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# Keratitis caused by *Aspergillus pseudotamarii*

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ABSTRACT

A male patient presented with complaints of redness, pain and defective vision in the left eye. The infiltrate healed completely after two weeks of topical natamycin administration. A polyphasic approach was used to identify the isolate as *Aspergillus pseudotamarii*, which produced aflatoxins in inducing medium.

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## 1. Introduction

Certain *Aspergillus* species, mainly *Aspergillus flavus*, *Aspergillus terreus, Aspergillus fumigatus* and *Aspergillus niger* have long been regarded as important pathogens in eye infections, especially keratitis [1]. The problem is prevalent also in South-India, which comprises largely of agrarian population. *Aspergillus* strains are among the most common organisms causing fungal keratitis in the case of rural agricultural workers. Most of the *Aspergillus* strains isolated from keratomycosis are being identified and reported at the genus level only. Their molecular identification at the species level is of great importance, as the pathogenic potential and antifungal susceptibilities may vary between different species [1]. Recent molecular studies revealed that the spectrum of *Aspergillus* species capable of causing mycotic keratitis is much wider than believed earlier, including *A. tamarii* [2], *A. nomius* [3], *A. tubingensis* [4], and *A. brasiliensis* [5]. In this report we describe a case of keratomycosis caused by *A. pseudotamarii,* an aflatoxigenic member of *Aspergillus* section *Flavi*.

## 2. Case

A 48 years old male patient presented to the Aravind Eye Hospital, Coimbatore, India in July, 2010 (day 0) with complaints of pain, redness and defective vision in the right eye for two months duration. The patient did not recall any trauma or injury to the eye, but might have had trivial injury during the course of his duties as a farmer. He had a history of Chikungunya fever 3 months back. On examination, his uncorrected visual acuity (UCVA) in the right eye was finger counting close to the face (FCF). Anterior segment examination of the right eye showed diffuse conjunctival congestion. Cornea showed a punched out epithelial defect with anterior stromal infiltrate. Surrounding the epithelial defect and the infiltrate there was a diffuse scarring of 1 × 1 mm with few bullae. Anterior chamber showed mild reaction with a 3 mm hypopyon. Lens appeared clear.

The patient was given topical itraconazole, chloramphenicol, 5% dexpanthenol eye ointments and oral itraconazole (100 mg) twice a day (day 0). Repeated scraping was done on day+3, which showed few fungal filaments in KOH and Gram's stain. The culture was positive for fungus (*Aspergillus*). Topical natamycin was started immediately and the epithelium started to heal well. The anterior chamber was quiet and the hypopyon resolved completely. The infiltrate completely healed and the patient was advised to reduce the dosage of natamycin eye drops and itraconazole eye ointment on day +14. By one month, cornea showed a macular

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grade scar and the eye remained quiet (day+30). The UCVA improved to 6/36. The patient was advised itraconazole eye ointment for a month.

The case isolate (isolate 763/10) was initially identified as *Aspergillus* sp. based on culture characteristics. Isolation of genomic DNA from mycelia grown in liquid YPG medium (1% Bacto yeast extract, 1% Bacto peptone, 1% D-glucose) for three days was performed by the Masterpure™ yeast DNA purification kit (Epicentre Biotechnologies, Madison, WI, USA) according to the manufacturer's instructions. A fragment of the calmodulin gene was amplified with primers cmd5 and cmd6 as described by Hong et al. [6]. DNA sequences were determined at Agowa GmbH, Berlin, Germany. Sequence analysis was performed by nucleotide–nucleotide BLAST similarity search at the website of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST) [7]. Homology searches revealed that the isolate belongs to the species *A. pseudotamarii*, an aflatoxin-producing member of *Aspergillus* section *Flavi* [12]. The analyzed 517 bp long sequence differed only in 4 positions from the sequences of strain CBS 766.97 (=NRRL 25517), the type strain of *A. pseudotamarii* [11–13] and strain CBS 765.97 (=NRRL 443) [11–13] (Table 1). The sequence of the calmodulin fragment was deposited in the GenBank database under the accession number of KC202290.

The isolate was subcultured on malt extract agar (MEA) and Czapek yeast autolysate (CYA) media for morphological examination. Fig. 1 shows the micromorphology and colony morphology of the case isolate. Conidial heads on CYA medium were orange brown eventually shifting to light brown in older colonies. The colony reverse was pale yellow brown, a diffusible pigment of the same colour could be seen in the agar medium. Sclerotia were dark brown to black, globose to subglobose, 1–2 mm in diameter. Conidial heads were globose, 500–770 μm in diameter and olive green on MEA medium. The isolate was deposited in the Szeged Microbiological Collection (SZMC, http://www2.sci.u-szeged.hu/microbiology/collection.htm) under the strain number of SZMC 3055.

Aflatoxin producing abilities of the *A. pseudotamarii* isolate were examined in 3 different culture media: liquid yeast extract-sucrose (YES) medium at 25 °C as well as RPMI and brain-heart infusion broth at 35 °C representing circumstances similar to the human body [14]. Aflatoxin extraction was performed with 2 ml of dichloromethane, the extracts were centrifuged at 10.000 × g for 10 min and the organic phases were dried and redissolved in 500 μl of acetonitrile. Aflatoxin contents of the samples were determined by reversed phase HPLC without derivatization, using water/acetonitrile/methanol (48/26/26) mobile phase and fluorescence detection at excitation and emission wavelengths of 360 nm and 455 nm, respectively [8]. The isolate was capable of aflatoxin

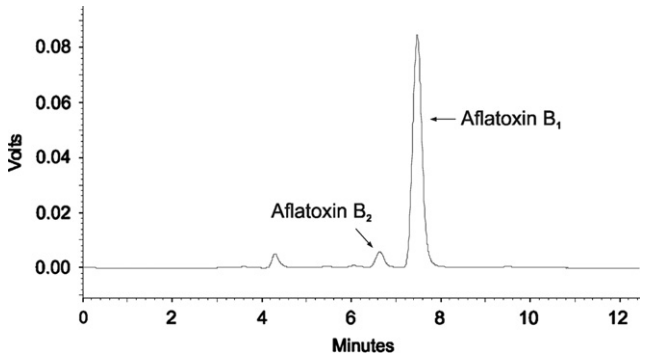


**Fig. 2.** HPLC chromatogram of the aflatoxins produced by the examined *Aspergillus pseudotamarii* isolate in yeast extract—sucrose medium.

**Table 1**
Similarity of the 517 bp calmodulin fragment of the case isolate (GenBank accession number: EF202030) to calmodulin sequences of related *Aspergillus* species from section *Flavi*

| Species | GenBank accession number | Strain designation | Number of overlapping bases | Sequence similarity (%) | Reference |
|---|---|---|---|---|---|
| *A. pseudotamarii* | AF255039 | NRRL 25517=CBS 766.97 (type strain) | 513/517 | 99.2 | 12 |
| *A. pseudotamarii* | AF255038 | NRRL 443=CBS 765.97 | 513/517 | 99.2 | 12 |
| *A. caelatus* | EF661522 | NRRL 25528=CBS 763.97 (type strain) | 502/517 | 97.1 | 13 |
| *A. caelatus* | EF661523 | NRRL 26100 | 502/517 | 97.1 | 13 |
| *A. tamarii* | EF661527 | NRRL 4911 (type strain) | 491/518 | 94.8 | 13 |
| *A. flavus* | EF661514 | NRRL 20521 | 486/517 | 94.0 | 13 |
| *A. flavus* | EF661513 | NRRL 4822 | 486/517 | 94.0 | 13 |

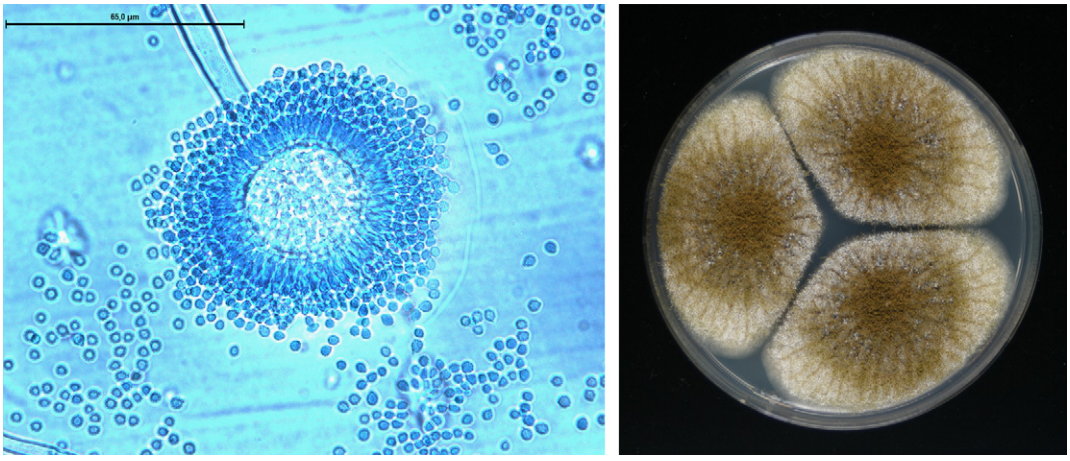


**Fig. 1.** Morphology of *Aspergillus pseudotamarii*. (A) Microscopic morphology of a conidial head. (B) Colony morphology of *Aspergillus pseudotamarii* on solid CYA medium. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

**Table 2**
Antifungal susceptibilities of the *A. pseudotamarii* isolate, *A flavus* and *A. tamarii* isolates (48 h). Minimal inhibitory concentration (MIC) values in μg/ml.

| | *A. pseudotamarii* SZMC 3055 | *A. flavus* ($n=24$) | *A. tamarii* ($n=4$) |
|---|---|---|---|
| Anidulafungin | 0.002[a] | 0.0025 [a] (0.002–0.008 μg/ml) | 0.004 [a] (0.002–0.008 μg/ml) |
| Amphotericin B | 0.19 | 5.29 (2–12 μg/ml) | 0.72 (0.38–1 μg/ml) |
| Micafungin | 0.002 [a] | 0.0023 [a] (0.002–0.004 μg/ml) | 0.0095 [a] (0.002–0.016 μg/ml) |
| Itraconazole | 1 | 1.32 (0.75–2 μg/ml) | 0.5 (0.38–0.75 μg/ml) |
| Fluconazole | > 32 [b] | > 32[b] | > 32 [b] |
| Caspofungine | 0.064[a] | 0.0134 [a] (0.008–0.023 μg/ml) | 0.086 [a] (0.016–0.25 μg/ml) |
| Posaconazole | 0.25 | 0.33 (0.19–0.5 μg/ml) | 0.34 (0.125–0.94 μg/ml) |
| Voriconazole | 0.19 | 0.45 (0.19–1 μg/ml) | 0.15 (0.125–0.19 μg/ml) |
| Natamycin[c] | 32 | ND | ND |
| Econazole[c] | 0.4 | ND | ND |
| Clotrimazole[c] | 0.7 | ND | ND |

n: number of isolates

[a] lawn of microcolonies within a discernable ellipse.
[b] homogeneously resistant.
[c] measured by the broth dilution method.

$B_1$ and $B_2$ production in YES medium only. The extract was first examined with TLC and the results were later confirmed by HPLC analysis (Fig. 2). The isolate produced 2.6 ng/ml aflatoxin $B_2$ and about 1 μg/ml aflatoxin $B_1$. However, the isolate was unable to produce aflatoxins under *ex vivo* conditions in either RPMI or BHI media at 35 °C, similar to other aflatoxin producing species like *A. flavus* or *A. nomius* [14].

The Etest method (BioMérieux SA, Lyon, France) for moulds was used to determine the antifungal susceptibility of the isolate to amphotericin B, fluconazole, itraconazole, voriconazole, micafungin, anidulafungin and caspofungin in accordance with the manufacturer's instructions [9,10]. The MICs of natamycin (Natamet; 5% suspension; Sun Pharmaceutical Ind. Ltd., Halol, India), econazole (Aurozole; 2% suspension; Aurolab, Madurai, India) and clotrimazole (Auroclot; 1% suspension; Aurolab, Madurai, India) were determined by the broth microdilution technique NCCLS M38-A [10]. Both the Etest and microdilution plates were incubated at 30 °C for 72 h. *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 ranges published previously for these isolates. Antifungal susceptibilities of the *A. pseudotamarii* isolate were compared to those of several *A. flavus* and *A. tamarii* isolates deriving from corneal ulcers (Table 2). The detected antifungal susceptibility values were mostly within the value ranges determined previously for *A. flavus* isolates [15,16]. However, the *A. pseudotamarii* isolate proved to be more susceptible to amphotericin B than either *A. flavus* or *A. tamarii* (Table 2).

## 3. Discussion

*A. flavus* is the most frequently occurring causative agent of mycotic keratitis, not only within *Aspergillus* section *Flavi*, but also within the entire genus *Aspergillus* [1,17]. However, *A. tamarii* [2] and *A. nomius* [3] were also reported from corneal ulcers during the recent years, indicating that further members of *Aspergillus* section *Flavi* have to be considered as potential causative agents of keratomycosis.

The species *A. pseudotamarii* was described in 2001 by Ito et al. [11], who elevated the genetically and morphologically different, aflatoxin-producing isolates of *A. tamarii* [18] to species rank. The new species was described based on the examination of two isolates, NRRL 443 (CBS 765.97) sent from Argentina in 1923 and NRRL 25517 (CBS 766.97) isolated in 1993 from tea field soil in Miyazaki, Japan. Among these two strains the latter was defined as the holotype of *A. pseudotamarii*. Isolates of the new species could be clearly differentiated from *A. tamarii* strains and other members of *Aspergillus* section *Flavi* by their internal transcribed spacer region of the rRNA gene cluster (ITS), partial β-tubulin and calmodulin gene sequences [11]. Unlike isolates of the closely related *A. tamarii* and *A. caelatus* species, the representatives of the new species proved to be able to produce B-type aflatoxins. Furthermore, similar to *A. caelatus, A. pseudotamarii* does not show any growth or conidial germination at 42 °C, while *A. tamarii* isolates do. *Aspergillus flavus*, *A. parasiticus* and *A. nomius,* the economically most important aflatoxin producing members of section *Flavi* grow moderately at 42 °C and have much smaller conidia than those of *A. pseudotamarii*, *A. tamarii* or *A. caelatus* [11–13,19,20]. HPLC-DAD analyses revealed that besides B-type aflatoxins, *A. pseudotamarii* is also capable of producing kojic acid and cyclopiazonic acid [13].

Besides Argentina and Japan, isolates of *A. pseudotamarii* could also be identified in soil samples collected in the Sukhothai Kiln, Khao Yai, Teak forest and Koh Samui Ubon regions of Thailand and from Brazil (J. C. Frisvad, presonal communication). The identification of an *A. pseudotamarii* isolate in India indicates a wide, possibly cosmopolitan distribution of this species.

## 4. Conflict of interest

No conflict of interest declared.

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