Direct sequencing of *Scedosporium apiospermum* DNA in the diagnosis of a case of keratitis

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The present report describes the diagnostic strategy followed in a case of keratomycosis. Together with conventional methods, a molecular strategy that involved the direct sequencing of an amplified portion of the genome encompassing the internal transcribed spacer 1 and 2 regions and sequence analysis was used. The data highlight the diagnostic role of molecular techniques, in parallel with conventional methods, in the management of ocular infections of fungal aetiology.

**Introduction**

The incidence of fungal infections of the eye has dramatically increased in the last few years for several reasons, including greater diffusion of immunosuppressive or broad-spectrum antibiotic therapies and improvement of laboratory detection systems (Klotz et al., 2000; Thomas, 2003). Mycotic aetiologies are involved in a high percentage of ocular infections, for example 16–37 % of keratitis cases (Ferrer et al., 2001) and 8–18.5 % of the more severe endophthalmitis. This makes rapid and specific diagnosis an important element in the clinical management of these conditions. Standard microbiological tests are useful in mycotic keratitis but often lack rapidity, usually taking longer than a week for appropriate growth and definitive identification of the aetiological agent (Han et al., 1996; Kunimoto et al., 1999a, b; Thomas, 2003). The identification of the infectious agent, at least at the genus level, is not just a mycological curiosity, but is an important step in the choice of an effective therapy because of the emergence of uncommon fungal pathogens constitutively resistant to some antifungal drugs. Moreover, a conventional mycological identification approach could be difficult in some cases, especially when morphological characteristics are not easily differentiated. In this context, the development of a culture-independent sequence-based approach could be of great help in all cases where a keratomycosis is suspected.

In this report, we describe a case of keratitis of mycotic aetiology managed with conventional culture approaches in parallel with molecular techniques.

**Case report**

A 42-year-old male presented at our hospital reporting severe pain, redness and foreign body sensation in his right eye. The cornea showed an ulcer of 4 × 3 mm with infiltrate and reduction of corneal thickness to about 30 %; hypopyon of 1.5 mm in the anterior chamber was present (Fig. 1a). Best corrected visual acuity was 0.2. Previously, he had been treated in another setting for 1 month with various eye drops including 0.3 % ofloxacin and 0.3 % tobramycin, with no significant clinical improvement. No trauma possibly related to this condition and no history of contact lens wear was reported; furthermore, immunodeficiency and drug or alcohol abuse could be excluded.

Topical therapy was withdrawn for 24 h before corneal scraping and the sample was directly inoculated onto chocolate agar and Sabouraud dextrose agar supplemented with chloramphenicol and gentamicin. Gram stain showed a moderate polymorphonuclear response with no signs of bacteria or fungi; calcofluor white did not evidence any hyphal structures. Treatment with 2 % tobramycin eye drops every hour (q.h.), 5 % cefazolin eye drops q.h., 0.2 % flucnazole eye drops q.h., 100 mg flucnazole by mouth twice per day (b.i.d.) and 1 % cyclopentolate eye drops b.i.d. was started.

**Abbreviations:** b.i.d., twice per day; q.h., every hour.
Only after 72 h of incubation (37 °C, 5% CO₂ for chocolate agar; 30 °C for Sabouraud dextrose agar) was a little grey colony of a filamentous fungus evident at the site of inoculation on chocolate agar but not on Sabouraud dextrose agar. The colony was subcultured on a new plate of Sabouraud dextrose agar and further incubated. After 48–72 h the colony turned darker in its basic structure but developed a cottony whiteish texture. Microscopy revealed ovoid conidial structures at the top of conidiophores, with several of them clearly separated from hyphae. Notwithstanding this, the overall structure did not evidence any distinctive characteristics even after several days of growth (Fig. 2). The isolate was sent after 2 weeks to a reference centre.

Meanwhile, a loopful of the colony was also resuspended in 100 μl lysis buffer (200 mM Tris/HCl pH 7.5; 0.5% SDS; 30 mM EDTA), vortexed, held at 100 °C for 15 min and processed for DNA extraction (Yamada et al., 2002). In brief, 100 μl 2.5 M potassium acetate was added to the solution, which was then incubated for 10 min at room temperature and subsequently centrifuged at 20,000 g for 10 min. One volume of phenol/chloroform/isoamyl alcohol (25:24:1) was mixed with the supernatant and centrifuged as above. The aqueous phase was precipitated with 1 vol. cold (−20°C) 2-propanol by centrifugation at 20,000 g for 10 min, then washed with 70% ethanol, air-dried and resuspended in 50 μl Tris EDTA (10 mM Tris/HCl, 1 mM EDTA pH 8). One microlitre purified DNA was amplified in a PCR reaction using a pair of universal fungal primers (V9D, 5′-TTAAGTCCCTGCCCTTTGTA-3′; LS266, 5′-GCAATTCCAAACAAACTCGACTC-3′; Pryce et al., 2003) encompassing conserved regions of fungal rRNA. The amplified product was visualized on agarose gel, purified and sequenced using two internal primers (ITS1, 5′-TCCGTAGGTGAACCTGCGG-3′; ITS4, 5′-TCCTCCGCTTATTGATATGC-3′; Pryce et al., 2003; White et al., 1990) encompassing the internal transcribed spacer 1 (ITS-1), 5.8 rRNA and ITS-2 regions. Although rRNA genes are quite conserved among fungi, allowing the use of universal primers, the ITS-1 and ITS-2 regions are divergent and distinctive especially at the genus level (Ferrer et al., 2001; Fujita et al., 2001; Iwen et al., 2002; Pryce et al., 2003).

DNA sequences were submitted to the BLAST database and yielded a 99% homology with several sequences of Scedosporium apiospermum, among which only those derived from reference strains were taken into consideration (AY213680, CBS 101.22; AY228112, CBS 108.54; AY228118, CBS 591.90; AF117943, ATCC 46173; AF117942, ATCC 64215). Moreover, a phylogenetic analysis of ITS1 and ITS2 sequences of fungal isolates described in different pathologic conditions of the eye (Thomas, 2003) was performed. A multiple-sequence alignment and the construction of a phylogenetic tree were carried out using MEGA v. 3 (molecular evolutionary genetic analysis; software freely available on www.megasoftware.net). More in detail, to perform the phylogeny and sequence comparison the UPGMA (unweighted pair group method using arithmetic averages) method was used, subjecting the
resulting tree to 100 bootstrap replications. UPGMA employs a sequential clustering algorithm, in which local topological relationships are identified in order of similarity, and the phylogenetic tree is built in a stepwise manner. Interestingly, in this analysis, besides the large genetic distances of ITS1 and ITS2 sequences among non-related fungal species, we noticed that those derived from S. apiospermum (AY213680; AY228112) were clearly differentiated also from those of Scedosporium prolificans (AJ842340; AF022484), a related multi-drug-resistant species reported in difficult-to-treat ocular infections that is not easily differentiated by the classical approach. Indeed, the two species were clustered with the highest value of bootstrap score (100%), but showed an evident nucleotide divergence (28%), indicating two distinct long branches on the phylogenetic tree and thus allowing easy identification at the species level.

After the preliminary culture results of mycotic aetiology, which were still not definitive at the genus level, antibiotic therapy was modified to 0.3% ofloxacin eye drops every 2-hourly keeping 0.2% fluconazole eye drops q.h. and 100 mg fluconazole by mouth b.i.d. Clinical presentation slightly improved, but the corneal thinning progressed into perforation after 3 weeks, requiring penetrating keratoplasty. On this occasion a corneal button was taken and sent to the microbiology laboratory. Gram stain showed an intense polymorphonuclear response but also in this phase no fungi or bacteria were evidenced; only a single septate hypha was visible on calcofluor white. DNA extraction was performed directly from the sample, following the same protocol described above, and the rRNA region was amplified and sequenced, obtaining the same results as the previous molecular identification starting from culture. Fungal colonies with the macroscopical and microscopical morphology above described were also seen on chocolate agar and Sabouraud dextrose plates after 72 h of incubation.

Postoperatively, after fungal identification, systemic fluconazole was substituted with 100 mg itraconazole b.i.d. No postoperative recurrence of infection or other complications occurred and antifungal therapy was withdrawn after 2 months. After 1 year the best corrected visual acuity was 1.0

Discussion

Among emerging uncommon fungal pathogens S. apiospermum is one of the species most frequently isolated from corneal samples in the course of keratitis burdened by a high risk of perforation (Bouza & Munoz, 2004; Diaz-Valle et al., 2002; Nulens et al., 2003; Rumelt et al., 2001; Saracli et al., 2003; Tanure et al., 2000; Wu et al., 2002). Because of the poor prognosis of ocular infections caused by this agent, rapid diagnosis may be crucial to preserve the eye (Diaz-Valle et al., 2002; Wu et al., 2002). S. apiospermum is considered a rapidly growing fungus, but definitive identification requires at least 3–4 days. Moreover, the case described and other reports (Guarro & Gene, 2002; Thomas, 2003) show unambiguously that the classical identification approach could be problematic. As an example, when submitted to a panel of laboratories as an NEQAS quality control, a S. apiospermum isolate was misidentified by more than 40% of participants (www.ukneqasmicro.org.uk/sa.htm). Several authors have suggested that molecular methods may usefully parallel classical techniques. Although clinical skill and conventional diagnostic mycology remain of crucial importance in the management of patients, an important diagnostic advantage can be gained from well-designed molecular assays. Indeed, we are aware of the problems still related to molecular diagnosis in mycology, including the need for standardized techniques and complete and well-referenced sequence databases. An additional aspect is the risk of subverting classical taxonomy, as has already happened in bacteriology. Notwithstanding this, the case reported as well as several larger studies performed in clinical settings or on database-derived reference isolates (Ferrer et al., 2001; Fujita et al., 2001; Voigt et al., 1999) show unambiguously the diagnostic potential of molecular methods in this field. In conclusion, the data suggest that direct sequencing and sequence analysis of fungal pathogens may effectively be used to complement conventional diagnostic techniques in cases of keratomycosis.

References


