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ORIGINAL ARTICLE

Otomycosis: The foremost aetiological agent causing otitis externa and the antifungal susceptibility pattern in North-Western Iran

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Abstract

Background: Otomycosis is considered a recurring fungal ear infection. The external auditory canal provides an appropriate and optimal situation for fungal growth.

Objectives: The study aimed to identify the causative agents of otomycosis and determine corresponding antifungal drug susceptibility patterns in north-western Iran.

Methods: From October 2020 until November 2021, 200 patients attended an otolaryngology referral centre with otitis externa, and their ear discharge and debris were examined and cultured. The identification of the fungal agents was implemented by polymerase chain reaction-restriction fragment length polymorphism and sequencing. In vitro antifungal susceptibility testing of the isolates was conducted in accordance with the CLSI broth microdilution protocols.

Results: The prevalence of otomycosis was measured 50.5% (n = 101/200). The majority of patients were in their forties (n = 35, 34.6%) and female (n = 57, 56.4%), and the most prevalent symptom was otalgia (56.4%). The most underlying factor was remarked manipulation employing a cotton swab (65.3%). Regarding fungus, *Aspergillus* section *Nigri* (58.57%) was the foremost isolate, followed by *Aspergillus* section *Flavi* (19.23%) and *Candida parapsilosis* (14.96%). The predominance of *Aspergillus* isolates had minimal in vitro sensitivity to tioconazole and nystatin. *Candida* species represented higher geometric mean minimum inhibitory concentrations (MIC) against nystatin. The MIC of three *Aspergillus* species isolates shown above the epidemiologic cut-off values (ECV) against itraconazole.

Conclusions: Otomycosis incidence surpassed in comparison with the previous study as the most common cause of otitis externa. The MIC distribution of *Aspergillus* species isolates against triazole antifungals is close to the defined ECVs and likely outrun it over time.

KEYWORDS
Aspergillus section *Nigri*, Iran, molecular identification, otitis externa, otomycosis, susceptibility testing

1 | INTRODUCTION

Mycotic otitis externa, also identified as otomycosis, is an infection of the external auditory canal that can be acute, subacute or chronic. This disease is one of the most pervasive problems encountered by Ear, Nose and Throat (ENT) specialists and general practitioners. It would be an acutely demanding and time-consuming treatment process for the intended physician since the patient's antibiotic therapy is partially discontinued when the symptoms disappear. It is worth stating that such an illness would be fatal in immunocompromised patients.[1–3] The infection can be spread through the Eustachian tube to the middle ear, resulting in inflammation of the middle ear.[4,5] Further, the accumulation of epithelial cells, waxy substances and other microorganisms create a proper environment for fungal growth in the ear canal. As the fungus congests this area, it gives rise to the potential of hearing loss and a feeling of fullness in the ear. Clinical symptoms manifest firstly as otorrhea and otalgia in the acute form, secondly, itching and otalgia in the subacute form, and at last, scanty discharge with itching and scaling in the chronic condition. Additionally, high humidity and dust in the living area are environmental factors that predispose to this disease.[3,6]

Poor hygiene, excessive usage of ear cleaners swab, history of trauma and foreign objects intrusion into the ear canal, manipulation of the ear canal, utilisation of oil drops, swimming, taking topical and oral antibiotics for the treatment of bacterial otitis and metabolic disorders such as diabetes are also among the major predisposing factors. Anatomical defects of the ear canal, eczema and seborrheic dermatitis, trisomy 21, allergic rhinitis and cystic fibrosis are all considered risk factors. Moreover, alteration in the pH of the ear epithelium, as well as quantitative and qualitative changes in ear wax, impair the ear's defence mechanisms and increase the likelihood of an individual developing otomycosis.[3,6–11]

Opportunistic fungi involved in otomycosis include *Aspergillus niger*, *Aspergillus fumigatus* and rare species of *Aspergillus*, in addition to the genera of *Penicillium*, *Mucor*, *Rhizopus*, *Scopulariopsis*, *Fusarium*, *Scedosporium*, Dermatophytes (primarily *Epidermophyton floccosum*, *Trichophyton mentagrophytes* and *Trichophyton violaceum*). However, some studies have labelled *Aspergillus niger* as the most frequent cause of otomycosis, followed by *Candida albicans* and *Candida tropicalis*.[7,12,13] Additionally, multidrug-resistant *Candida auris* yeast has emerged in both immunocompromised and healthy people. Given the increasing reports of otomycosis or ear colonisation due to *C. auris* in different parts of the world, particularly in our country,[14] its identification and screening have gained considerable attention. It is worth noting that *C. auris* identification is difficult to carry out by conventional methods.

In recent years, the prevalence of ear fungal infections has skyrocketed.[15] The elements of immediate and correct diagnosis and, afterwards, appropriate treatment can impede subsequent chronic disease, resistance to treatment, recurrence and other potential complications.[16]

It is vital to note that the treatment with antiseptic agents, antibiotics, corticosteroids and combinations of previously mentioned methods recommended in guidelines are currently prescribed solely based on clinical signs and symptoms, with no laboratory investigation. Likewise, treatment failure occurs when the causative is not correctly identified. In addition, the misuse of potentially toxic antimicrobial drugs may result in various complications for the patient.[17]

This study aimed to identify the fungi that provoke otomycosis in Guilan province in north-western Iran on a molecular scale, determine the antifungal drug susceptibility pattern of the isolates and compare the findings of this study to previous ones implemented in this region during the last decade.

2 | MATERIALS AND METHODS

2.1 | Patients

Patients with suspected otitis externa admitted to a tertiary referral ENT department in Guilan province, northwestern Iran, from October 2020 to November 2021, were included in a descriptive cross-sectional study. An ENT specialist clinically examined the patients who displayed one of the various clinical signs and symptoms, including pruritus, otalgia, ear fullness, tinnitus, hearing loss, and the discharge of dry and moist masses with the plug of hyphal matted or white and cheesy appearance during otoscopy. A patient with subtle clinical and mycological findings, including positive direct microscopy and culture, is defined as having otomycosis.[18] Additionally, individuals who used antifungals or antibiotics before the clinical examination at the time of the visit and who had a history of major chronic medical conditions, including cancer and TB, were excluded from this research.

2.2 | Sample collection and analysis

Using a buck ear blunt curette with otoscope guidance, the specimens, including debris and crust of the ear canal, were carried out by an ENT specialist while adhering to the strictest sterility standards and taking care not to touch the skin or any other areas, which was then transferred into microtubes containing sterile normal saline, and immediately transported to the laboratory for direct examination with 10% KOH solution.[19] CHROMagar Candida medium (CHROMagar) was used to culture yeast-positive samples for preliminary identification and possible multiple *Candida* species infections. Furthermore, all samples were cultured on a Sabouraud Dextrose Agar (SDA) medium (Condalab) with chloramphenicol and incubated for 2–7 days at 30°C. The macroscopic and microscopic characteristics of colonies were used to perform the primary identification of filamentous fungi using lactophenol cotton blue.

2.3 | Molecular identification

PCR-RFLP was used to identify yeasts species using ITS1 and ITS4 primers (ITS1 5'-TCC GTA GGT GAA CCT GCG G-3' and ITS4

5'-TCC TCC GCT TAT TGA TAT GC-3') and restriction enzymes *Msp*1 (Thermo Fisher Scientific),[20] and *Aspergillus* species using Bt2a and Bt2b primers (Bt2a 5'-GGT AAC CAA ATC GGT GCT TTC-3' and Bt2b 5'-ACC CTC AGT GTA GTG ACC CTT GGC-3') and *Alw*1 (Thermo Fisher Scientific) restriction enzymes, respectively, as described earlier[21] DNA was extracted using a combination of glass-bead and phenol-chloroform, whereas yeast DNA was extracted using boiling techniques.[22]

The intein-containing vacuolar ATPase precursor genes were used to identify the *C. parapsilosis* complex.[23] β-Tubulin gene sequencing was also used with an automated DNA sequencer (Applied Biosystems 3730 Genetic Analyser) to identify unknown *Aspergillus* species. The sequence results were exposed to nucleotide blast analysis, and the identification of the fungal species was based on the level of similarity to reference sequences in the NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) database.

2.4 | In vitro antifungal susceptibility testing

Antifungal susceptibility testing was performed using the broth microdilution method by 96-wells microplates, as specified in the Institute of Clinical and Laboratory Standards (CLSI) guidelines M38-A3, M27-S4, and the results were compared with reported ECV.[24–27]

Antifungal drugs and their final concentration ranges were as follows, amphotericin B (AMB) (Bristol-Myers-Squib); from 0.016 to 16 μg/ml, itraconazole (ITC) (Janssen); ketoconazole; from 0.016 to 16 μg/ml,

fluconazole (FLC) from 0.032 to 64 μg/ml, ketoconazole (KTC) from 0.016 to 16 μg/ml, miconazole (MCZ) from 0.016 to 16 μg/ml, nystatin (NYT) from 0.016 to 16 μg/ml, tioconazole (TCZ) from 0.016 to 16 μg/ml, voriconazole (VRC) from 0.016 to 16 μg/ml, terbinafine (TRB) from 0.002 to 4 μg/ml and tolnaftate (TNF) from 0.016 to 16 μg/ml, (Merck).

The antifungal agent stock solutions were prepared in 96-well microdilution plates using RPMI 1640 medium (Roswell Park Memorial Institute medium) (Gibco) buffered with MOPS (3-[N-morpholino] propane sulfonic acid) (Gibco).

For the preparation of fungal inoculum, conidia of moulds and colonies of yeasts were harvested from fungal cultures on SDA incubated at 35°C; the fungal stock suspensions were then diluted with sterile distilled water containing tween 20; then, the density of each of the suspensions was then adjusted to transmission in the range 80%–82% for moulds (density; 2.5–5 × 104 CFU/ml) and 75%–77% for yeasts (density; 2.5–5 × 103 CFU/ml) at a wavelength 630 nm. The final working suspension was obtained by diluting the stock suspension 50 times for moulds and 1000 times for yeasts with RPMI 1640 medium and inoculating it into the wells of the microdilution plates. The inoculated plates were incubated at 35°C, and the MIC results were read visually after 48 h (for moulds) and 24 h (for yeasts). The two standard strains, *Candida krusei* (ATCC 6258) and *Paecilomyces variotii* (ATCC 3630), were used for quality control. All tests were performed in duplicate. Both negative and positive control wells were performed for all drug columns.

2.5 | Statistical analysis

All experiment results were stored in the SPSS (IBM SPSS Statistics v26) and EXCEL (Microsoft Excel, 2019 version) Softwares, and the values were calculated and analysed. The amount of p-value <.05 was considered statistically significant.

3 | RESULTS

In this study, the prevalence of otomycosis was 50.5% (n = 101/200). The majority of otomycosis patients were in their fifth decade of life (32, 31.6%), followed by the sixth (23, 22.7%) and fourth (18, 17.8%) decades. There were 57 females (56.4%) and 44 males (43.5%), with 91 (90.1%) living in the cities and 11 (10.8%) living in rural areas. Unilateral ear involvement affected 80 (80.1%) patients. The most prevalent frequency of bathing among patients was three times per week (n = 36), while most of them had 0–4 baths per week (n = 83, 82.1%). The most common symptoms were otalgia (n = 57, 56.4%) and itching (n = 52, 51.4%) followed by ear fullness (n = 43, 42.5%), hearing loss (n = 20, 19.8%), tinnitus (n = 27, 26.7%) and scaling (n = 12, 11.8%). (Table 1).

A total of 92 saprophytic mould isolates were obtained. The dominant isolates were *Aspergillus* species (n = 89, 88%). *Aspergillus* section *Nigri* (n = 58, 57.4%), *Aspergillus* section *Flavi* (n = 19, 18.8%), *Aspergillus* section *Fumigati* (n = 12, 11.8%), *Mucor spp.* (n = 2, 1.9%) and *Syncephalastrum spp.* (n = 1, 0.9%), twenty-five yeast isolates containing, *Candida parapsilosis* (n = 14, 13.8%), *Candida orthopsilosis* (n = 6, 5.9%) and *Candida albicans* (n = 5, 4.9%), respectively. (Table 2) The sequence results have also been deposited in GenBank under accession numbers ON229044-ON229053.

The most common factor predisposing to the development of otomycosis was the overaggressive use of cotton swabs for ear cleansing, followed by manipulation of the ear with unusual tools (41, 40.6%), topical antibiotics usage (43, 42.6%), topical steroids usage (34, 33.7%), history of bacterial otitis (32, 31.7%) and swimming (9, 8.6%). Thirty-six (28, 27.7%) of the patients had previous fungal otitis. In six (5.9%) individuals, tympanic perforation was found. Fifteen (14.8%) patients had diabetes as an underlying disease.

A patient with underlying psoriasis was seen with a mixed infection of *A. niger* and *C. orthopsilosis*, as was another with a history of oral lichen planus from which *A. niger* was isolated. *Candida parapsilosis* was isolated from another patient who had used a hearing aid previously. This study's participants found no other underlying disease, such as cancer or immunosuppressive illnesses.

Fifteen (14.8%) patients identified fungal species from different genera. The most common combination was seen between *A. niger*/*C. orthopsilosis* (n = 4), followed by *A. niger*/*C. parapsilosis* (n = 3) and *A. flavus*/*A. niger* (n = 3). Ten patients had mixed fungal–bacterial

TABLE 1 Demographic features, signs and symptoms, underlying variables, and comorbidities of patients suspected of otitis externa referred to Amir Al-Momenin Hospital, Rasht, in north-western Iran by causative organisms

		Otomycosis	Bacterial Otitis	Mixed (Fungal – Bacterial)
Gender	Male ($n = 94$)	38	33	6
	Female ($n = 106$)	53	36	4
Age	<20 ($n = 10$)	4	4	1
	20–30 ($n = 23$)	11	7	0
	30–40 ($n = 45$)	15	18	3
	40–50 ($n = 59$)	29	17	3
	50–60 ($n = 39$)	21	15	2
	60> ($n = 24$)	11	8	1
Habitat	Rural ($n = 185$)	83	64	9
	Urban ($n = 15$)	8	5	1
Baths per week	0–4 ($n = 168$)	77	63	6
	4–8 ($n = 29$)	11	6	4
	8> ($n = 3$)	3	0	0
Ear involvement	Left ($n = 70$)	37	21	2
	Right ($n = 72$)	35	25	7
	Both side ($n = 58$)	19	23	1
Sign and symptoms	Itching ($n = 92$)	44	23	6
	Fullness ($n = 80$)	38	24	5
	Tinnitus ($n = 27$)	10	13	0
	Otalgia ($n = 113$)	51	42	5
	Hearing Loss ($n = 47$)	18	24	1
	Scaling ($n = 12$)	5	5	0
Predisposing factors	Swimming ($n = 28$)	9	15	0
	Using cotton swabs ($n = 133$)	51	44	8
	Manipulate the ear with unusual tools ($n = 96$)	38	33	3
	History of Bacterial Otitis ($n = 72$)	27	27	5
	History of Fungal Otitis ($n = 53$)	22	19	6
	Tympanic perforation ($n = 16$)	5	7	1
	History of topical antibiotic use ($n = 88$)	39	28	4
	History of systemic antibiotic use ($n = 45$)	17	12	7
	History of topical steroid use ($n = 67$)	31	22	3
	Earphone use ($n = 2$)	1	1	0
	Immunosuppressives use ($n = 1$)	1	0	0

infections, with five having *Aspergillus niger*, three having non-*albicans Candida* (NAC)s, and one each having *Aspergillus flavus* and *Candida albicans* co-infection with bacterial agents.

Except for tioconazole (GM = 5.5477), nystatin (GM = 2.1015) and terbinafine (GM = 1.6982), all antifungals tested were effective against the majority of *Aspergillus* isolates. Furthermore, nystatin (GM = 2.9485) and itraconazole (GM = 1.0867) had higher GM MICs against all *Candida* species isolates. In contrast, amphotericin B (GM = 0.07129) in *Aspergillus*, as well as ketoconazole (GM = 0.02570) and voriconazole (GM = 0.03686) in *Candida*, demonstrated the highest antifungal activity. Regarding the M59 document for ECV, one *A. niger* (MIC 8 μg/ml), *A. flavus* (MIC 2 μg/ml) and *A. fumigatus* (MIC 2 μg/ml) isolates were non-wild type against itraconazole; three *A. niger* non-wild type isolates with MIC 4 μg/ml against voriconazole were also examined (Table 3). There were three *Candida albicans* isolates with high itraconazole MICs (two 8 μg/ml and one 16 μg/ml) (Table 4).

According to the results of our investigation, all mixed fungal species had raised GM-MICs against AMB compared to single-agent isolated cases. Also, mixed fungal-bacterial strains that included AMB, NYT, MYC, TER and VOR consistently had higher GM-MIC elevations.

TABLE 2 Results based on direct examination, culture, and PCR-RFLP of patients suspected of otitis externa referred to Amir Al-Momenin Hospital Rasht, in north-western Iran

Direct examination with KOH solution (*n*)	Culture on SC/CHROMagar medium (*n*)	PCR-RFLP (*Msp*1, *Alw*1) (*n*)
Budding yeast (*n* = 10)	NAC *spp.* (*n* = 7)	*C. parapsilosis* (*n* = 6)
		C. orthopsilosis (*n* = 1)
	C. albicans Complex (*n* = 3)	*C. albicans* (*n* = 3)
Septate branching hyphae (*n* = 68)	*Aspergillus* section *Nigri* (*n* = 40)	*A. niger* (*n* = 40)
	Aspergillus section *Flavi* (*n* = 12)	*A. flavus* (*n* = 12)
	Aspergillus section *Flavi*/ *Aspergillus* section *Nigri* (*n* = 3)	*A. flavus*/*A. niger* (*n* = 3)
	Aspergillus section *Fumigati* (*n* = 8)	*A. fumigatus* (*n* = 8)
	Aspergillus section *Fumigati*/ *Aspergillus* section *Nigri* (*n* = 1)	*A. fumigatus*/*A. niger* (*n* = 1)
Septate branching hyphae with vesicle(*n* = 4)	*Aspergillus* section *Nigri* (*n* = 3)	*A. niger* (*n* = 3)
	Aspergillus section *Flavi* (*n* = 1)	*A. flavus* (*n* = 1)
Septate branching hyphae and bacteria(*n* = 5)	*Aspergillus* section *Nigri*/ Bacteria (*n* = 5)	*A. niger*/ND (*n* = 5)
Septate branching hyphae and budding yeast(*n* = 11)	*Aspergillus* section *Nigri*/NAC *spp.* (*n* = 7)	*A. niger*/*C. parapsilosis* (*n* = 3)
		A. niger/*C. orthopsilosis* (*n* = 4)
	Aspergillus section *Flavi*/NAC *spp.* (*n* = 1)	*A. flavus*/*C. parapsilosis* (*n* = 1)
	Aspergillus section *Fumigati*/ NAC *spp.* (*n* = 2)	*A. fumigatus*/*C. parapsilosis* (*n* = 2)
	Aspergillus section *Nigri*/NAC *spp.* (*n* = 1)	*A. niger*/*C. albicans* Complex (*n* = 1)
Budding yeast and bacteria(*n* = 5)	NAC *spp.*/Bacteria (*n* = 5)	*C. parapsilosis*/ND (*n* = 3)
		C. orthopsilosis/ND (*n* = 1)
		C. albicans/ND (*n* = 1)
Aseptate branching hyphae(*n* = 2)	*Syncephalastrum* spp. (*n* = 1)	ND (*n* = 2)
	No-growth (*n* = 1)	
Septate and aseptate branching hyphae(*n* = 1)	*Aspergillus* section *Nigri*/*Zygomycete* spp. (*n* = 1)	*A. niger*/ND (*n* = 1)
Negative(*n* = 25)	No-growth (*n* = 18)	–
	Saprophytic contamination (*n* = 7)	
Bacteria(*n* = 69)	Bacteria (*n* = 23)	ND (*n* = 69)
	No-growth (*n* = 46)	

Note: Non-albicans *Candida* (NAC) spp., ND: not determined.

4 | DISCUSSION

The present study aimed to demonstrate the prevalence of otomycosis in the Guilan Province, located in the southern-west coastal region of the Caspian Sea, with beaches and swimming facilities under hot and humid summer weather conditions. Notably, the prevalence of otomycosis increased from 43% in 2013 to 50.5% in the current conducted study in 2021.[28] In contrast to the investigation results presented by Nemati et al. (2013), all fungal isolates were identified at the species or section level using PCR methods, which allowed

TABLE 3 Antifungal susceptibility pattern of *Aspergillus* strains isolated from otomycosis patients suspected of otitis externa with otitis referred to Amir Al-Momenin Hospital, Rasht, in north-western Iran

		MIC (μg/ml)																	
Aspergillus species	Antifungal agents	0.016	0.031	0.063	0.125	0.25	0.5	1	2	4	8	16	32	≥64	MIC Range	MIC_{50}	MIC_{90}	G-Mean	Mode	ECV Wild type (%)/Non-wild type (%)
A. niger ($n = 58$)	TRB		1		3	3	9	14	12	16					0.032–4	1	4	1.1963	4	ND
	TNF			3	11	15	13	4	2	1		9			0.063–16	0.375	16	0.5635	0.25	ND
	NYT							8	39	11					1–4	2	4	2.0730	2	ND
	TCZ							1	3	8	20	26			1–8	8	16	8.9084	16	ND
	ITC	3	3	1	7	1	5	18	12	7	1				0.016–8	1	4	0.6502	1	86/14
	AMB	20	15	8	2	2	2	9							0.016–1	0.0312	1	0.0574	0.016	100/0
	VRC				2	7	11	21	14	3					0.125–4	1	2	0.8768	1	71/29
A. flavus ($n = 19$)	TRB	1	1	2	3	5	2	1	3	1					0.016–4	0.25	2	0.2789	0.25	ND
	TNF										3	16			8–16	16	16	14.3413	16	ND
	NYT								12	7					4–8	2	4	2.5819	2	ND
	TCZ	1				1	2	4	1	6	4				0.016–8	4	8	1.7283	4	ND
	ITC		2	5	4	1		1	5	1					0.032–4	0.125	2	0.2592	0.063	63/37
	AMB			2	10	7									0.063–0.25	0.125	0.25	0.1500	0.125	100/0
	VRC				2	11		6							0.125–1	0.25	0.5	0.2893	0.25	100/0
A. fumigatus ($n = 12$)	TRB			1			2	1	1	7					0.063–4	4	4	1.6818	4	ND
	TNF					1					1	9		1	0.25–64	16	49.6	11.986	16	ND
	NYT							4	6	2					1–4	2	4	1.7818	2	ND
	TCZ					1	2	1		1	4	3			0.25–16	8	16	3.5636	8	ND
	ITC			2	6	3			1						0.063–2	0.125	1.475	0.1668	0.125	92/8
	AMB		4	6	1		1								0.032–0.5	0.0625	0.3875	0.0625	0.063	100/0
	VRC				1	8	2	1							0.125–1	0.25	0.85	0.2973	0.25	92/8
All species ($n = 89$)	TRB	1	2	3	6	8	13	16	16	24					0.016–4	1	4	0.9178	4	ND
	TNF			3	11	16	13	4	2	1	4	34		1	0.063–64	1	16	1.6982	16	ND
	NYT							12	57	20					1–4	2	4	2.1015	2	ND
	TCZ	1				2	4	6	4	15	28	29			0.016–16	8	16	5.5477	16	ND
	ITC	3	5	8	17	5	5	19	18	8	1				0.016–8	1	4	0.4448	1	ND
	AMB	20	19	16	13	9	3	9							0.016–1	0.0625	1	0.0713	0.016	ND
	VRC				5	26	19	22	14	3					0.125–4	0.5	2	0.5981	0.25	ND

Abbreviations: A, *Aspergillus*, Antifungal agents; AMB, amphotericin B; G-Mean, geometric mean; ITC, itraconazole; MIC_{50}, Minimum Inhibitory Concentration required to inhibit the growth of 50% of organisms; ND, Not Determined; NYT, nystatin; TCZ, tioconazole; TNF, tolnaftate; TRB, terbinafine; VRC, voriconazole.

TABLE 4 Antifungal susceptibility pattern of *Candida* strains isolated from otomycosis patients suspected of otitis externa with otitis referred to Amir Al-Momenin Hospital, Rasht, in north-western Iran

		MIC (μg/ml)																	
***Candida* Species**	**Antifungal agents**	**0.016**	**0.031**	**0.063**	**0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**≥64**	**MIC Range**	**MIC_{50}**	**MIC_{90}**	**G-Mean**	**Mode**	**ECV Wild type (%)/Non-wild type (%)**
C. parapsilosis ($n = 14$)	FLC					2	7	3	2						0.25–2	0.5	2	0.6404	0.5	64/36
	KTC	5	7	2											0.016–0.063	0.031	0.063	0.0269	0.031	ND
	MCZ	1				4	1	7	1						0.016–2	1	1.5	0.4999	1	ND
	NYT								7	7					2–4	3	4	2.8284	2	ND
	TCZ	3	4	2	4		1								0.016–0.5	0.046	0.31	0.0538	0.031	ND
	ITC						5	9							0.5–1	1	1	0.7807	1	ND
	AMB		2	3	2	5	2								0.031–0.5	0.187	0.5	0.1380	0.25	100/0
	VRC	2	7	4		1									0.016–0.25	0.031	0.063	0.0400	0.031	14/86
C. orthopsilosis ($n = 6$)	FLC					2	1	2	1						0.25–2	0.75	ND	0.6299	1	ND
	KTC	2	4												0.016–0.031	0.031	ND	0.0248	0.031	ND
	MCZ						3	1	2						0.5–2	0.75	ND	0.8999	0.5	ND
	NYT								3	3					2–4	3	ND	2.8284	2	ND
	TCZ	1	1		1	1	2								0.016–0.5	0.187	ND	0.1249	0.5	ND
	ITC						2	2	2						0.5–2	1	ND	1	2	ND
	AMB		1	1	2	2									0.031–0.25	0.125	ND	0.0111	0.125	ND
	VRC	3	1	2											0.016–0.063	0.023	ND	0.0278	0.016	ND
C. albicans ($n = 5$)	FLC					3	1	1							0.5–1	0.25	ND	0.3789	0.25	60/40
	KTC	3	1	1											0.016–0.063	0.016	ND	0.0237	0.016	ND
	MCZ			4	1										0.063–0.125	0.063	ND	0.0718	0.063	ND
	NYT								2	2	1				2–8	4	ND	3.4822	2	ND
	TCZ	1	3	1											0.016–0.063	0.031	ND	0.0312	0.031	ND
	ITC						2				2	1			0.5–8	8	ND	3.0314	8	ND
	AMB			1	1	1	2								0.063–0.5	0.25	ND	0.2176	0.5	100/0
	VRC	1	2	1	1										0.016–0.125	0.031	ND	0.2454	0.031	20/80

(Continues)

TABLE 4 (Continued)

Candida Species	Antifungal agents	MIC (μg/ml)											MIC Range	MIC_{50}	MIC_{90}	G-Mean	Mode	ECV Wild type (%)/Non-wild type (%)
		0.016	0.031	0.063	0.125	0.25	0.5	1	2	4	8	16	32	≥64						
All *Candida* species ($n = 25$)	FLC					7	9	6	3						0.25–2	0.5	2	0.5743	0.5	ND
	KTC	10	12	3											0.016–0.063	0.031	0.063	0.0257	0.031	ND
	MCZ	1		4	1	4	4	8	3						0.016–2	0.5	2	0.3895	1	ND
	NYT								12	12	1				2–8	4	4	2.9485	2	ND
	TCZ	5	8	3	5	1	3								0.016–0.5	0.031	0.5	0.0591	0.031	ND
	ITC						9	11	2		2	1			0.5–16	1	8	1.0867	1	ND
	AMB		3	5	5	8	4								0.031–0.5	0.125	0.5	0.1436	0.25	ND
	VRC	6	10	7	1	1									0.016–0.25	0.031	0.087	0.0369	0.031	ND

Abbreviations: AMB, amphotericin B; *C*, *Candida*, Antifungal agents; FLC, fluconazole; G-Mean, geometric mean; ITC, itraconazole; KTC, ketoconazole; MCZ, miconazole; MIC_{50}, Minimum Inhibitory Concentration required to inhibit the growth of 50% of organisms; ND, Not determined' NYT, nystatin; TCZ, tioconazole; VRC, voriconazole.

accurate identification of the various causative fungal species. The antifungal susceptibility testing was also carried out with a more extensive selection of available antifungals, as in this study, fungi were distinguished as the most widespread aetiologic agents triggering otitis rather than bacteria. Taking this into account, increasing fungal infection and the emergence of antifungal resistance may be linked to global climate change during the past 10 years.[29,30] However, the influence of Guilan's humid subtropical climate on the rising trend in otomycosis incidence may be overlooked. For instance, Aboutalebian et al. reported an 80% prevalence in Isfahan province with a hot and dry environment in 2019. The reported prevalence of otomycosis varied between 11.4% and 92% in different regions of the world.[30–34] As a result, differences in the geographical-based study, inclusion criteria and typical patient predisposing variables may impact the inconsistency of prior research's various frequencies.

In the present study, similar to others, otomycosis was diagnosed more frequently in female patients (56%).[35–39] This could result from women being more concerned about their health and seeking medical advice. Some studies, however, have shown that men were more susceptible to the disease than women due to occupational issues and being more exposed to fungal agents.[40,41]

Otomycosis was most common in people in their fifth decade of life,[4,35] even though the majority of those involved in several studies were between the ages of 30 and 40.[42,43] Since the ageing process affects all of the body's organs, including the ear, the elderly were more susceptible to various ear ailments.

Although most of the patients lived in cities, rural regions had a higher rate of otomycosis (9 out of 15). People in rural areas would probably apply home remedies for ear infections and delay seeking medical treatments, exposing them to otomycosis.[19]

The majority of patients demonstrated clear signs of unilateral ear fungal infections, which was consistent with previous findings.[2,44] Unilateral otitis externa would indicate the presence of an underlying foreign body or neoplasia. Although bilateral involvement with these causes was feasible, unilateral involvement could occur with symmetric illnesses such as allergic dermatitis and ceruminous gland hyperplasia.[45]

According to Gharaghani et al, (Iran),[2] da Silva Pontes et al. (Brazil),[46] and Li et al. (China),[15] otalgia and itching were the most typical symptoms.

According to the results, the most common fungal isolate was *Aspergillus* section *Nigri* .[16,35,36,47] According to Ali et al., the most isolated mould species in Egypt were *Aspergillus niger* ($n = 52$, 50.9%), *Aspergillus flavus* ($n = 34$, 33.3%) and *Aspergillus terreus* ($n = 3$, 2.9%).[30] Kiakojuri et al. reported as follows: *Aspergillus flavus* ($n = 129$, 54.4%), *Aspergillus tubingensis* ($n = 26$, 11%) and *Aspergillus niger* ($n = 21$, 8.7%) for north-eastern Iran.[48] In this investigation, as reported for Isfahan with a hot and dry climate, *Candida parapsilosis* was isolated more than other yeast species,[36,49] contrary to previous findings that indicated *Candida albicans* was the predominant yeast species.[50–52] The ability of *C. parapsilosis* to adhere to epithelial cells and surfaces would be a factor that influenced the onset and recurrence of otomycosis.[53]

The history of using cotton ear swabs was evaluated as the most intense predisposing factor in the present research. An analogous statement was declared by Kiakojuri et al. and Aboutalebian et al. that cotton ear swabs played a critical role in incrementing the rate of infection and recurrences.[36,54] The vast majority of patients demonstrated background of bacterial ear infections (36.5%), often with a history of topical antibiotics (44%) and topical steroid usage (33.5%). Furthermore, the alteration of pH in the ear canal and elimination of any competing microorganisms induced remarkable influence on shifting the disease's genesis to fungal infections.[55,56]

Identifying mixed infections was taken under precise consideration in this study since it appeared that the combination of two different or distinct fungal species ($n = 15$) and fungal-bacterial ($n = 10$) species might form a harsher microenvironment and occasion a resistance to therapy by the development of a mixed infection.[57,58]

It is worth stating that the clinical presentation of a mixed bacterial and fungal infection could imitate a bacterial infection. If a proper laboratory investigation was not performed, the second fungal agents might not be appropriately identified. Consequently, the patient's treatment might fail with routine antimicrobial therapy due to prescribing inappropriate antifungals.[59]

The most common species in this study, *A. niger*, was highly sensitive to a wide range of drugs, including AMP, VRC, ITC, TRB and TNF; however, it has high MICs for some of the most commonly used antifungals for otitis externa, such as NYT and TCZ. Besides, these findings were consistent with those of Karaarslan et al.[13] despite the fact *Aspergillus niger*'s MIC_{50} for TNF was 0.37 μg/ml, nine isolates were found with a high MIC (16 μg/ml).

Candida parapsilosis, the most prominent yeast isolate in this study, was distinguished to be sensitive to a wide variety of antifungals, including KET, VRC, TCZ, AMB, MCZ, FLC and ITC. However, contrary to what Manguia et al. claimed, NYT contained high MICs and was not recommended as an effective drug.[60]

In conclusion, it can be noted that fungal agents were the most prevalent cause of otitis externa in Guilan Province with its humid subtropical climate. Thus, physicians need to take fungal infections for further evaluation. The findings of this study have a significant implication for clinical practice, particularly in such similar climates in developing countries. The MIC distribution of *Aspergillus* species isolates against triazole antifungals is analogous to the CLSI ECVs and likely to outrun it over time. Since the cryptic species of *Aspergillus* has different patterns of antifungal susceptibility, infection control might benefit from identification using more precise methods such as DNA sequencing for all isolated strains.[61] As investigating fungal otitis was prioritised, the determination of bacterial otitis was just limited to exercising direct examination and culture. Hence, species identification was not performed.

Although this study has demonstrated the causative agents of otomycosis and determined their antifungal drug susceptibility pattern in COVID-19 era, further research with larger sample size in other regions with similar climates and frameworks will be needed to evaluate the implications of global warming in the prevalence of otomycosis and their antifungal susceptibility patterns with focusing on identifying fungal-bacterial coexistence as mixed complexes. To provide timely administration of effective antimicrobials, reduction of the development of drug resistance and avoidance of treatment failure, implementing drug susceptibility testing is recommended by the physician before initiating therapy.

AUTHOR CONTRIBUTIONS

TSH designed the study; SHN, BR, MSH and AD collected the data. BR, AA, LF, IH and SM analysed the data. BR and TSH wrote the manuscript. TSH, MA and AMSA revised the article. The final manuscript was reviewed and approved by all authors.

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CONFLICT OF INTEREST

There are no conflicts of interest reported by the authors. This paper's content and writing are solely the responsibility of the authors.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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