

# Molecular Identification of *Aspergillus* Species, Antifungal Susceptibility, and Phenotypic Identification of Azole-Resistant Mutations in *Cyp51A* Gene Isolated from Xinjiang

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**Purpose:** This study aimed to determine the clinical distribution characteristics, in vitro antifungal susceptibility, and *cyp51A* mutation types of clinically isolated *Aspergillus* species in Xinjiang.

**Methods:** In this study, a total of 111 *Aspergillus* species were identified by sequencing the internal transcribed spacer (ITS) and  $\beta$ -tubulin (*BenA*) genes for molecular identification, performed antifungal susceptibility testing on these isolates using Sensititre YeastOne, selected azole-resistant isolates based on the antifungal susceptibility results and amplified the *cyp51A* gene for identification of the azole resistance mutation phenotype in the selected isolates.

**Results:** The most common *Aspergillus* species was *A. fumigatus* (40.54%), followed by *A. niger* (18.02%), *A. tubingensis* (16.22%), *A. terreus* (13.51%), *A. flavus* (6.31%), *A. welwitschiae* (2.70%), *A. fumigatiaffinis* (1.80%), and *A. lentulus* (0.90%). The antifungal susceptibility test results showed that *A. fumigatus*, *A. niger*, *A. tubingensis*, *A. flavus* and *A. terreus* were completely sensitive to itraconazole, with sensitivity rates of posaconazole and voriconazole were 99.10% and 88.29%, respectively. The sensitivity rate to amphotericin B was the lowest (62.16%). The MIC values of amphotericin B and voriconazole for the two cryptic *Aspergillus* species, *A. lentulus* and *A. fumigatiaffinis* with high (>1mg/L). The azole non-susceptible or non-wild type rate was (15/111, 13.51%). Eleven azole-resistant *Aspergillus* species had *cyp51A* mutations, while four strains did not have any *cyp51A* mutations.

**Conclusion:** In this study, the pathogenic *Aspergillus* species isolated from clinical cases in Xinjiang were diverse. Common pathogenic species showed the best in vitro antifungal activity against itraconazole, posaconazole, and echinocandins, whereas the MIC distribution of amphotericin B was significantly higher. Resistant strains may be mediated by point mutations in *cyp51A*, and phenotypic mutations are diverse. This information is of great significance for guiding the early diagnosis and antifungal therapy for aspergillosis.

**Keywords:** invasive aspergillosis, *Aspergillus fumigatus*, azole-resistance, antifungal susceptibility, *cyp51A* gene

## Introduction

Invasive fungal infections can cause a range of serious human diseases, but little is known about increasingly serious invasive fungal diseases. Among them, invasive aspergillosis (IA) is a systemic fungal infection that endangers patients' lives, mainly affecting patients with impaired immune function, with a mortality rate of up to 60%, and a mortality rate of around 20–30% when treated with voriconazole and isavuconazole as the first-line treatment.<sup>1</sup> Pathogenic *Aspergillus* species are the main cause of infections and deaths. Approximately 30 species of *Aspergillus*, with common ones including *A. fumigatus*, *A. flavus*, *A. niger* and *A. terreus*.<sup>2</sup> Among them, *A. fumigatus* has been reported to cause IA in human. Additionally, the warming climate and changes in the ecological niche of environmental fungi have led to the evolution and emergence of new *Aspergillus* pathogens, especially the emergence and spread of drug-resistant strains, which has exacerbated the difficulty in diagnosing and treating invasive aspergillosis.

Azole antifungal drugs are recommended as the first-line treatment and preventive drugs for *Aspergillus* infection.<sup>3</sup> However, increasing reports of *Aspergillus* resistance to azoles have emerged in recent years.<sup>4</sup> In recent years, it has also been reported that a number of *A. fumigatus* isolates from specimens of patients with novel coronavirus pneumonia-associated pulmonary *Aspergillus* have been reported to be resistant to multiple azole antifungal agents.<sup>5,6</sup> The failure rate of azole-resistant aspergillosis treatment is very high, with a mortality rate of up to 88%, which is 3–4 times that of patients with azole-sensitive aspergillosis.<sup>7</sup> Due to the limited types of antifungal drugs, some invasive aspergillosis, especially those affecting the central nervous system, will greatly increase the difficulty of clinical treatment if they are drug-resistant strains.

The mechanism of action of azoles is to inhibit ergosterol 14 $\alpha$ -demethylase (cyp51), blocking the ergosterol biosynthesis pathway, leading to a reduction in ergosterol synthesis, and exerting antifungal effects. The cyp51A gene mutation is the most common drug resistance mechanism of *A. fumigatus*,<sup>8–10</sup> the mutation types include point mutations, such as M220, G54, G138 and other hotspot amino acid substitutions, which reduce the affinity with azole antifungal drugs, these types of mutations are considered therapeutic drug-inducing mutations. The other type is tandem repeat non-synonymous mutations in the promoter region of cyp51A, such as TR34/L98H and TR46/Y121F/T289A, which are drug resistance mutations caused by exposure to environmental azole fungicides, and result in cross-resistance to multiple azole classes.<sup>11</sup>

Nevertheless, studies have reported that over 40% of *A. fumigatus* may be related to non-cyp51A mutations that lead to azole resistance among *A. fumigatus* isolates.<sup>12</sup> One of the issues is the mutations in the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase-encoding gene (*hmg1*) associated with triazole-resistance *A. fumigatus* isolates. Interestingly, a new study investigated the combination of *hapE* and *hmg1* mutations as potential contributors to azole resistance.<sup>13,14</sup> In addition to these mutations, other candidate genes such as *crd1B*, *MDR1*, *MDR2*, and *erg6* have also been extensively studied and are believed to play a role in non-cyp51A azole resistance. The growing number of resistant strains and the continuous emergence of new resistance mechanisms have aroused great concern worldwide.

Despite the increasing threat of invasive fungal infections caused by known or novel *Aspergillus* pathogens, there is still no research on the epidemiological characteristics of *Aspergillus* infections, in vitro antifungal drug susceptibility, or drug resistance in Xinjiang. Therefore, the present study was conducted to investigate the epidemiological distribution characteristics, drug sensitivity, and azole-resistant mutant phenotype of *Aspergillus* strains in Xinjiang to provide useful information for the early diagnosis and treatment of invasive fungal infections.

## Materials and Methods

### Sample Collection and Strain Culture

A total of 111 *Aspergillus* clinical strains were collected from 2011 to 2023 from the fungal specimen library of the Dermatology Laboratory of the First Affiliated Hospital of Xinjiang Medical University. After resuscitating the frozen preserved strains at  $-80^{\circ}\text{C}$  in a cryogenic refrigerator back to room temperature, a small amount of fungal liquid was collected with a sterile inoculation ring and inoculated onto Potato Dextrose Agar (PDA). The inoculated plates were incubated at  $28^{\circ}\text{C}$  for 3–5d. The colonies were observed for their macroscopic characteristics, including colony color, texture, and growth rate. For microscopic examination, a small portion of the colony was transferred to a glass slide and stained with lactophenol cotton blue. The slide was then observed under a light microscope at  $400\times$  magnification to examine the morphological features of the conidiophores, conidia, and vesicle structures.

### Aspergillus Isolates and Molecular Identification

DNA was extracted from isolates grown on PDA plates using the Ezup DNA Isolation Kit (Sangon Biotech, Shanghai, China) following the manufacturer's instructions. The clinical isolates were molecularly identified by PCR and sequencing of the ITS and *BenA* genes using the primer pair ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), and the primer pair BT2a (5'-GGTAACCAAATCGGTGCTGCTTTC-3') and BT2b (5'-ACCCCTCAGTGTAGTGACCCTTGGC-3'). The thermal cycling profiles for ITS and *BenA* amplification were as follows: 5 min at  $95^{\circ}\text{C}$ , followed by 30 cycles of  $94^{\circ}\text{C}$  for 30s,  $57^{\circ}\text{C}$  for 30s, and  $72^{\circ}\text{C}$  for 90s, with a final

**Table 1** The Clinical and Epidemiological Breakpoints of Different *Aspergillus* Species to Commonly Used Antifungal Drugs

Species	AMB	ITZ	VOR	POS	CAS	MCF
<i>A. fumigatus</i>	≤2	≤1	≤1	≤1	≤0.5	≤0.5
<i>A. flavus</i>	≤4	≤1	≤2	≤0.5	≤0.5	≤0.5
<i>A. niger</i>	≤2	≤4	≤2	≤2	≤0.25	≤0.25
<i>A. terreus</i>	≤4	≤2	≤2	≤1	≤0.12	≤0.25

**Abbreviations:** AMB, amphotericin B; ITZ, itraconazole; VOR, voriconazole; POS, posaconazole; CAS, caspofungin; MCF, micafungin.

extension step at 72°C for 10 min. The PCR reaction system is as follows: 1 µL of 10 mM dNTPs, 2.5 µL of 10X PCR buffer, 1 µL of 10 µM of each primer, 1 U Taq DNA polymerase, 1 µL genomic DNA, and DNase-free water, up to a final reaction volume of 25 µL. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel, the resultant PCR amplicons were purified and sequenced, and the obtained sequences were compared to reference sequences in GenBank.

## Antifungal Susceptibility Testing

Sensititre YeastOne YO10 (Thermo Fisher) was used for antifungal susceptibility testing with the seven antifungal agents, according to the manufacturer's instructions. The seven antifungal agents used in this study were amphotericin B (AMB), itraconazole (ITZ), voriconazole (VOR), posaconazole (POS), caspofungin (CAS), anidulafungin (AND), and micafungin (MCF). The antifungal agents were at a final concentration of 0.12–8 µg/mL for amphotericin B, 0.008–8 µg/mL for caspofungin, micafungin, voriconazole, and posaconazole, 0.015–16 µg/mL for itraconazole, and 0.015–8 µg/mL for anidulafungin. The conidial inoculum suspension was prepared at a turbidity of 0.5 McFarland units for the assay. With 48h of culturing (except for echinocandins, which were cultured for 24h), the antifungal drug sensitivity profiles of these inocula were determined according to the manufacturer's instructions. All isolates were tested according to Clinical and Laboratory Standards Institute (CLSI) M38-A3. Quality control was ensured using the strain recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST): *Candida Krusei* ATCC6258. The clinical and epidemiological breakpoint values of different *Aspergillus* species for azole antifungal drugs are shown in Table 1.

## Sequencing of cyp51A Gene

Azole-resistant strains were screened based on the results of the in vitro susceptibility testing. Genomic DNA of 15 strains of azole-resistant *Aspergillus* was extracted using a Fungus Genomic DNA Extraction Kit following the manufacturer's instructions. The cyp51A sequences of non-WT *Aspergillus* isolates were amplified using previously described PCR primers (Table 2). The cyp51A thermal cycling profile for amplification was as follows: 95°C for 5 min, followed by 30 cycles of 94°C for 30s, 63°C for 30s, and 72°C for 30s, with a final step of 72°C for 10 min. DNA sequences were compared with the cyp51A sequences of the reference strains of *A. fumigatus* (GenBank accession AF338659), *A. flavus* (GenBank accession NRRL3357), *A. niger* (GenBank accession ATCC 1015), *A. tubingensis* (GenBank accession F13880), and *A. terreus* (GenBank accession NIH 2624) to detect point mutations associated with azole resistance.

## Statistical Analysis

Statistical analyses and graphing were performed using SPSS 22 and Origin 2018 software. Count data were presented as frequencies or constituent ratios. A *P* value <0.05 was considered statistically significant for all statistical analyses.

**Table 2** PCR Primers Used to Amplify the *cyp51A* Gene

Species	Primer Name	Sequence (5'-3')	Reference
<i>A. fumigatus</i>	Cyp51A-5	5'-ATAATCGCAGCACCCTTCAGA-3'	[15]
	Cyp51A-7	5'-CCTTGTCAACGTCAGACGG-3'	
	Cyp51A-6	5'-TGGATGTGTTTTTCGACCGCTT-3'	
	Cyp51A-8	5'-CGGATCGGACGTGGTGTATG-3'	
	BT-2A	5'-GGTAACCAAATCGGTGCTGCTTTC-3'	
	BT-2B	5'-ACCCTCAGTGAGTGACCCCTTGGC-3'	
<i>A. terreus</i>	Cyp-AT-0	5'-GGTGGGAGAACTTTTCGTCTA-3'	[16]
	Cyp-AT-1	5'-ATTGGACCTCTACAACAACAATG-3'	
	Cyp-AT-2	5'-GAGGTCAGTGGTTTGTATGGAG-3'	
	Cyp-AT-3	5'-CTCCATACAAACCACTGACCTC-3'	
	Cyp-AT-4	5'-ACGGCAGCATGAAGTTGAT-3'	
	Cyp-AT-5	5'-CGAGTTTGCCGACCTCTAC-3'	
	Cyp-AT-6	5'-AGCGGGTTCTTCACCTTG-3'	
	Cyp-AT-7	5'-CCAGAATGTCGTCAAGGAGA-3'	
<i>A. niger</i>	Cyp-AT-8	5'-GCCGTTCTTACGCCTTGT-3'	[17]
	Ancyp51A F1	5'-CGACAACAACCTAGTACTTCAATGTCTTGC-3'	
	Ancyp51A F2	5'-CGTCCAGCTGATCGAAAAGGAACTCTCG-3'	
	Ancyp51A F3	5'-GCAGTATCAGGACCTTGACAAGCTGC-3'	
<i>A. tubingensis</i>	Ancyp51A R	5'-CGAGAGTTTCCTTTTCGATCAGCTGGACG-3'	[17]
	Atcyp51A F1	5'-ATGGCATATCTTGCTGTTGCAGGCGCCTAC-3'	
	Atcyp51A F2	5'-GTCCGACGTTGTGTACGACTG-3'	
	Atcyp51A F3	5'-CAAGAACCCAGACGAGGAGAAG-3'	
<i>A. flavus</i>	Atcyp51A R	5'-TTAGTTCAAGGACCCCTTGGAGTTGTC-3'	[18]
	Aflacyp51A F1	5'-CAAGAACAGCCTGCACAGAG-3'	
	Aflacyp51A R1	5'-GGGTGGATCAGTCTTATTA-3'	
	Aflacyp51A F2	5'-GCAATCATCGTCCTAAATC-3'	
	Aflacyp51A R2	5'-CTGTCCATTCTGTAGGTA-3'	
	Aflacyp51A F3	5'-GCATGAGGGAGATCTATATG-3'	
	Aflacyp51A R3	5'-CCTATAATTGCTGGTTTCG-3'	
	Aflacyp51A F4	5'-TGAAGCTATTCAATGTAGAC-3'	
	Aflacyp51A R4	5'-ACTGCTGATGGTGTGCTAAG-3'	

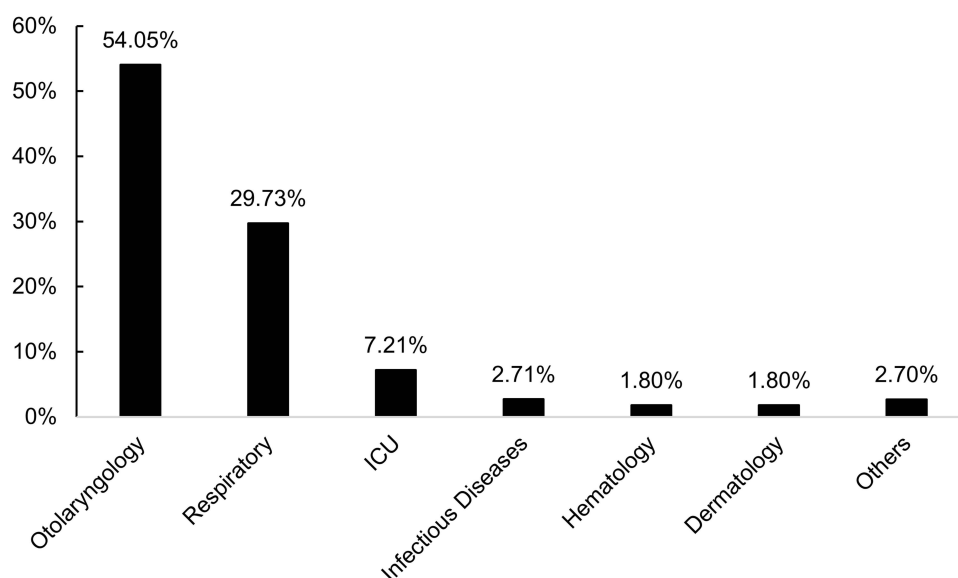
## Results

### Strain Clinical Information

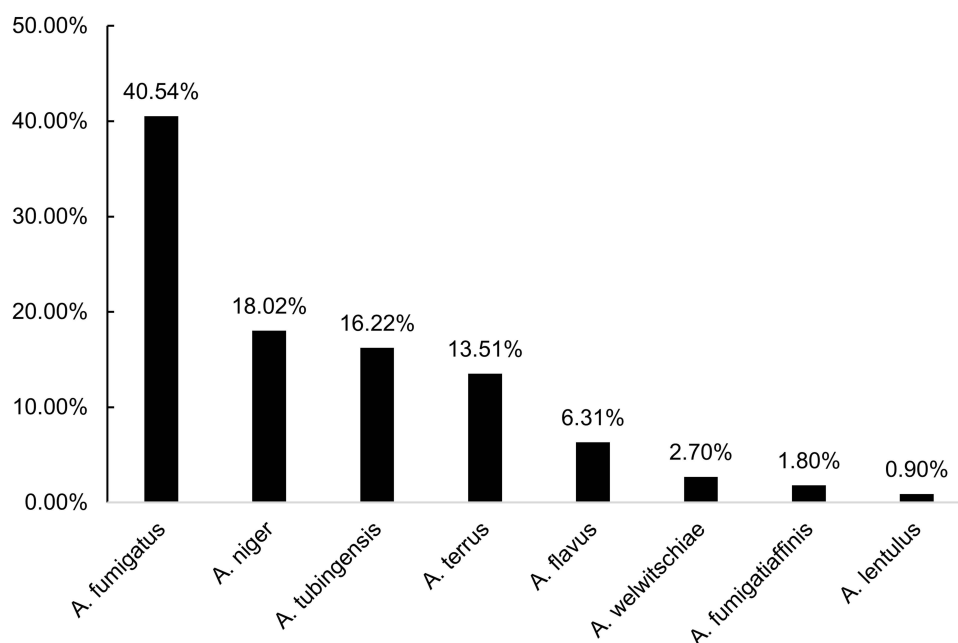
*Aspergillus* spp. were isolated from 111 patients with invasive fungal infections between 2021 and 2023. Of the 111 isolates examined, 54 were obtained from male patients, and the remaining 57 were obtained from female patients. The patients ranged in age from 20 to 95 (57.87±17.47) years, with a mean age of 59 years. Regarding clinical origin types, 55.86% of the *Aspergillus* isolates (62/111) were recovered from ear canal secretions, 40.54% (45/111 isolates) from sputum, 1.80% (2/111 isolates) from bronchoalveolar lavage fluid, and 1.80% (2/111 isolates) from other body fluid secretions. The detailed clinical departments of each patient are presented in Figure 1.

### Strain Identification and Department Distribution

Based on molecular identification, the most common species was *A. fumigatus* (n=45), followed by *A. niger* (n=20), *A. tubingensis* (n=18), *A. terreus* (n=15), *A. flavus* (n=7), *A. welwitschiae* (n=3), *A. fumigatiaffinis* (n=2), and *A. lentulus* (n=1) (Figure 2). In the otolaryngology department, *A. tubingensis* accounted for 30%, *A. niger* for 21.67%, *A. fumigatus* and *A. terreus* for 20%. In the respiratory department, *A. fumigatus* accounted for 60.60% and *A. niger* accounted for 18.18%. *A. fumigatus* accounted for 87.5% of patients in the department of intensive care medicine (Figure 3).



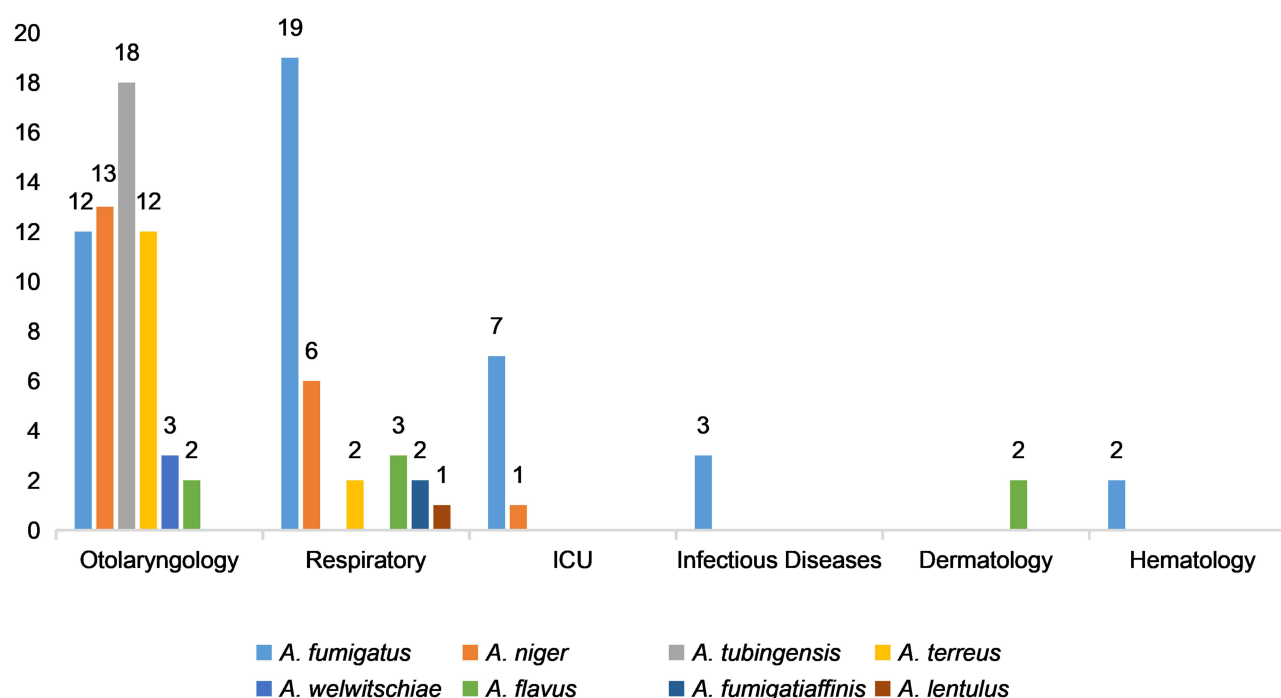
**Figure 1** Distribution of *Aspergillus* isolates in the clinical department.



**Figure 2** Molecular identification of *Aspergillus* species.

## Antifungal Susceptibility Testing

The antifungal resistance of the 111 *Aspergillus* isolates against seven active antifungals are shown in Table 3. For echinocandins, all *Aspergillus* isolates revealed lower MECs and Modal MECs  $\leq 0.25$   $\mu\text{g/mL}$  to the AND, CAS, and MCF. For AMB, AMB had significantly higher MICs for *Aspergillus* species than other antifungal drugs. According to EUCAST, all 111 strains of *Aspergillus* species had MICs  $\geq 2.00$   $\mu\text{g/mL}$  to AMB. *A. fumigatus* and *A. niger* were resistant to AMB at 2  $\mu\text{g/mL}$ , with 46.67% (21/45) *A. fumigatus* resistant to AMB and 45.00% (9/20) *A. niger* being resistant to AMB. *A. flavus* and *A. terreus* were resistant to AMB at 4  $\mu\text{g/mL}$ , 14.29% (1/7) of *A. flavus* isolates were resistant to AMB, and 0% (0/20) of *A. terreus* isolates were resistant to AMB. For azoles, the most clinical *Aspergillus* species had MICs  $\leq 0.50$   $\mu\text{g/mL}$  for POS (98.20%, n=109) and ITZ (91.90%, n=102). The prevalence of VOR resistance was 6.67%



**Figure 3** The source distribution of *Aspergillus* species specimens. The y axis shows the number of isolates.

in *A. fumigatus*, 28.57% in *A. flavus*, 16.67% in *A. tubingensis*, 10.00% in *A. niger*, and 20.00% in *A. terreus*, which are summarized in Figure 4. In addition, the drug susceptibility characteristics of the six cryptic *Aspergillus* species found in this study showed that the three strains of *A. welwitschiae* were sensitive to seven antifungal agents. *A. lentulus* and *A. fumigatiaffinis* are resistant to VOR, and the MIC for AMB is 2–8 µg/mL. Of the 111 isolates, 15 that showed azole-resistance to the tested antifungal drugs (3 *A. fumigatus*, 2 *A. flavus*, 3 *A. tubingensis*, 3 *A. terreus*, 2 *A. niger*, 1 *A. lentulus* and 1 *A. fumigatiaffinis*) were identified as azole-resistant.

**Table 3** MICs/MECs Range of Various Antifungal Agents Against Different *Aspergillus* Species

Species	Drug	Range	≤0.008	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16
<i>A. fumigatus</i>	AND	0.015–0.12		37	7		1							
	MCF	≤0.008–0.12	28	10	5		2							
	CAS	≤0.008–0.25	4	13	15	7	3	3						
	POS	0.03–2			2	5	17	19	1		1			
	VOR	0.25–4						17	24	1	2	1		
	ITZ	0.06–0.5				2	5	28	10					
	AMB	2–8									24	19	2	
<i>A. niger</i>	AND	0.015–0.12		9	7	2	2							
	MCF	≤0.008–0.12	8	3	5	2	2							
	CAS	≤0.008–0.25	1	2	2	8	3	4						
	POS	0.06–1				1	4	12	2	1				
	VOR	0.25–4						3	8	7		2		
	ITZ	0.25–1						5	12	3				
	AMB	2–4									11	9		

(Continued)

**Table 3** (Continued).

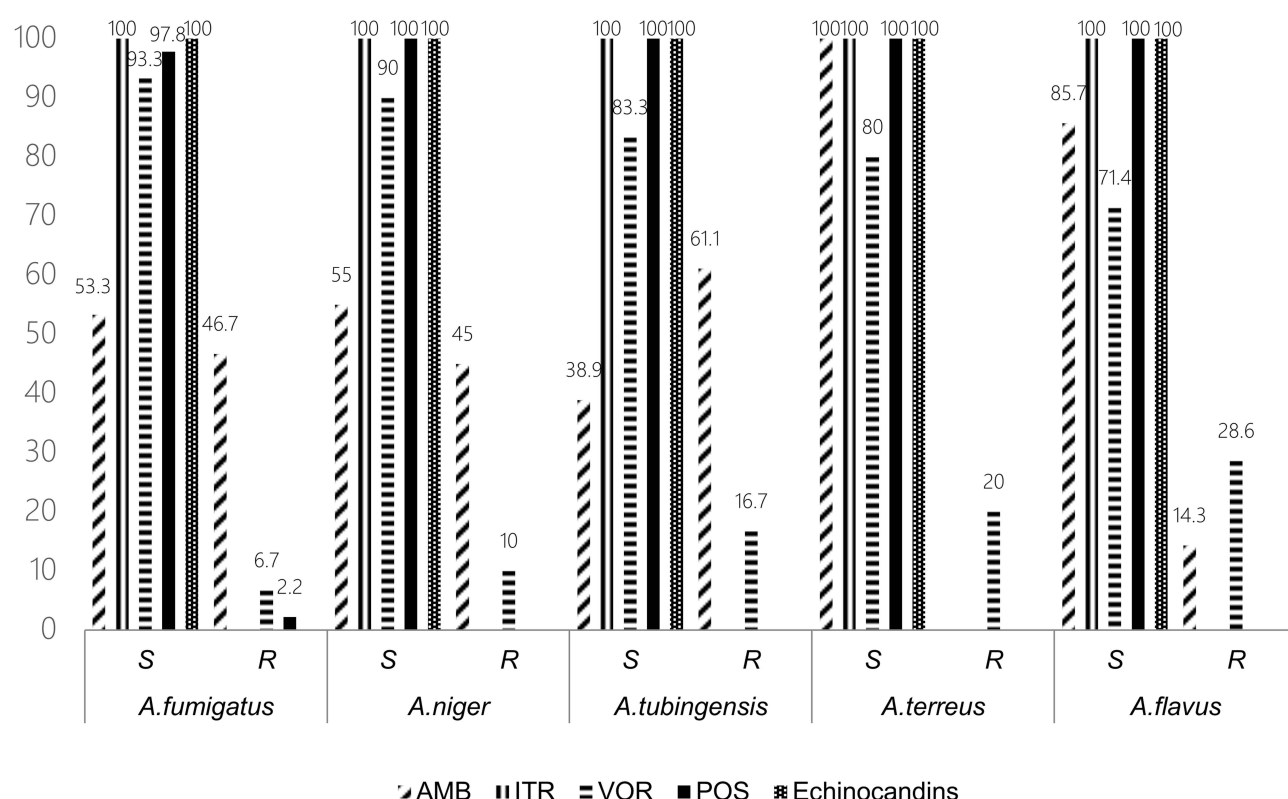
Species	Drug	Range	≤0.008	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16
<i>A. tubingensis</i>	AND	0.015–0.12		8	7	2	1							
	MCF	≤0.008–0.25	8	5	2		2	1						
	CAS	≤0.008–0.12	4	2	5	4	3							
	POS	0.06–0.5				1	3	10	4					
	VOR	0.25–4						5	7	3		3		
	ITZ	0.12–1					3	3	8	4				
	AMB	2–4									7	11		
<i>A. terreus</i>	AND	0.015–0.03		14	1									
	MCF	≤0.008–0.06	9	2	1	3								
	CAS	≤0.008–0.12	5	6	3		1							
	POS	0.03–0.25			1	4	5	5						
	VOR	0.25–4						1	9	2		3		
	ITZ	0.12–0.25					4	10	1					
	AMB	2–4									6	9		
<i>A. flavus</i>	AND	0.015–0.03		6	1									
	MCF	≤0.008–0.015	5	2										
	CAS	≤0.008–0.12	4	2			1							
	POS	0.03–0.25			1		1	5						
	VOR	0.25–4						1	3	1		2		
	ITZ	0.12–0.25					2	5						
	AMB	2–8									2	4	1	
<i>A. welwitschiae</i>	AND	0.015–0.06		1	1	1								
	MCF	0.03–0.25			1		1	1						
	CAS	0.12–0.25					1	2						
	POS	0.06–0.5				1		1	1					
	VOR	0.25–0.5						1	2					
	ITZ	0.12–0.5					1	1	1					
	AMB	2–4									2	1		
<i>A. fumigatiifinis</i>	AND	0.015		2										
	MCF	0.015		2										
	CAS	0.03–0.06			1	1								
	POS	0.5							2					
	VOR	2–4									1	1		
	ITZ	0.5–1							1	1				
	AMB	4–8										1	1	1
<i>A. lentulus</i>	AND	≥8											1	1
	MCF	≥8											1	1
	CAS	≥8											1	1
	POS	0.5							1					
	VOR	2									1			
	ITZ	1								1				
	AMB	4										1		

**Abbreviations:** MIC, minimum inhibitory concentration; MEC, minimum effective concentration; AMB, amphotericin B; ITZ, itraconazole; VOR, voriconazole; POS, posaconazole; AND, anidulafungin; CAS, caspofungin; MCF, micafungin.

## Cyp51A Mutation Phenotypes

Table 4 shows cyp51A mutations found in azole-resistant *Aspergillus* strains. Three *A. fumigatus* strains were azole resistant, had no tandem repeat in its cyp51A promoter region, but one of them (XJ-04) harbored the (F46Y, M172V, and D255E) mutation, while one strain (XJ-14) carried a G138 substitution, and another one (XJ-05) had no mutation in the cyp51A gene. Of the 3 non-WT *A. tubingensis* isolates, one (XJ-08) harbored the (G202, I335, and F345) mutation, two





**Figure 4** Antifungal susceptibility profiles of *Aspergillus* species according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) Clinical Breakpoints. **Abbreviations:** AMB, amphotericin B; ITZ, itraconazole; VOR, voriconazole; POS, posaconazole; S, susceptible; R, resistant; Echinocandins, anidulafungin, caspofungin and micafungin.

(XJ-01 and XJ-09) harbored the (G202 and F345) mutation. Two *A. niger* strains, one (XJ-11) had the T57A mutation, one (XJ-12) carried the (F29V and T57A) mutation in the *cyp51A* gene. In *A. terreus*, F200L were found only in (XJ-10) isolates, two (XJ-03, XJ-06) azole-resistant strains did not have mutations in the *cyp51A* gene. Likewise, two *A. flavus* isolates (XJ-02 and XJ-07) showed the amino acid substitution (P55, K130, F182, Y247F, and P388). Among the cryptic species, only *A. lentulus* showed the F46Y/N248T mutation combination.

**Table 4** Characterisation of *cyp51A* Amino Acid Substitutions in Fifteen Clinical *Aspergillus* Species Isolates With Triazole MICs Above the ECV

Isolate No.	Year of Isolation	Antifungal MIC (µg/mL)							Cyp51A Amino Acid Substitutions			
		ITZ	VOR	POS	AND	MCF	CAS	AMB				
<i>A. fumigatus</i>												
04	2015	0.25	2	0.5	0.03	0.12	0.25	2	F46Y	M172V	D255E	
05	2015	0.5	2	0.25	0.12	0.03	0.12	2	–	–	–	
14	2023	0.5	4	2	≤0.015	≤0.008	0.015	2	G138	–	–	
<i>A. tubingensis</i>												
01	2013	0.5	4	0.25	0.03	0.12	0.25	2	G202	–	F345	
08	2018	1	4	0.5	0.12	0.015	0.015	2	G202	I335	F345	
09	2018	1	4	0.5	0.03	≤0.008	0.015	4	G202	–	F345	

(Continued)



**Table 4** (Continued).

Isolate No.	Year of Isolation	Antifungal MIC (µg/mL)							Cyp51A Amino Acid Substitutions			
		ITZ	VOR	POS	AND	MCF	CAS	AMB				
<i>A. niger</i>												
11	2020	1	4	0.5	0.06	≤0.008	0.06	2	–	T57A		
12	2023	1	4	1	0.03	0.03	0.25	4	F29V	T57A		
<i>A. terreus</i>												
10	2019	0.25	4	0.25	≤0.015	≤0.008	0.015	4	F200L			
03	2014	0.25	4	0.25	≤0.015	0.03	≤0.008	4	–			
06	2016	0.25	4	0.06	≤0.015	0.06	0.12	4	–			
<i>A. flavus</i>												
02	2013	0.25	4	0.12	≤0.015	≤0.008	≤0.008	2	P55	K130	F182	P388
07	2016	0.25	4	0.25	≤0.015	≤0.008	0.015	4	–	Y247F	F182	P388
<i>A. lentulus</i>												
15	2011	1	2	0.5	≥8	≥8	≥8	4	F46Y	N248T		
<i>A. fumigati</i> affinis												
13	2022	1	2	0.5	≤0.015	0.015	0.03	8	–			

**Abbreviations:** AMB, amphotericin B; ITZ, itraconazole; VOR, voriconazole; POS, posaconazole; AND, anidulafungin; CAS, caspofungin; MCF, micafungin.

## Discussion

In this study, we report the findings from the survey of 111 *Aspergillus* species isolated from patients diagnosed with aspergillosis at the First Affiliated Hospital of Xinjiang Medical University. We characterized the species distribution and in vitro susceptibility to seven antifungal agents. For the azole-resistant *Aspergillus* species, we further determined the azole non-susceptible or non-wild type mechanisms by focusing on mutations in *cyp51A*. Our results provide a regional perspective on the management of patients with aspergillosis and pathogen characterization in China.

A total of 111 strains of *Aspergillus* species were collected and mainly isolated from ear secretion specimens, followed by sputum. The main sources of departments were otolaryngology department, respiratory department, and intensive medicine department. Sputum having *A. fumigatus* isolated in high proportion from specimens. The current study's finding is similar to the previously reported 20-year retrospective study from China, indicating the endemic nature of *A. fumigatus* as a respiratory tract pathogen in the region.<sup>19</sup> The *A. niger* and *A. tubingensis* were isolated in high proportions from ear secretion specimens. The association between *A. niger*, *A. tubingensis* and ear infection has been well documented in previous studies, which reported that almost more than half of otomycosis cases are caused by *A. niger* and *A. tubingensis*.<sup>20</sup> Data reported by Li from China<sup>21</sup> showed that *A. tubingensis* and *A. niger* were the main pathogenic fungi causing otomycosis in China, and there was no difference in their proportion. This may explain why the predominance of *A. tubingensis* in otomycosis in western China is likely to be due to dry, dusty, and windy environments.

In the present study, *A. fumigatus* was the most frequently isolated species, accounting for 40.54% of the isolates collected, similar to the rates observed in several epidemiological studies in other countries.<sup>22,24</sup> In our study, *A. niger* was the most common non-fumigatus *Aspergillus* species, which is in accordance with previous studies in Switzerland and Korea.<sup>25,26</sup> However, *A. flavus* was reported as the second most common species in the United States, Iran, and Brazil.<sup>27–29</sup> *A. terreus* and *A. niger* were the third and fourth most common isolates, respectively, indicating geographical variations in the prevalence of different species. For example, this could be due to the arid climate in Iran, which favors the growth of thermo-tolerant fungi such as *A. flavus*. This would lead to a higher prevalence of *A. flavus* in these regions, which is supported by the aspergillosis surveys conducted in these countries.

Regarding the antifungal susceptibility testing, echinocandins showed good activity against the *Aspergillus* isolates in this collection, while anidulafungin and micafungin can effectively inhibit the growth of more than 98% of strains when MEC is ≤0.12µg/mL, and CAS can effectively inhibit the growth of all strains when MEC is ≤0.25µg/mL. AND and MCF appeared to be more potent than CAS, which is consistent with the results of several previous studies.<sup>30,31</sup> Owing to the limited use of echinocandins in clinical settings in China, all three echinocandins presented ideal potency against

*Aspergillus* in vitro, and no resistant isolates were detected. Amphotericin B is widely used in hospitals as a salvage and last-line drug to treat severe and urgent cases of triazole-resistant *Aspergillus* infections.<sup>32,33</sup> In our study, the AMB MICs of all 111 isolates were  $\geq 2 \mu\text{g/mL}$ . Although little is known about the worldwide susceptibility of *Aspergillus* species to AMB, up to now, the identical incidence of high AMB MIC has been described in Korea and Canada.<sup>26,34</sup> Nonetheless, the reasons behind the emergence of high AMB resistance rates in these two geographic populations are still unknown. Resistance to AMB may involve two mechanisms. One is a mutation in the synthesis pathway that leads to a decrease in ergosterol concentration in the cell membrane and the other is an increase in the production of catalase, which can protect cells from oxidative stress caused by the drug. Resistance to AMB is usually due to a reduction in ergosterol or a change in the ergosterol biosynthesis pathway that results in a decrease in the target lipid on the plasma membrane, thereby reducing the ability to bind AMB,<sup>35</sup> spore germination upon UV irradiation,<sup>36</sup> and mutations in genes encoding sphingolipid FEN1 and SUR4.<sup>37</sup> In our study, 37.84% of the *Aspergillus* species were resistant to AMB. Therefore, the physicians in the region under study should carefully consider the prescription of AMB for *Aspergillus* infection treatment, and further exploration should be conducted to investigate the reasons for the widespread emergence of AMB resistance in this area. ITZ was the most efficient antifungal agent against this set of isolates (100.00% of the isolates were susceptible to ITZ), followed by POS (99.10%) and VOR (88.29%). The local rates of resistance to azoles in *Aspergillus* species was 13.51%, with 6.67% in *A. fumigatus*. In agreement with our findings, the susceptibility profiles of 227 clinical *Aspergillus* species collected from the northern Portugal also showed that most of the isolates were susceptible to ITZ (95.8%), VOR (97.4%), and POS (84.7%).<sup>38</sup> Moreover, in Anhui, China,<sup>39</sup> a reduced susceptibility to VOR was observed in 4.11% of the *Aspergillus* isolates, whereas none of them were resistant to POS and ITZ. This rate of resistance has been attributed to long-term azole therapy in patients with chronic aspergillosis in addition to cross-resistance to agricultural triazoles.

We also identified three other cryptic species that contributed 5.41% of all isolated *Aspergillus* species. It has been reported that in Iran, *A. welwitschiae* (former name *A. awamori*) in section Nigri serves as the pathogen of otomycosis,<sup>40,41</sup> and the species has also been isolated from human nails, causing onychomycosis.<sup>41</sup> *A. lentulus* and *A. fumigatiaffinis* are cryptic species of *A. fumigatus* complex. They have been observed to exhibit high MICs for all triazoles in several studies and are considered to have intrinsic azole resistance. In this study, the *A. lentulus* isolates had MICs of 0.5  $\mu\text{g/mL}$ , 2  $\mu\text{g/mL}$ , and 1  $\mu\text{g/mL}$  to POS, VOR, and ITR, respectively. Two *A. fumigatiaffinis* isolates were VOR resistant. Although these data were suggestive of intrinsic resistance to azoles in these species, it should not be overlooked that susceptibility varied among isolates. Notably, POS appears to retain remarkable antifungal activity against these cryptic species. This suggests the necessity of a surveillance program on azole resistance in non-*fumigatus* *Aspergillus* species as well as their genetic mechanisms.

Drug resistance mechanisms, such as mutations in *cyp51A*, increase in *cyp51A* expression, upregulation of efflux pumps, and other mechanisms, have been reported in *A. fumigatus*.<sup>33</sup> However, the emergence of non-synonymous hotspot mutations in the *cyp51* gene represents the main molecular mechanism associated with azole resistance. However, little information is available regarding other pathogenic *Aspergillus* species. Here, we attempted to understand the relationship between drug resistance and *cyp51A* mutations in *Aspergillus* species. In our study, we found amino acid substitutions (F46Y, M172V, D255E, and G138) in the *cyp51A* protein of resistant *A. fumigatus*. The resistance mutations (G138) tend to arise during prolonged treatment of chronic aspergillosis with azole drugs, which has been exemplified by several cases.<sup>42,43</sup> Other mutation-harboring *A. fumigatus* strains found in our study, including amino acid substitution (F46Y, M172V, and D255E) are scarcely related to azole resistance.<sup>44,45</sup> In addition, in one azole-resistant *A. fumigatus* isolate, none of the mutations were found, indicating that the elevated VRC MICs found for these strains could be due to other mechanisms. Three non-synonymous mutations (P55, K130, F182, Y247F, and P388) were found to be associated with drug resistance in the *cyp51A* sequence of resistant strains of *A. flavus*.<sup>18</sup> Regarding our results in the *A. niger*, sequence analysis of *cyp51A* revealed no general correlation between amino acid changes and azole resistance, since most of the mutations were present in both WT and non-WT strains. This is the case for F29V and T57A substitutions in *A. niger*, which were previously reported by others as well.<sup>17,46</sup> However, the G202, I335, and F345 amino acid substitutions might have a role in the triazole resistance of *A. tubingensis*, since the number of isolates tested in this study is limited, confirmatory studies should be performed in order to support our data. To our knowledge, the

mutations F200L found in these isolates have not yet been reported. However, it remains unclear whether they are the cause of azole resistance in these strains, and further studies are required to clarify their exact role. Regarding *A. terreus*, only one resistant isolate of *A. terreus* showed the amino acid substitution F200L. To the best of our knowledge, this is the first report of this point mutation in the *cyp51A* gene of *A. terreus*, and no mutations were found in the *cyp51A* gene of the two other azole-resistant *A. terreus* isolates.

A recently conducted multicentre study showed that azole-resistant *A. fumigatus* was found in 1.3% of environmental and 3.3% of clinical isolates. TR34/L98H mutations in the *cyp51A* gene were detected in 47.4% of the azole-resistant *A. fumigatus* isolates. However, in 52.6% of phenotypically resistant isolates, no mutations were detected within the *cyp51A* gene.<sup>47</sup> Moreover, the prevalence of azole-resistant *A. fumigatus* in a Danish national surveillance study was 6.1% (66/1083) at the patient level, and TR34/L98H was the most common alteration, but non-*cyp51A*-mediated resistance accounted for 19.7% (13/66).<sup>23</sup> In our study, 26.7% (4/15) *Aspergillus* strains did not find mutation in the *cyp51A* gene. To fill a gap in our understanding of the mechanism for azole resistance in the non-*cyp51A* strains, we highly recommend further and more extensive monitoring of the soil exposure to fungicides in agricultural and hospital areas, to determine trends in the rate of azole-resistant *Aspergillus* species and to investigate the other mechanisms of resistance such as overexpression of efflux pumps, gain-of-function mutations in transcription factors, mutations in regulatory and sterol biosynthesis elements, and mutations within the HMG-CoA reductase-encoding gene (*hmg1*), which encodes HMG-CoA reductase in *Aspergillus* species.

## Conclusion

In conclusion, this study provides a comprehensive analysis of the clinical distribution, antifungal susceptibility, and resistance mechanisms of *Aspergillus* species in Xinjiang, China. The findings highlight the predominance of *A. fumigatus* as the most common pathogenic species, alongside a diverse range of other *Aspergillus* species, including cryptic species with intrinsic resistance to azoles. The high prevalence of amphotericin B resistance observed in this region underscores the need for cautious use of this antifungal agent in clinical practice. Additionally, the identification of *cyp51A* mutations in azole-resistant strains suggests that these genetic alterations may play a significant role in mediating resistance, although further research is needed to fully elucidate the underlying mechanisms. Here, we emphasize the need for continuous surveillance of fungal infections in the hospital environment, which aids in overcoming the knowledge gap regarding the global fungal burden of infections and antifungal resistance, thus supporting public health interventions.

## Ethics Statement

The study was conducted in accordance with the Declaration of Helsinki. Ethical approval for the study was obtained from the Ethics Committee of the First Affiliated Hospital of Xinjiang Medical University. All patients consented to being involved in this study (Approval number: K202405-15).

## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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## Disclosure

The authors report no conflicts of interest in this work.

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