Identification problems with sterile fungi, illustrated by a keratitis due to a non-sporulating Chaetomium-like species

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A 39-year-old farm worker was injured in her right eye by a piece of wire, which resulted in a corneal ulcer unresponsive to antibiotic treatment. The clinical appearance was that of a corneal infiltrate with feathery borders resembling fungal keratitis. Corneal scrapings were collected and the patient was started on natamycin 5% eye drops, fluconazole 0.3% eye drops, and oral fluconazole. A non-sporulating fungus was isolated from the samples. Based upon macroscopic and microscopic morphologic features, it was provisionally identified as a Papulaspora species due to the fact that members of this genus generally do not form diagnostically useful conidia. However, it was found through the use of ITS sequencing that the isolate clustered within the ascomycete genus Chaetomium. The sequence did not fully match with any sequences of available ex-type strains of Chaetomium, Thielavia and Papulaspora and hence might belong to an undescribed specie. However, without diagnostic morphological features the taxon cannot be introduced as a novel member of the genus Chaetomium. Antifungal susceptibility testing was performed according to published standards. The corneal ulcer was successfully treated with six weeks of antifungal therapy.

Keywords fungal keratitis, Papulaspora, Chaetomium

Introduction

A relatively large proportion of melanized filamentous fungi that are isolated from human infections fail to produce diagnostic propagules in culture and have therefore been difficult to identify with classical, morphological parameters. During the years prior to the molecular era such cultures were either attributed to nondescript genera such as Madurella or Papulaspora, or were simply discarded as unidentifiable ‘mycelia sterilia’. Today we realize that this problematic section of the fungal kingdom includes a wide diversity of fungal species [1]. Poorly differentiated agents of disease may unexpectedly belong to divergent species or genera, and may even be phylogenetically remote at the ordinal level and above [2]. Such pathogenic fungi have systematically been neglected during 200 years of morphologic mycology and consequently will soon become a rapidly growing reservoir of hitherto unknown species [3]. The present paper describes an example of such a problematic identification of an isolate from a case of human keratitis.
Accurate diagnosis of etiologic agents is required in cases of keratomycosis since nearly one-third cannot be adequately treated or result in corneal perforation [4]. Worldwide, Aspergillus species represent the most common cause of fungal keratitis [5], but Candida and Fusarium species are also frequently encountered [5–7]. Numerous other filamentous fungi associated with soil and plant debris have the potential to cause aggressive infections, particularly when some type of traumatic implantation occurs. For environmental fungi no standard therapy is available and therefore establishment of their antifungal susceptibility profiles is essential to establish appropriate treatment.

Case report
A 39-year-old woman who wore soft contact lens was injured in her right eye with a piece of wire while working in a horse barn. After three days of a foreign body sensation, redness, and discharge from the eye, the patient presented to her local ophthalmologist. She was diagnosed with a corneal ulcer with a central infiltrate measuring 1 mm. Over the next two weeks, she was treated with ofloxacin 0.3% eye drops, tobramycin 0.3%/dexamethasone 0.1% eye drops, and gatifloxacin 0.3% eye drops, but no clinical improvement was observed.

On presentation to the Cornea Service at the University of New Mexico Health Sciences Center, the patient was complaining of decreased vision, light sensitivity, and foreign body sensation in her right eye. On initial examination, the patient’s visual acuity with existing spectacle correction was 20/400 in the right eye and 20/20 in the left eye. Slit lamp examination of the right eye revealed a mild conjunctival injection. She had a 3-mm corneal infiltrate with feathery borders with an overlying epithelial defect (Fig. 1A). Her right anterior chamber was remarkable for rare cell, but slit lamp examination of her left eye revealed nothing unusual. Dilated funduscopic examination was within normal limits.

Corneal scrapings were obtained as the clinical appearance of the corneal infiltrate raised the concern of microbial keratitis. The patient was started on wide-spectrum treatment with hourly natamycin 5% eye drops, atropine 1% eye drops twice daily, bacitracin/polymyxin ophthalmic ointment three times daily, and a two-week course of 200 mg of fluconazole orally every day. Two days later, she presented with a slightly larger corneal infiltrate measuring 3.2 mm accompanied by a new 1-mm hypopyon. The microbiology laboratory reported that a ‘mold’ was growing in cultures inoculated with portions of the scrapings. Therapy with fluconazole 0.3% eye drops every 2 h were initiated and alternated with natamycin 5% eye drops every 2 h.

Over the next week, the corneal infiltrate and hypopyon improved. The natamycin and fluconazole eye drops were decreased to six times daily. One week later, the corneal surface was debrided to facilitate antifungal penetration and the same mold was again isolated. Over the next four weeks, the topical antifungals were tapered and discontinued. The corneal ulcer was successfully treated with a total of six weeks of antifungal therapy (Fig. 1B). Despite a residual central stromal scar, her visual acuity with spectacle correction improved to 20/60.

Mycology
Since the fungal isolate, originally recovered on Sabouraud dextrose agar, failed to produce any identifiable morphologic diagnostic structures, even after prolonged incubation, a subculture was forwarded to the Fungus Testing Laboratory, Department of Pathology, the University of Texas Health Science Center at San Antonio, for identification.

Fig. 1 (A) Slit lamp photograph taken on initial presentation demonstrating a 3 × 3 mm corneal infiltrate with feathery borders and overlying epithelial defect. (B) After 6 weeks of antifungal treatment, the infiltrate was sterilized. A central stromal scar has limited her best-corrected spectacle vision to 20/60.
and antifungal susceptibility testing. There it was accessioned into their stock collection as UTHSC 03-1576 and subcultured onto potato flakes agar (PFA) [8], carnation leaf agar (CLA) [9] and V-8 juice agar [10], all prepared in-house, for examination of its macroscopic and microscopic morphologic features. It was subsequently subcultured to benomyl agar as an aid in determining potential basidiomycetous affinities [11]. Antifungal susceptibility testing was performed according to the previously published Clinical and Laboratory Standards Institute (formerly known as the National Committee for Clinical Laboratory Standards) M38-A methodology for filamentous fungi [12]. Briefly, a final inoculum of 0.4–5 × 10⁴ CFU/ml was employed for both the case isolate and the *Paecilomyces variotii* control strains UTHSC 90-459 (adjusted spectrophotometrically at 530 nm) with incubation at 35°C. RPMI 1640 medium (Angus Buffers and Biochemical, Niagara Falls, NY, USA) was used in evaluating the isolate’s susceptibility to ketoconazole (KETO), itraconazole (ITC, Janssen Research Foundation, Beerse, Belgium), fluconazole (FLC), and voriconazole (VRC, Pfizer, Inc., New York, NY, USA). Antibiotic medium 3 was used to test the mold’s sensitivity to amphotericin B (AMB, Bristol-Myers Squibb, Princeton, NJ, USA), natamycin (NAT, Alcon Laboratories, Inc., Fort Worth, TX, USA), and caspofungin (CAS, Merck & Co., Inc., West Point, PA, USA). Caspofungin endpoints were defined as the minimum effective concentration (MEC) as described for the echinocandins (Table 1) [13,14]. ITC, VRC, and CAS were tested subsequent to the case to assess susceptibility to other azoles, the newer triazole (voriconazole), and the echinocandin class of drugs. Results are included anecdotally.

Colonies incubated at 25°C on PFA and V-8 juice agar grew rapidly and were initially pale yellowish gray, becoming olivaceous after 7 days’ incubation. However, they remained sterile on these media. After 12 days incubation on CLA, colonies were effuse and pale olivaceous, with small black dots interspersed throughout the culture. A tease mount of these areas revealed hyaline hyphae consisting of both narrow (1.0–2.0 μm in diameter) and broad areas (up to 10 μm in diameter), with some sections becoming brown. No clamp connections were observed. Papulasporae originated from intercalary cells (between septa), were pale brownish yellow, becoming dark centrally and pale towards the periphery. Individual cells were globose to subglobose to angular, measuring approximately 8–12 × 10–15 μm. Papulasporae were elongate to subglobose and measured approximately 60–80 × 80–120 μm (Fig. 2A). Dark seta-like protrusions were seen projecting from some papulasporae (Fig. 2A and 2B, thick arrows), and from intercalary hyphal areas (Fig. 2B, thin arrow). After three weeks’ incubation, chains of what appeared to be phialoconidia were also evident (Fig. 2C, arrow).

### Table 1 In vitro antifungal susceptibility data.

<table>
<thead>
<tr>
<th>Antifungal drug</th>
<th>MIC (24 h/48 h) μg/ml</th>
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<tbody>
<tr>
<td>Amphotericin B</td>
<td>0.25/0.5</td>
</tr>
<tr>
<td>Natamycin</td>
<td>4/8</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>0.25/1</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>32/64</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>0.125/0.125</td>
</tr>
<tr>
<td>Voriconazole</td>
<td>0.5/0.5</td>
</tr>
<tr>
<td>Caspofungin</td>
<td>1/1</td>
</tr>
</tbody>
</table>

Blastn match of this sequence with any of the sequences deposited in GenBank. The nearest neighbors were
Thielavia hyalocarpa and Chrysosporium synchronum, both at 94% similarity. In the CBS database we found
the mold had similarity to Chaetomium cuculorum
CBS 156.52 at 99.0%, C. malaysiense CBS 669.82 at
97.9%, C. subcirrnatum CBS 177.84 at 97.9% and
C. virescens CBS 125.85 at 97.2%. Some 31 Chaetomium
and four Thielavia species had similarities between
97.0 and 94.0%. The nearest described Papulaspora
species was P. equi, ex-type strain CBS 573.89, at
16.9% ITS distance. In contrast, the isolate from keratiti
was 100% identical to an unidentified strain from
human skin, UTHSC 03-1315. The two isolates formed
a well-delimited cluster in the ITS tree at 100% boot-
strap support (Fig. 3).

Discussion
Morphologically the etiologic agent of the patient’s kerati-
tis was identified as a Papulaspora species. There have been
48 species described in the mitosporic genus Papulaspora
which is characterized by the presence of firm, well-formed,
frequently rounded masses of hypha-derived papulaspores
[17,18]. Such structures occur in widely divergent fungi.
Basidiomycetous fungi have been excluded [19] and reclas-
sified in Burgoa. Other members of the genus have been
re-identified as sterile forms of described, sporulating fungi,

Fig. 2  (A) Light microscopic photograph of the Papulaspora spp. demonstrating a cluster of papulaspores and a seta-like projection (arrow), lactophenol
cotton blue mount, 800 ×. (B) Light microscopic photograph of the Papulaspora spp. demonstrating dark seta-like protrusions from both papulaspores
(thick arrow) and intercalary hyphal areas (thin arrow), lactophenol cotton blue mount, 800 ×. (C) Light microscopic photograph of chains of phialoconidia
(thin arrow) occurring with the papulaspores, lactophenol cotton blue mount, 800 ×.
such as *P. manganica* (ex-type CBS 128.21) which is now known to be *Acremonium kiliense*. A morphological comparison of our isolate with described *Papulaspora* species suggested a similarity to *Papulaspora nishigaharanas* [20], based upon the appearance of dark seta-like protrusions from both immature individual cells and aggregates of cells representing the papulasores, as well as the production of chains of phialoconidia from long, solitary phialides. No material was available for sequencing.

The majority of *Papulaspora* species today are listed as non-sporulating forms of the ascomycete genus *Chaetomium* (www.indexfungorum.org), a member of the family *Chaetomiaceae*. Sequencing of the ribosomal ITS region of our isolate and of the *Papulaspora* generic type species, *P. sepedonioides*, proved that both strains did cluster amidst *Chaetomium*. Members of the related ascomycetous genus *Thielavia* may produce simple phialidic anamorphs similar to those observed in our *Papulaspora* isolate. Occasional cases of eye infection have been reported to have been caused by *Chaetomium* and *Thielavia* species [21,22].

The nearest neighbors of our *Papulaspora* strain within the ITS BLAST in GenBank were unnamed fungi,
while the closest named species was 6% different. We therefore sequenced 370 strains of *Papulaspora*, *Chaetomium* and *Thielavia* available in the CBS reference collection. An unidentified *Chaetomium* species recovered from human skin of a patient in Saudi Arabia, UTHSC 03-1315 was found to be 100% identical to our isolate. The branch (at 100% bootstrap support) was paraphyletic to the nearest named species, *Chaetomium cuniculorum* at 1.0% distance. No less than 37 named *Chaetomium* and *Thielavia* species, many of them being represented by their ex-type culture, were found closer than the first hit in GenBank, which was located at 94.0% similarity. The nearest *Papulaspora* species was *P. equi*, at 16.4% ITS distance. This species was isolated from an ocular infection in a horse [23], but appears to be unrelated.

Members of *Papulaspora*, *Chaetomium* and *Thielavia* are frequently isolated from animal feces, decaying plant matter, or soil. Almost half the strains of *C. cuniculorum* maintained at CBS (www.cbs.knaw.nl) originated from herbivore dung. Our patient was injured with a piece of wire while working in a horse barn. Other possible factors contributing to the development of fungal keratitis were her use of contact lens and her initial treatment with corticosteroid.

Based on the history and clinical appearance of the corneal infiltrate with feathery borders, the patient was started on our first line treatment for fungal keratitis, i.e., topical natamycin and oral fluconazole. Natamycin, a tetraene polyene which is considered to be fungicidal, is the only commercially available ophthalmic topical antifungal in the USA. We used oral fluconazole because it appears to be well-tolerated with few systemic side-effects and has been reported to have good corneal penetration [24]. Topical fluconazole was added in the treatment of our patient 48 h after presentation because of progression of the infiltrate. Although the natamycin 48 h minimum inhibitory concentration (MIC) of 8 mcg/ml was elevated, probably indicating in vitro resistance, this drug was found to be effective in vivo due to its high concentration when applied topically. The fluconazole MIC value of > 64 mcg/ml would typically indicate that the fungus was ‘resistant’ based upon normally achievable serum drug concentrations using standard dosing regimens. However, frequent topical fluconazole administration combined with the use of oral fluconazole probably achieved extremely high drug concentrations at the site of infection, allowing for appropriate treatment of the patient. Low MICs of the fungus to ITC, VRC and CAS (Table 1) indicated that these drugs had good in vitro activity and suggest potential clinical efficacy. This case, like several others concerning uncommon agents lacking defined breakpoints, illustrates the difficulty sometimes encountered in extrapolating in vitro antifungal susceptibility data.

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References

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