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Original Article

Species identification and antifungal susceptibility testing of *Aspergillus* strains isolated from patients with otomycosis in northern China

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KEYWORDS
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Abstract *Background/Purpose:* There are limited studies on species distribution and susceptibility profiles of *Aspergillus* strains isolated from patients with otomycosis in China.
Methods: A total of 69 confirmed *Aspergillus* species isolates were obtained from ear swabs of patients diagnosed with otomycosis from 2017 to 2018 in northern China. Identification of

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Aspergillus welwitschiae;
Antifungal susceptibility testing;
Northern China

these *Aspergillus* isolates at the species level was performed using conventional morphological methods and MALDI-TOF MS in combination with molecular sequencing, and *in vitro* susceptibility to nine antifungal agents was evaluated using the Sensititre YeastOne system.
Results: The *Aspergillus* section *Nigri* had the greatest distribution of *Aspergillus* isolates. *A. welwitschiae* (n = 25) was the most predominant isolate in section *Nigri*, followed by *A. tubingensis* (n = 12) and *A. niger* (n = 11). Other *Aspergillus* species were also isolated, including *A. terreus* (n = 11), *A. flavus*/*A. oryzae* (n = 8), and *A. fumigatus* (n = 2). Amphotericin B, posaconazole, and echinocandins were highly *in vitro* active against all the isolates tested. 2.9% (2/69) of the isolates were resistant to azoles in our study, including one *A. niger* isolate with a high MIC value for itraconazole (ITR) (16 mg/L) and one *A. tubingensis* isolate cross-resistant to both voriconazole (VOR) (MIC >8 mg/L) and ITR (MIC >16 mg/L). One *A. welwitschiae* and one *A. niger* isolate both had increased MIC values of 4 mg/L against VOR.
Conclusions: A. welwitschiae was the most prevalent *Aspergillus* species isolated from patients with otomycosis. Our findings also indicated that the azole-resistant *Aspergillus* section *Nigri* should be utilized to guide clinical medication for Otomycosis.
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Introduction

Aspergillus is a fungus found worldwide that can cause infections in primarily immunocompromised hosts and individuals with the underlying pulmonary disease. The primary route of aspergillosis is mainly through the respiratory tract. *Aspergillus* species, however, can invade other tissues such as skin, sinuses, central nervous system, eyes, nails, external ear, or become disseminated throughout the body.[1—5] Otomycosis, also known as fungal otitis externa, is a superficial fungal infection of the external auditory canal that sometimes invades the middle ear.[6—8] According to some case reports and data statistics, the incidence of otomycosis is higher in tropical and subtropical regions with hot, humid climates.[7] However, our study showed that there were also many cases of otomycosis in regions of northern China with relatively dry and cold climates. Patients with otomycosis usually exhibit ear itching, niffy otorrhea, ear fullness, and hearing loss.[7,9,10] Some studies have reported that *Aspergillus* spp. and *Candida* spp. are the most common pathogenic strains causing otomycosis.[11]

Although otomycosis is rarely life-threatening, it is a clinically troublesome disease because of the long-term treatment required and its tendency for recurrence.[7] With an overall objective to control filamentous fungal infection, rapid and accurate identification of the pathogenic agents responsible for this disease is essential. Because of some similar phenotypic characteristics among each *Aspergillus* species, it is difficult to identify them based on morphological criteria.[12] The present study combined three identification methods including conventional morphological method, matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS), and molecular sequencing method to improve the efficiency and accuracy of species identification.

Some topical antifungal agents, such as itraconazole (ITR) eardrops,[7] clotrimazole, and salicylic acid,[13] have been used to treat aspergillosis.[6] However, some studies have reported that *A. niger* and *A. tubingensis* are resistant to ITR,[14—16] which poses a huge challenge for treatment response in patients with otomycosis. In addition, some common *Aspergillus* spp., such as *A. fumigatus*, *Aspergillus* section *Nigri*, and *A. flavus*, have been reported to have lower susceptibility to voriconazole (VOR), the first choice for treating invasive aspergillosis.[14,17]

To the best of our knowledge, there have been limited studies on species identification and *in vitro* antifungal susceptibility testing (AST) of *Aspergillus* strains isolated from otomycosis cases in northern China. Our study aimed to perform effective and accurate identification and AST of 69 *Aspergillus* strains isolated from ear swabs of otomycosis patients in northern China.

Materials and methods

Ethics statement

This study was approved by the Human Research Ethics Committee of Peking Union Medical College Hospital (No. S-263). Written informed consent was obtained from all patients in the study and for permission to study the isolates cultured from them for scientific research.

Fungal isolates

A total of 69 *Aspergillus* strains were isolated from ear swabs of patients who were diagnosed with otomycosis under the China Hospital Invasive Fungal Surveillance Net — North China Program from 2017 to 2018. These isolates were originally collected from a total of 12 participating hospitals in the program from the Heilongjiang, Jilin, Liaoning, and Shaanxi Provinces in the northern regions of China. Morphological identification was performed at the Peking Union Medical College Hospital (PUMCH). Specimens were cultured on potato dextrose agar (PDA) (Thermo Fisher Scientific, Waltham, MA, USA) and incubated for 3—7 days at 28 °C. Isolates were initially identified using

conventional morphological methods, including colony characteristics and direct microscopic observations of spores and hyphae by magnification at 400 × and lactophenol cotton blue staining. Morphological identification was first performed at each of the cooperative hospitals for initial identification results and then repeated at PUMCH.

Identification using the MALDI-TOF MS system

All strains were identified using the VITEK® MS V3.2 system (bioMérieux, Marcy-l'Étoile, France), a mass spectrometer that uses MALDI-TOF technology. *Aspergillus* isolates were prepared for MALDI-TOF MS identification according to the manufacturer's instructions. All strains were cultured on Sabouraud dextrose agar (SDA) (Thermo Fisher Scientific) and incubated at 28 °C for 3–7 days. First, spores and hyphae from the *Aspergillus* isolates were gently collected using a sterile cotton swab pre-moistened with sterile deionized water. The collected components were then suspended in a sterile 2 mL round-bottomed tube containing 900 μL of 70% ethanol, vortexed, and centrifuged for 2 min at 14,000 *g*. After discarding all of the supernatant using a pipette, 40 μL of 70% formic acid and 40 μL of acetonitrile were added, and the samples were vortexed and centrifuged for 2 min at 14,000 *g*. Next, the supernatant was spotted on a single-use target and samples were required to dry completely. One microliter of VITEK mass spectrometry (MS)-α-Cyano-4-hydroxycinnamic acid matrix was added to each target slide spot, which was also required to dry completely. Finally, the target was run in the VITEK ® MS instrument, and the results were analyzed using the V.3.2 database.

Identification supplemented by molecular sequencing methods

For a small number of strains that failed to be identified by MALDI-TOF MS with 'no result' or without reaching the species level, molecular sequencing was carried out as a supplemental method to continue the accurate identification. First, these strains were cultured on SDA and incubated at 28 °C for 3–7 days until adequate growth of hyphae was observed. Then, plenty of hyphae were harvested and DNA was extracted using a QIAamp DNA mini kit (QIAGEN Co., Ltd, Hilden, Germany). Next, nucleotide sequencing of the internal transcribed spacer (ITS) regions of ribosomal DNA, as well as the β-tubulin (*BenA*) and calmodulin (*CaM*) genes of these strains was performed by Beijing Ruibio BioTech Co., Ltd (Beijing, China). Finally, these isolates were identified at the species level based on the ITS, *BenA*, and *CaM* sequence analyses, which were homologically aligned with reliable sequences in the GenBank database (https://www.blast.ncbi.nlm.nih.gov/Blast.cgi).

Antifungal susceptibility testing

In vitro antifungal susceptibility of the 69 isolates of *Aspergillus* spp. was determined using the broth microdilution method (BMD) with a Sensititre YeastOne (SYO) system, utilizing frozen commercial 96-well panels containing nine antifungal agents: anidulafungin (AND) 0.015–8 mg/L, micafungin (MF) 0.008–8 mg/L, caspofungin (CAS) 0.008–8 mg/L, flucytosine (5-FC) 0.06–64 mg/L, posaconazole (POS) 0.008–8 mg/L, voriconazole (VOR) 0.008–8 mg/L, itraconazole (ITR) 0.015–16 mg/L, fluconazole (Flu) 0.12–256 mg/L, and amphotericin B (AMB) 0.12–8 mg/L. A sufficient amount of conidia was collected from the SDA plates and was then suspended in 0.1% Tween-20 in saline (3 mL) with a turbidity of 0.5 McFarland (equivalent to (0.6–5) x 10^6 CFU/mL). Then, 100 μL of the conidial suspension was inoculated in SYO broth containing 11 mL RPMI-1640 medium to form a conidial working suspension with a final concentration range of 0.4×10^4–4×10^4 CFU/mL. Finally, the conidial working suspension was dispensed into the SYO 96-well panel with an inoculum size of 100 μL per well.

After incubating 96-well panels at 35 °C for 48 h, the minimum inhibitory concentrations (MICs) of POS, VOR, ITR, and AMB were visually measured at 48 h by means of a color change from red (growth) to blue (100% growth inhibition) in the antifungal solutions according to the SYO instruction. For AND, CAS, and MF, the minimal effective concentrations (MECs) were read at 24 h according to the CLSI M38-A3 document,[18] when there was a growth of small, round, compact hyphal formation in comparison to the hyphal growth observed in the growth control well.[14,18] *Candida parapsilosis* ATCC22019, *Candida krusei* ATCC6258, *A. fumigatus* ATCC®MYA-3627™, and *A. flavus* ATCC®204304™ were used as quality control and reference strains.

Data analysis

The ranges of MEC/MIC and median values of MIC (mMIC) or MEC (mMEC) were read and calculated for each *Aspergillus* spp. against the seven antifungal drugs, by the standard BMD based on the CLSI M38-A3 document.[18] Based on the CLSI M59 document,[19] epidemiological cut-off values (ECVs) have only been established for CAS, POS, VOR, ITR, and AMB against some major *Aspergillus* species., such as *A. fumigatus*, *A. flavus*, *A. niger*, *A. terreus*, and *A. versicolor*.

Results

Identification of the isolates

All 69 *Aspergillus* isolates cultured from the ear swabs could be correctly identified at the complex level by morphological methods at PUMCH, including 48 isolates of *Aspergillus* section *Nigri*, 11 isolates of *Aspergillus* section *Terrei*, 8 isolates of *Aspergillus* section *Flavi*, and 2 isolates of *Aspergillus* section *Fumigati* (Table 1). After 3–7 days of incubation at 28 °C, all isolates presented colonies of different sizes, shapes, colors, and textures on PDA. The microscopic characteristics of these filamentous fungi also varied depending on the species. Overall, the *Aspergillus* spp. grew rapidly and formed fluffy to velvet-like colonies, and some formed concentric circles of color while others were uniform in color.[20] Fig. 1 shows the different colony and microscopic characteristics of these *Aspergillus* species based on the molecular identification results. For example, the colonies of *A. niger* (Fig. 1(A-1)), *A. tubingensis*

Table 1 Comparison of identification results of 69 isolates of *Aspergillus* species recovered from patients with otomycosis using conventional morphological and MALDI-TOF MS methods.

Aspergillus section	*Aspergillus* species	No. of isolates identified by molecular sequencing method	Identification results using conventional morphological methods, no. (%)			Identification results using MALDI-TOF MS method, no. (%)			
			Correct to complex level	Unidentified	Misidentified	Correct to complex level	Correct to species level	Unidentified	Misidentified
Nigri	*A. welwitschiae*	25	48 (100.0)	0	0	47 (97.9)	0	1 (2.1)	0
	A. niger	11							
	A. tubingensis	12							
Terrei	*A. terreus*	11	11 (100)	0	0	10 (90.9)	0	1 (9.1)	0
Flavi	*A. flavus/A. oryzae*	8	8 (100)	0	0	8	8	0	0
Fumigati	*A. fumigatus*	2	2 (100)	0	0	2	2	0	0
Total		69	69 (100)	0	0	67 (97.1)		2 (2.9)	0

MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; PUMCH, Peking Union Medical College Hospital.

(Fig. 1(B-1)), and *A. welwitschiae* (Fig. 1(C-1)), displayed extremely similar characteristics, with white to yellow villous mycelia and dark brown to black conidia on PDA. In addition, it was also difficult to distinguish *A. niger* (Fig. 1(A-2)), *A. tubingensis* (Fig. 1(B-2)), and *A. welwitschiae* (Fig. 1(C-2)) based on the microscopic features, with both hyaline conidiophores and large, globose conidial heads radiating with phialides borne on metulae. Colonies of *A. flavus/A. oryzae* (Fig. 1(D-1)) were granular, flat, and yellow-green with radial grooves; the conidial heads typically radiated, later splitting to form loose columns, and conidiophore stipes were hyaline and coarsely roughened (Fig. 1(D-2)). *A. fumigatus* formed blue-green colonies with white margins and powdery to felt-like textures (Fig. 1(E—1)). Under the microscope (Fig. 1(E—2)), slightly roughened conidia of *A. fumigatus* were observed, and the conidial heads appeared to have an inverted flask shape. The colonies of *A. terreus* were cinnamon-buff to sand-brown in color (Fig. 1(F-1)), and the conidial heads had compact, columnar, and biseriate characteristics, with hyaline and smooth-walled conidiophores (Fig. 1(F-2)).

All 69 *Aspergillus* isolates were identified using the VITEK® MS V3.2 system (Table 1). Except for two isolates that failed to be identified with no ID generated, the majority of *Aspergillus* isolates tested (67/69, 97.1%) were correctly identified at the complex level. Of the 67 *Aspergillus* complexes, 10 isolates were correctly identified at the species level, including eight isolates of *A. flavus/A. oryzae*, and two isolates of *A. fumigatus*.

The 47 isolates of *Aspergillus* section *Nigri*, 10 isolates of *Aspergillus* section *Terrei*, 8 isolates of *A. flavus/A. oryzae*, and 2 unidentified isolates were further molecularly classified through sequencing analysis of the ITS, *BenA*, and *CaM* genes. Of the 65 isolates (Table 1), 25 were molecularly identified as *A. welwitschiae*, 12 as *A. tubingensis*, and 11 as *A. niger*, belonging to *Aspergillus* section *Nigri*; the other strains were identified as *A. terreus* ($n = 11$). Notably, eight isolates of *A. flavus* and *A. oryzae* were still difficult to distinguish from each other because of the confused alignment results from the GenBank database (Table 1).

Evaluation of the morphological identification of *Aspergillus* spp. from initial hospitals

Based on the results of the molecular sequencing, we evaluated the morphological identification of the tested *Aspergillus* spp. isolates from the initial hospitals (Table 2). Overall, the correct identification rate of *Aspergillus* spp. isolates in initial hospitals was 81.2% (56/69).

A total of 45 isolates were morphologically identified at the *Aspergillus* section *Nigri* level at the initial hospitals, but one isolate was correctly identified as *A. flavus/A. oryzae* based on molecular identification. Another four section *Nigri* strains were misidentified as other species, including one *A. niger* and two *A. tubingensis* isolates misidentified as section *Fumigati*, and one *A. welwitschiae* isolate misidentified as *Trichosporon asahii*. For the *Aspergillus* section *Terrei*, eight isolates were morphologically identified at the initial hospitals, and one isolate

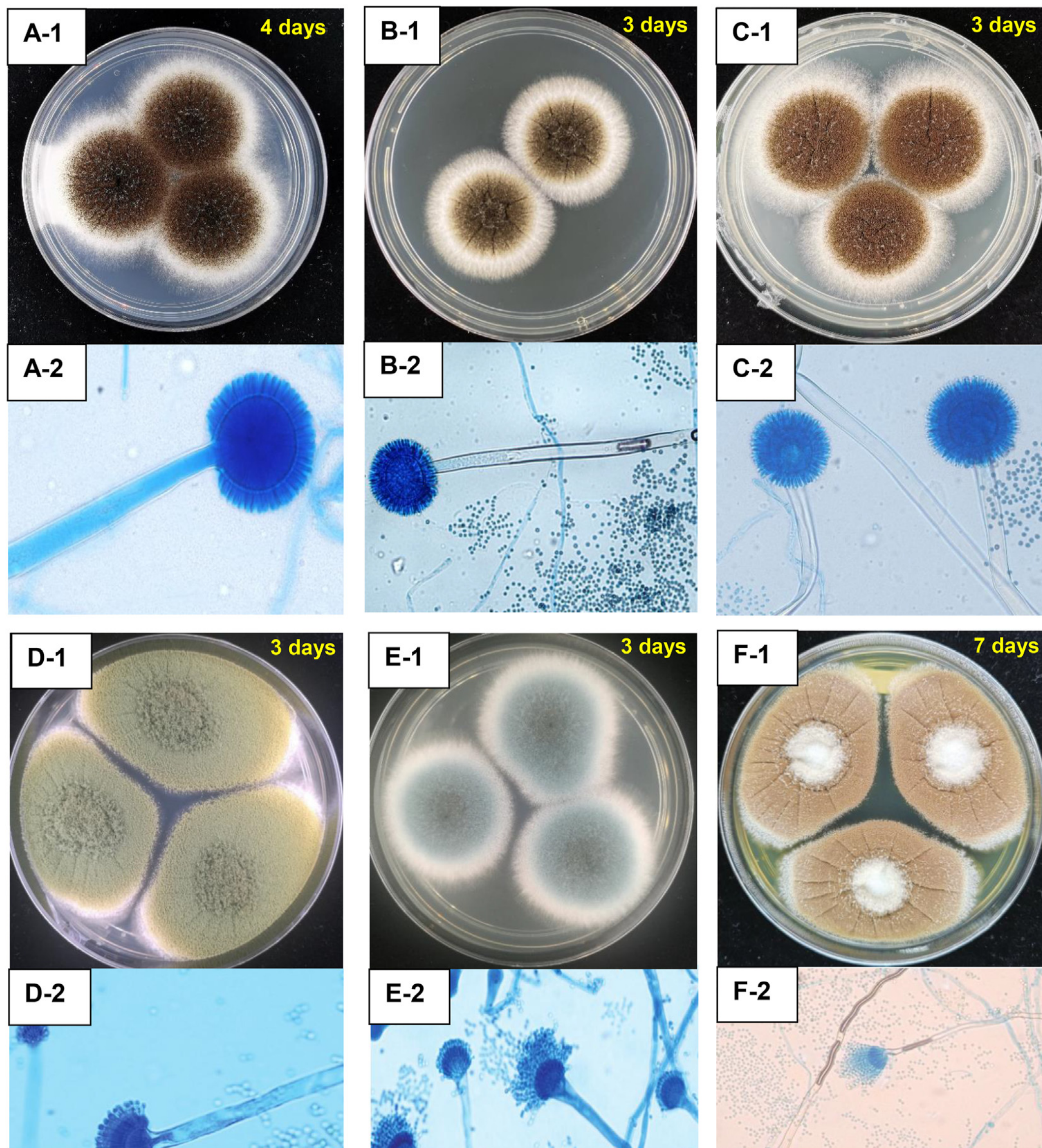

Figure 1. Morphology of *Aspergillus* spp. isolated from ear swabs of otomycosis patients. A-1, B-1, C-1, D-1, E−1, and F-1 are colonies, which were obtained after incubation on potato dextrose agar at 28 °C for 3−7 days, of selected strains of *A. niger*, *A. tubingensis*, *A. welwitschiae*, *A. flavus*/*A. oryzae*, *A. fumigatus*, and *A. terreus*, respectively. A-2, B-2, C-2, D-2, E−2, and F-2 are the slide microscope images of each isolate described above (magnification, 400 ×) obtained following lactophenol cotton blue staining.

was correctly identified as *A. flavus*/*A. oryzae*. According to the results of the corrected strains, another four isolates of section *Terrei* strains were misidentified as section *Fumigati* or only at the *Aspergillus* species level, with one strain unidentified. In addition, only three isolates of *Aspergillus* section *Flavi* were correctly identified at the initial hospitals. Most of the other section *Flavi* strains failed to be morphologically distinguished from section *Nigri* and section *Terrei* or were identified only at the *Aspergillus* species level.

Antifungal susceptibilities

The results for the quality control and reference strains were within the range of those provided in the SYO instruction and CLSI M61 document.[21] The susceptibilities of the 69 *Aspergillus* isolates to seven antifungal drugs by SYO are shown in Table 3. Table 4 shows the 69 isolates of *Aspergillus* spp. categorized as non-wild type to the indicated agents based on the ECVs of CLSI M59. The susceptibilities of *A. welwitschiae* and *A. tubingensis* to each antifungal drug in our study were in accordance with the ECVs proposed for *A. niger*. Overall, all seven antifungal drugs had high activities against most of the *Aspergillus* spp., except for 5-FC and Flu with inactivity to these isolates.[14,15] (data not shown in Table 3).

For AMB, all tested *Aspergillus* isolates among the different species had the same mMIC of 2 mg/L. Overall, AMB had high antifungal activities against 92.8% (64/69) of these isolates, but five isolates of *A. welwitschiae* were

Table 2 Evaluation of the morphological identification of *Aspergillus* species from initial hospitals based on molecular sequencing.

Morphology results	N	Based on molecular sequencing method		
		No. (%) of correct IDs	No. (%) of incorrect IDs and corrected strains (n)	
Aspergillus section *Nigri*	45	44	1	*A. flavus/A. oryzae* (1)
Aspergillus section *Terrei*	8	7	1	*A. flavus/A. oryzae* (1)
Aspergillus section *Flavi*	3	3	0	
Aspergillus section *Fumigati*	6	2	4	*A. niger* (1) *A. tubingensis* (2) *A. terreus* (1)
Aspergillus spp.	5	0	5	*A. flavus/A. oryzae* (3) *A. terreus* (2)
Trichosporon asahii	1	0	1	*A. welwitschiae* (1)
Unidentified	1	0	1	*A. terreus* (1)
Total	69	56 (81.2)	13 (18.8)	

N, number of isolates.

non-wild-type to AMB. For triazoles, POS showed the greatest antifungal activity against these isolates. Except for *A. fumigatus* without the ECV available to POS, other *Aspergillus* spp. isolates were all wild type to POS with the same mMIC of 0.12 mg/L. VOR showed greater antifungal activities against *A. terreus* in the testing, with a lower MIC range of 0.12—0.25 mg/L. However, *A. tubingensis* had a higher mMIC of 1.5 mg/L and a MIC range of 0.5—>8 mg/L against VOR than that of *A. niger* and *A. welwitschiae*, with mMICs of 1 mg/L and MIC ranges ≤4 mg/L. In addition, all tested *A. terreus*, *A. flavus/A. oryzae*, and *A. fumigatus* showed relatively lower MIC ranges ≤0.25 mg/L against ITR compared with that of section *Nigri* within 0.12—>16 mg/L.

One *A. niger* isolate and one *A. welwitschiae* isolate with increased MICs of 4 mg/L against VOR were found in our study. In addition, it was noted that there was one non-wild-type *A. niger* isolate with a higher MIC of ≥16 mg/L against ITR, regarded as resistance to ITR. Furthermore, there was also one cross-resistant *A. tubingensis* isolate against both VOR and ITR, with MICs of ≥8 mg/L and ≥16 mg/L, respectively.

Although the ECV was only established for CAS among some major *Aspergillus* spp. from the CLSI M59 document,[19] it was noted that 100% of the isolates were inhibited by 0.06 mg/L of AND, MF, and CAS, demonstrating their excellent antifungal potency and spectra. AND exhibited the lowest MEC range (≤0.015 mg/L) for all isolates. There was no significant difference in the MEC ranges against MF and CAS among the different species.

Discussion

According to many studies in different countries, *Aspergillus* spp. is the most predominant pathogen causing otomycosis, and section *Nigri* is the most common *Aspergillus* complex isolated from the ear swabs of otomycosis patients,[22,23] which is consistent with our present findings. In fact, otomycosis is very common in southern China, which has higher temperatures and a more humid climate. For example, a study from Shanghai indicated that 73.0% of fungi isolated from patients with otomycosis were *Aspergillus* spp., and section *Nigri* (75.0%) was isolated as the dominant section, followed by *A. terreus* (11.9%), *A. flavus* (8.3%), *A. fumigatus* (3.6%), and *A. versicolor* (1.2%). In addition, Li and He from Guangzhou also reported that section *Nigri* (n = 54) was the most frequent fungus in 75 isolated *Aspergillus* strains, followed by *A. terreus* (n = 12) and *A. flavus* (n = 9).[24] Of note, the species distribution of the top three *Aspergillus* spp. in our study was in agreement with both of the studies in Shanghai and Guangzhou, China, including the most common section *Nigri* (69.6%, 48/69), followed by *A. terreus* (15.9%, 11/69), *A. flavus/A. oryzae* (11.6%, 8/69), and *A. fumigatus* (2.9%, 2/69). Moreover, the number of *A. welwitschiae* isolates (n = 25) distinguished from section *Nigri* was greater than that of *A. tubingensis* (n = 12) and *A. niger* (n = 11). Our results were different from those in Japan, Egypt, and Turkey, where *A. niger* is the most common causative agent of otomycosis.[6,22,25] In contrast, *A. tubingensis* has been reported as the most predominant *Aspergillus* spp. causing ear infections in Iran and western China;[11,26] *A. flavus* is the most frequently isolated *Aspergillus* spp. in Spain.[27]

Numerous mycology laboratories in various countries have identified filamentous fungi based on morphological classifications.[7,22,23,27,28] However, the conventional morphological method has limitations in that it cannot accurately distinguish closely related species from each other. For example, *A. welwitschiae*, *A. tubingensis*, and *A. niger* are difficult to distinguish from each other owing to their similar colonies and microscopic features. In addition, the morphological classification of filamentous fungi requires professional microbiologists who are lacking in some primary hospitals. We combined the morphological method and VITEK® MS to identify filamentous fungi in our lab, confirming all our correct morphological identifications at the *Aspergillus* complex level. Molecular sequencing was used as a supplementary method in our lab to distinguish each species from section *Nigri* and

Table 3 Susceptibilities of 69 isolates of *Aspergillus* isolates to seven antifungal drugs using the Sensititre YeastOne method.

Aspergillus spp. (number of tested strains)	*Aspergillus* species (no. of isolates), mg/L											
	A. welwitschiae (25)		*A. tubingensis* (12)		*Aspergillus niger* (11)		*A. terreus* (11)		*A. flavus/A. oryzae* (8)		*A. fumigatus* (2)	
Antifungal drugs	MEC/MIC Range	mMIC/ mMEC	MEC/MIC Range	mMIC/ mMEC	MEC/MIC Range	mMIC/ mMEC	MEC/MIC Range	mMIC/ mMEC	MEC/MIC Range	mMIC/ mMEC	MEC/MIC Range	mMIC/ mMEC
AND	≤0.015	<0.015	≤0.015	<0.015	≤0.015	<0.015	≤0.015	≤0.015	≤0.015	≤0.015	≤0.015	≤0.015
MF	≤0.008−0.06	<0.008	≤0.008−0.03	0.015	≤0.008	<0.008	≤0.008−0.015	≤0.008	<0.008−0.03	0.0225	0.015	0.015
CAS	≤0.008−0.03	<0.008	≤0.008−0.06	0.015	≤0.008−0.03	<0.008	≤0.008−0.06	0.03	≤0.008−0.03	0.015	0.03	0.03
POS	0.06−1	0.12	0.06−1	0.12	0.06−0.5	0.12	0.06−0.12	0.12	0.06−0.12	0.12	0.03	0.03
VOR	0.5−4	1	0.5->8	1.5	1−4	1	0.12−0.25	0.12	0.5−2	1	0.5	0.5
ITR	0.12−2	0.25	0.12->16	0.75	0.12->16	0.25	0.06−0.25	0.12	0.06−0.12	0.06	0.06	0.06
AMB	1−4	2	1−2	2	0.5−2	2	1−2	2	2−4	2	2	2

MIC, minimum inhibitory concentration; MEC, minimal effective concentration; mMEC, median MEC; mMIC, median MIC; AMB, amphotericin B; AND, anidulafungin; CAS, caspofungin; ITR, itraconazole; MF, micafungin; POS, posaconazole; VOR, voriconazole.

section *Terrei*, which could only be identified at the *Aspergillus* complex level by VITEK® MS. According to the corrected morphological results of the initial hospitals with a correct identification rate of 81.2%, the level of morphological identification needs to be improved. MALDI-TOF MS-based identification of filamentous fungi was found to be less expensive and easier to conduct than morphological identification and molecular sequencing.[29] Furthermore, MALDI-TOF MS-based identification performed directly on clinical specimens could bypass fungal culture, as a result of the more rapid analysis performed in this system.[20,29]

Although we performed AST using SYO instead of the standard BMD, SYO is a commercially simple and reliable AST method for *Aspergillus* spp. based on the CLSI M38-A3 document,[18] with an accordance rate of 91.9% to BMD from a previous study,[30] which has been used in many laboratories for clinical AST. Overall, the antifungal susceptibility data in our study showed that triazoles, AMB, and echinocandins were active against most *Aspergillus* spp. Many studies have reported that *A. terreus* is innately resistant to AMB both *in vivo* and *in vitro*.[31,32] However, our data showed that AMB exhibited greater antifungal activity against *A. terreus* for unknown reasons; a similar result was also produced in a study by Li et al.[14] Flu and 5-FC were both inactive against all *Aspergillus* spp. in our study. This finding was in accordance with the results of Li et al.,[14] Yenisehirli et al.,[33] and Carrillo-Muñoz et al.,[15] indicating that this resistance should be noted in clinical practice to prevent treatment failure and recurrence of the disease.[11] Furthermore, one *A. niger* isolate found in our study was resistant to ITR with an MIC ≥16 mg/L, and one *A. tubingensis* isolate exhibited cross-resistance to both VOR (MIC ≥ 8 mg/L) and ITR (MIC ≥ 16 mg/L). Similar results reported in Japan showed a trend of *A. niger* and *A. tubingensis* displaying lower susceptibility to VOR and ITR.[16] Therefore, physicians should consider whether VOR and ITR can still be used as topical antifungal agents for aspergillosis treatment.

In conclusion, otomycosis also occurred in northern China in our multicenter study, although it was more prevalent in southern China due to a more favorable environment.

However, in addition to environmental factors, accumulation of cerumen and ear picking in unclear places, frequent water intake,[24] an immunocompromised host, and overuse of topical antibiotic/steroid ear drops are also considered predisposing factors for otomycosis.[34] Considering that *A. welwitschiae* was the major pathogen causing otomycosis in northern China, with *A. niger* and *A. tubingensis* being reported as the prevalent *Aspergillus* spp. in other areas, so it is crucial to develop a rapid identification method to distinguish species from those belonging to *Aspergillus* section *Nigri*. The results of *in vitro* AST indicated that AMB, POS, and echinocandins are potential drugs for the treatment of otomycosis caused by *Aspergillus* spp., but with undetermined clinical efficacy *in vivo*. The increased incidence of azole drug resistance among members from *Aspergillus* section *Nigri* and the mechanisms underlying their resistance remain to be investigated and explored to avoid outbreaks of the disease.

Table 4 *Aspergillus* species isolates (n = 69) categorized as non-wild type to the indicated agents based on the ECVs of CLSI M59.

Aspergillus species (no. of isolates)	AMB	Triazoles			Echinocandins		
		ITR	VOR	POS	AND	MF	CAS
A. welwitschiae (25)	5	0	1	0	NA	NA	0
A. tubingensis (12)	0	1	1	0	NA	NA	0
A. niger (11)	0	1	1	0	NA	NA	0
A. terreus (11)	0	0	0	0	NA	NA	0
A. flavus/A. oryzae (8)	0	0	0	0	NA	NA	0
A. fumigatus (2)	0	0	0	NA	NA	NA	0
Total (69)	5 (7.2)	2 (2.9)	3 (4.3)	0	NA	NA	0

ECVs, epidemiological cut-off values; AMB, amphotericin B; AND, anidulafungin; CAS, caspofungin; ITR, itraconazole; MF, micafungin; POS, posaconazole; VOR, voriconazole.

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Declaration of competing interest

The authors declare that they have no conflicts of interest.

References

1. Patterson TF, Thompson 3rd GR, Denning DW, Fishman JA, Hadley S, Herbrecht R, et al. Practice guidelines for the diagnosis and management of aspergillosis: 2016 update by the Infectious Diseases Society of America. *Clin Infect Dis* 2016;**63**: e1—60. https://doi.org/10.1093/cid/ciw326.
2. Lee JY, Yang PC, Chang C, Lin IT, Ko WC, Cia CT. Community-acquired adenoviral and pneumococcal pneumonia complicated by pulmonary aspergillosis in an immunocompetent adult. *J Microbiol Immunol Infect* 2019;**52**:838—9. http://10.1016/j.jmii.2019.05.014.
3. Lai CC, Wang CY, Hsueh PR. Co-infections among patients with COVID-19: the need for combination therapy with non-anti-SARS-CoV-2 agents? *J Microbiol Immunol Infect* 2020;**53**: 505—12. http://doi:10.1016/j.jmii.2020.05.013.
4. Tsai MH, Lin LC, Hsu JF, Lai MY, Huang HR, Chiang MC, et al. Rapid identification of invasive fungal species using sensitive universal primers-based PCR and restriction endonuclease digestions coupled with high-resolution melting analysis. *J Microbiol Immunol Infect* 2019;**52**:728—35. http://doi:10.1016/j.jmii.2019.06.001.
5. Chien SH, Liu YC, Liu CJ, Ko PS, Wang HY, Hsiao LT, et al. Invasive mold infections in acute leukemia patients undergoing allogeneic hematopoietic stem cell transplantation. *J Microbiol Immunol Infect* 2019;**52**:973—82. https://doi.org/10.1016/j.jmii.2018.09.006.
6. Hagiwara S, Tamura T, Satoh K, Kamewada H, Nakano M, Shinden S, et al. The molecular identification and antifungal susceptibilities of *Aspergillus* species causing otomycosis in Tochigi, Japan. *Mycopathologia* 2019;**184**:13—21. http://doi:10.1007/s11046-018-0299-9.
7. Jia X, Liang Q, Chi F, Cao W. Otomycosis in Shanghai: aetiology, clinical features and therapy. *Mycoses* 2012;**55**:404—9. http://doi:10.1111/j.1439-0507.2011.02132.x.
8. Szigeti G, Sedaghati E, Mahmoudabadi AZ, Naseri A, Kocsube S, Vagvolgyi C, et al. Species assignment and antifungal susceptibilities of black aspergilli recovered from otomycosis cases in Iran. *Mycoses* 2012;**55**:333—8. http://doi:10.1111/j.1439-0507.2011.02103.x.
9. Ismail MT, Al-Kafri A, Ismail M. Otomycosis in damascus, Syria: etiology and clinical features. *Curr Top Med Mycol* 2017;**3**: 27—30. http://doi:10.29252/cmm.3.3.27.
10. Sabz G, Gharaghani M, Mirhendi H, Ahmadi B, Gatee MA, Sisakht MT, et al. Clinical and microbial epidemiology of otomycosis in the city of Yasuj, southwest Iran, revealing *Aspergillus tubingensis* as the dominant causative agent. *J Med Microbiol* 2019;**68**:585—90. http://doi:10.1099/jmm.0.000948.
11. Kamali Sarwestani Z, Hashemi SJ, Rezaie S, Gerami Shoar M, Mahmoudi S, Elahi M, et al. Species identification and *in vitro* antifungal susceptibility testing of *Aspergillus* section *Nigri* strains isolated from otomycosis patients. *J Mycol Med* 2018;**28**:279—84. http://doi:10.1016/j.mycmed.2018.02.003.
12. Samson RA, Noonim P, Meijer M, Houbraken J, Frisvad JC, Varga J. Diagnostic tools to identify black aspergilli. *Stud Mycol* 2007;**59**:129—45. http://doi:10.3114/sim.2007.59.13.
13. Anwar K, Gohar M. Otomycosis; clinical features, pre-disposing factors and treatment implications. *Pak J Med Sci* 2014;**30**: 564—7.
14. Li Y, Wang H, Zhao YP, Xu YC, Hsueh PR. Antifungal susceptibility of clinical isolates of 25 genetically confirmed *Aspergillus* species collected from Taiwan and Mainland China. *J Microbiol Immunol Infect* 2020;**53**:125—32. http://doi:10.1016/j.jmii.2018.04.003.
15. Carrillo-Muñoz AJ, Quindós G, Ruesga M, del Valle O, Pemán J, Cantón E, et al. *In vitro* antifungal susceptibility testing of filamentous fungi with Sensititre Yeast One. *Mycoses* 2006;**49**: 293—7. http://doi:10.1111/j.1439-0507.2006.01250.x.
16. Hashimoto A, Hagiwara D, Watanabe A, Yahiro M, Yikelamu A, Yaguchi T, et al. Drug sensitivity and resistance mechanism in *Aspergillus* section *Nigr*i strains from Japan. *Antimicrob Agents Chemother* 2017;**61**:e02583. 16, http://doi:10.1128/aac.02583-16.
17. Liu W, Sun Y, Chen W, Liu W, Wan Z, Bu D, et al. The T788G mutation in the cyp51C gene confers voriconazole resistance in *Aspergillus flavus* causing aspergillosis. *Antimicrob Agents Chemother* 2012;**56**:2598—603. http://doi:10.1128/AAC.05477-11.
18. CLSI. *Reference method for broth dilution antifungal susceptibility testing of filamentous fungi*. Wayne, PA: Clinical and Laboratory Standards Institute; 2017. document M38-A3.
19. CLSI. *Epidemiological cutoff values for antifungal susceptibility testing*. Wayne, PA: Clinical and Laboratory Standards Institute; 2018. supplement M59.

20. Peng Y, Zhang Q, Xu C, Shi W. MALDI-TOF MS for the rapid identification and drug susceptibility testing of filamentous fungi. *Exp Ther Med* 2019;**18**:4865—73. http://doi:10.3892/etm.2019.8118.
21. CLSI. *Performance standards for antifungal susceptibility testing of filamentous fungi*. Wayne, PA: Clinical and Laboratory Standards Institute; 2017. document M61.
22. Kaya AD, Kiraz N. *In vitro* susceptibilities of *Aspergillus* spp. causing otomycosis to amphotericin B, voriconazole and itraconazole. *Mycoses* 2007;**50**:447—50. http://doi:10.1111/j.1439-0507.2007.01409.x.
23. Viswanatha B, Sumatha D, Vijayashree MS. Otomycosis in immunocompetent and immunocompromised patients: comparative study and literature review. *Ear Nose Throat J* 2012;**91**:114—21. http://doi:10.1177/014556131209100308.
24. Li Y, He L. Diagnosis and treatment of otomycosis in Southern China. *Mycoses* 2019;**62**:1064—8. http://doi:10.1111/myc.12979.
25. Abdelazeem M, Gamea A, Mubarak H, Elzawawy N. Epidemiology, causative agents, and risk factors affecting human otomycosis infections. *Turk J Med Sci* 2015;**45**:820—6. http://doi:10.3906/sag-1407-17.
26. Zhang L, Wang X, Houbraken J, Mei H, Liao W, Hasimu H, et al. Molecular identification and *in vitro* antifungal susceptibility of *Aspergillus* isolates recovered from otomycosis patients in Western China. *Mycopathologia* 2020;**185**:527—35. http://doi:10.1007/s11046-020-00448-7.
27. García-Agudo L, Aznar-Marín P, Galán-Sánchez F, García-Martos P, Marín-Casanova P, Rodríguez-Iglesias M. Otomycosis due to filamentous fungi. *Mycopathologia* 2011;**172**:307—10. http://doi:10.1007/s11046-011-9427-5.
28. Fasunla J, Ibekwe T, Onakoya P. Otomycosis in western Nigeria. *Mycoses* 2008;**51**:67—70. http://doi:10.1111/j.1439-0507.2007.01441.x.
29. Verwer PE, Van Leeuwen WB, Girard V, Monnin V, Van Belkum A, Staab JF, et al. Discrimination of *Aspergillus lentulus* from *Aspergillus fumigatus* by Raman spectroscopy and MALDI-TOF MS. *Eur J Clin Microbiol Infect Dis* 2014;**33**:245—51. http://doi:10.1007/s10096-013-1951-4.
30. Li Y, Wang H, Zhang G, Liu WJ, Xu YC. Comparative evaluation of Sensititre YeastOne colorimetric panel with broth microdilution method for *in vitro* antifungal susceptibility testing of *Aspergillus*. *Chin J Mycol* 2020;**15**:197—201.
31. Vahedi Shahandashti R, Lass-Flörl C. Antifungal resistance in *Aspergillus terreus*: a current scenario. *Fungal Genet Biol* 2019;**131**:103247. http://doi:10.1016/j.fgb.2019.103247.
32. Posch W, Blatzer M, Wilflingseder D, Lass-Flörl C. *Aspergillus terreus*: novel lessons learned on amphotericin B resistance. *Med Mycol* 2018;**56**:73—82. http://doi:10.1093/mmy/myx119.
33. Yenisehirli G, Bulut Y, Guven M, Gunday E. *In vitro* activities of fluconazole, itraconazole and voriconazole against otomycotic fungal pathogens. *J Laryngol Otol* 2009;**123**:978—81. http://doi:10.1017/S0022215109005489.
34. Stern JC, Lucente FE. Otomycosis. *Ear Nose Throat J* 1988;**67**:804—5. 09-10.