Infectious Keratitis Caused by Aspergillus tubingensis

László Kredics, PhD,* János Varga, DSc,† Sándor Kocsbé, MSc,* Revathi Rajaraman, MS,‡ Anita Raghavan, DO, FRCS,† Ilona Dóczy, PhD,§ Madhavan Bhaskar, MD,¶
Tibor Mihály Németh, BSc, Zsuzsanna Antal, PhD, Narendran Venkatapathy, DO, DNB,† Csaba Vágvölgyi, DSc, Robert A. Samson, DSc,‡ Manoharan Chockaiya, PhD,¶ and Manikandan Palanisamy, MSc, MPhil,‡\[102\]

Purpose: To report 2 cases of keratomycosis caused by Aspergillus tubingensis.

Methods: The therapeutic courses were recorded for 2 male patients, 52 and 78 years old, with fungal keratitis caused by black Aspergillus strains. Morphological examination of the isolates was carried out on malt extract agar plates. A segment of the β-tubulin gene was used for molecular identification. Antifungal susceptibilities were determined by the E test method for molds and the broth microdilution technique National Committee for Clinical Laboratory Standards M38-A.

Results: A 52-year-old man presented with complaints of pain and redness in the right eye. The patient was successfully treated with natamycin and econazole eyedrops, itraconazole, and oral ketoconazole. A 78-year-old man presented with total corneal necrosis in the right eye. A therapeutic keratoplasty was performed, and topical natamycin and econazole were applied. At the postoperative visit after 3 weeks, almost the full corneal graft was clear with formed anterior chamber. Black Aspergillus strains were isolated from the corneal scrapings of both cases and initially identified as Aspergillus niger based on culture characteristics. Sequence analysis of a segment of the β-tubulin gene revealed that the isolates are representatives of A. tubingensis.

Conclusions: Aspergillus tubingensis is closely related with A. niger, the differentiation of these 2 species is difficult by classical morphological criteria. To our knowledge, the presented cases of fungal keratitis are the first reports on ocular infection caused by A. tubingensis.

Key Words: Aspergillus tubingensis, corneal ulcer, fungal keratitis, keratomycosis

(Cornea 2009;00:000–000)

Filamentous fungi have replaced bacteria as the predominant cause of infectious keratitis in some developing countries. Keratomycosis, also known as mycotic, or fungal keratitis is a suppurative, usually ulcerative corneal disease. Aspergillus keratitis is frequent in agriculture-based geographical regions with hot, humid, tropical, or subtropical climates. Those at highest risk are young and middle-aged male agricultural workers as they are more exposed to the possibility of corneal trauma with fungus-contaminated material. Aspergillus flavus is the most commonly isolated fungal pathogen in Aspergillus keratitis cases followed by Aspergillus fumigatus, Aspergillus terreus, and Aspergillus niger. Other members of the genus less frequently occurring in keratitis include Aspergillus glaucus, Aspergillus ochraceus, and Aspergillus tamarii. Aspergillus tubingensis, a member of Aspergillus section Nigri, has not yet been reported from mycotic keratitis. Here, we describe 2 cases of fungal keratitis caused by A. tubingensis.

MATERIALS AND METHODS

The preoperative histories, surgical procedures, postoperative courses, and interventions were recorded for 2 patients with fungal keratitis (1 eye each) caused by black Aspergillus strains. As per the standard protocol, material obtained from scrapings of the leading edge and the base of the ulcer was inoculated directly onto 5% sheep’s blood agar (SBA), chocolate agar, and potato dextrose agar (PDA: 250 g of potato slices, 15 g of agar, 10 g of dextrose, and 1000 mL of distilled water) and into brain heart infusion broth. SBA and chocolate agar plates and brain heart infusion broth were purchased from Himedia Laboratories, Mumbai, India. Plates were incubated under aerobic conditions at 37°C, whereas the bottle containing PDA was incubated at 27°C. Microbial cultures were considered positive only if growth of the same
organism was demonstrated on 2 or more solid media or there was confluent growth at the site of inoculation on 1 solid medium with consistent direct microscopic findings. Samples inoculated on PDA and SBA resulted in fast-growing colonies with cottony aerial mycelia.

The isolates were subcultured on malt extract agar plates for morphological identification. For the purposes of molecular identification, mycelia grown on liquid media containing 0.5% yeast extract, 0.5% peptone, 1% glucose for 1 day were subjected to DNA isolation by the Masterpure yeast DNA purification kit (Epicentre Biotechnologies, Madison, WI) according to the manufacturer’s instructions. A segment of the β-tubulin gene was amplified using primers bT2a and bT2b. DNA sequences were determined using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems Inc, Foster City, CA) and an ABI 3100 DNA sequencer. Sequence analysis was carried out by BLASTN similarity search at the Web site of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST).

The E test method (AB BIODISK, Solna, Sweden) for molds was used to determine the minimal inhibitory concentration (MIC) values of the isolates to amphotericin B, fluconazole, itraconazole, ketoconazole, voriconazole, and caspofungin. In accordance with the manufacturer’s instructions, RPMI (Roswell Park Memorial Institute) 1640 agar (15 g in 1000 mL) supplemented with 20 g of glucose per 1000 mL of medium was used in the tests. The MIC of natamycin (Natamet, 5% suspension; Sun Pharmaceutical Industries Ltd, Halol, India), econazole (Aurozole, 2% suspension; Aurolab, Madurai, India), and clotrimazole (Auroclot, 1% suspension; Aurolab) were determined by the broth microdilution technique National Committee for Clinical Laboratory Standards M38-A. In initial dilution steps, dimethyl sulfoxide was used as a solvent to dissolve natamycin, econazole, and clotrimazole. Both the E tests and the microdilution plates were incubated at 30°C for 72 hours. Candida parapsilosis ATCC 22019 was used as the quality control for econazole, clotrimazole, ketoconazole, and amphotericin B during the susceptibility tests. Results obtained for these strains were in accordance with the quality control (QC) ranges published previously for these isolates.

**RESULTS**

**Case Reports**

**Case 1**

A 52-year-old man with no significant history presented with complaints of pain and redness in the right eye of 4 days’ duration. He had not taken any treatment for the same. On examination, his uncorrected vision was 6/12 in the right eye and 6/9 in the left eye. Slit lamp evaluation of the right eye was significant for the presence of a corneal infiltrate with an overlying epithelial defect in the paracentral cornea involving the anterior and mid stroma. A trace hypopyon was present. The left eye was normal in all respects. Plating of scrapings from the infiltrate revealed fungal colonies that were identified as black Aspergillus species (designated as strain 620/07). The patient was started on 5% natamycin half hourly, 2% econazole half hourly, 1% itraconazole eye ointment 3 times a day, Homide eyedrops 3 times a day, and 200 mg of tablets of ketoconazole twice a day. Five days later, the infiltrate was well demarcated, but the dimensions remained the same. The patient was asked to continue the same topical therapy. The ketoconazole tablets were terminated as the infiltrate was responding. After another 5 days, there was a very active subepithelial infiltrate. Inquiry revealed that the compliance with the medical regimen was poor. The need for adherence to the prescribed schedule was emphasized. As the lesion continued to be active, ketoconazole tablets were restarted. When seen 4 days later, the infiltrate had almost resolved. Topical antifungals were tapered to hourly application. The patient was seen 2 weeks later. Examination revealed a scar with no traces of activity.

**Case 2**

A 78-year-old man presented to the hospital with complaints of defective vision, pain, and redness in the left eye of 1 week’s duration. He could not recall any trauma to that eye. His medical history was relevant for cardiac disease. He had been treated with ofloxacin and atropine eyedrops elsewhere. On examination, his uncorrected visual acuity was 1/60 in the right eye, whereas in the left eye, there was light perception only. Slit lamp evaluation of the right eye was significant for an immature cataract. Slit lamp evaluation of the left eye was significant for lid edema, circumcorneal congestion, total corneal infiltrate with limbal involvement, and central thinning up to the Descemet membrane. Anterior chamber details could not be made out. Cultures from scrapings revealed a black Aspergillus (designated as strain 39/07). The very poor prognosis and the need for therapeutic keratoplasty were explained to the patient. He was admitted and started on topical 5% natamycin, 2% econazole, and 1% itraconazole eye ointment and 200 mg of ketoconazole tablets twice a day. Three days later, a therapeutic keratoplasty was performed. Postoperatively, the graft was clear with no evidence of residual infection. The patient was treated with tapering doses of antifungal medications.

**Molecular Identification and Antifungal Susceptibilities**

In both cases, black Aspergillus strains were isolated from corneal scrapings and initially identified as A. niger based on culture characteristics. Sequence analysis of a 376-bp fragment of the β-tubulin gene revealed that both isolates belong to the black Aspergillus species A. tubingensis. The resulting sequences were deposited in the GenBank database (accession numbers: EU600388 and EU600389 for strains 39/07 and 620/07, respectively). The β-tubulin sequence of isolate 39/07 proved to be completely identical with the corresponding sequences of the A. tubingensis type strain CBS 134.48, whereas that of isolate 620/07 differed from it in a single T deletion.

Figure 1 shows the micromorphology of the fungal isolate 39/07. Conidia of the fungal isolates (Fig. 1C) were smooth walled, similar to those of the A. tubingensis type strain CBS 134.48 (Fig. 1B). Living cultures of the isolates from case 1 and case 2 were deposited in the culture collection of the Department of Microbiology, Aravind Eye Hospital and Postgraduate Institute of Ophthalmology, Coimbatore, India (strain numbers: 620/07 and 39/07, respectively) and in the Centraalbureau voor Schimmelcultures (strain numbers: CBS 122719 and CBS 122725, respectively).

Table 1 shows the antifungal susceptibilities of the case isolates. Both of them were resistant to fluconazole (MIC > 256 μg/mL), and MIC values of clotrimazole were also higher than 32 μg/mL. MICs of natamycin proved to be similar (0.5 and 1 μg/mL for strains 620/07 and 39/07, respectively). MICs of other antifungal agents (itraconazole, ketoconazole, voriconazole, econazole, and amphotericin B) were higher than 2 μg/mL. The difference between the susceptibilities of the 2 keratitis isolates was 1 dilution step for ketoconazole, econazole, and natamycin, whereas the MIC values of...
strain 620/07 were 2 and 3 dilution steps higher for caspofungin and amphotericin B, respectively.

DISCUSSION

From Aspergillus section Nigri, only A. niger has been reported up to now as a possible causative agent of fungal keratitis. In a study from North India, Aspergillus niger was found to be the most common among the Aspergillus species causing keratitis, with 64 of 78 cases. However, the isolates in this study were identified on the basis of their macroscopic and microscopic colony morphology only, and the identifications were not confirmed by molecular techniques. In the cases described in this report, sequence analysis of a part of the β-tubulin gene revealed that the isolated strains belong to A. tubingensis.

Aspergillus tubingensis can be distinguished from other black Aspergilli with a number of techniques, including sequence analysis of the internal transcribed spacer (ITS) region, β-tubulin, calmodulin, and mitochondrial cytochrome B genes, amplified fragment length polymorphism analysis, mitochondrial DNA restriction fragment length polymorphism, detection of sequence variations of the calmodulin gene with fluorescence-based single strand conformation polymorphism (SSCP) analysis by capillary electrophoresis, ITS restriction fragment length polymorphism, and species-specific primers designed on the basis of ITS sequences.

Aspergillus tubingensis is known to produce pectinolytic, xylanolytic, and arabinoxylanolytic enzymes, opening the possibility for its application in biotechnological procedures. Dihydrocarbazole-containing alkaloids were also reported from A. tubingensis.

Data are available about the ability of certain A. tubingensis isolates to produce ochratoxin A as well. To our knowledge, the presented cases of fungal keratitis are the first reports on ocular infections caused by A. tubingensis. Furthermore, there is a lack of data in the literature about the involvement of A. tubingensis in any other kinds of human infections. However, the reidentification of culture collection strains may reveal further cases with this organism as the pathogen. As an example, the type strain of Aspergillus awamori var. hominis, isolated from the skin lesion of a patient in Brazil, 1954, has been reidentified as A. tubingensis strain CBS 107.55 = ATCC 12074. Howard et al examined 43 black Aspergilli derived from various clinical sources by sequence analysis of the ITS region of the ribosomal RNA gene cluster and partial calmodulin and β-tubulin sequences. According to their results, after A. niger, A. tubingensis was the second most common etiological agent with 18.6% of the cases. Most A. tubingensis isolates were found to be resistant (4 mg/L) to itraconazole in this study. Kano et al carried out sequence

<table>
<thead>
<tr>
<th>TABLE 1. MIC Values (µg/mL) of Antifungal Drugs Toward the 2 Aspergillus tubingensis Isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>Itraconazole*</td>
</tr>
<tr>
<td>Ketoconazole*</td>
</tr>
<tr>
<td>Voriconazole*</td>
</tr>
<tr>
<td>Amphotericin B*</td>
</tr>
<tr>
<td>Econazole†</td>
</tr>
<tr>
<td>Clotrimazole†</td>
</tr>
<tr>
<td>Fluconazole*</td>
</tr>
<tr>
<td>Natamycin†</td>
</tr>
<tr>
<td>Caspofungin*</td>
</tr>
</tbody>
</table>

* Determined by the E test method.
† Determined by the National Committee for Clinical Laboratory Standards broth microdilution method.

© 2009 Lippincott Williams & Wilkins

www.cornejrnl.com | 3
analyses of the same loci on 17 clinical isolates and observed that
12 of them belonged to the A. niger and 5 to the A. tubingensis
species. These studies indicate that although most of the reports
about the involvement of black Aspergilli in different human
diseases refer to the isolates simply as A. niger, other members of
Aspergillus section Nigri including A. tubingensis may also be
involved in human infections.

The presented cases also indicate that even the routinely
performed, morphology- and culture-based identification may be
misleading in the cases of Aspergillus species that are not
expected as keratitis pathogens because of the lack of literature
data about their involvement in corneal infections. Early and
accurate microbiological diagnosis coupled with appropriate
treatment is crucial for increasing the chances of complete
recovery. For a distinctive diagnosis of Aspergillus keratitis,
one has to depend on a combination of microscopy, culture
morphology, and molecular techniques.

REFERENCES
Wageningen, The Netherlands: Wageningen Academic Publishers;
2008:293–328.
6. Srinivasan M, Gonzales CA, George C, et al. Epidemiology and
aetiological diagnosis of corneal ulceration in Madurai, south India. Br
7. Glass NL, Donaldson GC. Development of primer sets designed for use
with the PCR to amplify conserved genes from filamentous ascomycetes.
9. National Committee for Clinical Laboratory Standards. Reference Method
for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi;
Committee for Clinical Laboratory Standards; 2002.
control isolates and tentative quality control ranges for in vitro
susceptibility testing of yeast isolates by National Committee for Clinical
Laboratory Standards proposed standard methods. J Clin Microbiol. 1994;
32:1650–1653.
11. Chowdhary A, Singh K. Spectrum of fungal keratitis in North India.
fragment length polymorphism analysis of Aspergillus carbonarius,
Aspergillus tubingensis, and Aspergillus niger strains isolated from grapes
and phylogeny of the Aspergillus section Nigri inferred from mitochondrial
cytochrome b gene. FEMS Microbiol Lett. 2001;200:
241–246.
fragment length polymorphisms in field isolates of the Aspergillus niger
15. Susca A, Stea G, Perrone G. Rapid polymerase chain reaction (PCR)-
single-stranded conformational polymorphism (SSCP) screening method
for the identification of Aspergillus section Nigri species by the detection
of calmodulin nucleotide variations. Food Addit Contam. 2007;24:
1148–1153.
180:191–196.
Aspergillus niger and other Aspergillus species belonging to section Nigri
and characterization of an exopolysaccharidase from Aspergillus
19. de Graaf LH, van den Broeck HC, Ooijen AJJ, et al. Regulation of the
20. Griekens MM, Visser J, de Graaf LH. Arabinosylan degradation by
fungi: characterization of the arabinoxylan-arabinofuranohydrolase
encoding genes from Aspergillus niger and Aspergillus tubingensis.
21. Sings HL, Harris GH, Dombrowski AW. Dihydrocarbazole alkaloids from
clinical black Aspergillus isolates and azole resistance. In: Clemons KV.
3rd Advances Against Aspergillosis Conference, 16–18 January 2008,
Miami; 2008: 29 (Abstract Book). San Diego, CA: University of
California San Diego School of Medicine.
24. Kano R, Perrone G, Peterson S, et al. Comparative sequence analy-
yses of multiple comparative loci reveal rare Aspergillus species in
transplant-recipients (TRANSNET study). In: Clemons KV. 3rd Advances
Against Aspergillosis Conference, 16–18 January 2008, Miami; 2008: 71
(Abstract Book). San Diego, CA: University of California San Diego
School of Medicine.