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Molecular epidemiology of otomycosis in Isfahan revealed a large diversity in causative agents

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Abstract

Purpose. To elucidate the clinical and microbial epidemiology of otomycosis in Isfahan, Iran.

Methodology. From January 2016 to January 2017 all patients clinically suspected of otomycosis at Al-Zahra Hospital, Isfahan, Iran were recruited. Specimens were taken using sterile swabs by an otorhinolaryngologist and subjected to culture and microscopy using potassium hydroxide and Giemsa stain. Isolated fungi were identified based on morphological and molecular characteristics.

Results. Otomycosis was confirmed in 97/120 patients (80.8 %). Females (72.2 %) and patients aged 30–39 years (33 %) were more commonly affected than others. Manipulation of ear canal (62.9 %) was the most common predisposing factor. Pruritus was observed in 84.54 % of the patients followed by hearing impairment (81.4 %), and most episodes were detected over the summer (50.5 %). Culture was positive for 81 (83.5 %) of confirmed cases and molds were the most prevalent causative agents (*n*=51, 63 %) followed by yeasts (*n*=19, 23.4 %) and yeast/mold mixes (*n*=11, 13.6 %). For the 16 remaining patients, no growth was seen in culture despite a positive result on direct examination. In total, 92 isolates (63 molds and 29 yeasts) were recovered in culture. Application of molecular methods showed 18 fungal species and the vast majority of them belonged to *Aspergillus* (*n*=53, 57.6 %) and *Candida* genus. Among the species involved, *Candida parapsilosis* (*n*=22, 22.7 %) and *Aspergillus tubingensis* (*n*=15, 15.5 %) were the most encountered species.

Conclusion. Outcomes from this study showed a different picture of prevalence, where *C. parapsilosis* and *A. tubingensis* but not *Aspergillus niger* were the most species encountered from patients suffering from otomycosis.

INTRODUCTION

Otomycosis, a superficial infection of the external ear caused by fungal pathogens, is a common condition in tropical and subtropical regions [1, 2]. It is usually accompanied by symptoms such as itching, otorrhea, otalgia, hearing loss and a sensation of ear fullness [2, 3]. In the absence of functional immune system, causative agents might penetrate into the inner ear and cause severe complications [4].

Otomycosis has been reported to be due mainly to infection by members of *Aspergillus* and *Candida* with a predominance of *Aspergillus niger* [4–8]. Following the application of DNA-based methods, it has become clear that fungi previously believed to be uncommon could be rather frequent etiological agents. This is exemplified by studies on black Aspergilli, which identified *Aspergillus awamori* [9] and *Aspergillus tubingensis* [10, 11] as species more commonly involved in otomycosis than *A. niger*. Thus, the use of molecular methods might change our knowledge regarding the microbial epidemiology of otomycosis. Moreover, the multidrug-resistant *Candida* species, *C. auris*, which is one of the most threatening fungal species [12] was isolated from an external ear discharge and owing

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Abbreviations: BLAST, basic local alignment search tool; ITS, internal transcribed spacer; NCBI, national center for biotechnology information; PCR, polymerase chain reaction; rDNA, ribosomal DNA; RFLP, restriction fragment length polymorphism; SDA, Sabouraud dextrose agar.

to application of molecular techniques it was correctly classified and recognized [13].

Despite the important role of molecular methods for precise identification of fungal isolates, morphological characteristics are more frequently used in studies on otomycosis [1, 3, 7, 8, 14–20].

The aim of this study was to elucidate the clinical symptoms of otomycosis in Isfahan, Iran, and to identity the causative agents of otomycosis using a molecular approach.

METHODS

Patients

This descriptive cross-sectional study was initiated in January 2016 and continued for 1 year. All patients clinically suspected of otomycosis visiting the otorhinolaryngology clinic of the Al-Zahra Hospital in Isfahan, Iran, were recruited. Demographic data and information on clinical manifestations were recorded using a questionnaire. Informed consent was provided by all the participants or their companions prior to enrolment in the study. Ethical approval of the study was obtained from the ethics committee of Isfahan University of Medical Sciences, Isfahan, Iran.

Specimen processing

Th otorhinolaryngologist took the clinical samples using sterile swabs. Direct microscopic examination was done for all the specimens using a 10 % potassium hydroxide (KOH) preparation and Giemsa staining. For each sample, culture was performed on two Sabouraud dextrose agar (SDA) plates (Biolife, Italy) supplemented with 50 mg l^{-1} chloramphenicol. Specimens were inoculated at three points on a SDA plate and streaked on the agar surface of another SDA plate for isolation of molds and yeasts, respectively.

Culture-based identification

Chromogenic Candida agar medium (Biolife, Italy) was used for preliminary identification of yeast isolates based on colony colour characteristics according to the instructions of the manufacturer. Colony-forming structures of mold isolates obtained by slide culture on SDA followed by lactophenol aniline blue staining were examined microscopically for primary identification at the genus/species level.

Molecular identification

DNA of yeast isolates were extracted using the boiling method [21]. Briefly, for each isolate, three–four colonies of overnight culture was transferred to a 1.5 ml tube containing 50 µl of sterile distilled water and placed in boiling water for 20 min, centrifuged for 5 min at 5000 r.p.m., and the supernatant was transferred to a new microtube and used as DNA template. For mold isolates, DNA was extracted using the phenol-chloroform method [22] following physical destruction of the cell wall by glass-bead manipulation.

Species identification of *Candida* isolates was confirmed by the internal transcribed spacer (ITS) PCR-RFLP method using *Msp*I restriction enzyme [23]. Isolates of the *Candida parapsilosis* complex and those yeasts not identified by PCR-RFLP were further identified by ITS rDNA sequencing using ITS1 and ITS4 primers according to the PCR conditions described previously [24].

For the isolates of *Aspergillus* spp. and *Penicillium* spp., a fragment of the β-tubulin gene was amplified and sequenced using bt2a and bt2b primers as described previously [10, 25]. For other mold isolates, the ITS region was targeted for sequence analysis.

After primary evaluation of DNA sequence quality using Chromas software version 2.6.6 (https://technelysium.com.au/wp/chromas/), all sequences were subjected to nucleotide BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and species identification was based on the degree of similarity to reference sequences in the National Centre for Biotechnology Information Database.

Statistical analysis

Descriptive data are shown as frequencies and percentages, and associations were evaluated using the chi-square test in SPSS version 22. A *P*-value of less than 0.05 was considered statistically significant.

RESULTS

Based on the results of direct microscopic examination, otomycosis was confirmed in 97 (80.8 %) out of 120 patients clinically suspected of the infection. Females (*n*=70, 72.2 %) were more frequently infected than males, and patients 30–39 years of age (*n*=32, 33 %) were more frequently infected than the other age categories (Table 1). Most patients with confirmed otomycosis were between 30 and 39 years old (*n*=32, 33 %). However, considering the total number of participants in each group, patients aged 40–49 years with 21 confirmed cases among 22 participants (95.5 %) were significantly more infected than other age groups (*P*=0.03). Thirty-six patients (37.1 %) had right ear involvement, and the remaining patients had either left (33 %) or bilateral (29.9 %) otomycosis. Manipulation of the ear canal (*n*=61, 62.9 %) was the most common predisposing factor followed by swimming (*n*=26, 26.8 %). Pruritus was the most frequent symptom reported, recorded for 82 patients (84.5 %). Hearing impairment was found in 79 (81.4 %) patients, of whom 44 underwent audiological examination; 20 (45.5 %) of these had lost >50 % of their hearing ability. Most otomycosis episodes (*n*=49, 50.5 %) were observed over the summer, followed by spring (22.7 %), winter (15.5 %) and autumn (11.3 %). The demographic data, predisposing factors/underlying diseases, symptoms, seasonal distribution and the affected ear(s) of the patients are summarized in Table 1.

No statistically significant difference was seen between occurrence of otomycosis and gender (*P*=0.86), season (*P*=0.11) and the side of the affected ear (*P*=0.52). Furthermore, no

Table 1. Distribution of 97 patients with otomycosis based on different characteristics

Characteristics	No. of patients (%)
Gender	
Male	27 (27.8)
Female	70 (72.2)
Age groups	
<20	3 (3.1)
20–29	17 (17.5)
30–39	32 (33)
40–49	21 (21.7)
50–59	8 (8.3)
60–69	10 (10.3)
≥70	6 (6.2)
Affected ear	
Right	36 (37.1)
Left	32 (33)
Right and left	29 (29.9)
Predisposing factors/underlying diseases	
Manipulation of ear canal	61 (62.9)
Swimming	26 (26.8)
Ototopical steroids/antibiotics	16 (16.5)
Diabetes	11 (11.3)
Clinical manifestations	
Pruritus	82 (84.5)
Hearing impairment	79 (81.4)
Otalgia	66 (68)
Inflammation	62 (63.9)
Suppuration	60 (61.9)
Tinnitus	51 (52.6)
Tympanic membrane perforation	19 (19.6)
Seasonal distribution	
Spring	22 (22.7)
Summer	49 (50.5)
Autumn	11 (11.3)
Winter	15 (15.5)

significant association was observed between having a history of swimming (P=0.44) or diabetes (P=0.71) with otomycosis. In contrast, manipulation of the ear canal (P=0.03) and using ototopical antibiotics/steroids (P=0.005) were significantly associated with the occurrence of otomycosis.

Direct microscopic examination was positive in 97 patients (80.8 %) by observing various yeast and filamentous elements (Fig. 1a–h). With regard to culture results, 51/97 cases were diagnosed as pure mold infections, 19 cases as pure yeast infections, and 11 cases as mixed mold and yeast infections. For the remaining 16 patients, no growth was observed in culture, despite the fact that 12 cases of *Malassezia* species and 4 cases of non-*Malassezia* species had been observed by direct examination. There was no significant clinical difference between mixed otomycosis infections (i.e. both yeasts and filamentous fungi) in terms of symptoms: otalgia (P=0.80), hearing impairment (P=0.29), pruritus (P=0.35), tinnitus (P=0.20), inflammation (P=0.12) and suppuration (P=0.54), as well as the clinical response (P=0.86).

All 92 isolates recovered from cultures, were identified using molecular tools (Table 2). Although *Aspergillus* (n=53, 57.6 %) was the genus most commonly involved in the disease, at species level, *C. parapsilosis* was the most frequent species (n=22, 22.7%), followed by *A. tubingensis* (n=15, 15.5 %).

Patients were treated using one or a combination of medications including clotrimazole, tolnaftate, Oticept (1 g hydrocortisone and 2 g glacial acetic acid per 100 ml), Myxacort (polymyxin B sulfate: 10 000 U ml^{-1}, neomycin sulfate: 5 mg ml^{-1}, hydrocortisone: 10 mg ml^{-1}) and fluconazole based on the results of clinical examination. Ear wax was removed for all the confirmed patients. In the 1-year period follow up, treatment resulted in complete recovery in 72 (74.2 %) patients, while in 25 patients (25.8 %), at least one episode of relapse was observed. Relapse occurred in 30 % (n=15) of pure mold infections, in 21.4 % (n=6) of pure yeast infections, and in 28.6 % (n=4) of mixed mold and yeast infections. Relapse courses were due to a divergent set of yeast and mold species among which *C. parapsilosis* was the most common species isolated in 24 % of cases. Surprisingly, no case of relapse due to *A. tubingensis* was observed.

DISCUSSION

Otomycosis is a common infection with various symptoms such as pruritus, otalgia and hearing loss, which may take a severe course, resulting in, e.g. fungal necrotizing otitis externa. Although the infection is usually easy to treat, it may be challenging in invasive or recurrent cases [5, 26].

Using direct microscopic examination and culture, 80.8 % of clinically suspected patients in this study were confirmed to have otomycosis, which is in line with findings of other studies confirming otomycosis in 81.3 % [19] and 83.6 % [15] of clinically suspected cases. In contrast, there are reports with higher and lower prevalences, ranging from 11.4 to 92 % [3, 8, 16, 17]. The discrepancy between the frequencies of various studies might highlight the variations in geographical origin of the studies and inclusion criteria of the patients.

In the present study, otomycosis was more commonly diagnosed in female patients (females, 72.2 % vs. males, 27.8 %), which is in agreement with other reports [3, 4, 8]. This might be due to better personal care of females and their visit of

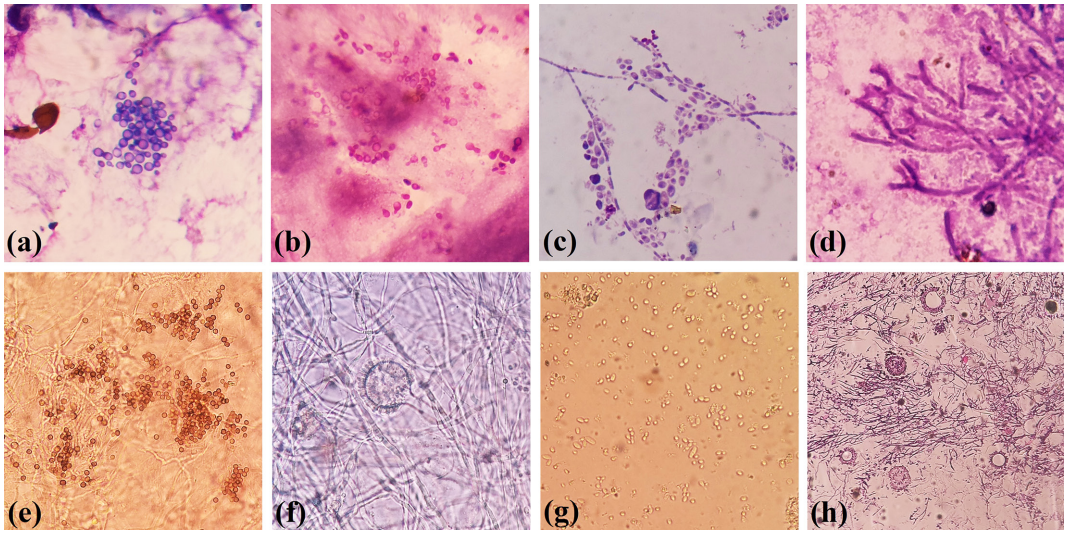

Fig. 1. Microscopy of *Malassezia* cells (a and b), *Candida* pseudohyphae (c) and *Aspergillus* filaments (d) in Giemsa-stained smears; conidia and vesicles of *Aspergillus* (e and f, respectively), and yeast cells of *Candida* (g) in a KOH wet mount followed by a histopathology section representing vesicles of *Aspergillus* species (h). (x400).

otorhinolaryngology clinics. In addition, head scarfs and 'hijab', which are commonly worn by women in Iran and result in decreased air circulation and accumulated humidity in ear canal, might be another attributable factor to the higher rate of female otomycosis [7]. Moreover, in our study, patients aged between 30 and 39 years constituted the most commonly affected group (33 %); other studies reported the age groups of 31–40 years [2], 21–40 years [14] and 35–44 years [18] as the most common age groups with otomycosis. However, in a study by da Silva Pontes *et al.* [27], 60 % of cases were found in patients between 2–20 years. In most studies, the age group with the highest frequency is reported as the most commonly affected group, regardless of the total number of patients in each age group. In the present study, of the 22 patients between 40 and 49, there were 21 cases of otomycosis (95.5 %), and hence, this age group reflected the group with the highest prevalence.

Ear canal manipulation and pruritus were the most common predisposing factor (62.9 %) and symptom (84.5 %), respectively. This finding adds support to other studies in which pruritus was observed in the majority of patients [1, 2, 14, 27, 28]. Hearing impairment was recorded for 81.4 % of patients. It can be a result of accumulation of fungal debris in ear canal or traumas caused by manipulation, which in turn lead to tympanic membrane perforation and defect in hearing ability.

Regarding seasonal distribution, more than half of the patients (50.5 %) were diagnosed over the summer and in a couple of other studies [1, 14], the highest number of patients were also diagnosed in this season. It appears that the summer season provides suitable conditions for the development of otomycosis by the dry dusty winds.

Species of *Aspergillus*, particularly *A. niger*, have been considered the most common filamentous cause of otomycosis in various studies that were based on morphological methods [7, 14, 15, 28]. However, several recent molecular studies demonstrated black *Aspergillus* species other than *A. niger* as the most common causative species [9, 10], which is in contrast with the currently accepted microbial spectrum of otomycosis. Accordingly, molecular studies are needed to provide accurate data regarding the microbial epidemiology of otomycosis. In this molecular study, *C. parapsilosis* was the leading species (23.9 %), and this finding is similar to the study of García-Agudo *et al.* [1]. This finding is directly connected to the use of molecular method for identification. If sequence analysis had not been used, based on morphology, all isolated *A. tubingensis* (*n*=15), *A. niger* (*n*=9), *A. awamori* (*n*=2), *A. foetidus* (*n*=2) and other *Aspergillus* spp. of section *Nigri* (*n*=3) would have been identified as *A. niger*, making this organism the most common species, rather than *C. parapsilosis*. One of the main reasons for no previous reports of *A. tubingensis* as the dominant pathogen may be greater due to misidentification as *A. niger* [29, 30]. This issue is well demonstrated in some studies in which *A. niger* as the most comment agent based on morphology is re-identified using molecular methods. Sarwestani *et al.* [10] re-identified 43 *A. niger* isolates, and surprisingly confirmed only 11 isolates (25.58 %) as *A. niger*, with the 32 remaining isolates (74.48 %) being *A. tubingensis*. Similarly, Szigeti *et al.* [9] identified 14 black *Aspergillus* isolates, among which, 11 and 3 isolates were identified as *A. awamori* and *A. tubingensis*, respectively; no *A. niger* was recorded.

In addition, we identified *Talaromyces funiculosus*, which is a rare cause of otomycosis or human infection. These findings are indicative of the superiority of molecular over morphological identification methods. Therefore, accurate pathogen

Table 2. Fungal species isolated from otomycosis patients using DNA-based methods listed by frequency

Causative organism	Frequency (%)	GenBank accession no.
Aspergillus species		
Aspergillus tubingensis	15 (16.3)	MK480618, MK480623, MK480624, MK480625, MK480629, MK480635, MK480636, MK480637, MK480638, MK480640, MK480642, MK480643, MK480659
Aspergillus flavus	14 (15.2)	MK480616, MK480619, MK480621, MK480630, MK480631, MK480634, MK480639, MK480644, MK480646, MK480647, MK480649, MK480658
Aspergillus niger	9 (9.8)	MK480617, MK480628, MK480645, MK480653, MK480655, MK480656, MK480657
Aspergillus oryzae	5 (5.4)	MK480648, MK480654, MK480660
Aspergillus section *Nigri*[a]	3 (3.3)	
Aspergillus awamori	2 (2.2)	MK480620, MK480651
Aspergillus foetidus	2 (2.2)	MK480650, MK480652
Aspergillus terreus	2 (2.2)	MK480622, MK480632
Aspergillus flavipes	1 (1.1)	MK391986
Candida species		
Candida parapsilosis	22 (23.9)	MK391984, MK391985, MK391987, MK391988, MK391990, MK391991, MK391992, MK391993, MK391994, MK391995, MK391996, MK391997, MK391999, MK392000, MK392001, MK392002, MK392003, MK392004, MK392005, MK392006, MK392008
Candida albicans	4 (4.4)	–
Candida tropicalis	1 (1.1)	–
Other		
Cladosporium cladosporioides	3 (3.3)	MK391989, MK392007, MK392009
Penicillium polonicum	2 (2.2)	MK480627, MK480641
Penicillium expansum	2 (2.2)	MK480626, MK480633
Cryptococcus spp.	2 (2.2)	–
Penicillium chrysogenum	1 (1.1)	MK480661
Alternaria tenuissima	1 (1.1)	MK391998
Talaromyces funiculosus	1 (1.1)	MK173043
Total	92 (100)	

a, These isolates could not be identified to the species level.

identification is critical to updating our knowledge regarding the role of different black *Aspergillus* species, as well as rare pathogens in the etiology of otomycosis.

Finally, this report represents one of the first molecular epidemiology studies on otomycosis, which unlike some previous studies [9, 10], is not limited to a specific group of pathogens, i.e. *Aspergillus* section *Nigri*. Accordingly, we found a wide variety of pathogens, including more than 19 different species. The demonstration of such diversity of pathogens involved in otomycosis is unprecedented.

We did not perform antifungal susceptibility testing for the isolated fungi, which is a limitation for present study.

Conclusion

In this study, a diverse set of species involved in otomycosis was observed. Following the identification of the majority of the black *Aspergillus* isolates as species other than *A. niger*, a shift from *Aspergillus* species to *C. parapsilosis* as the leading species involved in otomycosis was observed. Application of molecular approaches for identification of otomycosis-isolated fungi might change current knowledge on the causative agents of otomycosis.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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