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Epidemiology, antifungal susceptibility and biological characteristics of clinical *Aspergillus fumigatus* in a tertiary hospital

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Azole resistance in *Aspergillus fumigatus* poses a significant clinical challenge globally. In this study, we analyzed 307 clinical *A. fumigatus* isolates from General Hospital of Ningxia Medical University in China, collected between July 2023 and July 2024, to explore their susceptibility profiles, genotypic characteristics and biological traits. The overall frequency of azole resistance among clinical isolates in Ningxia province was found to be 1.20% (7 azole-resistant *A. fumigatus*, designated AF1-AF7), each exhibiting distinct phenotypes in terms of azole resistance, spore viability, and resilience to environmental stress. Among these strains, the *cyp51A* mutations predominantly included TR43/L98H, while the *hmg1* mutations were primarily S212P/Y564H. Additionally, novel mutations were discovered in *cyp51B*, specifically the t-215c point mutation and a base deletion (gatgccta) in the − 213 to − 206 region. The AF3 strain demonstrated the highest spore activity and anti-SDS efficacy. In contrast, the AF1 and AF2 strains were resistant to three azoles and also exhibited resistance to Menadione, similar to AF7. Notably, six out of the seven strains displayed resistance to NaCl and KCl, indicating a strong tolerance to saline conditions. These findings suggest that azole-resistant strains possess varying degrees of resistance to environmental stressors, implying that they may adapt to their surroundings through different evolutionary pathways, which could complicate clinical treatment strategies. Effective surveillance and control strategies are essential to control the widespread prevalence of azole-resistant *A. fumigatus* and to reduce the risk of infection in patients.

Keywords *A. fumigatus*, *cyp51A*, Azole resistance, Susceptibility profiles, Biological traits

Aspergillus fumigatus is the most ubiquitous airborne fungus, capable of causing life-threatening invasive aspergillosis (IA) and chronic pulmonary aspergillosis (CPA) in humans mainly by inhalation of its conidia[1]. Triazoles, including itraconazole (ITC), voriconazole (VRC), posaconazole (POS), and isavuconazole (ISV), are the first-line therapeutic and prophylactic drugs for IA and other Aspergillus-associated lung diseases[2,3]. However, with the widespread use of such drugs, the emergence and spread of azole-resistant *A. fumigatus* have been increasingly reported worldwide.

The mechanisms of resistance are primarily linked to modifications in the sterol biosynthesis pathway, which are caused by amino acid substitutions in the *Cyp51A* protein and the insertion of tandem repeats in the promoter region. These changes result in decreased affinity for azoles or overexpression of the *cyp51A* gene. According to the available data, the TR34-L98H is the dominant *cyp51A* gene substitutions. Additionally, mutations in 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) (*hmg1*, *hmg2*)[4–6], *cyp51B*[7] have also been confirmed to be associated with azole resistance. Recent studies have identified that abnormalities in mitochondrial function[8,9], accumulation of metabolites[10], and post-translational modifications of proteins[11] are involved in azole antifungal activity. Therefore, new resistance mechanisms continue to be explored.

In contrast to bacterial resistant rates, azole resistant rates in *A. fumigatus* have been steadily increasing worldwide, although the overall prevalence remains relatively low. The emergence of novel resistance mechanisms

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presents significant challenges for clinical management. The prevalence of azole-resistant *Aspergillus* species varies geographically. Data from 8 provinces in China showed 2.5% clinical and 1.4% environmental *A. fumigatus* isolates were identified as azole resistant[12], which is lower than the rates reported in Europe (9.5%) and North America (9.1%)[13]. In a previous study, we conducted the first antifungal susceptibility testing of *A. fumigatus* isolates in Ningxia, finding an azole resistance rate of 0.72%[14]. In the present study, we futher examined clinical *A. fumigatus* isolates from clinical microbiology group to assess their epidemiology and molecular characteristics. We also compared the morphological features and sensitivity to a range of oxidative and cell wall stress, to futher investigate the physiological differences among azole-resistant isolates mediated by different genetic mutations.

Results

Fungal isolates

This study analyzed 307 clinical isolates of *A. fumigatus*, including 7 strains that exhibited resistance to azole drugs. These isolates were obtained from the Clinical Microbiology Laboratory at the General Hospital of Ningxia Medical University, covering the period from July 2023 to July 2024. The isolates were recovered from patients with chronic obstructive pulmonary disease (COPD, $n = 55$, 17.92%), pulmonary aspergillosis ($n = 32$, 10.42%), coronavirus disease (COVID-19, $n = 23$, 7.49%), hematologic malignancies ($n = 4$, 1.30%), other tumors ($n = 9$, 2.93%), and various other infections and diseases ($n = 184$, 59.93%) (Fig. 1A). These patients were mainly hospitalized in the respiratory department ($n = 141$, 45.93%), emergency department ($n = 59$, 19.22%) and intensive care unit (ICU) ($n = 18$, 5.86%) (Fig. 1B). The strains were predominantly isolated from sputum

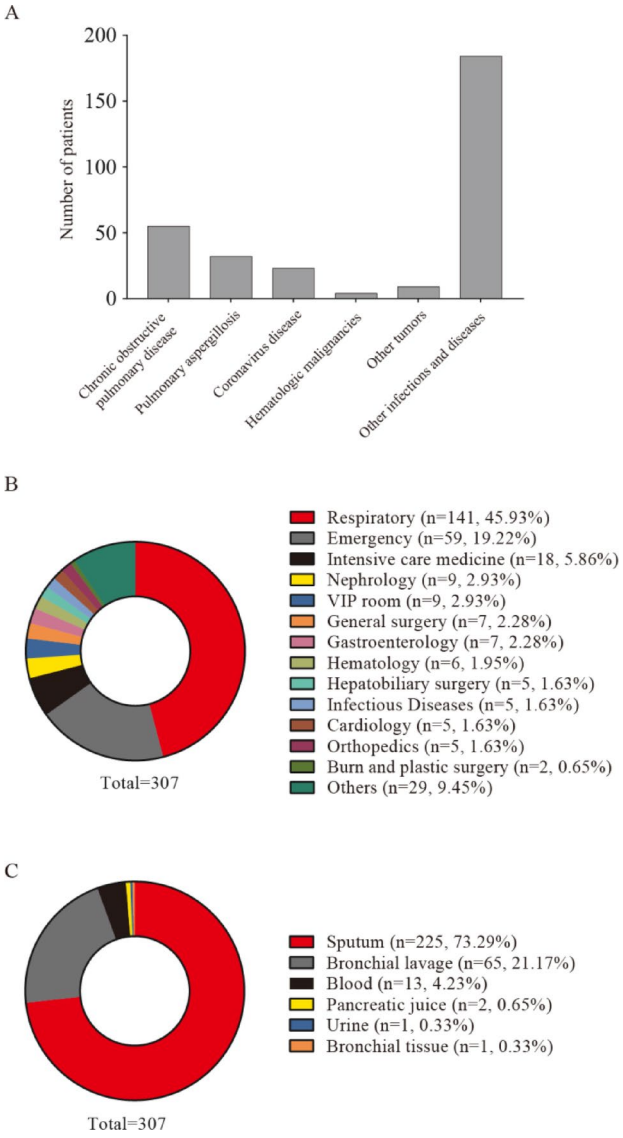

Fig. 1. Clinical distribution of *A. fumigatus* isolates: (**A**) The types of patient diseases associated with the isolation of *A. fumigatus*; (**B**) The distribution of isolates by department type; (C) The distribution of isolates by specimen type.

Antifungal	MIC_{50}	MIC_{90}	Range	ECVs	BP	WT (%)	Non-WT (%)
AND	≤0.015	0.03	≤0.015–0.03	–	–	–	–
MFG	≤0.008	0.03	≤0.008–0.12	–	–	–	–
CAS	0.03	0.06	≤0.008–0.12	0.5	–	307 (100.00%)	0
5-FC	>64	>64	4–>64	IR	–	–	–
POS	0.25	0.25	0.12–2	–	–	–	–
VRC	0.5	0.5	0.25–8	1	≥2	304 (99.02%)	3 (0.98%)
ITC	0.5	0.5	0.25–>16	1	–	303 (98.70%)	4 (1.30%)
FLC	>256	>256	256–>256	IR	–	–	–
AMB	1	2	0.25–8	2	–	305 (99.35%)	2 (0.65%)

Table 1. Susceptibility profiles of 307 *A. fumigatus* isolates. *VRC* voriconazole, *ITC* itraconazole, *POS* posaconazole, *CAS* caspofungin, *MFG* micafungin, *AMB* amphotericin B, *WT* wild type.

Case	Sex/age	Underlying condition	Strain	MICs(mg/L)			Mutation		
				VRC	ITC	POS	cyp51A	cyp51B	hmg1
1	male/74	Non-Hodgkin's lymphoma, diabetes, arrhythmia	AF1	8	>16	2	-	t-215c, -213_-206 gatgccta Del	S212P/Y564H
3	male/71	Chronic obstructive pulmonary disease with acute exacerbation, pneumonia, respiratory failure	AF3	4	2	1	TR34/L98H	–	S212P/Y564H
4	male/74	Chronic obstructive pulmonary disease with acute exacerbation, respiratory failure	AF4	1	16	1	TR34/L98H, S297T/F495I	t-215c, -213_-206 gatgccta Del	S212P/Y564H
5	male/70	Pneumonia	AF5	1	>16	1	TR34/L98H/S297T/F495I	–	S212P/S541G/Y564H
6	femal/61	Pneumonia	AF6	>8	0.5	0.5	TR46/Y121F/T298A	–	S212P/Y564H
7	femal/61	Chronic obstructive pulmonary disease with acute exacerbation, respiratory failure	AF7	4	>16	1	TR34/L98H	–	E105K/S212P/Y564H

Table 2. Characteristics of 7 azole-resistant strains and patients clinical profile.

specimens ($n=225$, 73.29%), followed by alveolar lavage ($n=65$, 21.17%), blood ($n=13$, 4.23%), pancreatic fluid ($n=18$, 5.86%), urine ($n=1$, 0.33%) and bronchial tissue ($n=1$, 0.33%) (Fig. 1C).

Susceptibility to antifungals and genes' mutations

The MIC values, MIC50, MIC90, range, ECVs, and categories, are summarized in Table 1. A total of 71.66% ($n=220$) of *A. fumigatus* isolates exhibited high MIC values for 5-FC (>64 μg/mL), while all isolates ($n=307$) demonstrated elevated MIC values of 256 or ≥256 μg/mL for FLC, indicating the intrinsic resistance of *A. fumigatus* to these antifungal agents. The MIC values for AND and MFG ranged from ≤0.015 to 0.03 μg/mL and ≤0.008 to 0.12 μg/mL, respectively. Regarding CAS, all strains were wild type (WT), with MIC values ranging from ≤0.008 to 0.12 μg/mL, and both MIC_{50} and MIC_{90} were 1 μg/mL. For AMB, one strain exhibited an MIC value of 8 μg/mL, categorizing it as non-WT, although it was sensitive to VRC, ITC, and POS. We identified a total of seven drug-resistant strains, designated as AF1 through AF7. Among the resistant isolates, AF1 and AF2 exhibited higher minimum inhibitory concentration (MIC) values of 2 μg/mL for POS, while four isolates (AF1, AF2, AF5, and AF7) demonstrated MIC values exceeding 16 μg/mL for ITC. Resistance to VRC was noted in AF1, AF2, AF3, AF6, and AF7, with MIC values of 8 μg/mL, 4 μg/mL, 4 μg/mL, >8μg/mL, and 4 ug/mL, respectively. Notably, AF1, AF2, AF3, and AF7 displayed resistance to at least two azoles (Table 2).

Mechanisms of Azole resistance mediated by gene mutations and expression

Amplification and sequencing of *cyp51A* and *cyp51B*, including their promoter regions, as well as *hmg1* in all isolated resistant strains in this study, along with two strains isolated in our previous study, revealed specific mutations (Table 3). All the isolates harbored the previously characterized *cyp51A* mutations (TR34/L98H, TR34/L98H, S297T/F495I, R46/Y121F/T298A), with the exception of strain AF1, which exhibited no changes in the coding sequence of *cyp51A* or its promoter. However, it contained a substitution at t-215c and a deletion (-213_-206 gatgccta Del) in the *cyp51B* promoter region (Fig. 2A), which were also found in the AF4 strain. In *hmg1*, all isolates carried S212P and Y564H mutations, whereas S541G and E105K were additionally detected in AF5 and AF7, respectively, all of which were located outside of sterol-sensing domain (SSD). Compared to the Af293, AF3 and AF6 exhibited a significant increase in the expression levels of the *cyp51A*, *cyp51B*, and *hmg1* genes. Additionally, the expression of the *hmg1* gene was significantly upregulated in strains AF2 and AF4; however, no significant changes were observed in the expression of their *cyp51* genes (Fig. 2B).

Primer name	Sequence (5′ to 3′)
cyp51A-F	GGTGCCGATGCTATGGCTTACGGC
cyp51A-R	GGTTCTGTTCGGTTCCAAAGCCG
cyp51B-F	GGGTCTCATCGCGTTCATTCTCGACG
cyp51B-R	GATACAGCGAGGATGGATAGTAGTCC
hmg1-F	ACCACCATCCTCTGCATCAA
hmg1-R	ATTCGGTCAGAGGCCAATCA
GAPDH1-F	GCCTCTTAAGGGTATCCTGACCTA
GAPDH1-R	TACCAGCTCACCAACTTCACGA

Table 3. Primers used for RT-qPCR.

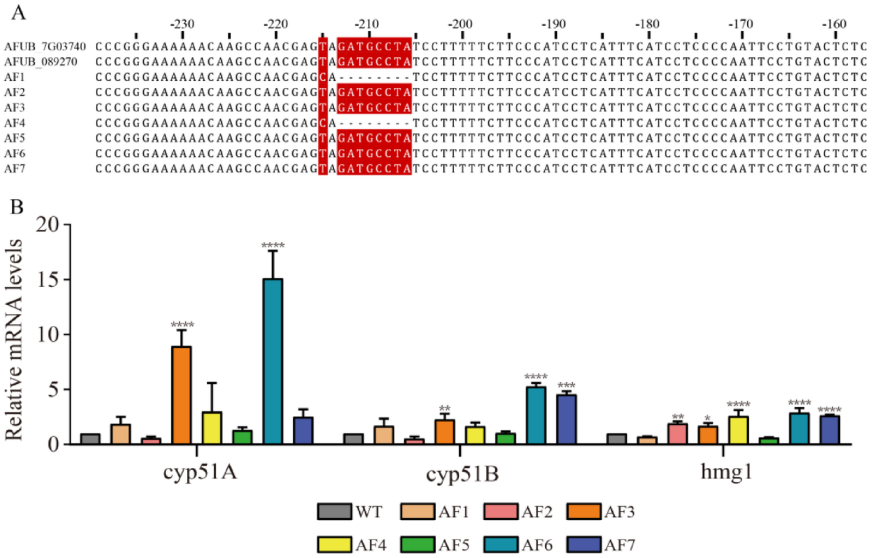

Fig. 2. Mutation sites of *cyp51B* and the mRNA levels of azole-resistance genes from AF1 to AF7. (**A**) Mutation sites located in the promoter region of *cyp51B*; (**B**) mRNA levels of the genes *cyp51A*, *cyp51B* and *hmg1* are shown. (*$P<0.05$; **$P<0.01$; ***$P<0.001$; ****$P<0.0001$)

Differences in radial growth and spore viability among azole-resistant strains

In comparison to strain Af293, no significant differences were observed in the colony color, morphology, or diameter among the seven azole-resistant strains (Fig. 3A and B). The assessment of conidia viability indicated a significant increase in strain AF3, while strains AF1 and AF4 exhibited significantly decreased viability. Furthermore, no significant differences were noted among strains AF2, AF5, AF6, and AF7 (Fig. 3C).

Differences in sensitivity to oxidative stress among azole-resistant strains

To further elucidate any differences in stress resistance among the various azole-resistant strains, we examined morphological changes of azole resistant *A. fumigatus* grown in media containing different oxidants or cell wall stress reagents. When cultured on a medium supplemented with menadione, the colony diameters of AF1, AF2 and AF7 increased compared to the wild-type at both 30 and 40 μM concentrations; surprisingly, AF5 was unable to survive at either concentration (Fig. 4).

Differences in sensitivity to cell wall stress among azole-resistant strains

To evaluate the tolerance of cell walls under stress conditions, strains were inoculated onto PDA medium enriched with various osmotic or ionic agents, including sodium dodecyl sulfate (SDS), potassium chloride (KCl), sodium chloride (NaCl), and sorbitol. Our results revealed that only AF3 was able to grow in 0.015% SDS, while the other strains could not. Compared to Af293, there were no significant differences in colony color, morphology, or colony diameter for AF3 at 0.005% and 0.01% SDS; however, the pigmentation of the other strains were slightly lower (Fig. 5A, B). In addition, for the sensitivity to various cell wall-perturbing agents, the colony diameters of all tested strains were unaffected by 1.2 M sorbitol. Conversely, the growth diameters of AF1, AF2, AF3, AF4, AF5 and AF6 were significantly increased compared to Af293 when exposed to 1.0 M KCl and 1.0 M NaCl, with only slight variations observed among the different strains (Fig. 5C, D).

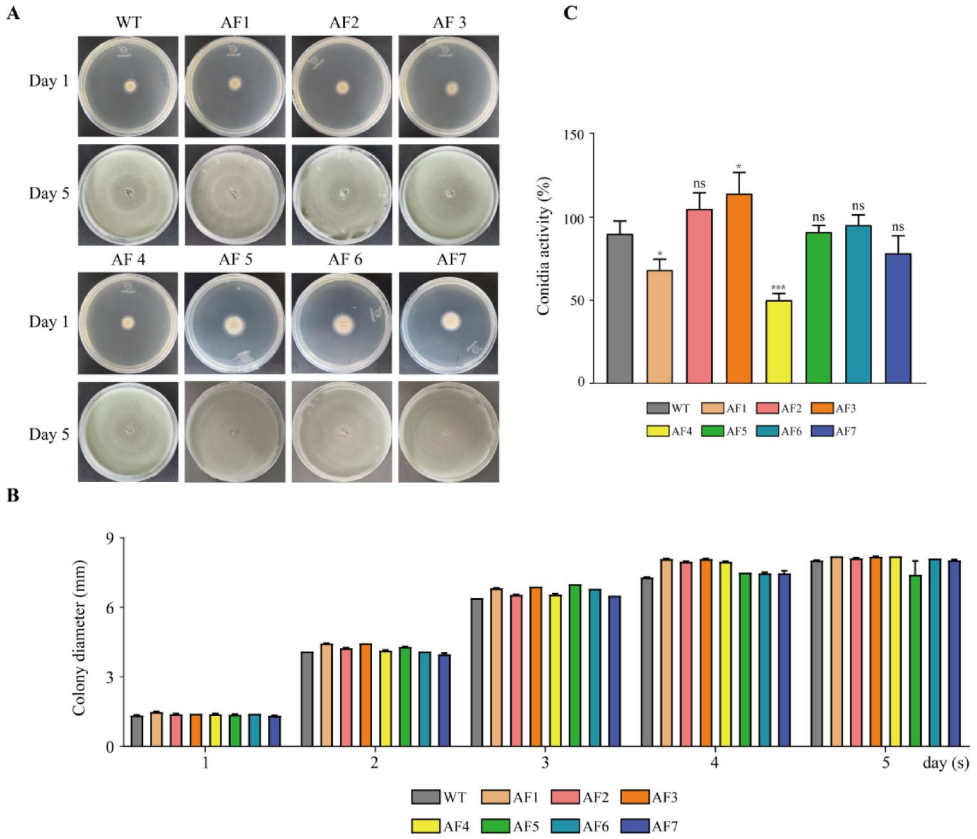

Fig. 3. Morphological analysis of azole-resistant *A. fumigatus*. (**A**) The morphologic images of azole-resistant *A. fumigatus* on PDA at day 1 and day 5; (**B**) Colony diameter at day 1 and day 5 statistics; (**C**) Spore viability statistics. (*$P<0.05$; **$P<0.01$; ***$P<0.001$; ****$P<0.0001$)

Discussion

A. fumigatus is a globally distributed environmental fungus and a major cause of aspergillosis, a serious and often fatal infection that primarily affects patients with compromised immune systems. Itraconazole, posaconazole, and voriconazole are the first-line treatment options for aspergillosis. However, over the past decade, numerous clinical and environmental reports have documented the emergence of azole-resistant *A. fumigatus*. The widespread occurrence of azole-resistant strains in the environment has resulted in the isolation of resistant strains from patients who have not previously been exposed to antifungal drugs[15,16]. Clinical isolates of azole-resistant *A. fumigatus* carrying these environmentally derived mutations are increasingly becoming the primary cause of resistance in patients exposed to azole treatments, indicating the significance of systematic resistance surveillance.

During the study period, the number of *Aspergillus* isolates gradually increased from July 2023 to July 2024, peaking in January, with counts ranging from 1.7 to 11.3 times higher than those observed in other months. This phenomenon may be related to the high prevalence of viral infections during this time. Notably, during the coronavirus disease 2019 (COVID-19) pandemic, COVID-19-associated pulmonary aspergillosis (CAPA) emerged as a significant complication, resulting in elevated mortality rates. The incidence of CAPA varies considerably based on factors such as country, region, and disease severity. Several studies have indicated that approximately 10–15% of patients with COVID-19 developed overlapping *Aspergillus* infections following comprehensive fungal examinations[17,18]. In a previous study, the incidence rate of CAPA among patients with severe to critical COVID-19 was reported to be about 0.4–2.7%. This rate may be influenced by several factors, including age, gender, chronic lung disease, the use of steroids and immunosuppressants, admission to the ICU, blood transfusions, and dialysis[19]. Additionally, another study identified elevated interleukin-6 (IL-6) levels, reduced CD4 + T-cell counts, and prolonged mechanical ventilation as independent risk factors for CAPA in COVID-19 patients[20]. Futhermore, azole resistance in *A. fumigatus* has increased over the past few decades. Based on our previous research findings[14], we monitored drug sensitivity for over two years and collected a total of 583 strains of *A. fumigatus*, of which seven were found to be resistant to azoles, indicating that the prevalence of azole resistance in Ningxia was approximately 1.20%. A previous study retrospectively evaluated the azole sensitivity of *A. fumigatus* isolates and reviewed literature on triazole resistant *A. fumigatus* published between 1966 and 2020 in China, demonstrating that the azole-resistant rate in China ranged from 2.5 to 5.56% for clinical isolates and from 0 to 1.4% for environmental isolates[21]. Data from the Danish National Surveillance Study over a two-year period revealed that the clinical azole resistance prevalence was 6.1% from 2018 to 2020[22], and 4.2% from 2020 to 2023 for environmental isolates[23]. A monitoring study conducted from 2011 to 2013

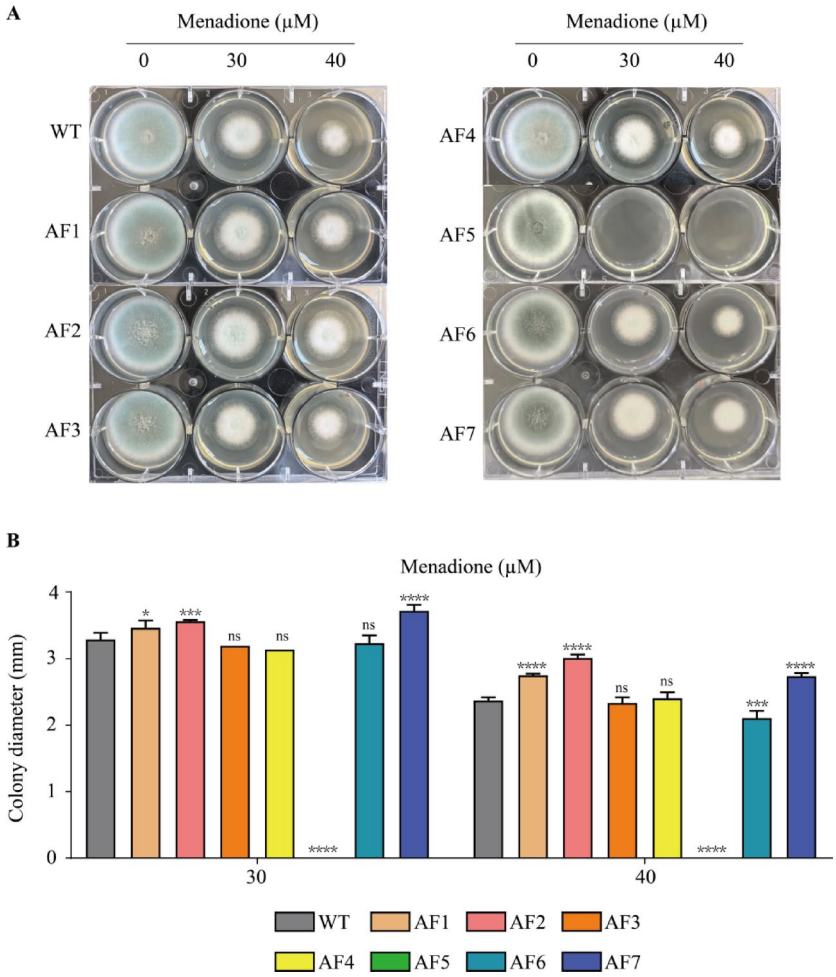

Fig. 4. Sensitivity of azole-resistant *A. fumigatus* to menadione. (**A**) Morphological images of strains exposed to various concentrations of menadione; (**B**) Statistical analysis of colony diameters depicted in Fig. 4A. (*$P<0.05$; **$P<0.01$; ***$P<0.001$; ****$P<0.0001$)

involving 1026 clinical isolates of A. fumigatus from 22 states in the United States showed no resistant strains and the MIC of azoles were generally low[24]. However, subsequent monitoring reports indicated an increase in the number of azole-resistant strains[25,26].

Cyp51 enzymes, known as 14-α sterol demethylases, are part of the cytochrome P450 family[27], and function as rate-limiting enzymes in the ergosterol biosynthesis pathway, which includes *cyp51A* and its homologue *cyp51B*[28]. The insertion of tandem repeats (TRs) in the promoter region of the *cyp51A* gene, along with amino acid substitutions in the coding region, has been identified as a predominant mechanism of azole resistance in *Aspergillus fumigatus*. Notable examples include TR34/L98H and TR46/Y121F/T289A, which confer resistance to itraconazole (ITC) and voriconazole (VRC), respectively, but typically result in a pan-azole resistance phenotype when assessed in vitro[29–31]. Strains AF3 and AF7 both possess the same *cyp51A* gene mutation (TR34/L98H); however, their azole resistance phenotypes differ. The AF7 strain demonstrates high resistance to ITC, exhibiting significantly elevated levels of *cyp51A*, *cyp51B*, and *hmg1* compared to AF3. This suggests that the development of azole resistance may involve various regulatory factors, and that the accumulation of multiple mutations can further influence enzyme activity and antibiotic affinity by altering the structure of *cyp51A*, potentially leading to distinct resistance phenotypes and gene expression profiles among strains. With the exception of the AF1 strain, all isolates exhibited *cyp51A* mutations previously shown to contribute directly to azole resistance. To further elucidate the resistance mechanism in AF1 strains, we investigated mutations in *cyp51B* and *hmg1*. Cyp51B is regarded as a significant complement to Cyp51A and plays a role in azole resistance. Buied et al. found that *cyp51B* was over-induced and constitutively expressed following azole exposure in an azole-resistant strain lacking *cyp51A* mutations[32]. Gonzalez-Jimenez I et al. identified a novel G457S mutation in *cyp51B* that was partially responsible for non-*cyp51A* associated resistance mechanisms[7]. In our study, we found only a single base mutation and an 8 bp deletion in the promoter region of *cyp51B* in strains AF1 and AF4. To further exclude the specificity of SNPs related to the genomic background, we also compared *cyp51B* in *A. fumigatus* A1163 (AFUB_089270) and obtained similar results. Mutations in this region have not been reported to date, and their potential association with azole resistance remains unclear, necessitating further investigation.

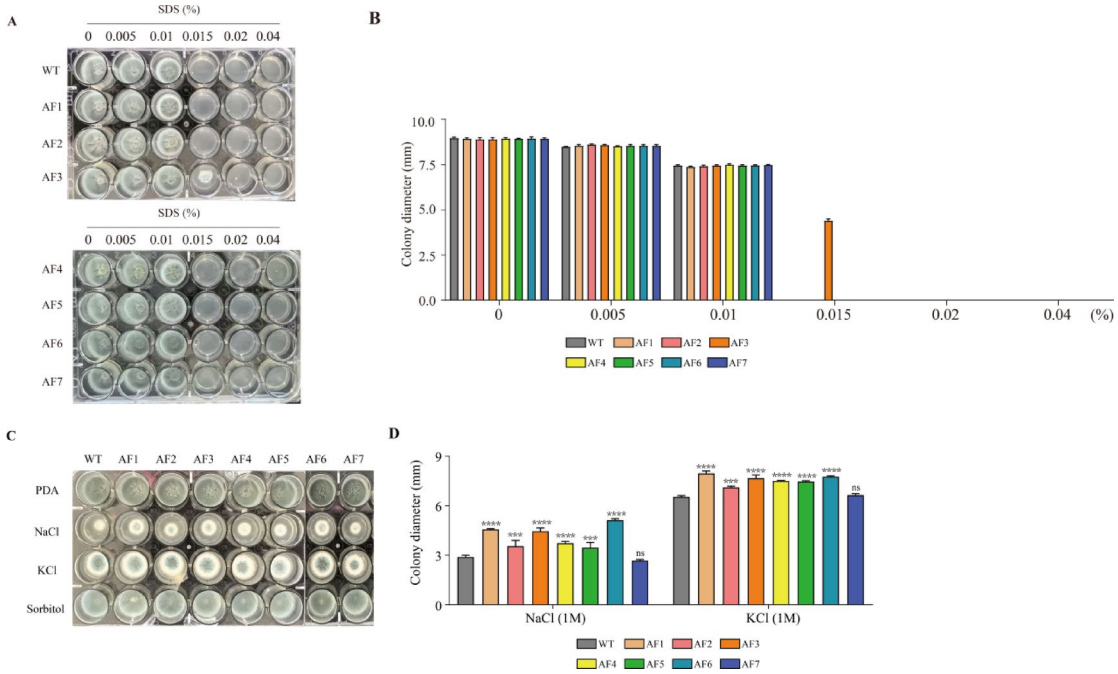

Fig. 5. Sensitivity to cell wall stress of azole-resistant *A. fumigatus*. (**A**) The morphologic images of azole-resistant *A. fumigatus* grown on PDA supplied with SDS ; (**B**) Colony diameter statistics. (**C**) The morphologic images of azole-resistant *A. fumigatus* grown on PDA supplied with NaCl, KCl and sorbitol; (**D**) Colony diameter statistics. (*$P<0.05$;**$P<0.01$; ***$P<0.001$; ****$P<0.0001$)

The Hmg1 enzyme in several eukaryotic organisms has been proven to catalyze the first committed step in ergosterol biosynthesis[33–35]. An analysis of *hmg1* polymorphism in the genomes of 170 *A. fumigatus* strains revealed that *hmg1* is a highly polymorphic gene[5]. Mutations located in the conserved sterol-sensing domain (SSD) of *hmg1*, such as D242Y, W272C, S269F, S305P, G307D, F390L, and I412S[4,5,36,37], have been identified to result in dramatically increased resistance to triazole agents via increased ergosterol production, accumulation of multiple ergosterol precursors downstream of Hmg1, or impair the protein's ability to sense sterols and signal for Hmg1 degradation[5,6,38]. Among these mutations, the S269F mutation has been reported to be associated with azole resistance, mainly due to an increase in ergosterol content in the cell wall[37]. Mutations D242Y, G307D, and P309L in Hmg1 were detected in azole-resistant strains that lacked any other known resistance mechanisms, indicating their potential role in resistance[5]. Similar to cyp51A-mediated resistance, triazole susceptibility profiles varied depending on the specific mutation sites in hmg1. Prior research indicates that the F390L mutation in Hmg1 may contribute to resistance against ITC and POS[39], while mutations S305P or G307D in Hmg1 were found to reduce susceptibility to VOR, and S269F mutation can confer multi-triazole resistance[36]. Amino acid changes located outside the SSD, such as E105K, S212P, Y564H, and S541G, which were detected in our study, were also found in azole-susceptible strains according to current studies[40,41], suggesting that these changes do not appear to be associated with azole resistance. The mRNA levels of *hmg1* in AF1 and AF5 were comparable to those of Af293; however, the mRNA levels of *hmg1* in AF2, AF3, AF4, AF6, and AF7 were significantly increased. The mechanisms by which these strains acquired *hmg1* mutations remain uncertain; some studies suggest that long-term antibiotic exposure is a contributing factor, while others report that most isolates with hmg1 mutations had no prior exposure to azoles[40]. Further research is necessary to elucidate these conflicting results.

In terms of basal growth analysis, our results indicated no significant differences in colony growth among the various resistant strains. During *A. fumigatus* infection, the host immune system triggers an increase in macrophage and neutrophil-mediated hyphal killing, primarily through the production of reactive oxygen species (ROS)[42]. This study also investigated the differences in resistance to oxidative stress among various azole-resistant isolates. The addition of menadione to the culture medium can mimic intracellular ROS production, thereby inducing oxidative stress in the strain. Although AF5 exhibits greater sensitivity to menadione stress compared to strain Af293, it shows significantly enhanced resistance to NaCl and KCl, while its resistance to SDS is comparable to that of Af293. This observation suggests that while AF5 has developed the capacity to resist salt stress, it may have compromised some of its ability to withstand oxidative stress. Experiments utilizing the *Galleria mellonella* larva infection model demonstrate no significant difference in virulence between AF5 and Af293 (Figure supplement 1), despite AF5 displaying resistance to azoles. Consequently, additional infection models are necessary to further investigate its responses to oxidative stress and virulence phenotypes[43]. The fungal cell wall is a dynamic organelle, and alterations to its structure represent a complex and tightly regulated process that is critical for pathogen survival by preventing cell lysis and shielding the fungus from environmental stress conditions[44,45]. Notably, AF3 demonstrates increased resistance in the presence of cell wall stress. Previous studies have established a correlation between cell wall integrity, stress resistance, strain virulence, and drug

resistance[46]. Furthermore, the distinctiveness of the cell wall components and the potential reorganization induced by azoles in AF3 warrant further investigation. In our study, we observed that azole-resistant strains exhibited variations in their mechanisms and phenotypes. Additionally, we further verified the variations in virulence using the Galleria mellonella infection model (Figure S1); however, no significant differences among these isolates were observed, indicating a complex interplay among host-*A. fumigatus* interactions.

However, this article has several limitations. First, we did not elucidate the mechanisms of resistance in non-*cyp51A* and *hmg1*-mediated strains, nor did we investigate the effects of *cyp51B* gene promoter deletion on resistance. Second, the molecular mechanisms underlying the differences in growth activity, antioxidant stress, and resistance to cell wall stress among these seven azole-resistant *Aspergillus fumigatus* require further investigation. Finally, the impact of these phenotypic characteristics on the host infection process remains unclear and warrants additional elucidation.

Conclusion

Our findings indicated that the prevalence of resistance in Ningxia was approximately 1.20%, primarily attributed to *cyp51A* mutations. Notable differences were observed in the growth, antioxidant responses, and cell wall stress phenotypes among various drug-resistant strains. This variation suggests that these strains exhibit a degree of adaptability, enabling them to survive under environmental stress. Therefore, effective surveillance and control strategies are essential to manage the widespread prevalence of azole-resistant *A. fumigatus* and to mitigate the risk of infection in patients.

Materials and methods

Strains, media, and culture conditions

The *A. fumigatus* strains used in this study were obtained from the clinical microbiology laboratory between July 2023 and July 2024. The isolates were cultured on Potato Dextrose Agar (PDA) plates at 37 ℃ for 5 days. Morphological identification was carried out based on both macroscopic and microscopic features. Molecular identification was performed by amplifying and sequencing the β-tubulin gene (*benA*) and the calmodulin gene (*CaM*). Conidia were harvested and cleaned with distilled water containing 0.05% Tween 80. The number of conidia were counted using a hemocytometer.

Antifungal susceptibility testing

Antifungal susceptibility testing was carried out using standard broth microdilution assays referring to the Clinical Laboratory Standards Institute (CLSI) M38-3rd guidelines. The drug concentration ranged from 0.03 to 16 μg/mL for amphotericin B (AMB), from 0.031 to 16 μg/mL for posaconazole (POS), voriconazole (VRC) and itraconazole (ITC), and from 0.015 to 8 μg/mL for micafungin (MFG) and caspofungin (CAS). Candida parapsilosis ATCC 22,019, and Candida krusei ATCC 6258 were used as the quality controls. The epidemiological cut-offs (ECVs) of AMB (2 μg/mL), CAS (0.5 μg/mL), ITC (2 μg/mL) and clinical breakpoint of VRC (≥ 2 μg/mL) refers to CLSI-M59 were used to define the isolates as wild type (WT)/susceptible (S) or non-WT/resistant (R).

Cyp51A, *cyp51B*, *hmg1* amplification and sequencing

The full coding sequences of the *cyp51A*, *cyp51B*, *hmg1* genes and *cyp51A* promoter region, were amplified and sequenced as previously described[5,7]. Short tandem repeats, single nucleotide polymorphism, and amino acid substitutions were identified by comparing the sequences with reference sequences from GenBank. To exclude the possibility of sequence alterations resulting from PCR-induced errors, each isolate was analyzed twice independently.

Genes expression analysis

Total RNA was extracted from mycelia using the RNAsimple Total RNA Kit according to the manufacturer's instruction (TIANGEN, Biotech, China), and cDNA was synthesized using a PrimeScript RT Master Mix kit (TaKaRa Biotechnology, China), The primer pairs used for qRT-PCR are listed in Table 3 to determine the expression of *cyp51A*, *cyp51B*, *hmg1*. Af293 was used as susceptible control. Relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method. Each sample were performed in triplicate.

Radial growth and spore viability analysis

Radial growth was assessed by point-inoculation of 3μL conidia (1×10^7conidia/mL) on the center of a PDA plate, incubated at 37 °C for 5 days, with colony diameter measurements taken every 24 h. For spore viability assays, conidia were collected after 2 days or 10 days of incubation. Approximately 1×10^2 conidia were spread on solid PDA plates incubated at 37 ℃ for 2 days to count the number of colonies formed. Conidial viability was calculated as the ratio of the number of viable colonies from 10-day-old conidia to the number of viable colonies from 2-day-old conidia.

Stress tolerance assay

To test sensitivity to oxidative and cell wall stress, 2 μL of a conidia suspension (1×10^6 conidia/mL) were spotted onto solid PDA plates supplemented with different concentrations of menadione (0 μM, 30 μM, 40 μM), sodium dodecyl sulfate (SDS) (0%, 0.005%, 0.01%, 0.015%, 0.02% and 0.04%) ,1.0 M KCl, 1.0 M NaCl, and 1.2 M sorbitol, each tested independently. The plates were incubated at 37 ℃ in the dark for 48 h. For oxidative stress agent hydrogen peroxide (H_2O_2), 100 μL of conidia (1×10^7conidia/mL) were spread on a PDA plate and 50 μL of different concentrations (30%, 15%, 7.5% and 3.75% v/v) of H_2O_2 were added to the center of the plate. After

incubation at 37 ℃ for 48 h, the diameter of the inhibition zone was measured. All the experiments were carried out in triplicate.

Galleria melonella infection experiments

Galleria mellonella larvae were procured from Huiyude Biotechnology Company based in Tianjin, China. Larvae weighing between 200 and 300 mg were selected for the experiment. Following the cultivation of Aspergillus fumigatus, its spores were harvested, diluted with PBS, and the concentration was adjusted to 1×10^6 conidia/mL. Each *Galleria mellonella* larva received an inoculum volume of 10 μL, with a total of 10 larvae per group. The operational procedures were conducted in accordance with previous studies[43,47]. After inoculation, the larvae were maintained in a dark environment at 37 ℃, and the mortality rate was assessed daily over the course of one week.

Data analysis

All statistical analyses were performed using Graphpad 8.0 with multiple comparisons evaluated through one-way analysis of variance. A P-value < 0.05 was considered statistically significant.

Data availability

Data from this study are available from the corresponding author upon request.

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Author contributions
Funding acquisition, Y.K.; conceptualization, Y.K., P.W., and W.J.; experiments, Y.K., W.M., and Q.L.; analysis, Y.K. and P.W.; writing-original draft, Y.K.; writing-review & editing, Y.K., P.W., and W.J.

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Declarations

Competing interests
The authors declare no competing interests.

Ethics approval
This study was approved by the Medical Science Ethics Institutional Review Board of General Hospital of Ningxia Medical University (KYLL-2024-0671, approved on 31 March 2024). This study follows the Declaration of Helsinki.

Informed consent
Informed consent was waived by the Medical Science Ethics Institutional Review Board of General Hospital of Ningxia Medical University.

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