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

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Received 12 July 2010/Returned for modification 17 September 2010/Accepted 22 September 2010

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 and MECs 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.

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Zygomycosis (mucormycosis) is a serious and often rapidly fatal infection, especially in immunocompromised hosts. Among zygomycetes, the species under the genera Rhizopus, Rhizomucor, Lichtheimia (Abisia), Mucor, Apophysomyces, Saksenaea, Cunninghamella, Cokeromyces, and Syncphalantrum 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, 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-orbital-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.

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† Published ahead of print on 29 September 2010.
**MATERIALS AND METHODS**

*Apophysomyces 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 Schimmelmicrobes (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 each antifungal agent 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 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 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 to a 530-nm wavelength to a transmission that ranged from 68 to 71% (0.6 × 10⁸ to 4 × 10⁹ 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 HpaCH4 IV adapter, 50 pmol of the MseI adapter, 2 U of HpaCH4 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 a 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′-CTG AAG TAC GGT TAC ACC-3′ and 5′-CGG TAC GGA GAT GTC-3′ for HpaCH4 IV, 5′-GAC GAT GTA CTG TA C-3′ and 5′-TAG TCA GAC TCT CAT-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 HpaCH4 IV primer with one selective residue (underlined) (5′-fluorescent-TGA TAC GGT TAC CCA TGC-3′), 1 μM MseI primer with four selective residues (5′-GAT GAG TCTG TCT GAAT TGA-3′), 0.2 mM each deoxynucleoside triphosphate (dNTP), and 1 U of Taq 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, Düsseldorf, Germany) 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 v5.0 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 demography, 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 strains 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 1. Clinical details and *in vitro* antifungal susceptibility patterns of *A. elegans* strains

<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-orbital-cerebral</td>
<td>Amphotericin B + surgery</td>
<td>Not known</td>
<td>2</td>
<td>&gt;16</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Surgery + amphotericin B</td>
<td>Expired</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>Nephrostomy + amphotericin B</td>
<td>Improved</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>Amphotericin B + extended surgery</td>
<td>Improved</td>
<td>4</td>
<td>&gt;16</td>
</tr>
<tr>
<td>NCCPF 102.36</td>
<td>42/M</td>
<td>Diabetes mellitus</td>
<td>Rhino-orbital-cerebral</td>
<td>Fluconazole + extended surgery</td>
<td>Expired</td>
<td>2</td>
<td>&gt;16</td>
</tr>
<tr>
<td>NCCPF 102.33</td>
<td>22/M</td>
<td>Diabetes mellitus</td>
<td>Rhino-orbital-cerebral</td>
<td>Extended surgery</td>
<td>Expired</td>
<td>1</td>
<td>&gt;16</td>
</tr>
<tr>
<td>NCCPF 102.34</td>
<td>59/M</td>
<td>Diabetes mellitus</td>
<td>Rhino-orbital-cerebral</td>
<td>Fluconazole + extended surgery</td>
<td>Improved</td>
<td>0.5</td>
<td>2</td>
</tr>
<tr>
<td>NCCPF 102.37</td>
<td>17/F</td>
<td>None</td>
<td>Bilateral kidneys</td>
<td>Nephrectomy + amphotericin B</td>
<td>Recovered</td>
<td>2</td>
<td>&gt;16</td>
</tr>
<tr>
<td>NCCPF 102.38</td>
<td>45/M</td>
<td>None</td>
<td>Right kidney</td>
<td>Nephrectomy + amphotericin B</td>
<td>Recovered</td>
<td>2</td>
<td>&gt;16</td>
</tr>
<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-orbital-cerebral</td>
<td>Extended surgery + amphotericin B</td>
<td>Expired</td>
<td>2</td>
<td>64.5</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>
<td>Cutaneous</td>
<td>Local debridement + amphotericin B</td>
<td>Recovered</td>
<td>2</td>
<td>&gt;16</td>
</tr>
<tr>
<td>NCCPF 102.43</td>
<td>35/M</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;16</td>
</tr>
<tr>
<td>NCCPF 102.44</td>
<td>37/M</td>
<td>Diabetes mellitus</td>
<td>Rhino-orbital-cerebral</td>
<td>Surgery + amphotericin B</td>
<td>Recovered</td>
<td>2</td>
<td>&gt;16</td>
</tr>
<tr>
<td>NCCPF 102.45</td>
<td>40/M</td>
<td>Diabetes mellitus</td>
<td>Rhino-orbital-cerebral</td>
<td>Amphotericin B</td>
<td>Recovered</td>
<td>2</td>
<td>&gt;16</td>
</tr>
<tr>
<td>NCCPF 102.46</td>
<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>32.25</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></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.25</td>
<td>4</td>
</tr>
<tr>
<td>CBS 477.78</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.5</td>
<td>&gt;64</td>
</tr>
</tbody>
</table>

**Note:** 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 variability was observed among the isolates regardless of their extent of genetic diversity among (7). In the present study, AFLP typing was used to estimate the markers was used to type 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 outcome analysis, as the MIC50 or MIC90 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.

ACKNOWLEDGMENTS
We thank Hamid Badali for his help with part of the susceptibility testing and Corina Bens for technical support. This study was partly supported by an educational grant from Basilea Pharmaceuticals International (Basel, Switzerland). J.F.M. has been a consultant to Astellas, Basilea, Merck, and Schering-Plough and received speaker’s fees from Gilead, Janssen Pharmaceutical, Merck, Pfizer, and Schering-Plough. No other authors have potential conflicts of interest.

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