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ORIGINAL RESEARCH

Epidemiology and Azole Resistance of Clinical Isolates of *Aspergillus fumigatus* from a Large Tertiary Hospital in Ningxia, China

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Purpose: The objective of this study was to determine the clinical distribution, in vitro antifungal susceptibility and underlying resistance mechanisms of *Aspergillus fumigatus* (*A. fumigatus*) isolates from the General Hospital of Ningxia Medical University between November 2021 and May 2023.

Methods: Antifungal susceptibility testing was performed using the Sensititre YeastOne YO10, and isolates with high minimal inhibitory concentrations (MICs) were further confirmed using the standard broth microdilution assays established by the Clinical and Laboratory Standards Institute (CLSI) M38-third edition. Whole-Genome Resequencing and RT-qPCR in azole-resistant *A. fumigatus* strains were performed to investigate the underlying resistance mechanisms.

Results: Overall, a total of 276 *A. fumigatus* isolates were identified from various clinical departments, showing an increasing trend in the number of isolates over the past 3 years. Two azole-resistant *A. fumigatus* strains (0.72%) were observed, one of which showed overexpression of *cyp51A, cyp51B, cdr1B, MDR1/2, artR, srbA, erg24A*, and *erg4B*, but no *cyp51A* mutation. However, the other strain harbored two alterations in the *cyp51A* sequences (L98H/S297T). Therefore, we first described two azole-resistant clinical *A. fumigatus* strains in Ningxia, China, and reported one azole-resistant strain that has the L98H/S297T mutations in the *cyp51A* gene without any tandem repeat (TR) sequences in the promoter region.

Conclusions: This study emphasizes the importance of enhancing attention and surveillance of azole-resistant *A. fumigatus*, particularly those with non-TR point mutations of *cyp51A* or non-*cyp51A* mutations, in order to gain a better understanding of their prevalence and spread in the region.

Keywords: *Aspergillus fumigatus*, azole resistance, *cyp51A* mutation, tandem repeat sequences

Introduction

Aspergillus fumigatus (*A. fumigatus*) is the most common *Aspergillus* species that can cause various serious diseases such as life-threatening invasive aspergillosis (IA) in immunocompromised individuals with hematopoietic stem cells and solid organ transplantation, acute leukemia, and receiving immunotherapy with/without corticosteroids.[1–3] Non-invasive infections in immunocompetent patients with underlying pulmonary conditions can lead to chronic pulmonary aspergillosis (CPA), and others can suffer from allergic bronchopulmonary aspergillosis (ABPA), fungal asthma, and *Aspergillus* bronchitis.[4–6] *A. fumigatus* has been identified as a critical priority fungal pathogen in the first-ever fungal priority pathogen list released by the World Health Organization (WHO) (https://www.who.int/publications/i/item/9789240060241). Recently, there has been a rise in reported cases of IA in patients with influenza[7] and coronavirus disease 2019 (COVID-19).[8,9] According to existing reports, the prevalence of aspergillosis varies across different provinces in China. A study conducted by Linna Huang et al found

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that 29.3% of patients with viral pneumonia in Beijing from December 2022 to February 2023 also had IA.[10] A retrospective cohort study revealed that between August 2016 and December 2019, a total of 617 adult viral pneumonia patients were admitted to six hospitals in China, with 14.7% patients being diagnosed with IPA.[11] In Nanjing, among clinically suspected patients, 26.6% of patients were ultimately diagnosed with invasive pulmonary aspergillosis (IPA),[12] which is lower compared to Shanghai, where 58.5% of patients were diagnosed with IPA.[13] However, there is currently a lack of epidemiological statistics regarding *A. fumigatus* infection in Ningxia and its surrounding areas.

Treatment for *A. fumigatus* has heavily relied on three classes of antifungal drugs: azoles, polyenes, and echinocandins.[3] However, the extensive use of azoles such as itraconazole (ITC), voriconazole (VRC), posaconazole (POS), and isavuconazole (ISV) over the past decades has led to the worldwide emergence of azole resistance in *A. fumigatus*. This increase in resistance, both environmentally and clinically, may be responsible for the failure of aspergillosis treatment and the consequent high mortality rates.[14,15]

Azole resistance in *A. fumigatus* is primarily caused by alterations to the sterol biosynthesis pathway, resulting from point mutations of *cyp51A* and the insertion of tandem repeats (TR) in the promoter region of *cyp51A* (such as TR34/L98H and TR46/Y121F/T289A).[16–18] However, there are increasing reports of *A. fumigatus* strains that are resistant to clinical azoles despite lacking *cyp51A* modifications.[19,20] Extensive research has been conducted on strains with multiple mutations in *hmg1* and *hmg2*, which encode the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase. These mutations have provided valuable insights into the mechanism of azole drug resistance in *A. fumigatus*.[20–25]

Several attempts have been made to correlate the mutations in *hapE*, which encodes one of the three subunits of the CCAAT box binding complex (CBC), with azole resistance. Interestingly, a new study investigated the combination of $hapE^{P88L}$ and $hmg1^{F262del}$ mutations as potential contributors to azole resistance.[26–28] 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.[1] The growing number of resistant strains and the continuous emergence of new resistance mechanisms have aroused great concern worldwide.[14,29]

The epidemiology of *aspergillus*-disease in Ningxia has not been previously reported, according to an extensive literature search available on PubMed. *A. fumigatus* infection in China exhibits regional differences.[30] General Hospital of Ningxia Medical University, the largest tertiary hospital in Ningxia with approximately 3500 beds, serves both local patients and those from surrounding areas. We believe that our hospital may be regionally representative to some extent. Furthermore, Ningxia, located in northwest China, has a unique geographical climate and lacks research on *A. fumigatus* infection and drug resistance. Therefore, our study focused on assessing clinical *A. fumigatus* isolates collected from the General Hospital of Ningxia Medical University from November 2021 to May 2023. We aim to evaluate the susceptibility of these isolates to different antifungal agents and to investigate potential resistance mechanisms, including *cyp51A* mutations and expression of genes related to azole-resistance.

Materials and Methods

A. fumigatus Strains and Molecular Identification

A. fumigatus isolates were obtained from individual patients hospitalized in the General Hospital of Ningxia Medical University between November 2021 and May 2023. Af293 was kindly donated by Professor Wei Liu of Peking University First Hospital. The fungal isolates were subcultured on sabouraud dextrose agar (SDA) at 25°C for 1 week. Morphological identification was performed based on macro and microscopic features, followed by matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS, bioMerieux, France). Molecular identification was performed by amplifying the β-tubulin gene (*benA*) and calmodulin gene (*CaM*) as previously described,[31,32] and the resulting PCR products were sequenced at Tsingke Biotechnology (Beijing, China). The obtained sequences were compared to reference sequences in GenBank. This study was approved by Medical Science Research Ethics Committee IRB of the General Hospital of Ningxia Medical University (2020989 approved on 09 October 2020). Written informed consent was obtained from all study participants (consent for research).

Antifungal Susceptibility Testing

The Sensititre YeastOne YO10 (Thermo Scientific, Cleveland, OH, United States) was used for antifungal susceptibility testing (AFST) with nine antifungals: anidulafungin (AND), micafungin (MFG), caspofungin (CAS), 5-flucytosine (5-FC),

posaconazole (POS), voriconazole (VRC), itraconazole (ITC), fluconazole (FLC), amphotericin B (AMB). The values of minimal inhibitory concentration (MIC) and minimum effective concentration (MEC) were determined according to the manufacturer's instructions. Briefly, conidia were collected from the SDA plates and adjusted to a McFarland standard of 0.5. A 100 μL conidial suspension was added to RPMI-1640 medium to achieve a final working concentration of $0.4–5\times10^4$ CFU/mL. 96-well plates with 100 μL working solution in each well were incubated at 35°C for 24–48 h. The minimum inhibitory concentration (MIC) values of POS, VCZ, ITC, and AMB were determined to be the lowest concentrations that produced 100% inhibition, indicated by the first blue or purple well after 48 h of incubation. The minimum effective concentration (MEC) values were determined as the lowest concentrations of AND, MFG, and CAS that produced a morphological change to small, rounded, compact hyphal forms compared to the growth control well after 24 h of incubation. The standard broth microdilution assays were performed in accordance with CLSI M38 third edition[33] to determine the isolates with MIC value(s) of VRC≥2 μg/mL or ITC>1 μg/mL base on YeastOne results. For CLSI methodology, the concentration ranges of antifungal agents were as follows: VRC, ITC, POS (0.031–16 μg/mL), CAS, and MFG (0.015–8 μg/mL). The antifungal drugs were purchased from Meilunbio, China. Candida parapsilosis ATCC 22019 and Candida krusei ATCC 6258 were used as quality controls. The epidemiological cut-offs (ECVs) of AMB (2 μg/mL), CAS (0.5 μg/mL), ITC (2 μg/mL), and the clinical breakpoint of VRC (≥2 μg/mL) referred to CLSI-M59 were used to define the isolates as wild type (WT)/Susceptible (S) or non-WT/Resistant (R).[34] MIC50 and MIC90 values were determined based on concentrations that inhibited 50% and 90% of the isolates, respectively. The calculation method used in this study can be referenced from the literature guide "Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi; Approved Standard Second Edition, M38-A2".

Whole-Genome Resequencing for Azole-Resistant *A. fumigatus*

In this study, a total of 276 strains of *A. fumigatus* from Ningxia were labeled as NYDZY 1 to NYDZY 276. Among these strains, NYDZY 162 and NYDZY 247 were identified as being resistant to azole. For the genome resequencing of NYDZY 162 and NYDZY 247, high-purity genomic DNA was extracted using QIAamp DNA Mini Kit (Qiagen). DNA qualification, library construction, and resequencing were executed at DNBSEQ platform.[35] High-throughput DNA sequencing (pair-end sequencing) was performed on Pacbio platform, with a read length of 300 bp at each end and an average of 3 Gb sequencing data for each library.

Determination of Azole-Resistance Related Genes Expression Levels by RT-qPCR

The expression levels of *cyp51A, cyp51B, cdr1b, atrR, atrF, srbA, erg4A/4B/24A/25A*, and *MDR1/2/3/4* were determined by real-time qPCR. Briefly, conidia (1.0×10^6 cells/mL) were collected from SDA plates and incubated at 35°C and 200 rpm for 48 h in 5 mL Sabouraud liquid medium. After centrifugation, the culture supernatant was discarded, mycelia were harvested, and they were washed twice with sterile distilled water. RNA extraction was performed using the RNAsimple Total RNA Kit (TIANGEN, Biotech, China) following the manufacturer's instructions. The concentration of RNA was determined using NanoDrop spectrophotometer (ThermoFisher), and cDNA was synthesized using a PrimeScript RT Master Mix kit (TaKaRa Biotechnology, China) according to the manufacturer's instructions. qPCR was conducted with specific primers (Table 1) using LightCycler 480 SYBR green I master mix (Roche). Af293 was used as susceptible control. The difference in gene expression was calculated using the 2-ΔΔCT method. Each sample was performed in triplicate.

Statistical Analysis

Statistical differences between multiple groups were performed by a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons tests, using GraphPad Prism 8.0 software. Data were presented as medians or means ± standard deviation. $P < 0.05$ was considered statistically significant.

Results

Fungal Isolates

A total of 276 strains identified as *A. fumigatus* using morphological and molecular methods and MALDI-TOF MS were collected in this study. The number of *A. fumigatus* isolates during the first 5 months of 2023 was 192, which is 3.4 times

Table 1 Sequence Information of the qPCR Primers Used in This Study

Primer Name	Sequence(5'to3')	Reference
cyp51A-F	*GGTGCCGATGCTATGGCTTACGGC*	[36]
cyp51A-R	*GGTTCTGTTCGGTTCCAAAGCCG*	
cyp51B-F	*GGGTCTCATCGCGTTCATTCTCGACG*	
cyp51B-R	*GATACAGCGAGGATGGATAGTAGTCC*	
atrR-F	*GAGTCTTTACTGCGTCTTTCTGG*	
atrR-R	*GAGCCGCTTGCATGATACCGG*	
srbA-F	*GTCCACCCCCGGCATTGGTGGG*	
srbA-R	*GGCAACGTCCGTACTTGATTGG*	
erg3-F	*GGATATTGTTCTTGAGATCTGGG*	
erg3-R	*CTGGTAATGAGACCGTCGAGGAG*	
erg24A-F	*GGCACCAAGAAAGGATTTCGAAG*	
erg24A-R	*GAATACAAACGCGCCGAGAGGG*	
erg25A-F	*GGACTCGCTCAATTCGTCCTATCCGC*	
erg25A-R	*GAGAGATGAGGTTGCTGCTGAGC*	
cdr1B-F	*GTCTCTTCTAGGGACGATAAATCC*	
cdr1B-R	*CGACCCTGGCGATTTCTCCTTCC*	
atrF-F	*TGCCCAGAGAAATCGACAAC*	[37]
atrF-R	*CCACCTCGTCGCAGATAGTC*	
MDR1-F	*GCTCTTCCCTTGTTCACAATTC*	
MDR1-R	*CGGCAATACCGAGATACACA*	
MDR2-F	*TGCCACATTCTTTAGCTCCAC*	
MDR2-R	*AAGACCGAACATGCTTGACC*	
MDR3-F	*GATGCATCCTGCAAAGTACG*	
MDR3-R	*AGGCTCCTTGGTGCTTGAC*	
MDR4-F	*CACTGAACGCAACTCCTGAA*	
MDR4-R	*TCTTTCTGGCTTCCTCCTCA*	
GAPDH1-F	*GCCTCTTAAGGGTATCCTGACCTA*	
GAPDH1-R	*TACCAGCTCACCAACTTCACGA*	
erg4A-F	*GTTCTTCGCTATTTCCTGG*	[38]
erg4A-R	*GCGGTTGATATCTCGTCTC*	
erg4B-F	*AGACTTTCCCTCAGCTCCC*	
erg4B-R	*CCAGTTCAAGGCGAAATAA*	

higher than the number during the same period of 2022. Notably, there was a significant increase in January and March of 2023 (Figure 1A). The *A. fumigatus* were collected from patients mainly distributed in department of respiratory (n=119, 43.12%), emergency department (n=49, 17.75%), intensive care medicine (ICU) (n=22, 7.97%), cardiology (n=12, 4.35%), geriatric (n=9, 3.26%), nephrology (n=7, 2.54%), hematology (n=7, 2.54%), and infection (n=6, 2.17%) (Figure 1B). In terms of specimen types, over 50% of the *A. fumigatus* isolates (n=246, 89.13%) were recovered from sputum, 9.78% (n=27) were from bronchoalveolar lavage fluid (BALF), 0.72% (n = 2) were from wounds, 0.37% (n = 1) were from puncture fluid (Figure 1C).

Antifungal Susceptibility Testing and Detection of *cyp51A*

The MIC50, MIC90, range, ECVs, and categories are summarized in Table 2. Among the *A. fumigatus* isolates, 49.28% (n=136) exhibited high MIC values of 5-FC (>64 μg/mL), while more than 50% of isolates (n=232, 84.06%) demonstrated higher MIC values for FLC (≥256 μg/mL). These findings suggest the inherent resistance of *A. fumigatus* to these antifungal agents. All strains were wild type (WT) to AMB, with MIC values ranging from 0.25 to 2 μg/mL, and both MIC50 and MIC90 = 1 μg/mL. The MIC values of CAS for three strains (NYDZY 39, NYDZY 65, NYDZY 200) were 8 μg/mL, 8 μg/mL, 4 μg/mL, respectively, which are categorized as Non-WT. However, all three strains showed low MIC values for VRC, ITC, and POS. Two isolates of *A. fumigatus* (NYDZY 162, NYDZY 247) exhibited higher MIC values

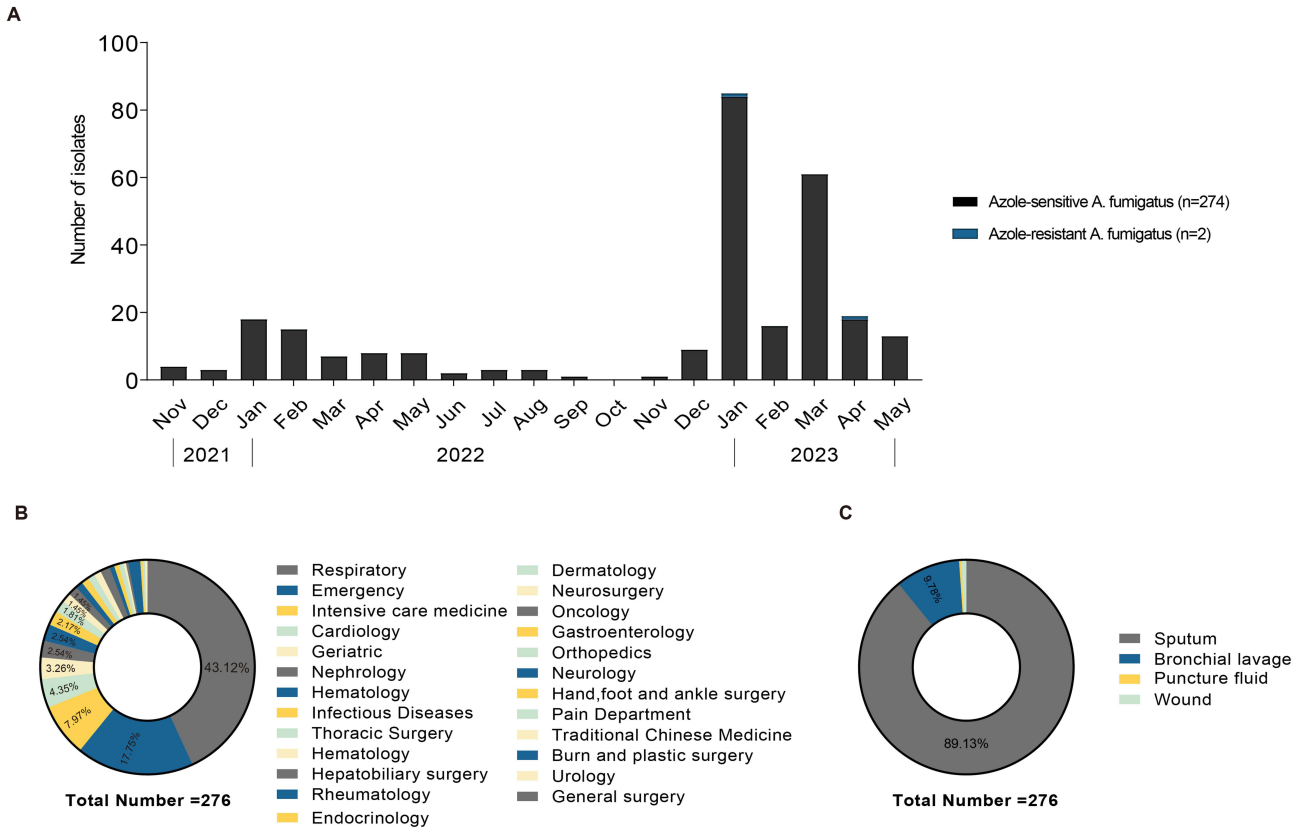

Figure 1 Distribution of the 276 *A. fumigatus* isolates from November 2021-May 2023: (**A**) Number of *A. fumigatus* isolates by month of detection; (**B**) Distribution by department type; (**C**) Distribution by specimen type.

of >16 μg/mL for ITC and 2 μg/mL for POS. In the case of VRC, NYDZY 162 exhibited high MIC values of 8 μg/mL, while NYDZY 247 showed values of 4 μg/mL, indicating azole resistance. The distribution of VRC, ITZ, and POS MICs for clinical *A. fumigatus* isolates is shown in Figure 2. The MIC/MEC values of VRC, ITC, POS, CAS, MFG, and AMB against two azole-resistant isolates, determined by YeastOne YO10 and CLSI methods, are summarized in Table 3. Both methods showed high categorical agreement. Compared to the CLSI method, NYDZY 162 exhibited one-dilution higher

Table 2 Susceptibility Profiles of 276 *A. Fumigatus* Isolates

Antifungal	MIC50 (μg/mL)	MIC90 (μg/mL)	Range (μg/mL)	ECVs (μg/mL)	BP (μg/mL)	Wild Type / Susceptible (%)[a]	Non-Wild Type / Resistance (%)[a]
AND	≤0.015	0.03	≤0.015–8	-	-	-	-
MFG	≤0.008	0.03	≤0.008–8	-	-	-	-
CAS	0.06	0.12	≤0.008->0.25	0.5	-	276 (100.00)	0
5-FC	>64	>64	0.06->64	IR	-	-	-
POS	0.25	0.25	0.03–2	-	-	-	-
VRC	0.25	0.5	0.06–8	1	≥2	274 (99.28)	2(0.72)
ITC	0.25	0.5	≤0.015->16	1	-	274 (99.28)	2(0.72)
FLC	>256	>256	64->256	IR	-	-	-
AMB	1	1	0.25–2	2	-	276 (100.00)	0

Notes: [a]Breakpoints are used to define isolates as susceptible or resistant, without established BP, The ECV is used to define the isolates as wild type or non-wild type.
Abbreviations: AND, anidulafungin; MFG, micafungin; CAS, caspofungin; 5-FC, 5-fluorocytosine; POS, posaconazole; VRC, voriconazole; ITC, itraconazole; FLC, Fluconazole; AMB, amphotericin B; ECVs, epidemiological cut-offs; BP, clinical breakpoint; ECV, not defined; IR, intrinsic resistance.

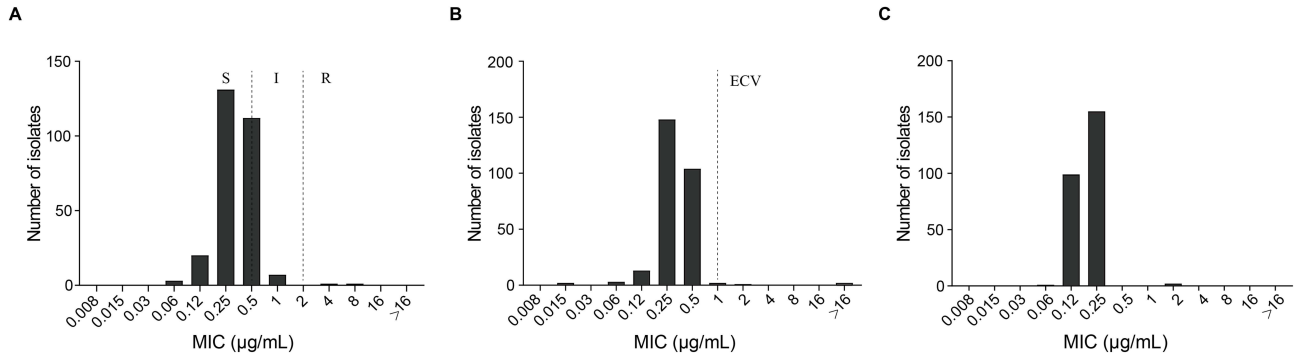

Figure 2 MICs' distributions of antifungals tested for 276 clinical *A. fumigatus* isolates with YeastOne YO10. (**A**) voriconazole; (**B**) itraconazole; (**C**) posaconazole.
Abbreviations: S, susceptible; I, intermediate; R, resistant; ECV, epidemiological cut-offs.

MIC values of VRC and one-dilution lower MIC values of AMB. Sequencing results revealed no mutations in the *cyp51A* coding and promoter region in NYDZY 162, but amid acid substitution was observed in NYDZY 247.

The Expression Levels of Azole-Resistance Related Genes

RT-PCR results revealed that *A. fumigatus* clinical isolates (NYDZY 162) resistant to triazoles showed increased expression of *cyp51A* ($P < 0.05$), *cdr1B* ($P < 0.0001$), *MDR1* ($P < 0.0001$), *MDR2* ($P < 0.01$), *artR* ($P < 0.01$), *srbA* ($P < 0.001$), *erg24A* ($P < 0.01$), and *erg4B* ($P < 0.01$) compared to the wild-type Af293. However, there was no significant difference in the expression of these genes in NYDZY 247 ($P > 0.05$). On the contrary, NYDZY 247 exhibited significantly lower levels of *erg24A* ($P < 0.001$) and *erg4B* ($P < 0.05$) expression compared to both Af293 and NYDZY 162. Additionally, the expression levels of genes *MDR3* ($P < 0.0001$, $P < 0.0001$), *erg25A* ($P < 0.01$, $P < 0.001$), and *erg4A* ($P < 0.0001$, $P < 0.0001$) significantly decreased in both NYDZY 162 and NYDZY 247 (Figure 3).

The Clinical Profiles of Patients with Azole-Resistant *A. fumigatum*

NYDZY 162 was isolated from the sputum of a severe COVID-19 patient (Case 1) who also had non-Hodgkin's lymphoma, diabetes, hypertension, arrhythmia, and other conditions. Chest CT scans revealed images of lungs with abnormal infiltrates, reticular and linear opacities, pleural thickening, and the accumulation of lung lesions on both sides. After receiving treatment with voriconazole and ceftazidime, the patient's condition improved significantly, leading to his discharge. NYDZY 247 was isolated from the sputum of a patient (Case 2) with novel coronavirus pneumonia who also had co-infection with bacteria and fungi during the acute exacerbation stage of chronic obstructive pulmonary disease. This patient had a history of old pulmonary tuberculosis, old bronchial tuberculosis, pulmonary hypertension, and other diseases. The chest CT scan results showed the accumulation of lung lesions on both sides, and features like reticular and linear opacities, nodule, pleural thickening, and pleural effusion. The condition of this patient improved after treatment with voriconazole, carbofenozin, imipenem/cilastatin, cefoperazone/sulbactam. The clinical profiles of patients with

Table 3 The MIC/MEC Values (μg/mL) of Different Antifungals Against Two A. Fumigatus Isolates as Determined by the YeastOne YO10 and CLSI Methods

Strain	Methods	VRC	ITC	POS	CAS	MFG	AMB
NYDZY 162	Yeastone YO10	8	>16	2	0.06	0.008	1
	CLSI method	8	>16	2	0.06	0.008	2
NYDZY 247	Yeastone YO10	4	>16	2	0.06	0.06	2
	CLSI method	4	>16	2	0.06	0.03	2

Abbreviations: VRC, voriconazole; ITC, itraconazole; POS, posaconazole; CAS, caspofungin; MFG, micafungin; AMB, amphotericin B; CLSI, Clinical and Laboratory Standards Institute.

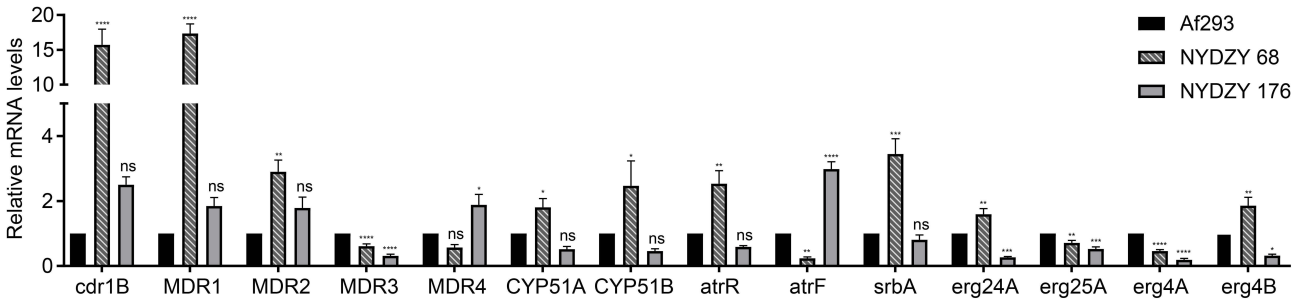

Figure 3 RT-qPCR of azole-resistance related genes' expression (*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001 in one-way ANOVA with Tukey's multiple comparison).
Abbreviation: ns, non-significant.

azole-resistant *A. fumigatus* was shown in Table 4. It is worth noting that the serum galactomannan (GM) tests were negative in both patients infected with azole-resistant *A. fumigatus* strains.

Discussion

A. fumigatus is a globally prevalent environmental mold that can cause a variety of human diseases, leading to notable mortality. Around 3,000,000 cases of chronic pulmonary aspergillosis (CPA) and invasive aspergillosis (IA) are reported worldwide annually.[39,40] Moreover, the extensive use of triazole antifungals has resulted in the emergence of azole-resistant *A. fumigatus*, which poses significant clinical and economic challenges.[41–44] Our study conducted in Ningxia province, China, observed an increase in the number of identified *A. fumigatus* strains from 2021 to 2023. Additionally, we discovered two azole-resistant strains during our research. These findings highlight a concerning trend of *A. fumigatus* infection in the area.

In our study, we collected a total of 276 strains of *A. fumigatus*. Among these, 192 strains were collected in 2023, which represents a more than 2.5 times increase compared to the previous year. This significant rise in isolates was strongly correlated with the surge in the number of COVID-19 infected patients following the lifting of epidemic control restrictions. This is similar to studies conducted in other provinces in China.[45] It has been reported worldwide that there is a high incidence of COVID-19-Associated Pulmonary Aspergillosis (CAPA) among critically ill COVID-19 patients,[46–49] which is believed to be caused by severe immunomodulation and lymphocyte depletion resulting from the virus and the subsequent use of immune-suppressive drugs.[50] The most common methods used for diagnosing CAPA include recovering *Aspergillus* spp. in culture media of bronchoalveolar fluid (BALF) and tracheal aspirate, as well as detecting conventional GM from BALF, tracheal aspirate, and serum specimens.[51] However, in our study, diagnosing and confirming CAPA in two patients with isolated azole-resistant *A. fumigatus* remains challenging due to the patients' serious underlying conditions, the overlap of clinical manifestations and imaging findings between COVID-19 and CAPA, the limited use of bronchoscopies to avoid the risk of virus transmission, and the imperfect sensitivity of serum GM.[1,52,53] Fortunately, both patients recovered and were discharged after receiving treatment with voriconazole and antibacterials, highlighting the importance of combination therapy and appropriate dosage for severe infections. But other researchers have indicated that patients infected with azole-resistant strains have a higher mortality rate compared to those infected with sensitive strains.[1,54] A 5-year retrospective cohort study showed a 21% higher day-42 mortality in azole-resistant invasive aspergillosis compared with wild-type infections cases.[55] In another multicentre retrospective study, it was also observed that the mortality rate at 6 and 12 weeks was higher in haematology patients with voriconazole-resistant cases compared to those with susceptible cases.[56] Therefore, it is an urgent issue to monitor drug resistance in clinical isolates and unravel the molecular mechanisms underlying azole resistance.

In addition to patients with associated diseases, strains with azole-resistance have also been identified in patients with no previous history of antifungal treatment.[57,58] Furthermore, environmental isolates have been found to develop azole-resistance due to the use of fungicides. Molecular epidemiological studies have demonstrated that people can acquire infections from their immediate surroundings.[59] The emergence of these resistant *A. fumigatus* in both natural and

Table 4 The Clinical Profile and Examinations of Patients Infected by Resistant *A. Fumigatus*

Case No.	Age/ Gender	Clinical Specimen	Clinical Profile						Strain	cyp51A Mutation
			Clinical Diagnosis	Underlying Condition	Antibacterial Treatment	Chest Computed Tomography	Laboratory Data	Outcome		
Case 1	74/male	Sputum	Novel coronavirus pneumonia	Non-Hodgkin's lymphoma, diabetes, hypertension, arrhythmia	Voriconazole 400mg q12h, Ceftazidime 2g q12h;	Bilateral lung lesions, Non-specific infiltration, reticular and linear opacities, pleural thickening	CRP, mg/L: 8.9 ALB, g/L: 30.3 WBC, 10^9/L:5.23 Neu%: 55.6 LYM%: 34.2 HB, g/L: 149 PLT, 109/L: 67 Serum GM: 0.235	Survived	NYDZY 162	No
Case 2	79/ female	Sputum	Novel coronavirus pneumonia	Chronic obstructive pulmonary disease with acute exacerbation, atelectasis, old pulmonary tuberculosis, old bronchial tuberculosis, pulmonary hypertension	Voriconazole 400mg q12h, Carbofenozin 50mg qd, Imipenem/ cilastatin 3g q8h, Cefoperazone/ sulbactam 6g q12h;	Bilateral lung lesions, reticular and linear opacities, nodule, pleural thickening, pleural effusion	CRP, mg/L:16.7 ALB, g/L 33.51 WBC, 10^9/L: 15.88 Neu%: 85.3 LYM%, %: 7.7 HB, g/L: 138 PLT, 10^9/L: 216 Serum GM: 0.291	Survived	NYDZY 247	L98H/ S297T

Abbreviations: CRP, C-Reactive Protein; ALB, Albumin; WBC, White blood cell count; Neu%, Neutrophil percentage; LYM%, lymphocyte percentage; HB, hemoglobin; PLT, platelet; Serum-GM, Serum galactomannan.

clinical settings has led to an expansion of the population of patients at risk, which has generated well-founded concerns in the face of limited treatment options.

The prevalence of azole-resistant *Aspergillus* species varies geographically. Our study found that 2 out of 276 samples (0.72%) were resistant to VRC and non-wild type for ITC, which is lower compared to Beijing (4.3%),[60] Shanghai (3.57–7.02%),[61,62] and Anhui (5.79%)[63] in China. In a separate study conducted in China, *A. fumigatus* isolates were collected from various regions, revealing a 4.4% frequency of azole resistance.[30] However, when compared to several other countries, the rates of azole-non-wild type *A. fumigatus* were found to be higher in Europe (9.5%) and North America (9.1%) than in Latin America and the Asia-Pacific region (5.3%) over a 5-year surveillance period.[42]

Azole resistance in *A. fumigatus* is primarily linked to mutations in *cyp51A* and overexpression of *cyp51A*, as well as overexpression of efflux pumps. Point mutations in the coding gene in *cyp51A* combined with tandem repeats in the promoter region have been frequently observed, such as TR34/L98H, which means that the sequence of tandem repeats 34 times, followed by the presence of the amino acid substitution from leucine (L) to histidine (H) at position 98. Mechanically, tandem repeats within the promoter region of *cyp51A* is associated with overexpression of *cyp51A* and the L98H substitution decreases hydrogen bonding between residue 98 and the polar side chains of neighboring residues, thereby preventing the triazole from fitting into the binding pocket.[64,65] Additionally, strains carrying a TR46 allele in conjunction with Y121F and T289A exchanges were also currently the most prevalent isolates worldwide.[1] The sequencing analysis results in our study revealed that NYDZY 247 carried L98H/S297T mutations in the *cyp51A* gene without the presence of TR in the promoter region (TR34 and TR46), which was quite different from other previous studies where the occurrence of such amino acid substitution was always accompanied by TR variations.[66–69] The mRNA level of *cyp51A* did not show any increase in NYDZY 247, which could be explained by the presence of non-TR mutations in *cyp51A*. Other single nucleotide polymorphisms (SNPs), such as G54, M220, and G448, in the *cyp51A* gene have been linked to drug resistance.[29] These polymorphisms are frequently observed in patients with chronic pulmonary aspergillosis, invasive aspergillosis bronchitis, aspergilloma, and chronic cavitary pulmonary aspergillosis (CCPA) who have undergone long-term treatment with azole antifungals and have been associated with several clinical treatment failures.[37,70–72] The specific amino acid substitution in the *cyp51A* sequence without tandem repeats determines the alteration in the interaction between 14α-demethylase and the azole drugs, leading to different patterns of azole cross-resistance.[73] Therefore, Non-TR mutation of *cyp51A* in *A. fumigatus* can still lead to drug resistance and have significant clinical consequences, which highlights the importance of closely monitoring such mutations.

Findings from several groups have shown that the transcription factors *AtrR* and *SrbA* play a significant role in azole resistance in *A. fumigatus*. These factors co-regulate the expressions of *cyp51A* and efflux transporter *cdr1B*,[36,74,75] which aligns with our main finding that NYDZY 247 exhibited co-upregulation of these genes. Moreover, several clinical azole-resistant *A. fumigatus* isolates have shown high levels of *cyp51B* expression, even in the absence of *cyp51A* mutations,[76,77] similar to what has been observed in NYDZY 162. Recent evidence suggests that *cyp51B* may act as a backup system when *cyp51A* expression is compromised.[78] Consistent with the results of other studies, our findings also showed higher levels of *cyp51A* and *cdr1B* expression in the azole-resistant strain NYDZY 162. However, there were some observed differences: NYDZY 162 exhibited a greater increase in cdr1B expression compared to *cyp51A*, whereas other studies reported a >500-fold induction of *cyp51A* and only a modest change in *cdr1B* expression (>5-fold).[79] These results imply that the increased expression of the efflux transporter gene in NYDZY 162 may be the primary cause of drug resistance.

Although antifungal susceptibility testing of *A. fumigatus* is not routinely performed in most clinical laboratories, regular assessment of it can be helpful in guiding clinical management and understanding changes in drug resistance. Prior to our study, no reports had been made regarding azole-resistant *A. fumigatus* in Ningxia. Although we only identified two azole-resistant strains, this finding represents the first occurrence in the entire northwest region of China. Given the increasing trend in the number of *A. fumigatus* isolates from 2022 to 2023, it is likely that more azole-resistant strains will be found in the future. Moreover, we report a novel resistance mechanism in one of the azole-resistant strains, which involves the non-TR L98H/S297T mutations of the *cyp51A* gene. However, it should be noted that our investigation is not exhaustive, and further research will be conducted to delve into the underlying mechanisms in our future work.

Conclusions

This study, for the first time, revealed a prevalence of azole resistance in *A. fumigatus* at 0.72% in Ningxia province of China. Among the isolates, NYDZY 162 and NYDZY 247 exhibited the same azole resistance phenotype with different resistance mechanisms. NYDZY 162 exhibited overexpression of *cyp51A* without any mutations, while NYDZY 247 showed non-TR point mutations (L98H/S297T) in *cyp51A* that have not been previously reported. Our study emphasized the severity of *A. fumigus* spread in the region and the emergence of azole resistance. Therefore, it is crucial to consistently enhance the surveillance of antifungal susceptibility in clinical strains and implement effective measures to eliminate transmission and nosocomial infections, which may be helpful to prevent further exacerbation of clinical azole resistance.

Data Sharing Statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author, Pengtao Wang, upon reasonable request.

Ethics Statement

This study was approved by Medical Science Research Ethics Committee IRB of the General Hospital of Ningxia Medical University (2020989, approved 09 October 2020). Written informed consent was obtained from all study participants (consent for research). This study follows the Declaration of Helsinki.

Author Contributions

All authors contributed significantly to the work reported, whether in terms of conception, study design, acquisition of data, analysis and interpretation, or all of these; participated in the drafting, revision, or critical review of the article; provided final approval of the version to be published; agreed on the journal to which the article was to be submitted; and agreed to take responsibility 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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