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 50s and MICs 90s for 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 Syncephalantrum 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 from 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, 27, 17, 25, 32, 38). A. elegans was believed to cause only cutaneous and mucocutaneous infection in immunocompetent hosts, 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

A. elegans isolates. A total of 18 A. elegans isolates were used 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, 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 M38-A2 (9). Stock solutions (3,200 mg/liter) of AMB, ISA, ITR, POS, and VOR were prepared by 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 antifungal concentration for each antifungal drug was 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 filter-sterilized 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 drug concentration at which abnormal hyphae were observed at 24 h of incubation. 

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'-CTCTAGATACGTGATC-3' and 5'-CGGTAGATTCAAAGTG-3') for HpyCH4 IV, 5'-GACGATGATCTCCAGTAC-3' and 5'-TAGTCAGACTGCGTACC-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 amplified in a volume of 25 μl under the following conditions: 1 μM HpyCH4 IV primer with one selective residue (underlined) (5'-fluorescent-TGATAGATACGTGATCC-3'), 1 μM MseI primer with four selective residues (5'-GATGAGTCTCTCAATAG-3'), 0.2 mM each deoxynucleoside 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 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 M38-A2 (9). Stock solutions (3,200 mg/liter) of AMB, ISA, ITR, POS, and VOR were prepared by 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 filter-sterilized 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.

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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, 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 strains of 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>
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<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;64</td>
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<tr>
<td>NCCPF 102.32</td>
<td>10/F</td>
<td>None</td>
<td>Bilateral kidneys</td>
<td>Nephroprostomy + amphotericin B</td>
<td>Improved</td>
<td>2</td>
<td>&gt;64</td>
</tr>
<tr>
<td>NCCPF 102.33</td>
<td>22/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;64</td>
</tr>
<tr>
<td>NCCPF 102.34</td>
<td>59/M</td>
<td>Diabetes mellitus</td>
<td>Rhino-orbito-cerebral</td>
<td>Fluconazole + extended surgery</td>
<td>Expired</td>
<td>1</td>
<td>&gt;64</td>
</tr>
<tr>
<td>NCCPF 102.35</td>
<td>17/F</td>
<td>None</td>
<td>Bilateral kidneys</td>
<td>Nephroprostomy + amphotericin B</td>
<td>Improved</td>
<td>0.5</td>
<td>64</td>
</tr>
<tr>
<td>NCCPF 102.36</td>
<td>45/M</td>
<td>None</td>
<td>Right kidney</td>
<td>Nephroprostomy + amphotericin B</td>
<td>Recovered</td>
<td>2</td>
<td>&gt;64</td>
</tr>
<tr>
<td>NCCPF 102.37</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;64</td>
</tr>
<tr>
<td>NCCPF 102.38</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>64</td>
</tr>
<tr>
<td>NCCPF 102.39</td>
<td>29/M</td>
<td>None</td>
<td>Cutaneous</td>
<td>Local debridement + amphotericin B</td>
<td>Recovered</td>
<td>2</td>
<td>&gt;64</td>
</tr>
<tr>
<td>NCCPF 102.40</td>
<td>35/F</td>
<td>Diabetes mellitus and intramuscular injection over left gluteal region</td>
<td>Cutaneous</td>
<td>Local debridement, insulin, antibiotics; no antifungal given</td>
<td>Expired</td>
<td>4</td>
<td>&gt;64</td>
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<td>NCCPF 102.41</td>
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<td>Rhino-orbito-cerebral</td>
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<td>Recovered</td>
<td>2</td>
<td>&gt;64</td>
</tr>
<tr>
<td>NCCPF 102.42</td>
<td>40/M</td>
<td>Diabetes mellitus</td>
<td>Rhino-orbito-cerebral</td>
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<td>Recovered</td>
<td>2</td>
<td>&gt;64</td>
</tr>
<tr>
<td>NCCPF 102.43</td>
<td>53/M</td>
<td>Diabetes mellitus</td>
<td>Cutaneous</td>
<td>Local debridement + itraconazole, posaconazole</td>
<td>Recovered</td>
<td>2</td>
<td>&gt;64</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;64</td>
</tr>
<tr>
<td>CBS 476.78</td>
<td>4582</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>4</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>0.5</td>
<td>&gt;64</td>
</tr>
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</table>

NCCPF, National Culture Collection of Pathogenic Fungi, Chandigarh, India; CBS, Centraalbureau voor Schimmelcultures 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 A. 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 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 upon 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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