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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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fungi (CLSI M38-A2) [24]. Drug stock solutions were prepared in RPMI-1640 medium (Sigma-Aldrich, Germany) buffered at pH value of 7.0 by 0.165 M morpholinepropanesulfonic acid (Sigma-Aldrich, Germany). Serial dilution was performed according to CLSI M38-A2 and the final 2 × drug suspensions were dispensed in 96-well microplates and stored at−70° C for future use. The final drug concentration range for NS and CLT was 0.0312–16 μg/mL and for FLU was 0.0312–64 μg/mL.

In order to prepare the conidia suspension, all the isolates were inoculated on SDA plates and incubated at 30° C. After sufficient sporulation, the conidia suspensions were prepared according to CLSI M38-A2 [24]. The density of the suspensions was adjusted to an optical density ranging from 0.09 to 0.13 at 530 nm by using a spectrophotometer device (Ultrospec 2000, Pharmacia Biotech) and the viability of the conidia was confirmed by culture on SDA plates. The 96-well test plates were incubated at 35° C and the minimum inhibitory concentrations (MICs) were visually determined by using a reading mirror. *Candida parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were used as quality control strains. All the experiments were performed in duplicate.

2.4. Statistical analysis

The susceptibility of *A. tubingensis* and *A. niger* isolates to each of the drugs was compared using the Mann–Whitney *U* test in SPSS software (version 15) and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Species identification

All the sequences received GenBank accession numbers (Table 1). Based on the analysis of the *β-tubulin* gene sequences, the majority of the isolates were identified as *A. tubingensis* (32/43, 74.42%) followed by *A. niger* (11/43, 25.58%). Therefore, the prevalence of *A. tubingensis* in this study was almost three times greater than *A. niger*. The evolutionary dendrogram of all the isolates is shown in Fig. 1.

3.2. Antifungal susceptibility

According to the results of the antifungal susceptibility tests, the widest MIC ranges were observed for CLT among both *A. tubingensis* (MIC range: 4– > 16 μg/mL) and *A. niger* (MIC range: 2–16 μg/mL) isolates. FLU had no activity against *A. niger* (GM > 64 μg/mL) and *A. tubingensis* (GM > 64 μg/mL) isolates. CLT exhibited lower MICs against *A. niger* (GM: 5.15 μg/mL) in comparison to *A. tubingensis* (GM: 9.72 μg/mL). According to the data analysis, this difference was statistically significant ($P < 0.05$). NS was almost similarly active against the *A. tubingensis* (GM: 4.65 μg/mL) and the *A. niger* (GM: 4.83 μg/mL) isolates. In general, the lowest MIC values in this study were observed for NS. The detailed results of the antifungal susceptibility-testing of the isolates are presented in Table 2.

4. Discussion

A. niger is a known cause of otomycosis and it has been reported as the most common etiology of this condition in different studies [1,4,25]. Gharaghani et al. [5], in their review on otomycosis in Iran, reported that among the 1527 Iranian cases with fungal otitis, 781 cases (51.15%) were due to *A. niger*. This report indicates the considerable role that these fungi play in the occurrence of otomycosis.

The application of molecular approaches in the identification of *Aspergillus* section *Nigri* clarified that all the black aspergilli isolates were not identical to *A. niger*. Therefore, black aspergilli are now considered as a complex known as *Aspergillus* section *Nigri* [9,12]. This section includes species which are highly similar in terms of morphology. Thus, their identification could not be performed solely based on their morphological characteristics [12].

To our knowledge, limited studies have been conducted on the sequence-based identification of *Aspergillus* section *Nigri* isolates from otomycosis patients thus far [2,26]. Therefore, we identified 43 isolates (four isolates had previously been identified [21]) using the sequence analysis of the *β-tubulin* gene.

In this study, *A. tubingensis* was the predominant cause of otomycosis (32/43, 74/42%) followed by *A. niger* (11/43, 25.58%). This finding is not in accordance with the results of Szigeti et al. [26]; they reported *A. awamori* as the predominant species (11 out of 14) in Hungary. Similarly, Howard et al. [27], in their study on 50 isolates including 20 isolates from ear swabs, reported *A. awamori* as the most frequent species. In addition, Szigeti et al. [2], in another study on seven black *Aspergillus* isolates from patients in the south-west of Iran (Ahvaz), reported *A. niger* (4/7, 57.14%) as the dominant species, followed by *A. tubingensis* (2/7, 28.57%), and *A. awamori* (1/7, 14.29%), which are not in agreement with our findings. These variations could be a result of the different geographical origins of these studies.

Molecular studies on *Aspergillus* section *Nigri* isolates from clinical sources other than otomycosis as well as environmental isolates have been conducted. Zarei et al. [21], in accordance to our results reported *A. tubingensis* as the most common species from clinical and environmental sources in Iran. Similarly, Iatta et al. [15], Alcazar-Fuoli et al. [17] and Li et al. [28] reported *A. tubingensis* as the dominant species in their studies. With regard to the dominance of species other than *A. niger* in the majority of the aforementioned studies, the results that are in agreement with our results, strongly highlight the need for the accurate identification of *Aspergillus* section *Nigri* isolates in order to prevent the misidentification of isolates. Correct identification could be performed using the sequence analysis of proper molecular targets. Furthermore, other methods, such as matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS), have been used with promising results [29]. The main limitations of MOLDI-TOF MS for this purpose include the lack of a comprehensive database, especially for rare species [14,30] and the interference of pigments [31]. Therefore, the development of simple and reliable procedures or the optimization of available methods needs further studies.

Different species within the section *Nigri* could harbor different drug susceptibility patterns [32]. Thus, antifungal susceptibility-testing seems to be beneficial for accurate therapy. However, the CLSI protocol of antifungal susceptibility-testing does not have interpretation breakpoints for certain commonly used antifungal drugs in otomycosis and this method is not available in every medical center. Accordingly, the optimization of this method or development of simplified alternative approaches for susceptibility-testing of common antifungal drugs in otorhinolaryngology clinics as well as the establishment of clinical relevance of in vitro susceptibility-testing results needs further studies.

In the present study, all the *A. tubingensis* and *A. niger* isolates were resistant to FLU (GMs: > 64 μg/mL) (Table 2). This finding is in accordance with the results of Szigeti et al. [2] and Yenisehirli et al. [33]; they reported a lack of in vitro activity for FLU against *Aspergillus* section *Nigri*. This resistance should be noted in clinical practice to prevent treatment failure and recurrence of the disease. However, Navaneethan and YaadhavaKrishnan [34], in their study on the therapeutic effects of three topical drugs, including CLT, FLU,

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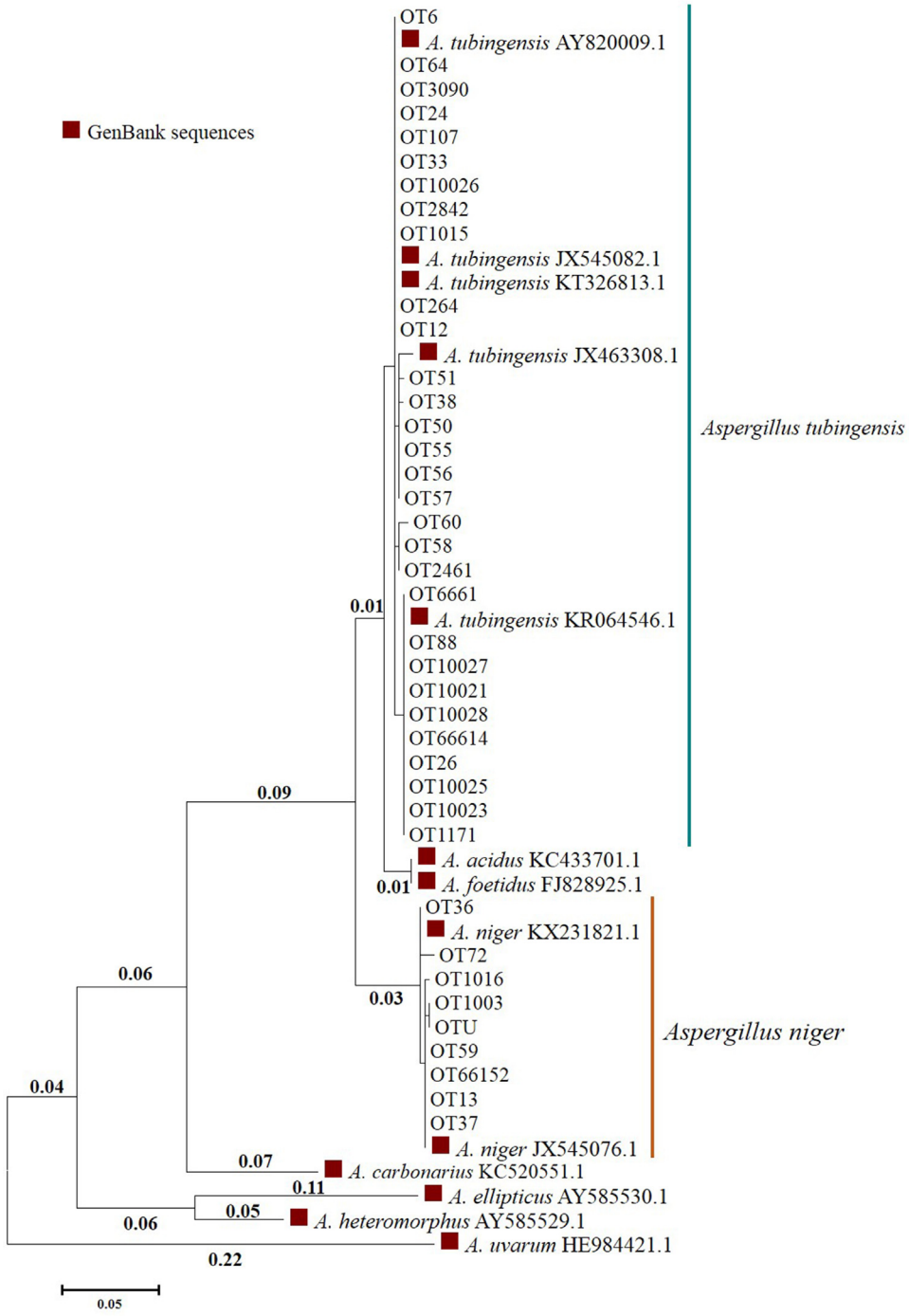

Fig. 1. The phylogenetic dendrogram of 39 strains of *Aspergillus* section *Nigri* isolated from otomycosis patients and GenBank sequences of some related species was constructed using the maximum likelihood method based on the Tamura-Nei model in MEGA 6.

and miconazole (MCN), found that CLT resulted in better clinical outcomes in the first week follow-up, followed by MCN and FLU. In the second week, however, surprisingly the best activity was recorded for FLU. These controversial results need more in vitro and in vivo investigations to provide a general view. Furthermore, although it has been shown that FLU is inactive against the majority of molds, this point is usually overlooked in clinical practice by empirical therapy without the aid of a laboratory. Therefore, in this study, we tried to provide laboratory evidence of FLU inactivity directly against otomycosis-isolated *Aspergillus* section *Nigri*, which we hope will be considered by otorhinolaryngologists.

CLT is a common topical antifungal drug used for the treatment of otomycosis, which was assessed in our study. *A. tubingensis* and *A. niger* isolates showed an almost wide range of MICs (4–> 16 μg/mL and 2–16 μg/mL, respectively) in agreement with the results of Nemati et al. [35]. However, in general, our MICs were higher than those reported by Nemati et al. [35]. This is indicative of intra-species variations of susceptibility to CLT, which highlights the necessity of antifungal susceptibility-testing.

The MIC values of CLT against *A. tubingensis* were significantly higher than the values of *A. niger* ($P < 0.05$). Similarly, higher MICs of an azole antifungal agent (itraconazole) against *A. tubingensis* isolates in comparison to *A. niger* and *A. awamori* isolates was

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Table 2
The minimum inhibitory concentration (MIC) values of three antifungal drugs against strains of *Aspergillus* section *Nigri* isolated from otomycosis patients.

Species ($n=45$)	Antifungal drugs	MIC values (μg/ml)								MIC Range	MIC50[a]	MIC90[b]	GM[c]
		2	4	8	16	>16	32	64	>64				
A. tubingensis (32)	Fluconazole							2	30	64–>64	>64	>64	>64
	Clotrimazole		5	14	12	1				4–>16	8	16	9.72
	Nystatin	1	23	8						2–8	4	8	4.65
A. niger (11)	Fluconazole								11	>64	>64	>64	>64
	Clotrimazole	1	6	3	1					2–16	4	8	5.15
	Nystatin		8	3						4–8	4	8	4.83

[a] The lowest concentration which inhibits 50% of isolates.
[b] The lowest concentration which inhibits 90% of isolates.
[c] Geometric mean.

reported by Iatta et al. [15]. However, in others studies a significant difference between the MICs of various species was not observed [26,36]. In general, scarce data on the in vitro activity of CLT against different species within the *Aspergillus* section *Nigri* are available. Therefore, more studies are required to prove inter-species variations in the susceptibility pattern to CLT.

NS is a topical drug with continuous use throughout time [19]. In our study, in comparison to FLU and CLT, the lowest MICs were observed for NS and there was no statistically significant difference between species ($P > 0.05$). However, due to a lack of the interpretative breakpoint for this drug in CLSI documents [24,37], the interpretation of the results and classification of the isolates as susceptible or resistant needs more investigation. Furthermore, as the data of treatment and the clinical outcomes are not available in this study, the relationship between the results of in vitro susceptibility-testing and clinical outcomes remains unclear.

In conclusion, our study indicated that *A. tubingensis* was about three times more prevalent in comparison to *A. niger* in otomycosis patients. However, these data are from a single center and cannot be extended to other parts of Iran. Therefore, accurate species identification is necessary to prevent misidentification of *Aspergillus* section *Nigri* isolates. FLU was inactive against both species in our study and this indicates the high possibility of treatment failure in cases of empirical therapy. Wide MICs and significant inter-species difference of CLT highlighted the need for antifungal susceptibility-testing before drug administration. Furthermore, although the lowest MICs were observed for NS, determination of the clinical relevance and definition of interpretative breakpoints needs further study.

Disclosure of interest

The authors declare that they have no competing interest.

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