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Species identification and in vitro antifungal susceptibility testing of *Aspergillus* section *Nigri* strains isolated from otomycosis patients

Z. Kamali Sarwestani [a], S.J. Hashemi [a], S. Rezaie [a], M. Gerami Shoar [a], S. Mahmoudi [a,b], M. Elahi [c], M. Bahardoost [d,e], A. Tajdini [c], S. Abutalebian [f], R. Daie Ghazvini [a,*]

[a] *Department of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Pour Sina st., Keshavarz Blvd., Tehran, Iran*
[b] *Students' Scientific Research Center, Tehran University of Medical Sciences, Tehran, Iran*
[c] *Department of Head and Neck surgery, AmirAlam Hospital, Tehran University of Medical Sciences, Tehran, Iran*
[d] *Colorectal research center, Iran University of Medical Sciences, Tehran, Iran*
[e] *Department of Epidemiology and Biostatistics, School of Medicine, Iran University of Medical Sciences, Tehran, Iran*
[f] *Department of Medical Parasitology and Mycology, School of Medicine, Isfahan University of Medical Science, Isfahan, Iran*

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ABSTRACT

Introduction. – *Aspergillus niger* is the most commonly reported etiology of otomycosis based on morphological characteristics. This fungus is a member of *Aspergillus* section *Nigri*, a set of morphologically indistinguishable species that can harbor various antifungal susceptibility patterns. The aim of this study was to accurately identify and determine the susceptibility pattern of a set of black aspergilli isolated from otomycosis patients.
Methods. – Forty-three black *Aspergillus* isolates from otomycosis patients were identified by using the PCR-sequencing of the β-*tubulin* gene. Furthermore, the susceptibility of isolates to three antifungal drugs, including fluconazole (FLU), clotrimazole (CLT) and nystatin (NS), were tested according to CLSI M38-A2. The data were analyzed using the SPSS software (version 15).
Results. – The majority of isolates were identified as *A. tubingensis* (32/43, 74.42%) followed by *A. niger* (11/43, 25.58%). The lowest minimum inhibitory concentration (MIC) values were observed for NS with geometric means (GM) of 4.65 μg/mL and 4.83 μg/mL against *A. tubingensis* and *A. niger* isolates, respectively. CLT showed wide MIC ranges and a statistically significant inter-species difference was observed between *A. tubingensis* and *A. niger* isolates ($P < 0.05$). FLU was inactive against both species with GMs > 64 μg/mL.
Conclusion. – Species other than *A. niger* can be more frequent as observed in our study. In addition, considering the low and variable activity of tested antifungal drugs, empirical treatment can result in treatment failure. Accurate identification and antifungal susceptibility testing of isolates is, however, recommended.

1. Introduction

Otomycosis is the condition which arises as a result of fungal involvement in the external ear; it is mainly caused by saprophytic molds, yeasts and rarely by dermatophytes. *Aspergillus* and *Candida* are the predominant genera and *Aspergillus niger* is the most frequent species isolated from cases of otomycosis in different studies [1–5].

In the majority of studies on otomycosis, all black aspergilli isolates are considered as *A. niger* [1,4–8]; black aspergilli are, in fact, a complex of species referred to as *Aspergillus* section *Nigri* [7]. This section includes at least 19 distinct species that are considered as common fungal agents of food spoilage with a wide global distribution [7,9]. Members of *Aspergillus* section *Nigri* are able to produce a panel of metabolites ranging from extracellular enzymes and organic acids, which could be used in food industries, to mycotoxins, which are a public health concern [7]. However, this section includes pathogenic species causing otomycosis, pulmonary aspergillosis, aspergilloma and onychomycosis [10,11].

Owing to the phenotypic similarities among species within *Aspergillus* section *Nigri*, their identification and classification,

* Corresponding author.
E-mail address: rdaie@tums.ac.ir (R. Daie Ghazvini).

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based on morphologic criteria, is very difficult [12]. Therefore, different methods have been proposed for the identification of these fungi, out of which sequence analysis of β-tubulin or calmodulin genes seems to be reliable techniques among them with superiority of calmodulin [12–14].

Using molecular techniques for the accurate identification of *Aspergillus* section *Nigri*, species other than *A. niger* were isolated from clinical sources. *A. tubingensis* was the dominant species in a study conducted by Iatta et al. [15]; Szigeti et al. [2], in an investigation on *Aspergillus* section *Nigri* strains isolated from otomycosis cases, found that the majority of strains were *A. awamori*. Furthermore, the clinical significance of other less-frequent species, such as *A. uvarum* and *A. acidus*, has been reported [16,17]. Therefore, regarding the dissimilarities among the susceptibility patterns of various *Aspergillus* species, the precise identification of *Aspergillus* section *Nigri* could be of great importance from a clinical point of view in order to prescribe adequate therapies [17]. Furthermore, the administration of antifungal drugs based on the susceptibility pattern of identified fungi has been recommended [18].

Except for patients with malignant external otitis concurrent with mastoiditis and/or meningitis, other patients with otomycosis should be treated with topical antifungal drugs along with the cleaning of the ear canal [3]. Clotrimazole (CLT) is one of the more commonly prescribed topical azoles in treatment of otomycosis [19]. In addition, fluconazole (FLU) and nystatin (NS) have a wide spectrum of activity among antifungal drugs [3]. In addition to good activity, the lack of ototoxic side effects is another advantage of certain azoles, including FLU [19].

To the best of our knowledge, there are limited studies (with limited sample sizes) on species identification and antifungal susceptibility testing of *Aspergillus* section *Nigri* strains isolated from otomycosis cases. Accordingly, the aim of this study was to accurately identify the species of 43 isolates of *Aspergillus* section *Nigri* by using the PCR-sequencing of the *β-tubulin* gene as well as to assess the susceptibility pattern of isolates to FLU, CLT and NS as broad-spectrum drugs of otomycosis.

Table 1
The demographic data of patients, identification results and GenBank accession numbers of *Aspergillus* section *Nigri* strains isolated from otomycosis patients.

Isolate	Patients data		Molecular identification (*β-tubulin* gene)	GenBank accession number
	Gender	Age		
OT59	Female	38	*A. niger*	KY990181
OT1016	Female	56	*A. niger*	KY990182
OT66152	Female	30	*A. niger*	KY990183
OT1003	Male	45	*A. niger*	KY990188
OT36	Male	57	*A. niger*	KY990192
OT37	Male	49	*A. niger*	KY990196
OT13	Male	32	*A. niger*	KY990194
OTU	Male	65	*A. niger*	KY990205
OT72	Female	43	*A. niger*	KY990217
OT58	Male	30	*A. tubingensis*	KY990180
OT38	Male	17	*A. tubingensis*	KY990184
OT12	Male	38	*A. tubingensis*	KY990185
OT264	Male	38	*A. tubingensis*	KY990186
OT60	Male	47	*A. tubingensis*	KY990187
OT51	Male	50	*A. tubingensis*	KY990189
OT50	Male	70	*A. tubingensis*	KY990190
OT55	Female	63	*A. tubingensis*	KY990191
OT2461	Male	43	*A. tubingensis*	KY990193
OT6661	Female	35	*A. tubingensis*	KY990195
OT57	Female	38	*A. tubingensis*	KY990197
OT1015	Male	43	*A. tubingensis*	KY990198
OT88	Female	23	*A. tubingensis*	KY990199
OT2842	Female	40	*A. tubingensis*	KY990200
OT10027	Female	43	*A. tubingensis*	KY990201
OT56	Female	44	*A. tubingensis*	KY990202
OT10021	Male	20	*A. tubingensis*	KY990203
OT10026	Male	53	*A. tubingensis*	KY990204
OT33	Female	32	*A. tubingensis*	KY990206
OT10028	Female	38	*A. tubingensis*	KY990207
OT66614	Male	23	*A. tubingensis*	KY990208
OT26	Female	53	*A. tubingensis*	KY990209
OT107	Female	62	*A. tubingensis*	KY990210
OT10025	Male	50	*A. tubingensis*	KY990211
OT10023	Male	51	*A. tubingensis*	KY990212
OT24	Female	42	*A. tubingensis*	KY990213
OT3090	Male	59	*A. tubingensis*	KY990214
OT64	Female	24	*A. tubingensis*	KY990215
OT6	Male	54	*A. tubingensis*	KY990216
OT1171	Male	34	*A. tubingensis*	MF166857

2. Materials and methods

2.1. Fungal isolates

Forty-three isolates of *Aspergillus* section *Nigri* were included in this study. Out of these, 39 isolates were previously recovered over 10 months from patients with otomycosis at a referral center in Tehran, Iran, and were identified as *A. niger* while performing routine morphological examinations [20]. Four strains, including *A. niger* (2 strains) and *A. tubingensis* (2 strains), were previously isolated from ear swabs and identified based on the sequencing of the *β-tubulin* gene [21]. The characteristics of the isolates are presented in Table 1.

2.2. Molecular identification

All the isolates were cultured on sabouraud dextrose agar (SDA, Merck, Germany) plates and incubated at 30° C until sufficient growth of colonies took place. Before the pigmentation of the colonies, mycelia were harvested and DNA was extracted using a high pure PCR template preparation kit (Roche, Germany) according to the recommended instructions of the manufacturer. A fragment of the *β-tubulin* gene was amplified using Bt2a (5́-GGT AAC CAA ATC GGT GCT GCT TTC-3′) and Bt2b (5́-ACC CTC AGT GTA GTG ACCCTT GGC-3′) primers in the following thermal conditions: an initial denaturation period of 5 min at 95° C, followed by 35 cycles of 30 seconds at 94° C, 45 seconds at 56° C, and 45 seconds at 72° C; this was followed by a final extension of 5 minutes at 72° C. The PCR products were subjected to single-direction sequencing by using a forward primer (Bioneer, South Korea). The results were visually checked by using Chromas (version 2.5.1) (http://www.technelysium.com.au/wp) and were deposited in the GenBank. The species of each isolate was identified in comparison to the reliable sequences of the GenBank by using the basic local alignment search tool of the National Center for Biotechnology Information (https://www.blast.ncbi.nlm.nih.gov/Blast.cgi). The phylogenetic dendrogram was constructed using the maximum likelihood method based on the Tamura-Nei model [22] in the Molecular Evolutionary Genetics Analysis software (version 6) [23]. The *β-tubulin* gene sequence of certain related species, including *A. ellipticus* (AY585530.1), *A. heteromorphus* (AY585529.1), *A. acidus* (KC433701.1), *A. foetidus* (FJ828925.1) and *A. uvarum* (HE984421.1), were considered as well.

2.3. Antifungal susceptibility

The in vitro activity of antifungal drugs, including NS (Merck, Germany), CLT (Behvazan Pharmaceutical Co., Iran), and FLU (Merck, Germany), against the isolates of *Aspergillus* section *Nigri* were determined according to the standard protocol of the Clinical and Laboratory Standards Institute (formerly NCCLS) for filamentous

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