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Two cases of fungal keratitis caused by *Metarhizium anisopliae*

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ABSTRACT

We present two cases of keratitis due to Metarhizium anisopliae in geographically separated areas of the United States. The isolates were microscopically similar but morphologically different and were identified by ribosomal DNA sequencing. Both isolates had low minimum inhibitory concentration (MIC) values to caspofungin and micafungin, but high MIC values to amphotericin B. The morphologic and antifungal susceptibility differences between the two isolates indicate possible polyphylogeny of the group.

1. Introduction

Metarhizium anisopliae is an entomopathogenic filamentous fungus that is a plant symbiont and is found in soil throughout the world [1]. It causes disease in a wide variety of insects and other arthropods and is used commercially as an agricultural pesticide in many countries, including the United States (US) [1]. Previously, *M. anisopliae* was not considered to be pathogenic to humans due to its optimal growth temperature of 25 °C [2], however, human infection has been reported in the literature. Most of the cases have been ophthalmic infections with additional cases of fungal sinusitis and disseminated fungemia. The first reported case occurred in Columbia, South America in 1997 and was a fungal keratitis in an 18-year-old man [3]. There have now been four reported cases of fungal keratitis [3–6] and two cases of sclerokeratitis [7,8]. Here we report two additional cases of *Metarhizium* keratitis in soft contact lens wearers.

2. Case

The first case is a 47-year-old woman from Georgia, US, who presented to Emory University Hospital (Day 0) with severe pain, foreign body sensation, and decreased visual acuity in her left eye that started 10 days prior and got progressively worse. The patient was a soft contact lens wearer who reports poor lens hygiene and that she routinely wore her contact lenses overnight. The patient had no history of ocular disease or underlying systemic disease.

On examination (Day 0), the affected eye had an approximately 2 × 2 mm fluffy white infiltrate slightly superior and to the left of the visual axis surrounded by scattered stromal subepithelial infiltrates, 1 + edema, 1 + scleral injection and a large (10 mm) epithelial defect. Visual acuity was 20/20 in the right eye and 20/200 in the left eye.

The differential diagnosis was herpes simplex virus, *Acanthamoeba*, or bacterial infection, but because of the appearance of the corneal infiltrates the infection was presumed to be fungal. Treatment with topical voriconazole eye drops (10 mg/mL, not commercially available, prepared by compounding pharmacy in-house) every hour plus gatifloxacin eye drops (5 mg/mL, Zymaxid®, Allergan, Irvine, CA) four times per day was started on Day 0 along with oral acyclovir (1 g twice daily, Zovirax®, GlaxoSmithKline, Philadelphia, PA). Scrapings from the corneal lesion were taken and were directly inoculated onto tryptic soy agar with sheep blood (Remel, Lenexa, KS) and Sabouraud dextrose agar (Remel) plates and sent to the Clinical Microbiology Laboratory for bacterial and fungal culture. A Gram stain was not performed but corneal scrapings evaluated in the Ophthalmic Pathology Laboratory showed epithelial cells and septate fungal elements.

The culture plates were incubated at 30 °C in ambient air and within three days the plates began to show growth of compact velvety colonies along the inoculation streaks. The colonies were initially white and gradually turned dark green on the surface and brownish orange on the reverse (Fig. 1A, B). Tease preparations and slide culture showed numerous microconidia (Fig. 2). Based on morphologic and growth characteristics, the isolate was presumptively identified as *Metarhizium* species (isolate B10964).

After three weeks (Day 21), oral voriconazole (200 mg every 12 h)

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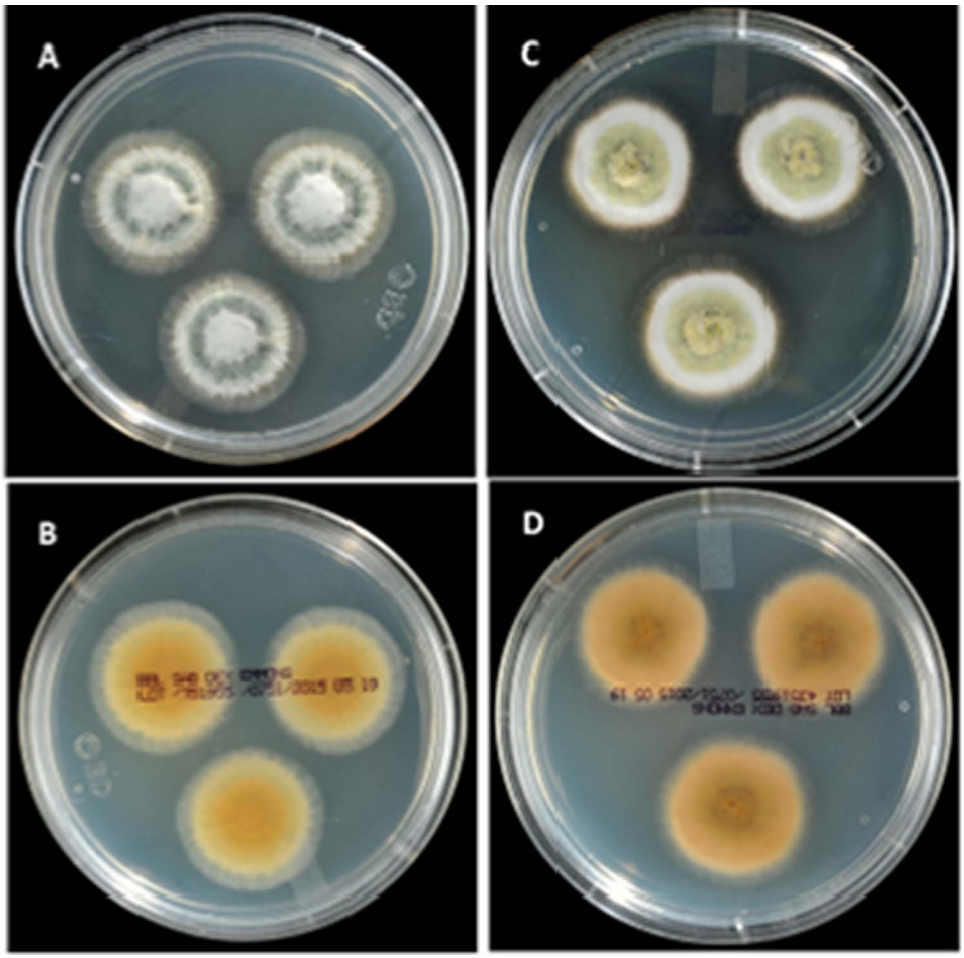

Fig. 1. Morphologically different colony types of the B10964 (A, B), Georgia isolate, and B11022 (C, D), Missouri isolate, *Metarhizium anisopliae* isolates after 7 days of growth on Sabouraud dextrose agar at 25 °C (BBL, Becton, Dickinson and Company, Franklin Lakes, NJ). Colonies are floccose with a dense heaped center that was either light with a dark green ring (B10964) or yellow-green (B11022), both with a white fringe. The reverse of both isolates was a brownish orange color.

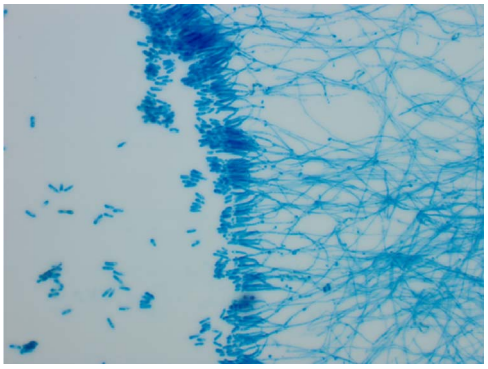

Fig. 2. Lactophenol cotton blue stain of slide culture of the Georgia *Metarhizium anisopliae* (B10964) isolate, showing slender conidiophores with verticilliate branching ending with clavate, parallel phialides and cylindrical microconidia in chains (original magnification, 400 ×).

was added and the frequency of voriconazole eye drops was decreased to four times daily. The acyclovir was discontinued due to determination of a fungal infection. Natamycin eye drops (50 mg/mL, NATACYN®, Alcon, Fort Worth, TX) every hour were administered early in the treatment regimen, however the patient was unable to tolerate them.

At 34 days after presentation, the patient developed inflammation-associated glaucoma with intraocular pressure as high as 50 mm Hg (normal range10–21 mm Hg) and visual acuity was reduced to light perception in the left eye. The inflammation-associated glaucoma was treated with trans-scleral diode laser cyclophotocoagulation.

Approximately 12 weeks after the initial visit (Day 84), superior thinning was noted, but the infiltrate was improving and the size of the epithelial defect had decreased to 4.5 × 2.5 mm. Approximately one week later new infiltrates were noted and corneal smears showed occasional fungal elements. Again, scrapings from the corneal lesion were taken and directly inoculated onto tryptic soy gar with sheep blood and Sabouraud dextrose agar plates and sent to the Clinical Microbiology Laboratory for bacterial and fungal culture, and once more a mould (isolate B11041) was identified with the same characteristics as the initial isolate (isolate B10964).

A corneal transplant was performed 13 weeks (Day 91) after the initial visit due to persistence of fungal keratitis. There were no signs of continued fungal infection but a cataract developed and was subsequently removed.

The second case is a 50-year-old man from rural Missouri, US, who presented to his primary care provider with left eye pain (Day 0). The pain was acute in onset (approximately one week in duration) and the patient experienced photophobia and vision loss in the left eye. On exam, the primary care provider noted a cloudy white area in the left eye suspicious for fungal infection, and referred the patient to Barnes-Jewish Hospital for further work up.

The patient reported using disposable contact lenses designed to be worn during the day each day for two weeks, and recounted sleeping with the lenses in place approximately three times per week. Of note, he had been in a motor vehicle accident approximately 30 years prior and

experienced lacerations of the left eyelid at that time, and reported that occasionally small pieces of glass emerged from this area. The patient was initially treated with moxifloxacin (5 mg/mL, Vigamox®, Alcon, Fort Worth, TX) and tobramycin/dexamethasone (3 mg/mL tobramycin/1 mg/mL dexamethasone, Tobradex®, Alcon) drops every hour.

At Barnes-Jewish Hospital, bacterial, viral, fungal, and acid-fast bacillus cultures were performed, as well as culture for *Acanthamoeba*. The bacterial culture grew one colony of *Staphylococcus epidermidis*. Each of the fungal, bacterial, and acid fast bacillus cultures grew a mould (Day 3) that was subsequently identified as *Metarhizium anisopliae* by sequence analysis at the University of Texas at San Antonio Fungus Testing Laboratory (Fig. 1 C, D) (isolate B11022).

The patient's left eye was treated with gatifloxacin ophthalmic solution (5 mg/mL, Zymaxid®) every three hours while awake and every four hours while asleep, prednisone four times per day, scopolamine ophthalmic drops twice a day, and preservative free artificial tears four times per day. Details of antifungal treatment are not known. The patient was discharged home five days following presentation to Barnes-Jewish Hospital in stable condition.

Approximately two months later (Day 46), the patient returned to Barnes-Jewish Hospital for follow up with persistent eye pain. A corneal scraping from the left eye was obtained and submitted for bacterial, fungal, and *Acanthamoeba* culture. *Metarhizium anisopliae* was again recovered from the bacterial, fungal and mycobacterial cultures. The cornea was resected and a half-button was submitted for histopathology, which revealed abundant filamentous fungi accompanied by a conspicuous lack of inflammatory cells. The patient was subsequently followed by an outside provider.

Since the isolates from the two cases were microscopically similar but the colonies were morphologically different, molecular identification and phylogenetic analysis of the obtained sequences was performed to further explore their relatedness. Fungal genomic DNA was extracted from the initial isolate from Georgia (B10964) and the Missouri isolate (B11022), the internal transcribed spacer (ITS) and D1/D2 regions of the ribosomal DNA were amplified using PCR, and big dye sequencing was performed as previously described [9]. The ITS and D1/D2 sequences were aligned and the combined sequence was used for species identification using the BLAST function of the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov). The resulting sequence alignments were used to construct a neighbor-joining tree using Geneious 8.1.6 (Biomatters Ltd., San Francisco, CA). Sequences have been deposited in the NCBI database (accession numbers MF435927 and MF435928).

Phylogenetic analysis showed the isolates from both cases fit into a group consistent with *Metarhizium anisopliae* (Fig. 3), although the high degree of sequence variation within the group indicates this may not be a monophyletic group and could be divided in the future with the isolates from the two cases belonging to separate clades.

Antifungal susceptibility testing was performed using standardized Clinical and Laboratory Standards Institute broth microdilution methodology as described in document M38-A2 [10] with the exception of amphotericin B which was assayed using gradient diffusion (Etest; bioMérieux, Durham, NC). Due to poor growth at 37 °C, minimum inhibitory concentration (MIC) values were recorded after 48 h of incubation at 30 °C. Reference strains *Aspergillus fumigatus* ATCC® MYA 3626 and *Purpurocilium lilacinus* ATCC® MYA 3630 were tested concurrently and the resultant MIC values for the antifungal agents tested were within the expected ranges for quality control.

There is a considerable difference between the susceptibility profiles of the Georgia (B10964 and B11041) and Missouri (B11022) isolates (Table 1). The isolates from both locations had low MIC values to caspofungin, micafungin, and anidulafungin, but very high MIC values to amphotericin B. The MIC values for voriconazole and posaconazole for all isolates tested were between 0.12 and 4 μg/mL, with the Missouri isolate (B11022) exhibiting lower MIC values than the Georgia isolates

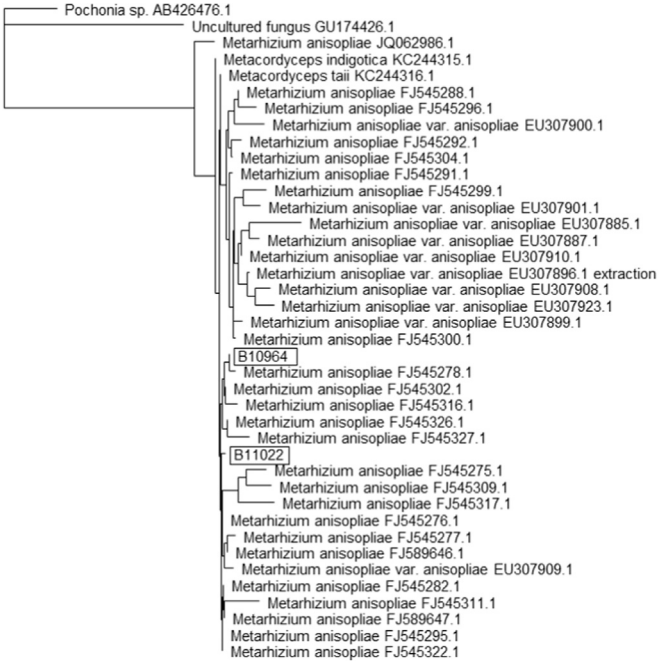

Fig. 3. Neighbor-joining tree of combined ITS and D1/D2 regions of the ribosomal cistron. Alpha-numeric numbers are NCBI accession numbers.

Table 1
Antifungal susceptibility testing results for the *Metarhizium anisopliae* isolates.

Strain	MIC values (μg/mL)[a]							
	AMP B	FLU	VORI	ITRA	POSA	CASPO	ANID	MICA
B10964	> 32	> 256	2	> 16	2	0.06	1	< 0.008
B11041	> 32	> 256	2	> 16	4	0.06	1	0.015
B11022	> 32	4	0.25	0.5	0.12	0.06	1	< 0.008

Abbreviations: AMP B, amphotericin B: FLU, fluconazole: VORI, voriconazole; ITRA, itraconazole; POSA, posaconazole; CASPO, caspofungin; ANID, anidulafungin; MICA, micafungin.

[a] Antifungal susceptibility testing performed using broth microdilution, except for amphotericin B gradient diffusion testing.

(B10964 and B11041). It is interesting that the Missouri isolate had a low MIC value to fluconazole (4 μg/mL), which should have no activity against moulds, but the Georgia isolate had a very high MIC value (> 256 μg/mL). Similarly, the Missouri isolate had a low MIC value to itraconazole (0.5 μg/mL) but the Georgia isolate had a very high MIC value (> 16 μg/mL). These data are again consistent with the possible future separation of the *Metarhizium* group.

3. Discussion

Metarhizium anisopliae is a ubiquitous fungus capable of saprophytic growth in soil and parasitic growth in many insect species [1]. Due to its use as an agricultural pesticide, exposure may be common. However, no cases of *M. anisopliae* infection have been associated with its use as a pesticide and how human infection with *M. anisopliae* becomes established is not clear [2].

It was noted by Eguchi et al. that all cases of *M. anisopliae* keratitis and sclerokeratitis have occurred in extratropical climates which is unusual as most fungal keratitis infections develop in tropical climates [8]. This may be due to the lower optimal growth temperature of 25 °C (range 15–30 °C) for *M. anisopliae*, although some isolates survive temperatures up to 35–40 °C [11]. Our isolates all grew at 30 °C but failed to grow at 37 °C. Ophthalmic infections are the most common human infection for *M. anisopliae* occurring to date, possibly due to the

lower normal corneal temperature, which ranges from 32.5 °C to 36.5 °C [5,8]. Additionally, there have been two documented cases of sinusitis, with the normal temperature in the maxillary sinus around 34 °C [12]. This may suggest that *M. anisopliae* is better able to thrive and establish an infection in areas of the body with a lower basal temperature. Prior injury does not appear to be required for inoculation, however, both patients described here and the majority of previous reports occurred in soft contact lens wearers which may suggest this increases vulnerability to *M. anisopliae* keratitis.

There have been three reported cases of cutaneous *M. anisopliae* infection, all occurring in immunocompromised individuals (two children and one adult) [11–13]. Disseminated infection was suspected in two of these cases (one child and one adult) as evidenced by multiple culture-positive skin lesions [13,14]. Brain and lung abscesses consistent with fungal infection were seen on a computed tomography scan in one of the cases and were presumed to be caused by *M. anisopliae* [13]. No obvious portal of entry was identified in either case.

Topical natamycin is usually considered the most effective treatment for ocular *M. anisopliae* infections [8], although there is no clinical or microbiological evidence that it is any more effective than amphotericin B. Many of the cases of *M. anisopliae* prior to identification were initially treated with amphotericin B. Antifungal susceptibility testing on the three corneal isolates from the cases presented here as well as in previous studies on an ocular isolate, a skin lesion isolate, two isolates causing sinusitis, and three isolates recovered from bronchoalveloar lavage specimens revealed high MIC values to amphotericin B ($\geq$ 16 μg/mL) [8,11,15]. Additionally, when tested, 5-fluorocytosine (5-FC) also had high MIC values ($>$ 64 μg/mL) [8,15]. Consistent with these *in vitro* findings, one case of disseminated *M. anisopliae* treated with amphotericin B and 5-FC did not result in clinical improvement [13]. Interestingly, a tablet diffusion method (Neo-Sensitabs, Rosco Diagnostica, Taastrup, Denmark) of susceptibility testing suggested that the organism was susceptible to amphotericin B but resistant to azole agents [13]. Both reported cases of sclerokeratitis had unfavorable responses to multidrug antifungal therapy even though natamycin was included in the regimen, and ultimately required therapeutic corneal transplant [7,8]. Corneal transplant was also required in the two keratitis cases reported here as well as in one previously reported case [6].

Caspofungin and micafungin may be effective agents for ocular and other infections since corneal penetration is adequate after systemic administration [16,17], and susceptibility testing in the current study and in previous reports demonstrated MIC values (0.25 μg/mL or lower) that suggested *M. anisopliae* would be susceptible [8,11]. Anidulafungin also had low MIC values (1 μg/mL) for the isolates in the current study but was not evaluated in other studies.

The azoles (itraconazole, voriconazole, posaconazole, miconazole, and fluconazole) and SCH 56592 had variable MIC values and antifungal susceptibility testing may help to inform individual treatment decisions if these agent are considered [8,11,15]. Intravenous voriconazole failed to improve a skin lesion due to an isolate that exhibited an MIC value of 2 μg/mL, and the lesion was subsequently excised [11]. Itraconazole did produce a clinical response in a case report of a cat with invasive disease, however relapse occurred after discontinuation of therapy [18].

The poor treatment outcomes emphasize that early diagnosis and increased understanding of the antifungal susceptibility profile of *M. anisopliae* isolates is required. The phenotypic and genotypic differences between the isolates described here indicate that *M. anisopliae* may be a species complex and the differential susceptibilities are an indication that a distinction of isolates within the complex may have clinical implications.

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Conflict of interest

There are none.

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