Multilocus microsatellite typing for \textit{Rhizopus oryzae}

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\textit{Rhizopus oryzae} is the most frequent causative agent of zygomycosis. Although zygomycosis causes considerable morbidity and mortality in immunocompromised patients, the epidemiology of the disease is not well studied and no standard molecular typing method has been described for any of the causative agents. Here we describe a multilocus microsatellite typing (MLMT) method for \textit{R. oryzae}. \textit{R. oryzae} genome sequences were downloaded from the Fungal Genome Initiative database (Broad Institute). The intergenic regions and ORFs of approximately 5.7 Mb were screened for repeat regions with the help of the online repeat search tool Repeat Masker. Of the 30 microsatellite loci identified, 3 microsatellites [RO3, (CCT)$_n$; RO4, (TA)$_n$; and RO8, (GAA)(GGA)$_n$] were selected after validation of the ability to amplify them and their size variation in 8 randomly selected clinical isolates of \textit{R. oryzae}. Nucleotide sequence analysis of these loci demonstrated polymorphism in the microsatellite repeat number. The capabilities of these microsatellite loci were assessed for strain differentiation on 30 clinical isolates, based on fragment size determination in an automated capillary electrophoresis using fluorescent labelled primers. These three polymorphic microsatellite loci were found to have good discriminatory power (\textit{D} (RO3, \textit{D}=0.846; RO4, \textit{D}=0.747; RO8, \textit{D}=0.742; with a combined \textit{D}=0.986) and stability for seven subcultures. It was also confirmed that the MLMT method may be applied to both \textit{R. oryzae} and \textit{Rhizopus delemar} (a proposed new species), although MLMT analysis could not differentiate them into two clusters. The MLMT system, described here for what is believed to be the first time for a zygomycotic fungus, holds promise as a powerful tool for the strain typing of \textit{R. oryzae}.

INTRODUCTION

Classically, the class Zygomycetes has been subdivided into two orders, Mucorales and Entomophthorales, although recently a modified classification of these fungi has been proposed, wherein the classical Zygomycota has been classified into the phylum Glomeromycota and four subphyla comprising Mucormycotina, Kickxellomycotina, Zoopagomycotina and Entomophthoromycotina (Hibbett, 2007). The fungi in the order Entomophthorales produce indolent subcutaneous and mucocutaneous mycoses in healthy hosts, whereas those in the Mucorales produce rapidly fatal infections in immunocompromised hosts (Ribes et al., 2000). An alarming rise in the incidence of invasive zygomycosis in recent years is a matter of concern worldwide (Greenberg \textit{et al.}, 2004; Kauffman, 2004; Roden \textit{et al.}, 2005), with special interest in India due to the large case series reported (Chakrabarti \textit{et al.}, 2006; 2009; Nithyanandam \textit{et al.}, 2003; Sundaram \textit{et al.}, 2005). Occasionally, outbreaks of zygomycosis have been reported that were linked with excavation, construction or contaminated air-conditioning filters. In hospitals, nosocomially acquired zygomycosis due to \textit{Rhizopus oryzae} had also been reported (Keys \textit{et al.}, 1978; Mead \textit{et al.}, 1979). Nosocomial zygomycosis has been associated with immunosuppression, antifungal prophylaxis and a variety of procedures, and has been related to the use of devices including bandages or medication patches, intravenous catheters and tongue depressors (Keys \textit{et al.}, 1978; Mead \textit{et al.}, 1979; Ribes \textit{et al.}, 2000; Spellberg \textit{et al.}, 2005).

\textit{R. oryzae} is the most common Mucorales to cause zygomycosis (Chakrabarti \textit{et al.}, 2006; Ribes \textit{et al.}, 2000; Roden \textit{et al.}, 2005). The fungus is identified by its microscopic morphology and growth temperature. Recently, \textit{R. oryzae} strains have been divided into two species based on

Abbreviations: 6-FAM, 6-carboxyfluorescein; AFLP, amplified fragment length polymorphism; MLMT, multilocus microsatellite typing; MLST, multilocus sequence typing; NCCPF, National Culture Collection of Pathogenic Fungi.

The GenBank/EMBL/DDBJ accession numbers for the microsatellite sequences of RO3, RO4 and RO8 are GU132512–GU132525.
organic acid production: R. oryzae (lactic acid producers) and Rhizopus delemar (fumaric/malic acid producers) (Abe et al., 2007). To understand the molecular epidemiology of R. oryzae and R. delemar, molecular strain typing is essential. For strain differentiation in fungi, various pattern-based typing techniques, such as random amplified polymorphic DNA and RFLP analysis have been used, but a major problem with such techniques is the poor inter-laboratory reproducibility and difficulty in exchanging the results obtained by these techniques (Gil-Lamaignere et al., 2003). Multilocus sequence typing (MLST) has been used as a reproducible typing method for a few fungal agents (Bougnoux et al., 2003; Dodgson et al., 2003), but its strain discriminatory power is poorer than the multilocus microsatellite typing (MLMT) method (Klaassen, 2009). Moreover, MLST is a laborious and expensive technique. The MLMT method has been used successfully for strain typing in Aspergillus fumigatus, Penicillium marneffei, Candida albicans and Candida glabrata (de Valk et al., 2007a; Dodgson et al., 2003; Fisher et al., 2004; Sampaio et al., 2005). The method targets multiple loci that contain di-, tri- or tetrancleotide repeats, and has the potential to be a highly discriminatory and reproducible typing method. Here, we describe what is believed to be the first report of development of the MLMT method for strain typing of R. oryzae and R. delemar.

METHODS

Isolates. Thirty clinical isolates of R. oryzae (identified on the basis of microscopic morphology and growth temperature) stored at ~80 °C in National Culture Collection of Pathogenic Fungi (NCCPF) (previously the Mycology Culture Collection Laboratory) at the Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, India, were used in this study (Table 1). The isolates were isolated from patients with invasive zygomycosis who attended the Nehru Hospital, PGIMER, Chandigarh, India. The isolates were considered unrelated because they were isolated from multiple wards at different time intervals. After growth of the isolates, their identity was confirmed by morphological features (broad, aseptate, ribbon-like hyphae having sporangiophores and rhizoids; sporangiophores and rhizoids borne from creeping aerial hyphae known as stolons; sporangiophores, single or in tufts, brown, mostly unbranched, sometimes with brownish swelling; brownish rhizoids sparingly branched; spherical sporangia with ellipsoidal columellae; apophysis short or absent; greyish-green sporangiospores, longitudinally striated and rhomboid to lemon-shaped) and by the sequencing of the internal transcribed spacer region of the rRNA gene of the isolates.

Identification of lactic acid- and fumaric/malic acid-producing strains of Rhizopus species. The organic acids produced by the strains were determined by the method described by Abe et al. (2007) with certain modifications. Fermentation of the isolates was carried out in 10 ml organic acid production medium containing 50 g glucose L⁻¹, 6.7 g yeast nitrogen base without amino acid (Difco) L⁻¹ and 5 g casamino acids L⁻¹. Just before inoculation, calcium carbonate (sterilized in an oven at 180 °C for 5 h) was added to a final concentration of 2.5% (w/v). The medium was inoculated with a small amount of mycelia, pre-grown on Sabouraud dextrose agar (SDA; Hi Media), and incubated at 27 °C on an incubator shaker (90 r.p.m.) for 3 days. The organic acids (lactic acid and fumaric/malic acid) in the culture supernatant were analysed by HPLC (Shimadzu). After 3 days of incubation, the fermentation medium was centrifuged, and the culture supernatant filtered through a 0.22 µm membrane filter and injected into the HPLC column. The HPLC used a C-18 column with 0.1% phosphoric acid solvent, at a flow rate of 1 ml min⁻¹ and a column temperature of 40 °C, with a UV detector.

Isolation of genomic DNA. Whole-cell DNA from the mycelia of each isolate was extracted following a slightly modified protocol of the small-scale fungal DNA extraction method developed by Lee & Taylor (1990). Briefly, spores harvested from SDA were allowed to grow in Sabouraud dextrose broth (HiMedia) at 37 °C on a rotary shaker (HT Infors) at 120 r.p.m. for 3–5 days. The mycelial mat was recovered by filtration and washed with sterile saline. Approximately 0.2–0.3 g mycelial mat was ground in the presence of liquid nitrogen, and the resultant powder was transferred to a 1.5 ml microcentrifuge tube containing 600 µl lysis buffer [100 mM Tris/HCl (pH 8.0), 50 mM EDTA, 3% SDS]. The tubes were vortexed briefly and protease K (Sigma) was added to a final concentration of 20 µg ml⁻¹. The tubes were incubated at 56 °C for 1 h. Finally, the DNA was extracted using a phenol:chloroform extraction procedure. The DNA was precipitated with an equal volume of 2-propanol in the presence of 3 M sodium acetate. The pellet was washed with 70% alcohol, dissolved in 100 µl TE [10 mM Tris/HCl (pH 7.5), 1 mM EDTA] and stored at ~20 °C for further use.

Identification of microsatellites and PCR primer design. To identify microsatellite repeats, the available R. oryzae genome sequences were downloaded from the website of the Fungal Genome Initiative (Broad Institute) (www.broad.mit.edu/annotation). The intergenic regions and ORFs of approximately 5.7 Mb were screened for microsatellites with the help of an online bioinformatics tool, Repeat Masker (www.repeatmasker.org). Di-, tri- and tetrancleotide repeats were selected on the basis of loci with the highest repeat numbers and counterselected on loci containing two or more repeat sequences within the boundaries of potential PCR primer regions. Interrupted repeats, which might have a lower chance of displaying inter-strain repeat number variation, were not taken into consideration.

Screening of microsatellites by PCR and sequencing. Each individual microsatellite locus was amplified by PCR in the presence of 2 mM MgCl₂, 200 µM each dNTP (Bangalore Genie), 0.25 µM each primer (Integrated DNA Technologies), 0.25 U Taq polymerase (Bangalore Genie) and 5–10 ng fungal genomic DNA in a total volume of 10 µl. The amplification reactions were performed in a thermocycler (Eppendorf Mastercycler). PCR cycling conditions consisted of an initial denaturation step for 5 min at 94 °C, followed by 35 cycles of 94 °C for 1 min, 55–59 °C for 30 s (varied according to microsatellite loci) and 72 °C for 1 min, with a final extension step at 72 °C for 5 min. The annealing temperature for the three selected microsatellite loci was 58 °C for 30 s. The amplified products were separated in an 8% polyacrylamide gel to ascertain the amplicon band sizes. The presence of all microsatellite loci was screened in eight randomly selected R. oryzae isolates by apparent size variation on PAGE. The microsatellite loci that were available in all isolates (confirmed by amplification), and having better discrimination power, were subjected to DNA sequencing to confirm whether the apparent size variation seen on PAGE was due to changes in the repeat numbers of the microsatellites. Sequencing reactions were performed with a BigDye terminator cycle sequencing kit, version 3.1/1.1 (Applied Biosystems). All the sequencing reactions were purified and analysed on an ABI 3130 genetic analyser (Applied Biosystems).

Automated capillary electrophoresis. The chosen microsatellites of R. oryzae were analysed further by fragment size analysis in an

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automated capillary electrophoresis system for all 30 isolates. Individual microsatellite loci were amplified by PCR as described above using a fluorescently labelled primer. The primer for RO8 was labelled with the fluorescent dye 6-FAM, whilst RO3 and RO4 were labelled with VIC and NED, respectively. After PCR, a 2 μl aliquot of each sample was diluted 200-fold and added to 10 μl Hi-Di formamide containing 0.5 μl GeneScan 500 6-carboxytetramethyl-rhodamine size standards (Applied Biosystems). Amplicons were denatured at 95 °C for 5 min and rapidly chilled on ice. Denatured samples were resolved by capillary electrophoresis with a 35 cm capillary filled with POP-7 polymer (Applied Biosystems) in an ABI Prism 3130 genetic analyser (Applied Biosystems). The molecular sizes of the amplified alleles were automatically analysed and calculated using GENESCAN analysis software (version 2.1; Applied Biosystems). As a result of the length polymorphisms, isolates were assigned to different microsatellite genotypes based on one or more differences in band size.

Discriminatory power and stability of the markers. The discriminatory power of the microsatellite markers was calculated using Simpson’s index of diversity (D). This index is a statistical measure that any two randomly chosen isolates are of the same genotype for a given marker(s). A D value of 1 indicates that all isolates are unique, whereas a D value of 0 indicates that all isolates are identical. The stability of the typing method was evaluated by analysing the DNA preparations after each passage for up to seven passages. Briefly, in this experiment, a Rhizopus species spore suspension (3 × 10^6 spores ml^{-1}) was prepared and approximately 100 μl of this suspension was plated on SDA and incubated at 37 °C. A single non-sporulating colony was then picked and placed on a new SDA plate and incubated until sporulation. The spores were collected and a portion was used to inoculate Sabouraud dextrose broth for DNA extraction, whilst the rest was plated on SDA to obtain growth of the fungus with spores. This procedure was repeated seven times, and the extracted DNA in each passage was used for microsatellite analysis.

Data analysis. Typing data were imported into BioNumerics version 5.0 software (Applied Maths). Microsatellite data were analysed using the multistate categorical similarity coefficient.

Table 1. *R. oryzae* and *R. delemar* isolates included in the study, fragment sizes of the three microsatellite loci and acid production patterns

<table>
<thead>
<tr>
<th><em>R. oryzae</em> NCCPF no.</th>
<th>Age (years)/ sex of patient</th>
<th>Month and year of isolation</th>
<th>Source of isolate</th>
<th>Fragment size (bp)</th>
<th>Acid production</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>RO3</td>
<td>RO4</td>
</tr>
<tr>
<td>710016</td>
<td>35/M August 2001 Nasal tissue</td>
<td>285</td>
<td>195</td>
<td>303 LA</td>
<td></td>
</tr>
<tr>
<td>710024</td>
<td>60/M October 2002 Nasal tissue</td>
<td>285</td>
<td>189</td>
<td>313 LA</td>
<td></td>
</tr>
<tr>
<td>710031</td>
<td>11.5/F March 2003 Nasal tissue</td>
<td>279</td>
<td>191</td>
<td>303 FA</td>
<td></td>
</tr>
<tr>
<td>710034</td>
<td>60/F May 2003 Nasal tissue</td>
<td>288</td>
<td>189</td>
<td>313 LA</td>
<td></td>
</tr>
<tr>
<td>710050</td>
<td>36/F August 2004 Nasal tissue</td>
<td>285</td>
<td>189</td>
<td>333 FA</td>
<td></td>
</tr>
<tr>
<td>710056</td>
<td>50/M December 2004 Maxillary tissue</td>
<td>289</td>
<td>189</td>
<td>306 FA</td>
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<tr>
<td>710058</td>
<td>35/M February 2005 Nasal tissues</td>
<td>297</td>
<td>196</td>
<td>333 FA</td>
<td></td>
</tr>
<tr>
<td>710059</td>
<td>19/F April 2005 Nasal crust</td>
<td>278</td>
<td>189</td>
<td>303 LA</td>
<td></td>
</tr>
<tr>
<td>710060</td>
<td>45/M June 2005 Nasal tissue</td>
<td>278</td>
<td>191</td>
<td>303 FA</td>
<td></td>
</tr>
<tr>
<td>710068</td>
<td>36/F December 2005 Nasal tissue</td>
<td>288</td>
<td>191</td>
<td>303 FA</td>
<td></td>
</tr>
<tr>
<td>710070</td>
<td>12/F December 2005 Nasal tissue</td>
<td>283</td>
<td>195</td>
<td>303 FA</td>
<td></td>
</tr>
<tr>
<td>710073</td>
<td>2/F May 2006 Nasal crust</td>
<td>288</td>
<td>189</td>
<td>309 LA</td>
<td></td>
</tr>
<tr>
<td>710078</td>
<td>25/M August 2006 Nasal tissue</td>
<td>298</td>
<td>186</td>
<td>309 LA</td>
<td></td>
</tr>
<tr>
<td>710079</td>
<td>28/M August 2006 Nasal tissue</td>
<td>285</td>
<td>189</td>
<td>303 FA</td>
<td></td>
</tr>
<tr>
<td>710083</td>
<td>30/F October 2006 Palate ulcer</td>
<td>298</td>
<td>186</td>
<td>309 FA</td>
<td></td>
</tr>
<tr>
<td>710084</td>
<td>64/M October 2006 Nasal swab</td>
<td>285</td>
<td>194</td>
<td>335 FA</td>
<td></td>
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<tr>
<td>710085</td>
<td>NA November 2006 Nasal tissue</td>
<td>286</td>
<td>186</td>
<td>303 FA</td>
<td></td>
</tr>
<tr>
<td>710086</td>
<td>30/M April 2007 Nasal tissue</td>
<td>293</td>
<td>189</td>
<td>313 FA</td>
<td></td>
</tr>
<tr>
<td>710088</td>
<td>50/M August 2008 Nasal tissue</td>
<td>297</td>
<td>196</td>
<td>333 LA</td>
<td></td>
</tr>
<tr>
<td>710089</td>
<td>35/M January 2008 Nasal crust</td>
<td>278</td>
<td>191</td>
<td>303 FA</td>
<td></td>
</tr>
<tr>
<td>710093</td>
<td>6/M January 2008 Nasal tissue</td>
<td>288</td>
<td>189</td>
<td>313 LA</td>
<td></td>
</tr>
<tr>
<td>710094</td>
<td>25/M August 2008 Nasal wash</td>
<td>283</td>
<td>191</td>
<td>303 LA</td>
<td></td>
</tr>
<tr>
<td>710097</td>
<td>28/M June 2008 Nasal tissue</td>
<td>285</td>
<td>195</td>
<td>313 FA</td>
<td></td>
</tr>
<tr>
<td>710099</td>
<td>50/M September 2008 Nasal tissue</td>
<td>283</td>
<td>189</td>
<td>303 FA</td>
<td></td>
</tr>
<tr>
<td>710100</td>
<td>2/F November 2008 Palatal ulcer</td>
<td>278</td>
<td>186</td>
<td>306 LA</td>
<td></td>
</tr>
<tr>
<td>710101</td>
<td>45/F January 2009 Nasal crust</td>
<td>285</td>
<td>195</td>
<td>303 FA</td>
<td></td>
</tr>
<tr>
<td>710103</td>
<td>52/F February 2009 Nasal tissue</td>
<td>288</td>
<td>189</td>
<td>309 FA</td>
<td></td>
</tr>
<tr>
<td>710116</td>
<td>45/F February 2009 Nasal crust</td>
<td>285</td>
<td>186</td>
<td>335 FA</td>
<td></td>
</tr>
<tr>
<td>710117</td>
<td>50/F January 2009 Nasal tissue</td>
<td>278</td>
<td>197</td>
<td>303 ND</td>
<td></td>
</tr>
<tr>
<td>710118</td>
<td>60/M July 2009 Tissue from cutaneous lesion</td>
<td>293</td>
<td>189</td>
<td>303 LA</td>
<td></td>
</tr>
</tbody>
</table>

F, Female; FA, fumaric acid; LA, lactic acid; M, male; ND, not detected.
RESULTS

After analysing 30 R. oryzae isolates (identified by morphology and growth temperature) for organic acid production, the isolates were classified into 17 R. delemar isolates (fumaric/malic acid producers), 12 R. oryzae isolates (lactic acid producers) and one isolate that could not be classified as either of the above as there was no organic acid production by this isolate (Table 1).

Computational analysis of R. oryzae (the strain identified later as R. delemar on the basis of organic acid production) genome sequences yielded 30 microsatellite loci with the help of the online tool Repeat Masker. A total of 10 of these microsatellites were intergenic, whilst the other 20 were within the putative coding region. The nomenclature used for the microsatellite loci was RO for R. oryzae, followed by a number. The intergenic microsatellites could not be amplified in all isolates tested. Of the 20 putative coding-region microsatellite loci, 3 that could be amplified with good discrimination from 8 randomly selected isolates were selected for analysis of 30 clinical isolates of R. oryzae. These microsatellite loci were: RO3 [(CCT)]n from ORF RO3G 04330 (R. oryzae predicted protein); RO4 [(TA)]n from ORF RO3G 04103 (R. oryzae hypothetical protein); and RO8 [(GAA)(GGA)]n from ORF RO3G 13155 (R. oryzae predicted protein). The PCR products of RO3, RO4 and RO8 showed apparent size variation by PAGE (data not shown).

Sequence analysis confirmed the presence of the microsatellites and demonstrated polymorphism in the microsatellite repeat number. The sequence information for the various alleles of these three microsatellite loci was submitted to GenBank (accession numbers given in Table 2). Accurate sizes of the selected loci were determined by an automated capillary electrophoresis system for all the clinical isolates of R. oryzae (Fig. 1). The PCR products of RO3, RO4 and RO8 varied in the range of 278–298, 186–197 and 303–335 bp, respectively (Table 1). These 3 microsatellite loci identified 21 genotypes among the 30 R. oryzae isolates.

The discriminatory power (D) was highest for locus RO3 (0.846), whereas for RO4 and RO8 the D value was 0.747 and 0.742, respectively. The combined D value was 0.986 (Table 2). All three microsatellite loci were found to be reasonably stable, as they produced the same genotypes even after seven passages. A dendrogram of the 30 R. oryzae isolates included in the MLMT analysis using these three microsatellite loci showed clustering of the isolates into four groups. (Fig. 2)

DISCUSSION

This study describes what is believed to be the first report of the development of a stable and discriminatory MLMT system for R. oryzae. Microsatellite markers have been used successfully for strain typing and population genetics studies of several fungal species because of their hypervariable nature, ease of PCR amplification and interpretation, co-dominance and potential use in automated assays. The ideal MLMT scheme should amplify the same loci from all studied isolates and should demonstrate sufficient repeat number diversity to develop a discriminatory typing scheme (Klaassen, 2009). Recently, sequencing and assembly of the entire R. oryzae genome has been completed (Ma et al., 2009). This has provided the opportunity to search for more potential microsatellite loci in silico by using software available in the public domain. The Rhizopus genome is approximately 46 Mb. As the genome sequence was quite large, we started screening sequences with a National Center for Biotechnology Information tool, which yielded ten intergenic (non-coding) microsatellites. When these intergenic microsatellites failed to qualify as suitable markers for strain typing, we screened approximately 5.7 Mb of ORFs. Although we found 20 microsatellite loci in the coding region, only three – RO4 [(TA)]n, RO3 [(CCT)]n and RO8 [(GAA)(GGA)]n – fulfilled the criteria for an ideal microsatellite marker. In general, it is believed that non-coding region microsatellites are far more discriminatory than those in coding regions, and these have been utilized for the typing of various fungi (Fisher et al., 2004; de Valk et al., 2007a; Sampaio et al., 2005). However, in the present study, the non-coding region microsatellites could not be amplified in most of the isolates tested. This may have been due to high levels of sequence variation in the primer-binding sites. In seven out of ten cases, attempts to amplify the non-coding region microsatellites generated a number of non-specific bands (results not shown). Thus, we suggest that the non-coding region microsatellites are not good candidates for an MLMT system for R. oryzae. The three coding-region microsatellites described here were found to have reasonably good discriminatory power and stability. The discriminatory power of the assay was greater when all three loci were used for analysis, compared with the

Table 2. Salient characteristics of selected microsatellite loci

<table>
<thead>
<tr>
<th>Microsatellite</th>
<th>No. of genotypes</th>
<th>Size (bp)</th>
<th>Repetitive motif</th>
<th>D value</th>
<th>Dye label</th>
<th>GenBank accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>RO3</td>
<td>7</td>
<td>278–298</td>
<td>(CCT)ₙ</td>
<td>0.846</td>
<td>VIC</td>
<td>GU132512–GU132516</td>
</tr>
<tr>
<td>RO4</td>
<td>5</td>
<td>186–196</td>
<td>(TA)ₙ</td>
<td>0.747</td>
<td>NED</td>
<td>GU132517–GU132519</td>
</tr>
<tr>
<td>RO8</td>
<td>6</td>
<td>303–335</td>
<td>(GAA)(GGA)ₙ</td>
<td>0.742</td>
<td>6-FAM</td>
<td>GU132520–GU132525</td>
</tr>
</tbody>
</table>
discriminatory powers of individual loci. A combined discriminatory power of 0.986 was achieved for *R. oryzae*, which is in good agreement with reports for typing of other fungi by microsatellite analysis (de Valk *et al.*, 2007a; Sampaio *et al.*, 2005). For the pathogenic yeast *C. albicans*, the discriminatory power based on microsatellite analysis

![GENESCAN profile showing the results of automated fragment sizing for microsatellite analysis. Each microsatellite locus is represented by a different colour: RO4 (black), RO3 (green) and RO8 (blue). Size standards (GeneScan LIZ 500) are shown in orange.](image1)

**Fig. 1.** GENESCAN profile showing the results of automated fragment sizing for microsatellite analysis. Each microsatellite locus is represented by a different colour: RO4 (black), RO3 (green) and RO8 (blue). Size standards (GeneScan LIZ 500) are shown in orange.

![Dendrogram based on the three microsatellite loci RO4, RO3 and RO8. The results denote the NCCPF strain numbers, sex of the patients, age of the patients (years), the year of isolation and body site source of isolate. F, Female; M, male; RhOrySTR, Rhizopus oryzae short tandem repeats MLMT.](image2)

**Fig. 2.** Dendrogram based on the three microsatellite loci RO4, RO3 and RO8. The results denote the NCCPF strain numbers, sex of the patients, age of the patients (years), the year of isolation and body site source of isolate. F, Female; M, male; RhOrySTR, *Rhizopus oryzae* short tandem repeats MLMT.
has been reported to range from 0.87 to 0.97 for the typing of 114 isolates (Sampaio et al., 2005). Likewise, a high degree of discrimination (D=0.989) was achieved using polymorphic microsatellite markers for typing 100 A. fumigatus isolates (de Valk et al., 2007a). In the present study, we evaluated only 30 isolates, so it is possible that there would be an improvement in discriminatory power when a larger numbers of isolates are included and more microsatellite loci are analysed. As we screened only about 5.7 Mb of the genome, there remains scope for identifying many more ideal microsatellite markers by screening the rest of the genome and validating any potential markers.

The ability to assign an identical genotype to the same isolate after multiple passages defines in vitro stability. This is another important criterion for evaluating a typing system (Gil-Lamagnere et al., 2003). The reproducibility as well as the in vitro stability of the present MLMT method was found to be 100 % for up to seven passages. Recently, Sabino et al. (2010) described the stability of microsatellite markers for Candida parapsilosis for 300 generations (Sabino et al., 2010). As the genotypes were the same for up to 300 generations, they predicted a mutation rate of less than 3.33 x 10^{-3} for these microsatellites. However, for Aspergillus species, the stability of microsatellites has only been determined for 14–30 passages (Balajee et al., 2007; de Valk et al., 2005; Lasker & Run, 2004). For organisms with a short generation time, it may be necessary to assess the stability of the markers for up to several hundred generations, whereas for filamentous fungi, which have a longer generation time, it remains to be seen whether they have to be followed for a similarly high number of generations. The level of stability of the markers obtained in the current study may be sufficient to use them to answer epidemiological questions concerning outbreaks in a hospital or in a given geographical location. However, future studies are required to assess the stability of microsatellite markers in filamentous fungi. In addition to good reproducibility and discrimination power, microsatellite analysis has other advantages. Because the procedure is PCR based, MLMT analysis requires relatively small amounts (~30–40 ng per reaction) of template DNA compared with other methods. Large numbers of isolates may be typed easily, as preparation is simple and does not require the tedious and labour-intensive procedures needed for highly purified DNA. Using a standard panel of microsatellite loci and test isolates, inter-laboratory comparisons and creation of databases are feasible (de Valk et al., 2009).

At present, for molecular strain typing of fungi, three methods are popular: MLST, MLMT and amplified fragment length polymorphism (AFLP) analysis (Bougnoux et al., 2003; de Valk et al., 2005, 2007b; Dodgson et al., 2003). Each has its advantages and disadvantages. Klaassen (2009) compared MLST and MLMT techniques and pointed out that the different microsatellite markers may display much heterogeneity with regard to stability. Within one locus, alleles with a low number of repeat units may be more stable than the alleles with a high number of repeats. In contrast, the stability of single-nucleotide polymorphisms in housekeeping genes makes MLST a more reliable typing method for population genetic studies, although the discriminatory power of MLST is expected to be lower than MLMT. However, MLST is laborious and time-consuming. MLMT is versatile for strain discrimination because of its inherent instability compared with the single-nucleotide polymorphisms in housekeeping genes. The degree of stability offered by the presence of microsatellite markers in the coding regions should be sufficient to balance the stability versus its variability that gives rise to many genotypes. This balancing phenomenon is only a feature of MLMT. In comparison with the AFLP method, the MLMT approach is found to be highly biased towards arbitrarily selected loci, whereas AFLP provides an overview of the entire genome variability. Furthermore, the AFLP technique is applicable to any organism without the need for prior genome sequence information. In contrast, MLMT is usually species specific and requires species sequence information for the identification of microsatellite loci (de Valk et al., 2007b). MLMT also requires specialized reagents, software and expensive equipment, which may not be available in many laboratories. This problem may partially be circumvented by running the PCR products in a high-resolution agarose gel system or using denaturing PAGE.

Abe et al. (2007) have divided R. oryzae isolates into two species based on organic acid production, R. oryzae (lactic acid producers) and R. delemar (fumaric/malic acid producers). They proposed R. delemar as a new species. The R. oryzae genome sequence information available from the database of the Fungal Genome Initiative is actually from a R. delemar strain (later confirmed by organic acid production). We wanted to see whether the protocol worked with both species and thus isolates of both species were included in this study. The microsatellite markers worked for members of both species but could not distinguish them into two clusters. Inclusion of additional microsatellites in the future may help in species differentiation.

In conclusion, the present study describes a MLMT system for R. oryzae and R. delemar that seems to have a good degree of discriminatory power, stability, ease of use and interpretation, typeability and high-throughput potential. The method may be improved further with the use of additional microsatellites, and it could be used to address important epidemiological issues such as tracking the transmission of infection and strain specificity for a specific disease manifestation.

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Apophysomyces elegans: Epidemiology, Amplified Fragment Length Polymorphism Typing, and In Vitro Antifungal Susceptibility Pattern


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Apophysomyces elegans is an emerging pathogen in India. We planned the present study to analyze the clinical pattern of the disease, to perform molecular strain typing, and to determine the in vitro activities of eight antifungal drugs against A. elegans. A total of 16 clinical and two environmental A. elegans isolates were included in the study. The clinical histories of the patients were noted. MICs or minimum effective concentrations (MECs) were determined for antifungal drugs by microdilution testing in accordance with CLSI standard M38-A2 guidelines. Of 16 patients, seven had rhino-cerebral, five had cutaneous, and three had renal zygomycosis. One patient had osteomyelitis. Uncontrolled diabetes was observed in 63% of the patients. Amplified fragment length polymorphism (AFLP) analysis divided the strains into two clearly different clades. The fingerprints of the environmental strains (including the type strain) were clearly different from those of the clinical strains. The MICs of amphotericin B, itraconazole, posaconazole, and isavuconazole were 2 and 4, 1 and 2, 0.5 and 1, and 2 and 4 μg/ml, respectively. The strains had high MICs for fluconazole, voriconazole, and echinocandins. The study indicates a possible change in the clinical pattern of zygomycosis due to A. elegans in India. The fungus caused not only cutaneous or subcutaneous infection but also other deep-seated infections, and the disease is commonly associated with uncontrolled diabetes. The AFLP patterns show a clear difference between environmental and clinical strains. Posaconazole is the most active drug against the isolates, followed by itraconazole. The MICs of amphotericin B against A. elegans were higher than those of the other drugs.

Zygomycosis (mucormycosis) is a serious and often rapidly fatal infection, especially in immunocompromised hosts. Among zygomycetes, the species under the genera Rhizopus, Rhizomucor, Lichtheimia (Absidia), Mucor, Apophysomyces, Saksenaea, Cunninghamamella, Cokeromyces, and Syncephalas-trum have been reported to cause invasive zygomycosis, and the species under Rhizopus, Lichtheimia, and Rhizomucor are the more commonly reported pathogens (5, 25, 32, 33). However, Apophysomyces elegans, once considered a rare pathogen, has increasingly been isolated from patients in tropical and subtropical climates over the last 2 decades (5, 25, 32). Patients with A. elegans infection have been documented in India, the southern United States, Australia, Mexico, Caribbean islands, Colombia, and Venezuela. However, of nearly 100 cases published in the literature, the majority (~60%) were from India (5–7, 12, 17, 25, 32, 38). A. elegans was believed to cause only cutaneous and mucocutaneous infection in immunocompetent patients, but it has been implicated in serious deep-seated infections (rhino-orbito-cerebral and renal zygomycosis) in recent years (5–8, 22, 23, 32, 35). Wound contamination with soil (possibly harboring A. elegans spores) after an accident is considered the single most important risk factor for cutaneous or subcutaneous zygomycosis due to A. elegans (25, 32). However, it is not clear how A. elegans acquisition occurs in patients with rhino-cerebral or renal zygomycosis. To understand the epidemiology of the disease, molecular strain typing is important. No serious attempt has been made to perform strain typing of A. elegans except our previous attempt to type the strains using two microsatellites (7).

As with other cases of zygomycosis, amphotericin B and its lipid formulations have been the mainstay of therapy in patients with A. elegans infections (32, 33), though there continues to be a need for developing new treatment strategies due to the limitations of amphotericin B caused by its toxicity. In search of an alternative therapy, in vitro antifungal susceptibility testing was performed for large collections of zygomycetes. The results showed that the zygomycetes consist of a heterogeneous group with differing antifungal susceptibilities (1, 2, 4, 10, 21, 30, 34). However, it is difficult to comment on A. elegans, as only 11 isolates have been subjected to in vitro antifungal susceptibility testing to date, and they were tested against a limited number of antifungal agents (2, 10, 34). Therefore, to address both problems, we evaluated the in vitro activities of eight antifungal agents, including isavuconazole, a new triazole drug, against 18 A. elegans strains and typed those A. elegans strains by amplified fragment length polymorphism (AFLP) analysis.
**Materials and Methods**

*Apophysomyces elegans* isolates. A total of 18 *A. elegans* isolates were included in the present study. A collection of 15 clinical isolates were obtained from the National Culture Collection of Pathogenic Fungi, Chandigarh, India, and three strains were obtained from the Centraalbureau voor Schimmelcultures (CBS) Fungal Biodiversity Centre, Utrecht, Netherlands (two environmental isolates, CBS 476.78 and CBS 477.78, and one clinical isolate, CBS 658.93). The identity of each isolate was verified based on taxonomic criteria by conventional procedures (16) and then confirmed by DNA sequencing of internal transcribed spacer regions (ITS1 and -2) and the 5.8S region. The clinical details of the 16 patients from whom the isolates were obtained are presented in Table 1.

**Antifungals tested.** Antifungals used in this study included amphotericin B (AMB; Bristol Myers Squibb, Woerden, Netherlands), itraconazole (ITR; Janssen Cilag, Tilburg, Netherlands), fluconazole and voriconazole (FLU and VOR, respectively; Pfizer Central Research, Kent, United Kingdom), posaconazole (POS; Schering-Plough Corp., Kenilworth, NJ), and anidulafungin (ANI; Pfizer Central Research).

**Antifungal susceptibility testing.** The MICs or minimum effective concentrations (MECs) (for CAS and ANI) were determined by the broth microdilution method, in accordance with the guidelines of CLSI document M27-A2 (9). Stock solutions (3,200 mg/liter) of AMB, ISA, ITR, POS, and VOR were prepared using dimethyl sulfoxide solution (DMSO), while FLU, CAS, and ANI were dissolved in sterile distilled water to a final stock concentration of 3,200 mg/liter. Final concentrations of each antifungal solution were made using water-sterilized (0.22-μm filter) RPMI 1640 medium with l-glutamine (Difco, Breda, Netherlands). Growth was assessed visually after incubation in ambient air at 35°C for 24 h. To induce sporulation, we used a previously reported method (27). Two 1-cm² agar blocks with hyphal growth on Sabouraud dextrose agar were cut and transferred to a petri dish containing 20 ml of sterile distilled water. Three drops (0.2 ml) of a fertilizer-stereiled 10% yeast extract solution were added to each plate. The plates were incubated in the dark at 35°C. After 5 days of incubation, sporulation appeared. The spore concentrations were adjusted spectrophotometrically at a 530-nm wavelength to a transmission that ranged from 68 to 71% (0.6 × 10⁶ to 4 × 10⁶ CFU/ml). MICs for AMB and the azoles corresponded to a 100% reduction in growth relative to that of control wells lacking an antifungal. MECs for CAS and ANI, assessed microscopically, corresponded to the lowest concentration at which abnormal hyphae were observed at 24 h of incubation. *Paecilomyces variotii* (ATCC 22319) and *Candida krusei* (ATCC 6258) were included in the study as quality control strains. Values for MIC₅₀ and MIC₉₀ were obtained by ordering the MIC data for each antifungal in ascending arrays and selecting the median and 90th percentile, respectively, of the MIC distribution.

**AFLP analysis.** Approximately 50 ng of genomic DNA was subjected to a combined restriction-ligation procedure with a mixture containing 50 pmol of the HpyCH₄ IV adapter, 50 pmol of the MseI adapter, 2 U of HpyCH₄ IV (New England Biolabs, Beverly, MA), 2 U of MseI (New England Biolabs), and 1 U of T4 DNA ligase (Promega, Leiden, Netherlands) in a total volume of 20 μl of 1× reaction buffer for 1 h at 20°C. Next, the mixture was diluted five times with 10 mM Tris-HCl (pH 8.3) buffer. Adapters were made by mixing equimolar amounts of complementary oligonucleotides (5'-CTGATAGACTGCCGTACC-3' and 5'-GGGTTGGCGACGTC-3' for HpyCH₄ IV; 5'-GACGATGACTCTCTAGGC-3' and 5'-TAGTCAGGGACTCATG-3' for MseI) and heating them to 95°C for 2 min, with subsequent slow cooling to ambient temperature. One microliter of the diluted restriction-ligation mixture was used for amplification in a volume of 25 μl under the following conditions: 1 μl HpyCH₄ IV primer with one selective residue (underlined) (5'-fluorescent-TGATAGCTGCCGTACCAGT-3'), 1 μM MseI primer with four selective residues (5'-GATGAGTCTCTAATQTAGAG-3'), 0.2 mM each dioxynucleoside triphosphate (dNTP), and 1 U of Tag DNA polymerase (Roche Diagnostics) in 1× reaction buffer containing 1.5 mM MgCl₂. Amplification was performed as follows. After an initial denaturation step for 4 min at 94°C, we applied to the first 20 cycles a touchdown procedure consisting of 15 s of denaturation at 94°C and 15 s of annealing at 66°C, with the temperature for each successive cycle lowered by 0.5°C, followed by 1 min of extension at 72°C. Cycling was then continued for a further 30 cycles, with an annealing temperature of 56°C. After completion of the cycles, incubation at 72°C for 10 min was performed before the reaction mixtures were cooled to room temperature. Reaction products were diluted 10-fold with distilled water. One microliter of diluted products was combined with 0.25 μl of the ET400-R size marker (GE Healthcare, Diegem, Belgium) and 8.75 μl of distilled water. After a 1-min denaturation step at 94°C, the samples were quickly cooled to room temperature and injected onto a MegaBACE 500 automated DNA analysis platform equipped with a 48-capillary array, as recommended by the manufacturer (GE Healthcare). Typing data were imported into BioNumerics v 5.0 software (Applied Maths, Sint-Martens-Latem, Belgium) and analyzed by using clustering by the unweighted-pair group method using average linkages (UPGMA) and the Pearson correlation coefficient.

**Results**

The details of the 16 patients (including demographic, site of infection, risk factors, therapy, and outcome) and the sources of the two environmental strains are presented in Table 1. All patients were from India except one (case 16) (Table 1), who was from the Caribbean. It is of interest to note that of the 16 patients, seven patients had rhino-cerebral, five had cutaneous, and three had renal zygomycosis. In one patient, *A. elegans* was isolated from bone tissue, which was reported earlier (26). Uncontrolled diabetes was a risk factor in 10 patients, intraocular injection in two patients (one with diabetes mellitus), and application of occlusive plaster in one patient; no risk factors could be ascertained in three patients. All patients except one (who expired before surgery could be performed) had extensive debridement or surgery to remove necrotic masses or the affected kidney. Amphotericin B deoxycholate was prescribed for the majority (75%) of patients; one patient received a lipid formulation of amphotericin B, one patient received three antifungal drugs (at first, amphotericin B, and then itraconazole after development of toxicity because of amphotericin B, followed by posaconazole), one patient had fluconazole (the patient had associated invasive candidiasis), and the other two patients expired before institution of any antifungal agent. In the outcome analysis, 40% of the patients with a known course of disease had a fatal outcome. The outcome was poorer (67% fatality) in patients with rhino-orbito-cerebral zygomycosis.

The detailed results of *in vitro* susceptibility testing against each antifungal agent are presented along with the isolates in Table 1. Summaries of the results in the form of MIC/MEC range, geometric mean MIC/MEC₅₀, and MIC/MEC₉₀ are tabulated and compared with those from previously published series (2, 10, 34) in Table 2. The strains in the present series had high MICs of fluconazole, voriconazole, and the two echinocandins. The MIC₅₀ and MIC₉₀ of amphotericin B for the strains were 2 and 4 μg/ml; and the MIC₅₀ and MIC₉₀ results for itraconazole, posaconazole, and isavuconazole were 1 and 2, 0.5 and 1, and 2 and 4 μg/ml, respectively. The single patient who developed infection due to an *A. elegans* strain with a MIC of <1 μg/ml for amphotericin had a positive outcome, whereas of those patients infected with MICs of ≥1 μg/ml, 43% had a poor outcome.

The AFLP fingerprints obtained with MseI and HpyCH₄ IV contained multiple bands in the range of 50 to 250 bp (Fig. 1). The dendrogram yielded a clear separation between clinical and environmental isolates. The clinical isolates had an average similarity of greater than 90%. The two environmental isolates (CBS 476.78 and CBS 477.78) were 95% similar, but they were less than 20% similar to the clinical isolates. A clinical isolate from the Caribbean was clustered in the same clade as the clinical isolates from India. One other sample (102.37) yielded a faint fingerprint but still clustered well within the remainder of the isolates.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Patient age (yr)/sex</th>
<th>Risk factor(s)</th>
<th>Type or site(s) of disease</th>
<th>Therapy</th>
<th>Outcome</th>
<th>MIC (µg/ml)</th>
<th>MEC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCCPF 102.24</td>
<td>40/M</td>
<td>Diabetes mellitus</td>
<td>Rhino-orbito-cerebral</td>
<td>Amphotericin B + surgery</td>
<td>Not known</td>
<td>2</td>
<td>&gt;16</td>
</tr>
<tr>
<td>NCCPF 102.31</td>
<td>30/F</td>
<td>Diabetes mellitus</td>
<td>Cutaneous</td>
<td>Surgery + amphotericin B</td>
<td>Expired</td>
<td>2</td>
<td>&gt;16</td>
</tr>
<tr>
<td>NCCPF 102.32</td>
<td>10/F</td>
<td>None</td>
<td>Bilateral kidneys</td>
<td>Nephrostomy + amphotericin B</td>
<td>Improved</td>
<td>2</td>
<td>&gt;16</td>
</tr>
<tr>
<td>NCCPF 102.36</td>
<td>42/M</td>
<td>Diabetes mellitus</td>
<td>Rhino-orbito-cerebral</td>
<td>Amphotericin B + extended surgery</td>
<td>Improved</td>
<td>4</td>
<td>&gt;16</td>
</tr>
<tr>
<td>NCCPF 102.33</td>
<td>22/F</td>
<td>Diabetes mellitus</td>
<td>Rhino-orbito-cerebral</td>
<td>Fluconazole + extended surgery</td>
<td>Expired</td>
<td>2</td>
<td>&gt;16</td>
</tr>
<tr>
<td>NCCPF 102.34</td>
<td>59/M</td>
<td>Diabetes mellitus</td>
<td>Rhino-orbito-cerebral</td>
<td>Extended surgery</td>
<td>Expired</td>
<td>1</td>
<td>&gt;16</td>
</tr>
<tr>
<td>NCCPF 102.37</td>
<td>17/F</td>
<td>Bilateral kidneys</td>
<td>Nephrectomy + amphotericin B</td>
<td>Improved</td>
<td></td>
<td>0.5</td>
<td>2</td>
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<tr>
<td>NCCPF 102.38</td>
<td>45/M</td>
<td>None</td>
<td>Right kidney</td>
<td>Amphotericin B</td>
<td>Recovered</td>
<td>2</td>
<td>&gt;16</td>
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<tr>
<td>NCCPF 102.39</td>
<td>42/M</td>
<td>Intramuscular injection in gluteal region</td>
<td>Cutaneous</td>
<td>Local debridement + amphotericin B</td>
<td>Improved</td>
<td>2</td>
<td>&gt;16</td>
</tr>
<tr>
<td>NCCPF 102.41</td>
<td>79/M</td>
<td>Diabetes mellitus</td>
<td>Rhino-orbito-cerebral</td>
<td>Extended surgery + amphotericin B</td>
<td>Expired</td>
<td>2</td>
<td>0.5</td>
</tr>
<tr>
<td>NCCPF 102.42</td>
<td>29/M</td>
<td>None (plaster-of-paris cast applied at the site of fracture)</td>
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<td>Local debridement + amphotericin B</td>
<td>Recovered</td>
<td>2</td>
<td>&gt;16</td>
</tr>
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<td>35/F</td>
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<td>Cutaneous</td>
<td>Local debridement, insulin, antibiotics; no antifungal given</td>
<td>Expired</td>
<td>4</td>
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<td>Recovered</td>
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<td>&gt;16</td>
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<td>Diabetes mellitus</td>
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<td>Expired</td>
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<td>&gt;16</td>
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<td>53/M</td>
<td>Diabetes mellitus</td>
<td>Cutaneous</td>
<td>Local debridement + amphotericin B, itraconazole, posaconazole</td>
<td>Recovered</td>
<td>2</td>
<td>&gt;16</td>
</tr>
<tr>
<td>CBS 658.93</td>
<td>69/M</td>
<td>None</td>
<td>Osteomyelitis humerus</td>
<td>Lipid amphotericin B (cumulative, 13 g) + surgery</td>
<td>Recovered</td>
<td>2</td>
<td>&gt;16</td>
</tr>
<tr>
<td>CBS 476.78</td>
<td>0.25</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4</td>
<td>0.125</td>
</tr>
<tr>
<td>CBS 477.78</td>
<td>0.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&gt;64</td>
<td>2</td>
</tr>
</tbody>
</table>

NCCPF, National Culture Collection of Pathogenic Fungi, Chandigarh, India; CBS, Centraalbureau voor Schimmecultures Fungal Biodiversity Centre, Utrecht, Netherlands; M, male; F, female; AMB, amphotericin B; FLU, fluconazole; ITR, itraconazole; VOR, voriconazole; POS, posaconazole; ISA, isavuconazole; CAS, caspofungin; ANI, anidulafungin. The MICs (for AMB, FLU, ITR, VOR, and POS) or minimum effective concentrations (MECs) (for CAS and ANI) were determined by the broth microdilution method, in accordance with the guidelines of CLSI document M38-A2 (9).
DISCUSSION

A. elegans in tropical and subtropical countries has been described as an emerging pathogen, known to cause cutaneous infection after traumatic inoculation (5–8, 12, 17, 22, 25, 28, 32, 33, 35, 38). However, in the present series, only 31% of patients had cutaneous infection, in comparison to 44% of patients with rhino-cerebral involvement. The third (19%) common presentation was renal zygomycosis. Further, it has been assumed that the majority of patients with A. elegans infection demonstrate no underlying immune dysfunction. Only a few patients have been reported with risk factors like diabetes, severe burns, renal transplantation, myelofibrosis, and corticosteroid use (5, 32). However, among our patients, 63% had uncontrolled diabetes as a risk factor. All these patients except one were diagnosed in India from 2004 through 2008. The epidemiology of zygomycosis in India has been observed to be different from that of developed countries (25). A phenomenal increase in the number of cases of zygomycosis has been reported from this country in patients with uncontrolled diabetes (5, 6). The number of cases with uncontrolled diabetes is so overwhelming that other factors are overshadowed (25). It seems that the epidemiology of A. elegans infections in India is also different from that in other countries. A. elegans is reported to produce rhino-cerebral and renal zygomycosis in a considerable number of patients, and uncontrolled diabetes is strongly associated with such patients. In cutaneous A. elegans infection, local wound contamination after an accident or injury represents the common method of spread of the disease (25), but it is not clear how the acquisition of A. elegans occurs in patients with rhino-cerebral or renal zygomycosis. In a review of seven cases of rhino-cerebral zygomycosis due to A. elegans, three patients had predisposing facial or head trauma, but the majority had no predisposing condition. As with other zygomycetes, the infection may be acquired in those patients via inhalation of spores (22). Similarly, in the cases with primary renal zygomycosis due to A. elegans, the lung was the possible route of entry.

AFLP analysis, a high-resolution robust fingerprinting assay,
is a promising tool for strain typing of fungi (19). The technique not only has high sensitivity, reproducibility, and resolution but also has the ability to amplify between 50 and 100 fragments from random locations scattered throughout the genome, with no prior sequence information needed. The technique has been used to type Candida and Aspergillus species (11, 19) but has never been used to type zygomycetes, including Apophysomyces elegans. Fingerprinting using two microsatellite markers was used to type A. elegans strains with limited success (7). In the present study, AFLP typing was used to estimate the extent of genetic diversity among A. elegans isolates and to determine any specific genotypes associated with specific clinical types of the disease. AFLP analysis revealed that most of the isolates had very similar fingerprints with relatively little genomic variation. However, based on the presence or absence of variable bands in the AFLP fingerprints, the majority of clinical isolates were grouped into a single clade. The genetic variability was observed among the isolates regardless of their clinical types or the year of isolation. Interestingly, the banding patterns of the environmental isolates were found to be clearly different from those of the clinical isolates. AFLP analysis has been shown to be useful not only for strain typing but also as a powerful tool to distinguish between closely related fungal species (19). Therefore, our results question the homogeneity of A. elegans species. Recently, Alvarez et al. also suggested heterogeneity in the strains of A. elegans after sequencing the histone 3 gene, the internal transcribed spacer region of ribosomal DNA (rDNA), and domains D1 and D2 of the 28S rRNA gene (3). Therefore, it would be pertinent to analyze larger collections of A. elegans strains to understand their taxonomic position and molecular epidemiology.

Amphotericin B is the most commonly used drug in patients with zygomycosis, and it also shows the best in vitro activity against the majority of zygomycetes. However, variation in susceptibility among strains under each species is observed (1, 2, 4, 10, 21, 23, 30, 34). In the two reported series where limited strains of A. elegans were tested (2, 34), the MIC50 of amphotericin B were 0.03 to 0.125 µg/ml (Table 2). However, our strains were comparatively more resistant, as the MIC50 and MIC90 were 2 and 4 µg/ml, respectively. The methods of in vitro susceptibility testing might have some bearing on the difference, as we used RPMI 1640, in contrast to antibiotic medium 3 (Difco), which was used by the other two studies (2, 33). However, a correlation was observed in our in vitro susceptibility results with our in vivo outcome analysis, as the patient having a strain with a MIC of <1 µg/ml for amphotericin B recovered, whereas 43% of the patients infected with strains having MICs of ≥1 µg/ml succumbed to their illnesses. Still, with our study being retrospective, it may not be ideal for in vitro and in vivo correlations.

Although itraconazole is not preferred as a zygomycete-active compound, the in vitro susceptibility data show that itraconazole is reasonably active against zygomycetes (1, 2, 18, 20, 21, 34). Even some cases of zygomycosis were treated successfully with itraconazole (13, 29). After the in vitro susceptibility testing results of itraconazole against A. elegans were analyzed, it appears that itraconazole may be useful in patients infected with a susceptible strain.

Posaconazole is the first drug among azoles demonstrating good antizygomycete activity, with a MIC50 of <1 µg/ml (1, 2, 10, 34). A similar observation was made for this collection of A. elegans isolates (Table 2). The drug has been recommended as salvage therapy in patients with zygomycosis (13, 36) and has also been used to treat patients with A. elegans infection (14, 28). A patient with complicated rhino-orbital A. elegans zygomycosis was successfully treated with posaconazole after failure of liposomal amphotericin B therapy (14). However, failure after posaconazole therapy was also noted in a patient with a brain abscess due to A. elegans and a basidiomycete species (31). Isavuconazole demonstrated potent in vitro activity against Aspergillus sp. (15, 24) and limited antifungal activity against zygomycetes (15, 24, 30, 37). In comparison to the results with itraconazole, ravuconazole, and voriconazole, isavuconazole showed potent activity against zygomycetes, especially against Rhizopus species (15, 30, 37). The drug was never evaluated against A. elegans strains. In the present study, the MIC50 for isavuconazole was 2 µg/ml. Therefore, the drug may be an alternative treatment for A. elegans infection.

The study confirms the emerging trend of invasive zygomycosis due to A. elegans. The fungus caused not only cutaneous or subcutaneous infection but also rhino-orbital-cerebral and renal zygomycosis. Uncontrolled diabetes is strongly associated with the disease. The AFLP pattern raises serious doubt about the homogeneity of A. elegans species. In vitro susceptibility testing demonstrates similar susceptibility patterns, as in other zygomycetes. Increased MICs of amphotericin B were observed in the majority of strains. Posaconazole is a promising drug to treat such patients, and isavuconazole or itraconazole are possible alternatives in patients having infection due to susceptible strains.

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Cavitary Pulmonary Zygomycosis Caused by Rhizopus homothallicus

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We report the first two proven cases of cavitary pulmonary zygomycosis caused by Rhizopus homothallicus. The diagnosis in each case was based on histology, culture of the causal agent, and the nucleotide sequence of the D1/D2 region of the 28S ribosomal DNA.

CASE REPORT

Case 1 involved a 47-year-old male with a history of type II diabetes mellitus for 13 years who developed diabetic nephropathy leading to end-stage renal disease. He had undergone renal transplantation and was on triple-drug immunosuppression (cyclosporine, azathioprine, and prednisolone). One month posttransplantation, he developed an intermittent fever with chills and pleuritic chest pain. A chest X-ray revealed a cavitary lesion in the right upper lobe with bilateral nodular infiltrates, for which he was treated with a primary drug regimen of antituberculosis therapy (ATT), although multiple sputum examinations were negative for acid-fast bacilli. The patient did not respond to the ATT and was referred to our institute (Postgraduate Institute of Medical Education and Research [PGIMER], Chandigarh, India). At our institute, contrast-enhanced computed tomography (CT) of the thorax revealed a thick, smooth-walled cavitary lesion of the posterior segment of the right upper lobe and multiple nodules in both lungs (Fig. 1). No pleural effusion was noticed. Echocardiography ruled out endocarditis. His blood sugar levels ranged from 153 to 226 mg/dl. While he was at the hospital, he was treated with human insulin therapy. ATT was continued along with vancomycin and tazobactam. Ultrasound-guided fine-needle aspiration of the cavitary lesion of the lung was performed. Direct microscopic examination of calcofluor white-stained smears of the aspirated fluid was done. Part of the fluid was cultured on Sabouraud dextrose agar (SDA) and brain heart infusion agar (HiMedia, Mumbai, India). Direct microscopy of the aspirated fluid did not reveal any fungal elements, nor did the cultures yield any fungal colonies during 4 weeks of incubation. As the patient did not improve until the 20th day of his hospital stay, an open-lung biopsy was performed. It revealed pus in the pleural cavity with a large cavitary lesion in the right upper lobe. No apparent mass was noticed. A wedge-shaped biopsy sample was taken from the site of the lesion. Tissue slides stained by the hematoxylin-and-eosin and periodic acid-Schiff procedures showed dense acute inflammatory infiltrates across the interstitial septa, a dense fibrosis surrounding the alveoli, extensive necrosis with necrotic debris and broad, aseptate, ribbon-like hyphae (Fig. 2). The causal fungus was observed invading the vessel wall. Culture of the biopsy tissue on SDA (HiMedia, Mumbai, India) grew a fast-growing, floccose white colony turning golden brown. Microscopic examination of the colony showed broad, aseptate hyphae with lateral not-well-developed sporangiophores bearing globose sporangia containing a small number of sporangiospores. In addition, a large number of golden brown zygospores with stellate walls and with unequal suspensor cells were observed. The isolate was presumptively identified as Rhizopus homothallicus Heseltine and Ellis (8). Liposomal amphotericin B (Fungisome; Life-care Innovations, India) was given at a dose of 1.5 mg/kg of body weight/day. Follow-up after a cumulative dose of 5.0 g of liposomal amphotericin B therapy showed a striking improvement of the patient both clinically and on radiological investigation. No relapse or recurrence of the infection was noticed during 5 months of follow-up after recovery.

Case 2 involved a 70-year-old male who presented at the King George Medical University Hospital, Lucknow, India, with a history of fever, cough, chest pain, mucopurulent expectoration, and recurrent hemoptysis for 25 days. On general examination, he had signs of glossitis and stomatitis. Examination of his respiratory system revealed bronchial breath sound over the left mammary area, and the rest of the physical examination was unremarkable. Chest radiography revealed the presence of air space consolidation with eccentric cavitation in the midzone of the left lung. CT of the thorax revealed a large, thick-walled cavity in the left upper lobe abutting the chest wall and encroaching upon the arch of the aorta. On routine investigation, uncontrolled blood sugar levels (range, 232 to 360 mg/dl) were noted. Sputum examination did not reveal acid-fast bacilli. The patient was treated with oral antibiotics (625 mg of amoxicillin-clavulanic acid twice daily and 150 mg of clindamycin four times a day for 2 weeks). He did not show clinical or radiological improvement. The patient refused to
undergo bronchoscopy. Transthoracic needle aspiration of the left cavitary lesion revealed ribbon-like, broad, coenocytic hyphae on direct KOH examination, as well as smears subjected to periodic acid-Schiff staining and Gomori’s methenamine silver staining procedure. Fungal culture of the aspirate on SDA (HiMedia, Mumbai, India) grew white, cottony colonies. Direct examination of a teased mount of the colony stained with lactophenol cotton blue showed numerous golden brown, globose, spiny zygospores with suspensor cells. The isolate was tentatively identified as \textit{R. homothallicus}. The patient was treated with injectable insulin for glycemic control and intravenous conventional amphotericin B (50 mg/day) for 15 days. After a cumulative dose of 750 mg of amphotericin B, the patient developed acute renal failure. After the withdrawal of amphotericin B, his renal function improved rapidly. However, he refused subsequent treatment with amphotericin B and left the hospital against medical advice. He returned after 3 months with massive hemoptysis and succumbed to his illness rapidly before any treatment with antifungal agents could be initiated.

Colonies of both isolates on SDA were fast growing, white, floccose, and devoid of pigmentation on the reverse side. Within 10 to 14 days of incubation at 28 to 30°C, colonies turned grayish. Slide culture mounts of the isolate from case 1 stained with lactophenol cotton blue (PGIMER MCCL [Mycology Culture Collection Laboratory] 710076) on SDA incubated at 30°C for 10 days showed broad, hyaline, aseptate, branching hyphae producing very few sporangiophores opposite poorly developed rhizoids. The sporangiophores measured 5 to 27 μm in diameter and 50 to 150 μm in length bearing...
globose to subglobose sporangia measuring 50 to 150 μm in diameter. The sporangiophores were angular-globose, grayish, 3.5 to 5.0 μm in length, and 4.0 to 6.5 μm in width. The striking feature was abundant homothallic, thick-walled zygospores that were reddish brown in color and measured 40 to 100 μm in diameter, including stellate spines. Suspensor cells were uneven in size, the larger ones being globose (Fig. 3).

The isolate from case 2 (MCCL 710099) was studied at the CDC. Slide cultures on potato dextrose agar and malt extract agar after 2 weeks at 25°C did not show any fertile sporangia containing sporangiospores. Sporangiophores were poorly developed opposite poorly developed rhizoids in tufts of two to five at several locations. Due to the lack of any sporulation, agar blocks (2 by 2 cm) with mycelial growth from the culture plates were transferred aseptically to a plate containing 20 ml of sterilized distilled water to which 3 to 5 drops of 15% filter-sterilized yeast extract solution was added. After 5 days of incubation at 37°C, examination of growth over the surface of water showed abundant globose, golden brown, spiny zygospores supported by uneven-size suspensor cells. Both isolates were thermotolerant and grew at 46 to 48°C. Both isolates were identified as *R. homothallicus* Hesseltine and Ellis (8).

The identities of both isolates were further confirmed by nucleotide sequencing of the 28S ribosomal DNA (rDNA) region. Whole-cell DNA was extracted from the isolates by a slightly modified version of the small-scale fungal DNA extraction method described by Lee and Taylor (10). Briefly, pure cultures of the isolates were grown in Sabouraud dextrose broth (HiMedia, Mumbai, India) and incubated at 37°C on a rotary shaker (HT Infors, Germany) at 120 rpm for 3 to 5 days. The mycelial mat (0.4 to 0.5 g) was prepared and crushed to a fine powder with a mortar and pestle in the presence of liquid nitrogen and lysis buffer. The DNA was extracted by the standard phenol-chloroform (25:24) extraction method. DNA precipitation was carried out using 2 volumes of chilled absolute alcohol and 1/5 volume of 10 M-ammonium acetate, followed by washing with 70% alcohol. The DNA pellet was dissolved in 100 μl of TE buffer. DNA preparations were stored at −20°C until use. PCR was performed in a reaction mixture of 10 μl containing 2 mM MgCl₂, 200 μM each deoxynucleoside triphosphate (Bangalore Genie, Bangalore, India), 0.25 μM each primer NL1 (5’-GCATATCAATAAGCGGAGGAAAAG) and primer NL4 (5’-GGTCCGTGTATTCAAGACGG) (Integrated DNA Technologies, Inc., Coralville, IA), 0.25 U of Taq polymerase (Bangalore Genie), and 10 ng of fungal genomic DNA. The amplification reactions were performed in an Eppendorf Mastercytler (Eppendorf, Hamburg, Germany). PCR amplification was performed by 36 cycles of annealing at 52°C, extension at 72°C for 2 min, and denaturation at 94°C for 1 min. PCR products were purified with a gel extraction kit (Qiagen Hilden, Germany), and both strands were sequenced by the BigDye terminator cycle sequencing ready reaction kit, version 3.1 (Applied Biosystems, Foster City, CA) with primers NL1 and NL4. The reaction products were analyzed on Genetic Analyzer 3130 (Applied Biosystems). The basic local alignment search tool (BLAST) was used to compare the sequences obtained with those in the GenBank database and to see the similarity of the two isolates. The sequences of both isolates gave 99% identity with each other and 98% identity with the ex-type strain of *R. homothallicus* (AB 250198, NRRL 2538 = CBS 336.62). The two Indian isolates have been deposited in the CBS Fungal Biodiversity Centre, Utrecht, Netherlands, with the following accession numbers: MCCL 710076 = CBS 125071; MCCL 710099 = CBS 125072.

Antifungal susceptibility testing of both isolates was performed by the microdilution broth technique following the protocol of Clinical and Laboratory Standards Institute document M38A (5). The MICs for both isolates were similar:
amphotericin B, 0.5 μg/ml; flucytosine, >64.0 μg/ml; fluconazole, 64.0 μg/ml; itraconazole, >16.0 μg/ml; voriconazole, 4.0 μg/ml; caspofungin, 16 μg/ml.

Among the different agents of zygomycosis, Rhizopus spp. are the most commonly implicated agents causing human infections, and R. oryzae is the predominant species, being implicated in 90% of the reported cases of invasive zygomycosis (3, 4, 17). The other Rhizopus spp. less commonly reported as causal agents are R. microsporus (16), R. azurescens (6), R. schipperae (2), and R. stolonifer (7). To our knowledge, the present report describes the first two cases of invasive zygomycosis caused by R. homothallicus. Hesseltine and Ellis described R. homothallicus in 1961 based on the ex-type strain (NRRL 2538) isolated from a soil sample from Guatemala in 1956 (8). Subsequently, the species was isolated in India from soil samples from several areas, from dung, and from stored grains of Triticum sp. (http://www.cabri.org/CABRI/srs-doc/index.html). When isolated from soil or other environmental sources, R. homothallicus closely resembles R. microsporus in general morphology, especially asexual sporangiophores, sporangia, sporangiogspores, and maximum growth temperatures (19). However, strains maintained under laboratory conditions often lose their sporulation ability, including the ability to form zygospores. According to Schipper and Stalpers, the ex-type strain of R. homothallicus (NRRL 2538 = CBS 336.62) no longer produces zygospores (19). In 1970, Scholer attempted to produce experimental infection in mice using R. homothallicus but was not successful. The reasons for this failure were considered to be an insufficient number of sporangiogspores in the inoculum and a failure to inject large-size zygospores intravenously (20).

Schipper (18) classified Rhizopus spp. into three groups, namely, the R. stolonifer group, the R. oryzae group, and the R. microsporus group, based on phenotypic characters and maximum growth temperatures (18, 19). Recent studies by Abe et al. (1) based on the molecular phylogeny of Rhizopus spp. have concurred with Schipper’s treatment of Rhizopus sp. groups.

Earlier observations by Scholer (20) and recent observations by Jennessen (9) have stressed that in R. homothallicus, rhizoids, sporangiogspores, sporangia, and sporangiogspores are often poorly developed. We also observed similar findings in our two isolates. Production of abundant zygospores was the main phenotypic character that was helpful in the identification of the isolate from case 1. The isolate from case 2 failed to produce zygospores when grown on routinely used potato dextrose agar and malt extract agar. A low-nutrient medium had to be used to induce zygospore production (13). R. homothallicus can also be confused with another homothallic species, namely, R. sexualis, which also produces abundant zygospores. However, R. homothallicus grows at temperatures as high as 46°C, while R. sexualis does not grow at 37°C.

Given the limitation of phenotypic identification methods, rDNA-based gene sequences have been used extensively for the molecular identification of fungi, including zygomycetes (1, 11, 23). The rDNA comprises a small-subunit gene (18S), a large-subunit gene (28S), and internal transcribed spacer (ITS) regions (ITS1 and ITS2). The ITS region is generally used for the species identification of fungi, as the sequences of closely related taxa can be aligned with confidence. To obtain similar resolution with the 18S and 28S genes, a large portion of the DNA must be sequenced. In the present study, many attempts to sequence the ITS region of the rDNA failed (results not shown). Hence, sequencing of the D1/D2 region of the 28S rDNA was performed. The failure to obtain pure sequence of the ITS region may be attributed to the homothallic nature of the fungal species under study, which led to multiple distinct ITS regions in a single strain. The presence of multiple bands did not allow proper analysis of the sequences. The presence of multiple distinct ITS regions in another homothallic Rhizopus species, R. sexualis, has been described earlier. Therefore, sequencing of the D1/D2 region of the 28S rDNA may be a more useful and easy method for the identification of homothallic R. homothallicus (1, 11, 23).

Pulmonary zygomycosis is considered second in frequency after the rhino-orbital-cerebral type among different categories of zygomycosis and has rarely been reported without any predisposing factor. Coughing, fever, and pleuritic chest pain are the common presenting symptoms of patients with pulmonary zygomycosis (22). Pulmonary zygomycosis may have a wide variety of lesions, including an isolated solitary nodule, lobar involvement, and cavity or disseminated lesions (14, 15, 22). Pulmonary consolidation, cavitation, or an effusion is less frequently seen (15, 22). Both patients in the present report had cavitary lesions. Tedder et al. (22), in a review of 156 cases of pulmonary zygomycosis, observed that only 6.0% had radiographic findings of cavitation of the lung. In our earlier two series of reports on zygomycosis from PGIMER, 25 patients had pulmonary zygomycosis and none had cavitary lesions (3, 4), although a contrasting claim of approximately 40% cavitary lesions among patients with pulmonary zygomycosis has been made (12). However, it is not clear whether the type of lesion depends on the virulence of the causal agent, the host’s immune status, or both. It was suggested that such cavities represent liquefaction of pulmonary infarcts (15). A correlation between the type of fungi of Mucorales isolated from the pulmonary lesion and the type of lesion has never been established. Interestingly, a few species such as R. stolonifer (7) and Cunninghamamella bertholletiae (24) have been isolated from patients with cavitary lesions.

Hemoptysis in patients with zygomycosis may be fatal and may occur due to erosion of the cavitary lesion into the bronchus (23). The patient in case 2 had a similar fate. Sputum or bronchoalveolar lavage analysis, though frequently employed, rarely leads to confirmation of the diagnosis. Procedures such as open-lung biopsy, surgical extirpation, and thoracosternal needle aspiration provide better samples for diagnosis (22). In the present two cases, invasive procedures helped in the definitive diagnosis.

Without prompt therapeutic management, invasive zygomycosis invariably proves fatal. Aggressive surgical treatment, appropriate medical therapy, and control of predisposing factors are of vital importance in the treatment of such cases (3). Amphotericin B is the first-line drug of choice for most of the cases of zygomycosis. In both of the cases presented here, the patients were treated with amphotericin B using either a conventional or a liposomal formulation. The patient in case 1 responded well to the therapy. The second patient succumbed.
to the infection, possibly due to inadequate treatment. Both isolates of \textit{R. homothallicus} had amphotericin B MICs of 0.5 μg/ml. The MIC patterns observed with the two isolates of \textit{R. homothallicus} were consistent with those reported for other \textit{Rhizopus} species (21).

**Nucleotide sequence accession numbers.** Nucleotide sequence data for the 28S rDNA regions of both isolates were submitted to GenBank (accession no. EU128745 for MCCL sequence data for the 28S rDNA regions of both isolates were relevant to this report.

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None of us has an association that might pose a conflict of interest relevant to this report.

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Medical Mycology

Allergic fungal rhinosinusitis caused by Neosartorya hiratsukae from India
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PLEASE SCROLL DOWN FOR ARTICLE
Case report

Allergic fungal rhinosinusitis caused by Neosartorya hiratsukae from India

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We report here a case of allergic fungal rhinosinusitis caused by Neosartorya hiratsukae. This fungus was never previously been isolated from any case with fungal rhinosinusitis. The identification of this agent was confirmed by comparing the nucleotide sequence of the ITS region of ribosomal DNA with that in the GenBank DNA database. Identification of N. hiratsukae on the basis of colony morphology and microscopic feature may be difficult due to similarities with a few Aspergillus species. Scanning electron microscopy or DNA sequence analysis is essential for the accurate identification.

Keywords allergy, fungal rhinosinusitis, Aspergillus, Neosartorya, rDNA sequence, diagnosis

Introduction

Fungal rhinosinusitis (FRS) once considered a rare disorder has been reported with increasing frequency worldwide. Though confusion exists regarding classification of FRS, the commonly accepted system divides FRS, based on histopathological evidence into invasive and non-invasive forms. There are three types of invasive FRS, i.e., acute, chronic, and granulomatous. The two non-invasive FRS disorders are fungal ball and fungus-related eosinophilic FRS that includes allergic fungal rhinosinusitis (AFRS) [1]. There is still a great deal of controversy as to the definition of the latter. Bent and Kuhn [2] proposed the following five diagnostic criteria; type I hypersensitivity, nasal polyposis, characteristic CT scan findings, positive fungal stain or culture, allergic mucin with fungal elements and no tissue invasion. Despite this situation, it appears that AFRS is a distinct entity that requires not only the presence of eosinophilic mucin with hyphae, but also the presence of atopy.

It is known that a diverse array of fungi are associated with AFRS, with the most common being melanized fungi, such as Bipolaris species, Curvularia species, and Alternaria species [1,3]. Interestingly in India, Aspergillus flaveus was isolated in more than 80% cases of AFRS [4,5]. We report here the recovery of Neosartorya hiratsukae from a patient with AFRS, which represents the first such occurrence.

Case report

A 40-year-old male medical practitioner presented to our out-patient department (Postgraduate Institute of Medical Education and Research, Chandigarh, India) complaining of nasal blockage, headache, and protrud-
ing left eyeball. The illness started with headache, which was holocranial, progressively increasing in intensity and associated with thin nasal discharge. He had no significant past history and was apparently healthy before this episode. Examination revealed deviated nasal septum to the right side and nasal discharge, along with mild proptosis of the left eyeball. However, he had no diplopia and visual acuity was intact and tympanic membranes were clear. Examinations of the respiratory system, cardiovascular system and gastrointestinal tract were unremarkable. A computed tomography scan of the sinuses without any contrast revealed soft tissue density in the left sphenoid and posterior ethmoid sinuses without any signs of mucoperiosteal change.

The nasal endoscopic examination revealed nasal polyposis and thick secretions in the nasal cavity. Endoscopic ethmoidosphenoidotomy of the left side was performed. Copious amount of thick mucus in the left sphenoid and posterior ethmoid was found. A pocket of pus was also noticed in the sphenoid sinus and inflammatory polypoidal mucosa in the left sphenoid and ethmoidal gallery. There were no signs of bony erosion or bony sequestrum. The polypoidal mass and the mucus were completely removed. Postoperatively the sinus was irrigated with physiological saline. The excised tissue and the mucus were sent for histopathological and mycological examination. The patient was put on oral steroids, 0.75 mg/kg/day for two weeks and steroidal nasal spray for one month. In the 18-month follow-up no recurrence of disease was observed and the patient did not complain of any relevant symptoms. The endoscopic examination did not show any further pathogenic lesions and on follow up CT scan no abnormality was detected.

Histopathological section of the excised tissue (mucus was inadvertently not included) showed edema in the subepithelium and mixed inflammatory cell infiltrate comprising eosinophils, plasma cells and lymphocytes. No invading fungal hypha could be seen in the tissue. Examination of potassium hydroxide (10%) wet mounts of the surgical specimen along with mucus revealed hyaline septate hyphae. Mycelial growth was detected in all Sabourad dextrose agar cultures which were incubated at 37°C or 25°C. The colonies were initially white, but turned to pale yellow after 10 days of incubation. Lactophenol cotton blue preparation of the fungus revealed hyaline septate hyphae with smooth walled conidiophores and spherical vesicles. Conidiogenous cells were uniserate covering upper two third of vesicle having globose or subglobose slightly rough walled conidia (3–5µm). Based on the morphological features the isolate was initially identified as *Aspergillus* species. To obtain the identity of the species, the isolate was subcultured on Czapek agars (CA) and Malt extract agars (MEA) and incubated at 25°C in dark. On CA the colonies were restricted, velvety, irregularly folded, and white to greenish white, and light brown reverse. On MEA the colonies were velvety, radially folded, white to greenish white, with pale reverse (Fig. 1). Microscopic examination of the lactophenol cotton blue mount of the isolate revealed a large number of cleistothecia and conidial heads. Cleistothecia were non-ostiolate, globose to subglobose, measuring about 100–600 µm in diameter. The peridium was thin and membranous. The ascospores were hyaline, one celled, lenticular with equatorial crests (Fig. 2). The vesicles, conidiophores and conidia were similar to those described above. To identify the isolate, internal transcribed region (ITS) including ITS1, 5.8S and ITS2 of the ribosomal DNA was amplified using universal primers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCTTCCGCTATTGATATGC). Sequencing reactions were performed with Big Dye Terminator Cycle Sequencing Kit, Version 3.1(Applied Biosystems, CA, USA) for both the strands. All the sequencing reactions were purified and analyzed on ABI 3130 Genetic Analyzer (Applied Biosystems). The sequences obtained were compared with those in the GenBank DNA database. The sequence of our isolate gave 100% identity with *Neosartorya hiratsukae*. The nucleotide sequence of ITS1, 5.8S and ITS2 of our isolate have been deposited in the GenBank with accession number EU593904. The pictures of the isolate and the sequence data were also sent to Centraalbureau voor Schimmelcultures, The Netherlands for their expert opinion. The isolate was identified as *Neosartorya*
hiratsukae. Our isolate is deposited at Mycology Culture Collection Laboratory, Postgraduate Institute of Medical Education and Research, Chandigarh, India (MCCL 840003).

Antifungal susceptibility testing of the strain was performed to determine the minimum inhibitory concentration (MIC) through the use of the approved protocol of Clinical Laboratory Standard Institute (CLSI) document M-38A [6]. The results were read after 48 h of incubation: amphotericin B 0.25 μg/ml; flucytosine 8 μg/ml; fluconazole >64 μg/ml; itraconazole >16 μg/ml; voriconazole 0.25 μg/ml; and capsofungin >16 μg/ml.

Discussion

Aspergillus fumigatus, an ubiquitous fungus, is an important human pathogen. The teleomorphs of section Fumigati are placed in the genus Neosartorya (family Trichocomaceae), which includes more than 20 species but only a few of them known to cause human infections [7,8]. The latter species have been implicated in pulmonary and cerebral aspergillosis, osteomyelitis, endocarditis, mycotic keratitis, and wound infection [9–15]. N. fischeri and N. pseudofischeri are the most common agents responsible for these diseases. Neosartorya hiratsukae is a rare fungus and was isolated for the first time in Japan from air and from pasteurized aloe juice [16]. Guarro et al. [5] reported cerebral aspergillosis caused by Neosartorya hiratsukae in a 75-year-old woman from Brazil. The diagnosis was based on the isolation of the fungus from specimens obtained from frontal and occipital lesions. N. hiratsukae has also been isolated from the skin, brain and bronchoalveolar lavage specimens of patients in Seattle, USA [15], skin and oropharyngeal lesions from Spain [17] and from continuous ambulatory peritoneal dialysis fluid in a patient with end stage renal disease in New Zealand [18]. The present report describes the first case of fungal rhinosinusitis caused by N. hiratsukae from India.

Neosartorya species are possibly under reported due to laboratory practice of discarding the non-sporulating or slowly sporulating white mycelia as laboratory contaminants [8,9]. Under reporting may also be attributed to the difficulty in identification of the species of Aspergillus isolated in a routine clinical microbiology laboratory. Traditionally the identification of a member of section Fumigati is done by observation of the colony pattern and the microscopic morphology of the conidiogenous structures, conidia, ascomata and ascospores. Ascospores ornamentation under scanning electron microscopy is essential in differentiating several species of Neosartorya showing morphological similarity on the light microscope [7,8]. In the present case, the isolate was initially identified as Aspergillus hollandicus (Eurotium amstelodami) based on anamorphic and teleomorphic morphology. We did not perform any scanning electron microscopic studies. However, DNA sequencing of the ITS region of rDNA helped us in the identification of N. hiratsukae. Besides ITS region, sequencing of the calmodulin gene, β tubulin gene, actin gene, rodlet A gene are used to differentiate species within the section Fumigati [15].

The present case is categorized as AFRS on the basis of nasal polyposis, characteristic CT scan findings, positive fungal stain and culture, allergic mucin with fungal elements and no tissue invasion. The detection of fungi in allergic mucin is considered important in the diagnosis of AFRS [19]. However, if the mucus is not included along with the tissue, hyphae may be missed. The same thing happened in our histopathological examination where polypoidal tissue was processed without the mucus. The hyphae could be detected in the mucus in the mycology laboratory. This finding confirms the observations reported earlier that mucus should always be included for histopathological or mycological examination in the diagnosis of AFRS [20,21].

At present, the diagnosis of each category of FRS is important in order to establish optimum therapy and to predict the course of treatment. As the present case was categorized as AFRS, the patient was managed with steroid therapy only, without any antifungal agent and the patient responded to a two week post-surgical course of oral steroid therapy and one month use of steroid nasal spray. Though high rate of recurrence is
reported in AFRS group, the patient in the present case did not have any recurrence in an 18 months follow up.

In conclusion this is the first report of FRS caused by Neosartorya hiratsukae. The fungus may be missed, as the identification of this agent is difficult based only on conventional macroscopic and microscopic observations. DNA sequencing or scanning electron microscopy is essential to identify this isolate.

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