Species assignment and antifungal susceptibilities of black aspergilli recovered from otomycosis cases in Iran

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Summary

Black aspergilli are among the main causative agents of otomycosis worldwide. In this study, the species assignment of black aspergilli isolated from otomycosis cases in Iran was carried out using sequence analysis of part of the calmodulin gene. The results indicate that Aspergillus niger is not the only black Aspergillus species involved in otomycosis cases in Iran: Aspergillus awamori and Aspergillus tubingensis are also able to cause ear infections. Antifungal susceptibility tests were carried out against five antifungal drugs including amphotericin B, fluconazole, itraconazole, ketoconazole and terbinafine. All isolates were highly susceptible to terbinafine, while they exhibited moderate susceptibilities against amphotericin B, fluconazole and ketoconazole. Aspergillus niger and A. awamori were found to have higher minimal inhibitory concentrations for azoles than A. tubingensis, in accordance with previous findings.

Key words: Antifungal agents, Aspergillus niger, Iran, molecular typing, otomycosis.

Introduction

Otomycosis, also known as fungal otitis externa, has been used to describe a fungal infection of the external auditory canal and its associated complications, sometimes involving the middle ear. Many fungal species have been identified as infectious agents in otomycosis, with Aspergillus and Candida species being the most common. In tropical and subtropical regions, Aspergillus is considered the predominant causative organism, with Aspergillus niger as the most frequently described species.1–3 Several studies have been performed on otomycosis cases also in Iran, and most studies identified A. niger as the most frequent pathogen.4–8 Vennewald & Klemm [2] suggested that inflammatory conditions of the ear canal1,9,10 predispose patients to otomycosis most commonly caused by thermotolerant Aspergillus and Candida species.

Black aspergilli identified in otomycosis cases are usually referred to as A. niger.1,8,11 However, black aspergilli (Aspergillus section Nigri)12 are one of the most difficult groups concerning classification and identification. Several species assigned to this section cannot be reliably distinguished based on morphological or physiological methods.13 Recent data indicate that sequence-based methods can be used successfully for species assignment in this group of microorganisms.13–15 Regarding black aspergilli isolated from other forms of human infections, recent molecular studies clarified that A. niger is not the only agent among black aspergilli which is able to cause various forms of aspergilloses. For example, Balajee et al. [16] found that six of the 19 isolates which came from invasive aspergillosis belong to Aspergillus tubingensis, while Alcazar-Fuoli et al. [17] identified three species (A. niger, A. tubingensis and Aspergillus acidus) from clinical sources. It is important to identify the causal agent of otomycosis to gain proper
information about the clinical relevance of a given species, and from a practical point of view, it is also highly recommended that the antifungal treatment chosen should be based on the susceptibility of the identified species.18

To the best of our knowledge, black aspergilli causing otomycosis have not yet been reliably identified to species level using a sequence-based approach. In this study, we examined the species assignment of black Aspergillus isolates from otomycosis cases from Iran using sequence analysis of part of the calmodulin gene. Antifungal susceptibility profiles of the isolates have also been recorded, and compared to those obtained in previous studies.

Materials and methods

Isolates

The strains were isolated from patients with symptoms of external otitis in Ahvaz (Iran) in 2008–2009.8 All patients showed one or more of the aural symptoms (itching, otalgia, and hearing loss). Secretion and pus were collected from the ear lesions by two sterile cotton wool swabs. One swab was used for direct microscopy and the other for culture examination. Direct examination of the samples was carried out by staining the smears with methylene blue technique. Otomycosis was confirmed by the presence of septate branching mycelium, fungal conidia, fruiting bodies, yeast cells and pseudohyphae on direct microscopy. The presence of fungal elements in stained smears was confirmed by fungal culture. Swabs were rolled over the surface of Sabouraud’s dextrose agar with chloramphenicol (SC) plates (Merck, Germany). Cultures were incubated aerobically at ambient temperature (25–27°C) for 1 week. Isolated aspergilli were identified on the basis of colonial morphology and slide cultures.

Genotypic studies

The fungal cultures used for the molecular studies were grown on malt peptone (MP) broth as described previously.13 The cultures were incubated at 25 °C for 7 days. DNA was extracted from the cells using the Masterpure™ yeast DNA purification kit (Epicentre Biotechnologies, Madison, WI, USA) according to the instructions of the manufacturer. Amplifications of the partial calmodulin gene were set up as described previously.19 Sequence analyses were performed with the Big Dye Terminator Cycle Sequencing Ready Reaction Kit for both strands. Sequences were analysed on the ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA).

The DNA sequences were edited with the DNASTAR computer package. Alignments of the sequences were performed using MEGA version 4.20 Alignment gaps were treated as fifth character state, and all characters were unordered and of equal weight. The MP tree was obtained using the Close-Neighbour-Interchange algorithm with search level 3 in which the initial trees were obtained with the random addition of sequences (10 replicates). The tree is drawn to scale, with branch lengths calculated using the average pathway method. To assess the robustness of the topology, 1000 bootstrap replicates were run by Maximum Parsimony.21 Other measures including tree length, consistency index, retention index and composite index were also calculated. An Aspergillus flavus isolate was used as out-group in these experiments.

Antifungal susceptibility tests

The in vitro antifungal activities of the antifungal agents were determined using a broth microdilution method, which was performed in accordance with the Clinical and Laboratory Standards Institute (formerly the National Committee for Clinical Laboratory Standards) guidelines.22 Minimal inhibitory concentration (MIC) values were determined in 96-well flat-bottomed microtitre plates by measuring the OD of the fungal cultures at 620 nm (Jupiter HD; ASYS Hitech GmbH, Eugendorf, Austria). The test medium was RPMI 1640 (Sigma-Aldrich Co., St. Louis, MO, USA) containing L-glutamine, but lacking sodium bicarbonate, buffered to pH 7.0 with 0.165 M MOPS (Sigma-Aldrich Co.). Conidial suspensions of moulds were prepared from 5 days old cultures grown on yeast peptone dextrose agar slants. The suspensions were diluted in RPMI 1640 to give a final inoculum of 5 × 10⁴ conidia ml⁻¹. All antifungal agents (all from Sigma-Aldrich Co.) were dissolved in DMSO. The final concentrations for itraconazole, terbinafine and amphotericin B in the wells were 0.031–16 µg ml⁻¹, while ketoconazole and fluconazole concentrations were 0.062–32 µg ml⁻¹ and 0.125–64 µg ml⁻¹, respectively. All experiments were repeated at least three times. The MIC values have been determined by taking MIC-50 (the lowest drug concentration causing 50% inhibition of visible fungal growth) and MIC-100 (the lowest drug concentration causing 100% inhibition of visible fungal growth) after 48 h incubation at 35 °C.
Results

Species assignment of black aspergilli causing otomycosis

Overall, black Aspergillus strains were isolated from seven clinical specimens (Table 1). During phylogenetic analysis of the calmodulin sequence data, 478 positions were in the final dataset, out of which 196 were parsimony informative. Figure 1 depicts one of the 41 most parsimonious trees (tree length: 577, consistency index: 0.662722, retention index: 0.783818, composite index: 0.551525). Based on the phylogenetic analysis, four isolates were found to belong to A. niger, two isolates to A. tubingensis and one isolate to the A. awamori species.

Antifungal susceptibility tests

Antifungal susceptibilities of the isolates were studied against five drugs: amphotericin B, fluconazole, itraconazole, ketoconazole and terbinafine. All isolates were found to be resistant to fluconazole (>64 μg ml⁻¹; data not shown). The MIC50 and MIC100 values of amphotericin B, itraconazole, ketoconazole and terbinafine of the isolates are listed in Table 2.

Discussion

Aspergillus niger is cited as one of the most frequent causative agents of otomycosis in Iran⁴⁻⁸ and in other parts of the world.²,¹¹ However, A. niger cannot be reliably distinguished from several other members of Aspergillus section Nigri using morphological and physiological criteria alone.¹³ To our knowledge, this report is the first attempt to correctly identify black aspergilli

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Table 1 Species assignment of the black Aspergillus isolates recovered from otomycosis cases in Iran.

<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>Gender</th>
<th>Age</th>
<th>Species assignment¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 = SZMC 2640²</td>
<td>Female</td>
<td>26</td>
<td>Aspergillus niger</td>
</tr>
<tr>
<td>1 = SZMC 2641</td>
<td>Female</td>
<td>32</td>
<td>Aspergillus tubingensis</td>
</tr>
<tr>
<td>2 = SZMC 2642</td>
<td>Male</td>
<td>38</td>
<td>A. niger</td>
</tr>
<tr>
<td>2 = SZMC 2643</td>
<td>Female</td>
<td>29</td>
<td>A. tubingensis</td>
</tr>
<tr>
<td>3 = SZMC 2644</td>
<td>Male</td>
<td>42</td>
<td>A. niger</td>
</tr>
<tr>
<td>4 = SZMC 2645</td>
<td>Male</td>
<td>40</td>
<td>Aspergillus awamori</td>
</tr>
<tr>
<td>4/1 = SZMC 2646</td>
<td>Male</td>
<td>36</td>
<td>A. niger</td>
</tr>
</tbody>
</table>

¹Species assignments were based on calmodulin sequence data according to Samson et al. [13] and Varga et al. [15].
²SZMC: Szeged Microbiological Collection, University of Szeged, Szeged, Hungary.

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Figure 1 Maximum parsimony tree of type strains and Iranian isolates came from otomycosis cases (in bold) of Aspergillus section Nigri based on calmodulin sequence data. Bootstrap values >70% are indicated on the branches.
isolated from otomycosis cases using a sequence-based approach. Sequence analysis of part of the calmodulin gene was carried out, since this region was suggested previously to be useful for species identification among black aspergilli.\textsuperscript{13,14} Sequence data indicated that besides \textit{A. niger}, two other black \textit{Aspergillus} species, \textit{A. tubingensis} and \textit{A. awamori} are also capable of causing otomycosis in Iran. \textit{Aspergillus awamori} has recently been found to represent a phylogenetic species closely related to \textit{A. niger} based on a multilocus sequence approach and AFLP analysis,\textsuperscript{23} while \textit{A. tubingensis} is a widespread species related to \textit{A. niger}.\textsuperscript{13}

Antifungal susceptibility data indicate that there are no significant differences in the susceptibilities of \textit{A. niger} and \textit{A. awamori} to either of the antifungal tested (Table 2). All isolates were resistant to fluconazole; this is in agreement with previous findings.\textsuperscript{24} On the other hand, all isolates were found to be highly susceptible to terbinafine. Previous studies also indicated that black aspergilli are relatively susceptible to this antifungal drug.\textsuperscript{17,25–27} Regarding itraconazole, MIC\textsubscript{100} values were between 1 and 2 μg ml\textsuperscript{-1} (Table 2). Previously, Karaarslan et al.\textsuperscript{[25]} observed that \textit{A. niger} isolates exhibited MIC values of 0.21 μg ml\textsuperscript{-1} for itraconazole. However, other studies found much higher MIC values.\textsuperscript{28–30} Alcazar-Fuoli et al.\textsuperscript{[17]} observed three different antifungal patterns based on the itraconazole MIC values, including isolates with low and high itraconazole MICs, and a third group showing an uncommon, so-called paradoxical effect to this antifungal drug. Paradoxical or Eagle effect is used for the phenomenon when a drug administered at a given concentration has the opposite effect to that which would normally be expected. It was described long time ago for antibacterial drugs,\textsuperscript{31} and also for antifungal substances.\textsuperscript{17,12}

The examined isolates exhibited high MIC values both for amphotericin B and ketoconazole (Table 2). However, \textit{A. tubingensis} isolates exhibited slightly lower MIC values for ketoconazole than those for \textit{A. niger} and \textit{A. awamori} strains. This observation is in agreement with those of Kredics et al.\textsuperscript{[33]} However, regarding the susceptibility values of \textit{A. tubingensis} to the antifungal drugs used in this study, previous reports are highly controversial (Table 3). For example, MIC values ≥8 μg ml\textsuperscript{-1} were observed by Pagiotti et al.\textsuperscript{[34]} and in this study, while much lower MIC values were detected in other studies.\textsuperscript{16,33} More isolates of this species should be examined to clarify the background and significance of these contradictory observations/discrepancies.

In conclusion, \textit{A. niger} is not the only black \textit{Aspergillus} species involved in otomycosis cases in Iran: \textit{A. awamori} and \textit{A. tubingensis} are also able to cause ear infections. Several of the ‘\textit{A. niger}’ isolates identified in previous studies\textsuperscript{4–8} could also represent other black \textit{Aspergillus} species. Previous studies together with our results indicate that \textit{A. niger} and \textit{A. awamori} MICs for azoles are slightly higher than those of \textit{A. tubingensis} and \textit{A. acidus}.\textsuperscript{17} These differences in the antifungal susceptibility profiles of black aspergilli underline the importance of sequence-based species identification among human pathogenic black aspergilli.

| Table 2: Antifungal susceptibilities of the black \textit{Aspergillus} isolates recovered from otomycosis cases in Iran. |

<table>
<thead>
<tr>
<th>Species</th>
<th><em>Itraconazole</em> (μg ml\textsuperscript{-1})</th>
<th><em>Ketoconazole</em> (μg ml\textsuperscript{-1})</th>
<th><em>Terbinafine</em> (μg ml\textsuperscript{-1})</th>
<th><em>Amphotericin B</em> (μg ml\textsuperscript{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC\textsubscript{100}</td>
<td>MIC\textsubscript{50}</td>
<td>MIC\textsubscript{100}</td>
<td>MIC\textsubscript{50}</td>
</tr>
<tr>
<td>\textit{Aspergillus niger}</td>
<td>2</td>
<td>1</td>
<td>32</td>
<td>8</td>
</tr>
<tr>
<td>\textit{Aspergillus awamori}</td>
<td>2</td>
<td>1</td>
<td>16</td>
<td>4</td>
</tr>
<tr>
<td>\textit{Aspergillus tubingensis}</td>
<td>1</td>
<td>0.25–0.5</td>
<td>8</td>
<td>0.5</td>
</tr>
</tbody>
</table>

\textsuperscript{[2]} Determined by the E-test method.

| Table 3: Antifungal susceptibility values of \textit{Aspergillus tubingensis} isolates based on previously published reports. |

<table>
<thead>
<tr>
<th>Drug</th>
<th>Number of isolates examined</th>
<th>MIC ranges (μg ml\textsuperscript{-1})</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphotericin B</td>
<td>2</td>
<td>0.032–0.25\textsuperscript{1}</td>
<td>[33]</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>&gt;8</td>
<td>[34]</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.125–0.25</td>
<td>[16]</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>0.12–0.25</td>
<td>[17]</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>2</td>
<td>1–2\textsuperscript{1}</td>
<td>[33]</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>&gt;16</td>
<td>[34]</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.5–1</td>
<td>[16]</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>0.5–11</td>
<td>[17]</td>
</tr>
<tr>
<td>Terbinafine</td>
<td>6</td>
<td>8–16</td>
<td>[33]</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>0.25–1.17</td>
<td>[17]</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>2</td>
<td>0.5–1\textsuperscript{1}</td>
<td>[33]</td>
</tr>
</tbody>
</table>
Acknowledgments

This study was supported by OTKA grant No. K 84077.

References

31. Eagle H, Musselman AD. The rate of bactericidal action of penicillin in vitro as a function of its concentration, and its

