. (13), stated that there is a 19% incidence of PCM, 34% of those due to traumatic inoculation, and only 3% associated to car crashes. However, Chakrabarti et al. (14) and
Chakrabarti (15) reported the prevalence of PCM associated to car accidents to be higher.

In the present case, despite administering amphotericin B and fluconazole and carrying out extensive surgical debridement, the infection spread extremely rapidly. The reported therapeutic response of *Apophysomyces* spp. to antifungals is highly variable. Most cases had previously been managed similarly to our patient, with with amphotericin B and surgical debridement treatment. Recent reports have shown that posaconazole has a good *in vitro* response (MIC 0.1g / L) (16, 17, 18) as well as *in vivo* (3, 16, 18), although in the cases of mucormycosis due to *Apophysomyces trapeziformis*, as with the Joplin tornado, only 6 out of 13 patients responded to treatment with that compound (6, 19).

The phenotypic characterization of the isolate CBS 136361 allowed us to find certain differences from the other species of the genus, such as the production of verrucose sporangiophores (smooth-walled in the other species of the genus), an intermediate spore shape between *A. ossiformis* and *A. trapeziformis*, and the assimilation of potassium gluconate, while *L*-arabinose, *D*-cellobiose, and *D*-melezitose were not assimilated. However, the pattern of nitrogen sources, also assimilating the resistance to NaCl, MgCl₂ and to cycloheximide, was similar to those of the other species of the genus (1). The ITS, D1/D2 and H-3 nucleotide sequences, as well as the phylogenetic tree inferred from them, show that our isolate is clearly separate from the other species of *Apophysomyces*. Consequently, we consider this isolate represents a new species of the genus.

Although only the present isolate of *A. mexicanus* so far exists, it is important to be
aware of the high MIC of amphotericin B against this strain, since this drug is recommended for the treatment of mucormycosis. An experimental study tested different isolates of Apophysomyces variabilis, the most common species of the genus, showing better susceptibility to posaconazole compared to amphotericin B (18).

Nucleotide sequence accession numbers. The ITS, D1/D2 and H-3 sequence data of the case isolate have been deposited into GenBank under accession numbers HG974255, HG974256 and HG974254 respectively.

REFERENCES


Fig. 1. A. The initial phase of the lesion. B. Dry ulcer expansion. C. Broad, coenocytic, dichotomously branched hyphae seen on a 10% KOH direct mounting (bar, 10 μm). D. Hematoxylin-eosin staining of biopsy material, showing many wide, coenocytic hyphae (bar, 10 μm). E. An ulcer after the second surgical debridement. F. *Apophysomyces mexicanus* (CBS 136361), sporangiophore (scanning electron microscope) (bar, 15 μm). G. *Apophysomyces mexicanus* (CBS 136361), sporangiospores (Nomarski) (bar, 10 μm)

Fig. 2. Maximum-likelihood (ML) tree based on Kimura two-parameters among the combined data set (ITS, D1/D2 and H3) sequences of our isolate and type and reference strains of other *Apophysomyces* spp used in a previous study (1). Numbers on the branches are bootstrap ML values above 65. Branch lengths are proportional to distance. Type strains of the different species are indicated with T. The new species proposed in this study is indicated in boldface.
A. variabilis (GMCH 480/07)
A. variabilis (UTHSC 03-3644)
A. variabilis (UTHSC 06-4222)
A. variabilis (GMCH 211/09)
A. variabilis (IMI 338332)
A. variabilis (IMI 338333)
A. variabilis (CBS 658.93T)
A. elegans (CBS 477.78)
A. elegans (CBS 476.78T)
A. ossiformis (UTHSC 04-838T)
A. ossiformis (UTHSC 07-204)
A. mexicanus (CBS 136361T)
A. trapeziformis (UTHSC R-3841)
A. trapeziformis (UTHSC 08-2146)
A. trapeziformis (UTHSC 04-891)
A. trapeziformis (UTHSC 06-2356)
A. trapeziformis (UTHSC 08-1425T)
Saksenaea vasiformis (FMR 10131)