Identification and Susceptibility of *Aspergillus* Section *Nigri* in China: Prevalence of Species and Paradoxical Growth in Response to Echinocandins

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Molecular identification and *in vitro* antifungal susceptibility tests of 43 *Aspergillus* section *Nigri* isolates from China were performed. *Aspergillus niger* and *Aspergillus tubingensis* were present in almost equal numbers. All of the isolates had low MIC/MECs (minimum effective concentrations) for the 7 common antifungals, and a paradoxical effect was observed for the first time in response to caspofungin and micafungin.

A spargillus section Nigri, which includes 26 species of black *Aspergillus*, is important in medical mycology, food science, and biotechnology (1). More importantly, in the clinical setting, *Aspergillus niger*, which is the most commonly described species of *Aspergillus* section *Nigri*, is the third most common pathogen that causes invasive pulmonary aspergillosis and the most frequent etiological agent of otomycosis (2, 3). However, *Aspergillus* section *Nigri* is one of the most difficult groups to classify; species that belong to this group are typically identified as *A. niger* based on morphological observations in the clinical laboratory (4). In recent years, sequence-based molecular methods have been used successfully for species identification in this group, particularly methods using the calmodulin gene, which can distinguish all species within section *Nigri* (4–6). Molecular studies indicated that several species in addition to *A. niger* were able to cause human infections; different regions had different species of pathogenic black *Aspergillus*, and *A. niger* and *Aspergillus tubingensis* were the most frequently identified pathogens in previous studies (2, 7–14). Additionally, it is worth noting in a clinical context that azoles exhibited different activity against the two species and that isolates from different geographical regions exhibited remarkable differences in susceptibility to azoles and amphotericin B (1–13, 15). In light of this situation, accurate identification and susceptibility testing are necessary in the clinic.

To date, no reports are available concerning the identification and antifungal susceptibility testing of black *Aspergillus* in China. The aim of this study was to reidentify black *Aspergillus* isolates from Chinese clinical patients and environments based on sequence analysis of the calmodulin gene. Furthermore, *in vitro* drug susceptibility was tested to evaluate the same isolates.

A total of 43 isolates that belong to *Aspergillus* section *Nigri* and had been preidentified as *A. niger* based on morphology were collected nationally by the Research Center for Medical Mycology at Peking University between 1997 and 2014. These isolates included 27 from clinical samples and 16 from the environment. For details, see Table S1 in the supplemental material. Isolates were cultured on 2% malt extract agar (MEA) at 28°C for 5 days and were subsequently investigated using molecular analysis and antifungal susceptibility tests.

Genomic DNA was extracted and purified according to the instructions provided with the DNeasy plant minikit (Qiagen, Hilden, Germany), with modifications: the cells were disrupted using glass beads (Sigma-Aldrich, Saint Louis, MO, USA) and the Tissuelyser II system (Qiagen). The fungal isolates were identified via PCR amplification and sequencing of the partial calmodulin gene and the partial beta-tubulin gene when necessary using the CL1 and CL2A primer pair (16) and the Bt2a and Bt2b primer pair (17), respectively; the sequence data were adjusted using SeqMan Pro software (DNASTar, Madison, WI, USA); then they were used to conduct alignment analysis for preliminary species identification in the NCBI genomic database (http://blast.ncbi.nlm.nih.gov/) and CBS database (http://www.cbs.knaw.nl/Collections/BioloMICSSequences.aspx?file=all).

Additional GenBank sequences for *Aspergillus* section *Nigri* calmodulin and beta-tubulin genes were subsequently incorporated for comparison and reference. Phylogenetic trees were prepared from alignments using the MEGA 6.06 program (18–20). The maximum-parsimony tree was obtained using the Tree-Bisection-Regrafting (TBR) algorithm with search level 2, in which the initial trees were obtained via the random addition of sequences (10 replicates). The support for each clade was determined using bootstrap analysis with 1,000 replications. *Aspergillus flavius* CBS 100927 was used as an outgroup in this analysis. All sequences from our isolates were deposited in the NCBI GenBank database.

*In vitro* susceptibility testing was performed in triplicate based on CLSI document M38–A2 (21). The final concentration of...
spores dispensed into the wells was adjusted to approximately $1.5 \times 10^4$ CFU/ml, as determined by quantitative spore counting using a hemocytometer. All antifungal drugs were obtained as standard powders. The final concentrations of amphotericin B (Sigma-Aldrich), itraconazole (Shouguang Pharm, Shandong, China), voriconazole (Shouguang Pharm), posaconazole (Merck, Rahway, NJ, USA), caspofungin (Sigma-Aldrich), and micafungin (Astellas Pharma, Tokyo, Japan) ranged from 0.031 to 16 $\mu$g/ml.
TABLE 1 MIC/MECs for black *Aspergillus* isolates recovered in China

<table>
<thead>
<tr>
<th>Drug</th>
<th>MIC/MEC for:</th>
<th>Geometric mean</th>
<th>Range</th>
<th>50%</th>
<th>90%</th>
<th>Geometric mean</th>
<th>Range</th>
<th>50%</th>
<th>90%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>A. niger</em> (20)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td><em>A. tubingensis</em> (23)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ITR</td>
<td>0.925</td>
<td>0.5–1</td>
<td>1</td>
<td>1</td>
<td></td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>VOR</td>
<td>0.369</td>
<td>0.125–0.5</td>
<td>0.25</td>
<td>0.5</td>
<td></td>
<td>0.62</td>
<td>0.25–2</td>
<td>0.5</td>
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<tr>
<td>POS</td>
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<td>0.125–0.25</td>
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<td>0.25</td>
<td></td>
<td>0.239</td>
<td>0.25–1</td>
<td>0.25</td>
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<tr>
<td>CAS</td>
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<td>0.031–0.5</td>
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<td>0.25</td>
<td></td>
<td>0.071</td>
<td>0.031–0.25</td>
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<td>MCFG</td>
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<td>0.25</td>
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<td>0.032</td>
<td>0.031–0.063</td>
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<td>AMB</td>
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<td>0.5</td>
<td>1</td>
<td></td>
<td>0.63</td>
<td>0.5–1</td>
<td>0.5</td>
<td>1</td>
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<tr>
<td>TRB</td>
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<td>0.25</td>
<td></td>
<td>0.402</td>
<td>0.25–1</td>
<td>0.25</td>
<td>0.5</td>
</tr>
</tbody>
</table>

a: ITC, itraconazole; VOR, voriconazole; POS, posaconazole; CAS, caspofungin; MCFG, micafungin; AMB, amphotericin B; TRB, terbinafine.

b: Numbers in parentheses are numbers of isolates.

and the final concentration of terbinafine (Novartis, Basel, Switzerland) ranged from 0.008 to 4 μg/ml. The isolates *Candida parapsilosis* ATCC 22019, *Candida krusei* ATCC 6258, *Aspergillus flavus* ATCC 204304, and *Trichophyton mentagrophytes* ATCC MYA 4439 were included in each assay run as quality controls.

After 48 h of incubation at 35°C, the MICs were determined visually by comparing the growth in the wells containing the drug to that in the wells containing the drug-free control. Amphotericin B, itraconazole, voriconazole, and posaconazole were found to require the lowest drug concentrations to prevent any discernible growth (100% inhibition), whereas terbinafine required the lowest concentration for ≥80% inhibition. The minimum effective concentration (MEC) was defined as the lowest concentration of drug causing the growth of small, rounded, compact hyphal forms in comparison to the hyphal growth observed in the growth control well for caspofungin and micafungin. The Etest assay was performed at the same time to further confirm the activity of the drugs. No isolate had an MIC that exceeded the epidemiological cutoff values (ECVs) for azoles (itraconazole, 2 μg/ml; voriconazole, 2 μg/ml; posaconazole, 0.5 μg/ml) (22). Additionally, 8 isolates, including both species, exhibited paradoxical responses to itraconazole, such that the isolates grew at concentrations of 8 μg/ml and/or 16 μg/ml. Interestingly, 15 and 18 isolates also exhibited this effect in response to caspofungin and micafungin, respectively. The Etest results verified again that caspofungin had good activity against the isolates, but the colony number increased at concentrations ranging from 2 μg/ml to 32 μg/ml.

The geometric mean (GM) MIC/MECs, MIC<sub>50</sub>/MEC<sub>50</sub> (MECs at which 50% of isolates are inhibited), MIC<sub>90</sub>/MEC<sub>90</sub>, and ranges of MIC/MECs for both *A. niger* and *A. tubingensis* are presented in Table 1. Both species had low MIC<sub>90</sub> for the 7 antifungal drugs. No isolate had an MIC that exceeded the epidemiological cutoff values (ECVs) for azoles (itraconazole, 2 μg/ml; voriconazole, 2 μg/ml; posaconazole, 0.5 μg/ml) (22). Additionally, 8 isolates, including both species, exhibited paradoxical responses to itraconazole, such that the isolates grew at concentrations of 8 μg/ml and/or 16 μg/ml. Interestingly, 15 and 18 isolates also exhibited this effect in response to caspofungin and micafungin, respectively. The Etest results verified again that caspofungin had good activity against the isolates, but the colony number increased at concentrations ranging from 2 μg/ml to 32 μg/ml.

The intraspecific and interspecies differences in the antifungal susceptibilities of *Aspergillus* section *Nigri* from previous studies were inconsistent (7–13), primarily for the azoles and amphotericin B. Our study also observed the above differences among voriconazole, terbinafine, and amphotericin B, but all the MICs were low for both species. Interestingly, besides itraconazole, the paradoxical effect was observed for the first time in echinocandins on *Aspergillus* section *Nigri*.

In conclusion, *A. niger* and *A. tubingensis* are the main black *Aspergillus* species present in clinical and environmental samples in China. Both of these species had low MIC/MECs for common antifungal drugs in vitro, and some of the isolates exhibited a paradoxical effect in response to itraconazole, caspofungin, and micafungin.

**Nucleotide sequence accession numbers.** The calmodulin sequences determined in this study were deposited in GenBank under accession numbers KM593192 to KM593235, which includes the number KM593222 for the isolate CBS 121.55.
ACKNOWLEDGMENTS

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We declare that we have no relevant conflicts of interest.

REFERENCES


