Case Report

Saksenaea erythrospora infection following a serious sailing accident

Silvia Relloso,1,2 Vanesa Romano,2 Maria Fernanda Landaburu,1 Fabian Herrera,2 Jorgelina Smayevsky,2 Cecilia Vecino1 and Maria Teresa Mujica1

Correspondence
Maria Teresa Mujica
mtmujica@gmail.com

1Instituto de Microbiología y Parasitología Médica, Universidad de Buenos Aires – Consejo Nacional de Investigaciones Científicas y Técnicas (IMPaM, UBA-CONICET), Argentina
2Centro de Educación Médica e Investigaciones Clínicas ‘Dr Norberto Quirno’ (CEMIC), Buenos Aires, Argentina

Saksenaea erythrospora is a species of the order Mucorales recently described and reported as a cause of human mucormycosis. We report a case of S. erythrospora in a man involved in a serious sailing accident causing deep skin and soft tissue contamination with soil and water. Direct microscopic examination of the clinical sample with Giemsa stains showed hyaline and non-septate hyphae belonging to the order Mucorales. Fungal identification was performed by culture of biopsy material on SDA, and identification of species by floating an agar block containing the fungus in a nutritionally deficient medium consisting of sterile distilled water supplemented with 0.05 % yeast extract; and by sequencing the ITS region of the rDNA. This is the first report to our knowledge of infection with S. erythrospora in Argentina, confirming the presence of this fungus in this country.

Introduction
Species of the genera Rhizopus, Mucor and Lichtheimia are the most common agents causing human mucormycosis. Species of the genera Saksenaea and Apophysomyces have been included among the agents of these infections (Gomes et al., 2011).

Mucormycosis may manifest as rhinocerebral, pulmonary, abdominal, pelvic, cutaneous, and disseminated forms. Cutaneous and subcutaneous infections by species of the genera Saksenaea and Apophysomyces are likely to be associated with soil contamination of skin lesions in immunocompetent individuals (Chakrabarti et al., 1997; Alvarez et al., 2010; Mayayo et al., 2013). The use of classical morphological characteristics for the microbiological identification of mucormycosis remains difficult since it is time-consuming and may require the expertise of a reference laboratory. Additionally, some filamentous isolates from human infections fail to produce diagnostic propagules in culture.

Molecular-based identification tools have been evaluated for several groups of medically important fungi (Iwen et al., 2002). The internal transcribed spacer region (ITS) of the nuclear rDNA has been proven to be a good phylogenetic marker in Mucorales (Balajee et al., 2009; Guarro, 2012; Alvarez et al., 2010; Mayayo et al., 2013; Walther et al., 2013). A recent taxonomic revision of Saksenaea based on morphological as well as on molecular characters revealed that the morphologically defined species Saksenaea vasiformis includes two additional species, Saksenaea erythrospora and Saksenaea oblongispora (Alvarez et al., 2010).

Up to now, approximately 40 cases of Saksenaea infections, mostly cutaneous infections, have been reported around the world (Vega et al., 2006; Mayayo et al., 2013), although the actual number of clinical cases is likely to be underestimated. So far there has been only a single case report on a human infection caused by S. erythrospora (Hospenthal et al., 2011). In this case the patient became infected as a consequence of a combat trauma. Lawhon et al. (2012) described a veterinary case in a premature bull calf.

We describe a case of myositis by Saksenaea erythrospora in a patient who suffered a serious sailing accident. Furthermore, phenotypic and molecular methods used for the identification of Saksenaea are also discussed.

Case report
In September 2011, a 55-year-old man was referred to our hospital because of severe injuries. Two weeks previous to
hospitalization he had had a sailing accident in a river and suffered deep skin and soft tissue injuries, with muscular avulsion. He had been successfully treated with ampicillin–sulbactam (1.5 mg/6 h) and ciprofloxacin (400 mg/12 h) for 15 days in another hospital owing to a soft tissue poly-microbial infection by *Enterococcus faecalis*, *Enterobacter cloacae* and *Aeromonas* spp.

The patient was afebrile and in good general condition when admitted to our hospital. However, he presented an open, large and deep wound, with muscles and fascia exposure in the inner region of the right thigh. During surgical debridement, purulent discharge was observed, and *Pseudomonas aeruginosa* was isolated. He was treated with piperacillin–tazobactam (4.5 g intravenously every 6 h) for 12 days. Twelve days after admission he presented fever and thick caseous discharge from soft tissues and muscles. An abscess on the thigh with inflammatory involvement of quadriceps, adductor and internal straight muscles was observed by magnetic resonance imaging (MRI).

Several samples were collected for microbiological studies. Microscopic examination using Giemsa stains showed hyaline and non-septate hyphae with right-angle branching. *Mucormycosis* was diagnosed and treatment with liposomal amphotericin B (L-AmB) 5 mg kg<sup>−1</sup> day<sup>−1</sup> was initiated. Culture of tissue samples on SDA (Sabouraud–glucose agar; Britainia) grew a white aerial mycelium after 5 days at 30 °C and 37 °C. Microscopic examination showed non-septate sterile hyphae from the culture.

Debridement was performed every other day revealing the same discharge of hyaline and non-septate hyphae found in all cases by microscopic examination but cultures were always negative.

Hyperbaric oxygen therapy was then administered after day 28 of L-AmB treatment and day 20 of successive local debridement to remove devitalized tissues. After 15 sessions of hyperbaric oxygen therapy, a new MRI showed further muscle inflammatory involvement. Samples collected from muscle discharge were scarce and did not show hyphae. The patient was discharged on posaconazole treatment 400 mg every 12 h, but admitted again within 10 days due to fever. Direct examination showed hyaline and non-septate hyphae, suggesting *Mucorales*. MRI revealed persistent inflammatory changes in quadriceps. Treatment with interferon-gamma was added. The clinical response consisted of improvement in muscle inflammatory changes at MRI and negative microbiological results.

Isolate recovered from caseous discharge of soft tissues and muscles (SRM) was cultured on different media to study growth characteristics. Colonies on SDA are fast-growing and white with no pigment on the reverse. The fungus grew as sterile mycelia in malt extract agar, potato dextrose agar, SDA, Czapek–Dox agar (Becton Dickinson), and in an agar block with sterile distilled water and salt water (0.85 % NaCl). Sporulation was achieved by floating the agar block containing fungal culture in a nutritionally deficient medium solution for 7 days at 37 °C (Padhye & Ajello, 1988). A few typical flask-shaped sporangia (Fig. 1) arising singly from erect sporangiophores were observed. Sporangiophores were hyaline at first but soon became light brown with dichotomously branched and darkly pigmented rhizoids at their base. Sporangiospores were mostly ellipsoid but biconcave in the lateral view. A mucilaginous plug (Fig. 1a) was observed on the apex of immature sporangia. The characteristics described identify the isolate as a member of the genus *Saksenaea*.

Molecular identification was based on PCR amplification and sequencing of ITS. Briefly, the isolate was incubated in YPD broth (1 % yeast extract, 2 % peptone, 2 % glucose). The mycelial mass was centrifuged and washed with 1 M sorbitol. Genomic DNA extraction and purification were done using the QIAmp DNA Mini kit (Qiagen). DNA quality and quantity were estimated by spectrophotometry (SmartSpec 3000 spectrophotometer; Bio-Rad).

The ITS was amplified by PCR using primers ITS1 and ITS4 (White *et al.*, 1990). Amplicons were purified using a QIAquick PCR Purification kit (Qiagen) and then bidirectionally sequenced using an ABI Prism 3100/3100-Avant Genetic Analyzer (Applied Biosystems) with the same primers as used for PCR amplification. The nucleotide sequences obtained were compared with those available in the GenBank database using BLASTN [National Center for Biotechnology Information (NCBI) Internet homepage, http://blast.ncbi.nlm.nih.gov/Blast.cgi].

![Fig. 1. Microscopic characteristics of the isolate of *S. erythrosora* cultured in a nutritionally deficient medium consisting of sterile distilled water supplemented with 0.05 % filter-sterilized yeast extract. (a) Incipient sporangium and sporangiospores. The apex of the sporangium presents a mucilaginous plug (bar, 100 μm). (b) Typical flask-shaped sporangium containing sporangiospores. Columella are seen at the base of the sporangium. Light brown sporangiophores arise from darkly pigmented rhizoids (bar, 100 μm). (c) The arrow shows an ellipsoid, smooth-walled, biconcave sporangiospore, in a lateral view (bar, 10 μm).](image-url)
Alignment of over 456 bp corresponding to ITS1 (partial sequence), the 5.8S ribosomal gene (complete sequence) and ITS2 (partial sequence) with S. erythrospera FR687331 and S. vasiformis EU182902 showed 96% similarity, query coverage 100% and an E-value of 0.0, and 96% identity, query coverage 92% and E-value 0.0, respectively. Sequence obtained in this study was submitted to GenBank.

Sequences of the ITS of the SRM isolate were aligned with sequences belonging to Saksenaea spp. and related species deposited in GenBank using the CLUSTAL W program. Neighbour-joining (NJ) phylogenetic trees were reconstructed using the MEGA package, version 5.05 (Tamura et al., 2011).

In the phylogenetic tree, the SRM isolate recovered from our patient was included in the S. erythrospera cluster (Fig. 2).

Discussion

Mucormycosis caused by Saksenaea spp. most often occurs after traumatic implantation of the fungus with contamination with soil, but can also be due to inhalation of spores, a tattoo, spider bites, insect stings, and the use of indwelling catheters (Garcı´a-Martı´nez et al., 2008; Chakrabarti et al., 1997; Lechevalier et al., 2008; Oberle & Penn, 1983; Parker et al., 1986; Mayayo et al., 2013). The rapid progression of the disease highlights the importance of correct diagnosis, identification of the aetiological agent and appropriate treatment.

The microbiological identification of agents of mucormycosis is difficult to obtain. Culture may not be requested if the diagnosis is not suspected. Certain methods of processing, such as the use of a tissue grinder, may damage the coenocytic mycelium, thus losing its viability. Isolates may fail to grow on subculture or fail to sporulate, and therefore they are not reliable for species identification.

Only one of several specimens collected from this patient was positive for the mucoralean isolates, and sporangia were not detected in either primary culture. We observed the key features of the genus Saksenaea only by means of floating an agar block containing fungal culture in a nutritionally deficient medium. As multiple methods of inducing sporulation of Saksenaea spp. have met with variable degrees of success (Bearer et al., 1994; Blanchet et al., 2008; Blanchet et al., 2013). The rapid progression of the disease highlights the importance of correct diagnosis, identification of the aetiological agent and appropriate treatment.

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the need for reliable tools to improve diagnosis, epidemiological investigations, and treatment evaluation of mucormycosis has led to the development of molecular tools (Guarro, 2012; Balajee et al., 2009). We could not reliably identify our strain with a BLASTN search because we observed two strains of two different species with the same similarity. The sequence EU182902 obtained from a strain that caused a cutaneous lesion (Blanchet et al., 2008) probably also belongs to S. erythrospora, but in 2008, when the sequence was deposited in GenBank, S. erythrospora had not been recognized. A simple BLASTN search does not always allow species identification because of misidentifications or obsolete names in GenBank. Phylogenetic analyses including ex-type sequences of all currently accepted species allowed identification of our strains as S. erythrospora. In our case, the molecular approach allowed a faster identification of the isolate than the conventional method based on morphological traits.

The clinical relevance and incidence of infections caused by S. erythrospora remain unknown, although a fatal case caused by this species has been reported recently (Hospenthal et al., 2011). To our knowledge, this is the first report of infection with S. erythrospora in Argentina and confirms the presence of this fungus in our country.

As our patient had relapse of mucormycosis, appropriate treatments for the eradication of the S. erythrospora were administered, including multiple surgical debridement, combined therapy with L-AmB and posaconazole (Mayayo et al., 2013; Rogers, 2008; Kontoyiannis & Lewis, 2011), the use of a hyperbaric chamber and the use of interferon-gamma (Spellberg et al., 2005; Rogers, 2008; Kontoyiannis & Lewis, 2011).

It is noteworthy that the clinical features of the patient were very important for diagnosis. However, microscopic examination of the sample, culture of the mucoralean isolates on media that promote the reproductive structure production, and sequencing the ITS led us to make a definitive diagnosis of mucormycosis by S. erythrospora. We must emphasize that the molecular identification of mucoralean isolates accelerated the time of fungal identification.

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