Infection and Drug Resistance

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ORIGINAL RESEARCH

Epidemiology of Clinically Significant *Aspergillus* Species from a Large Tertiary Hospital in Shanghai, China, for the Period of Two Years

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Background: *Aspergillus* species are becoming a major public health concern worldwide due to the increase in the incidence of aspergillosis and emergence of antifungal resistance. In this study, we surveyed all *Aspergillus* species isolated from aspergillosis patients in Zhongshan Hospital Fudan University, Shanghai, China, from 2019 to 2021.

Methods: We characterized the susceptibility profiles of these *Aspergillus* species to medical azoles (voriconazole, itraconazole and posaconazole) using YeastOne™ broth microdilution system. To determine the underlying antifungal resistance mechanisms in azole-resistant *A. fumigatus* (AR*Af*) isolates, we characterized mutations in the *cyp51A* gene. Genotypic diversity of sampled *A. fumigatus* was investigated using CSP-typing.

Results: A total of 112 *Aspergillus* isolates (81 *A. fumigatus*, 17 *A. flavus*, 5 *A. niger*, 2 *A. terreus*, 2 *A. lentulus*, 2 *A. oryzae*, 1 *A. nidulans*, 1 *A. versicolor* and 1 *A. sydowii*) from 105 patients diagnosed with aspergillosis (including proven or probable invasive aspergillosis, chronic pulmonary aspergillosis, allergic bronchopulmonary aspergillosis and cutaneous aspergillosis) were obtained. Eight isolates (7 *A. fumigatus* and 1 *A. niger*) from seven patients were either azole non-susceptible or non-wild type. Azole non-susceptible or non-wild type rate was 7.1%/isolate and 6.7%/patient analysed. Four AR*Af* harbored TR34/L98H mutation, whereas one carried TR46/Y121F/T289A allele. The 81 *A. fumigatus* isolates were spread across 8 CSP types with t01 to be the predominant type (53.1%). AR*Af* isolates were distributed over CSP types t01, t02, t04A and t11.

Conclusion: Results from this study provided us with an understanding of the antifungal resistance and related characteristics of *Aspergillus species* in Eastern China. Further comparisons of our results with those in other countries reflect potential clonal expansion of *A. fumigatus* in our region. Further surveillance study is warranted to guide antifungal therapy and for epidemiological purposes.

Keywords: *Aspergillus fumigatus*, aspergillosis, antifungal susceptibility profile, non-susceptible, non-wild type, CSP-typing

Introduction

Aspergillus species are a group of globally distributed filamentous ascomycete molds which grow ubiquitously in nature.[1] Over 800 *Aspergillus* species have been recorded and described.[2] Infections with *Aspergillus* species lead to aspergillosis which contains a broad spectrum of illnesses including allergic bronchopulmonary aspergillosis (ABPA), chronic pulmonary aspergillosis (CPA) to life-threatening invasive aspergillosis (IA).[3] The annual incidence of CPA and IA in the world is estimated to be 3 million cases and over 250,000 cases, respectively.[4] The case-fatality rate associated with aspergillosis has been reported to be 20%–72% depending on the underlying conditions of the patients.[5,6] Aspergillosis has become a public health concern worldwide, while routine antifungal susceptibility testing for *Aspergillus* species has not been a common practice in clinical microbiology laboratories in many places including China. In addition, the susceptibility data of *Aspergillus* species to commonly used antifungals as well as the association to clinical outcomes are still lacking, especially for infections with azole-resistant

Received: 19 April 2023
Accepted: 12 July 2023
Published: 17 July 2023

A. fumigatus (AR*Af*). In 2022, WHO developed its first fungal priority pathogens list with *A. fumigatus* ranked the highest for perceived public health importance.[7]

Azole agents target and inhibit the biosynthesis of cytochrome P450 sterol 14α-demethylase (CYP51A), which in turn inhibits the cell growth.[8] Multiple azole resistance mechanisms have been characterized in *A. fumigatus* with the most well-described mechanism, mutations in the *cyp51A* gene. Mechanisms with tandem repeats (TR) in the promoter region of *cyp51A* gene, including TR34/L98H and TR46/Y121F/T289A were considered to be of environmental origin, whereas azole-resistance development after long-term medical azole therapy was considered as patient route.[9] Currently, aspergillosis and the causal pathogens, *Aspergillus* species, have been investigated in different regions from many countries with a focus on their antifungal resistance against empirically used medical azoles and genotypic diversities.[10–22] These reports suggested a high diversity in these fungal characteristics, which differ across geographical regions. For example, across countries in Europe, the detected azole-resistance frequencies have been found a 28-fold difference, ranging from 1.1% to 28%.[14,15] A similar large difference was also observed in other continents including America, Asia, Africa and Oceania, ranging from 0.6% to 13.9%.[10,16,17] The surveillance studies conducted in China reported the resistant rate to be lower than 10% with AR*Af* concentrated in the eastern and southeastern provinces.[18–22] Even within the same city, the prevalence of AR*Af* varies between individual hospitals.[18,19] With these highly diverse regional characteristics in *Aspergillus* species, it is necessary to develop surveillance programs on the antifungal resistance and genetic diversity of *Aspergillus* species at the hospital level and between different patient populations. Zhongshan Hospital Fudan University is one of the largest tertiary referral hospital in Eastern China with about 2005 beds and 104 ICU beds. Over 150,000 patients were admitted and around 4 million outpatient visits to this hospital per year. This study could provide a snapshot of the clinically significant *Aspergillus* species profile from Eastern China.

In this study, we surveyed all *Aspergillus* species isolated from aspergillosis patients in Zhongshan Hospital Fudan University from 2019 to 2021. We aimed to evaluate their susceptibility profiles to medical azoles and determine the potential underlying antifungal resistance mechanisms in AR*Af*. Furthermore, we characterized the genotypic diversity of sampled *A. fumigatus* using CSP-typing.

Materials and Methods

Clinical Data Collection and Definitions Used

One hundred and twelve *Aspergillus* species were isolated and collected from one hundred and five patients diagnosed with aspergillosis at Zhongshan Hospital Fudan University, Shanghai, China, from July 2019 to June 2021. Among these patients, seven patients were coinfected by two different isolates. Repeatedly isolated *Aspergillus* strains from the same patients were ruled out. The following data were collected for each patient: age, sex, underlying diseases, potential risk factors for fungal infections, previous exposures to azole antifungals, antifungal therapy received, and clinical outcome. Proven/probable invasive aspergillosis (IA), chronic pulmonary aspergillosis (CPA), and allergic bronchopulmonary aspergillosis (ABPA) were classified according to the European organization for research and treatment of cancer and the mycoses study group (EORTC/MSG) definitions proposed in 2019, the European Society for Clinical Microbiology and Infectious Diseases, the European Confederation of Medical Mycology and the European Respiratory Society Joint Clinical Guidelines (ESMID-ECMM-ERS) proposed in 2017 and new clinical diagnostic criteria for ABPA/Mycosis published in 2020, respectively.[23–25] Prior antifungal exposure was defined as having received an azole agent in the 3 months prior to *Aspergillus* species detection.

Isolates Identification

All specimens and *Aspergillus* isolates were processed and cultured according to routine clinical mycological procedures.[26] Briefly, all specimens were processed and inoculated on Sabouraud Dextrose Agar (Oxoid, UK). The inoculated plates were incubated at 35°C for at least 7 days. We first used Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-ToF-MS) (bioMerieux, France) to identify *Aspergillus* species, followed by molecular identification for azole non-susceptible or non-wild type *A. fumigatus* isolates. Partial *β-tubulin* genes were amplified and sequenced as previously described.[27]

In vitro Susceptibility Testing

Antifungal drug susceptibility was evaluated on all *Aspergillus* isolates against three commonly used medical azoles, itraconazole, posaconazole, and voriconazole, using Sensititre™ YeastOne™ broth microdilution system (YO10 panel, Thermo Fisher Scientific, USA) according to the manufacturer's instruction. Briefly, well-isolated colonies (over three different colonies per sample) from a 7-day culture of *Aspergillus* isolate were inoculated into the sterile saline water. The conidial suspension was then adjusted to 0.5 McFarland (0.6–5 × 10^6 CFU/mL) and 100uL of the suspension was added to 11mL YeastOne™ inoculum broth (Thermo Fisher Scientific, USA) and mixed thoroughly. A 100uL of the mixture was further inoculated into each well of the YeastOne™ microdilutions 96-well plate (Thermo Fisher Scientific, USA). The reference *A. fumigatus* isolate ATCC 204305 was used as the control. The minimal inhibitory concentration (MIC) values of isolate P53-27660 were further determined by the broth microdilution method according to the criteria of M38-A3 established by Clinical and Laboratory Standards Institute (CLSI).[28] The antifungal drugs were purchased from Sigma-Aldrich, USA. The reference *Candida parapsilosis* isolate ATCC 22019 was used as the control. The results of susceptibility tests were compared with the MIC breakpoints and epidemiological cutoff values (ECVs) published by the CLSI.[29,30] *A. fumigatus* isolates were regarded as resistant, intermediate and susceptible when the MIC was ≥2 ug/mL, 1 ug/mL and ≤0.5 ug/mL for voriconazole, respectively. On the other hand, *A. fumigatus* isolates with the MIC values above 1 ug/mL were considered to be non-wild type for itraconazole. For *A. niger*, isolates were classified as non-wild type if they had itraconazole, posaconazole and voriconazole MIC values above 4 ug/mL, 2 ug/mL and 2 ug/mL, respectively. *A. flavus* isolates were regarded as non-wild type if they had itraconazole, posaconazole and voriconazole MIC values above 1 ug/mL, 0.5 ug/mL and 2 ug/mL, respectively. For *A. terreus*, isolates were classified as non-wild type if they had itraconazole, posaconazole and voriconazole MIC values above 2 ug/mL, 1 ug/mL and 2 ug/mL, respectively. In this study, AR*Af* was referred to voriconazole non-susceptible (intermediate and resistant) and/or itraconazole non-wild type *A. fumigatus* isolates.

Detection of cyp51A Mutations

To determine the potential genetic mechanisms underlying antifungal resistance in the seven azole non-susceptible or non-wild type *A. fumigatus* isolates, we sequenced both the promoter region and the entire coding region of the *cyp51A* gene using Sanger sequencing following the protocols described previously.[31,32] The sequences were aligned with the *cyp51A* gene in the azole-susceptible strain (GenBank accession number AF38659) using CLC Main Workbench (QIAGEN bioinformatics, Germany).

Determination of CSP1 Types and Genetic Diversity

We also sequenced the *csp1* region and determined the genetic diversity of all sampled *A. fumigatus* isolates.[33] The CSP types of these isolates were assigned according to the CSP-typing nomenclature described by Klaasen et al in 2009. Simpson's index of diversity (D) was used to analyze the genetic diversity between these isolates, where n_j is the total number of *A. fumigatus* isolates of the particular jth type, N is the total number of *A. fumigatus* isolates in the test population, and s is the total number of different CSP types described.[34] MEGA 11.0 software was used to construct the maximum likelihood phylogenetic tree based on *csp* gene sequences to illiterate the distribution of AR*Af* isolates.

$$D = 1 - \frac{\sum_{j=1}^{s} n_j(n_j - 1)}{N(N-1)}$$

Statistical Analysis

The prevalence of CSP types between different hospital wards and departments was compared using the Fisher's exact test. Two-sided p-values of <0.05 were considered statistically significant. R studio version 4.2.1 software was used for statistical analysis.

Ethics Statement

The study protocol was approved by the Ethics Committee of Zhongshan Hospital Fudan University (approval number: B2022-376R).

Results

Specimen Origin and Characteristics of Aspergillosis Patients

In total, 112 *Aspergillus* isolates from 105 patients diagnosed with aspergillosis were obtained from July 2019 to June 2021. They were recovered from sputum (90.2%), bronchoalveolar lavage (7.1%), lung biopsy (0.9%), fluid from the surgical wound (0.9%), and peritoneal dialysis fluid (0.9%). The majority of the *Aspergillus* isolates were recovered from the Department of Respiratory Medicine (32.1%), Surgical ICU (22.3%), and Department of Infectious Diseases (16.1%). In addition, twelve (10.7%) and seven (6.3%) isolates were obtained from outpatients and patients who went to the emergency room, respectively.

All patients aged from 53 to 72 years old (median = 65) and the majority were males (75.2%). Out of 105 patients, 102 of them received antifungal therapy and azoles were administered as the empirical treatment for 91 patients. Fifteen patients had azole exposure before *Aspergillus* species detection. The most prevalent underlying diseases were chronic lung diseases (48.6%), hematological or solid malignancies (23.8%), and hematopoietic cell or solid organ transplantation (19.0%) (Table 1). Fifty-five (52.4%) patients were diagnosed with IA (2 proven, 53 probable). Thirty-seven (35.2%) and twelve (11.4%) patients were diagnosed with CPA and ABPA, respectively. The remaining patient was diagnosed

Table 1 Demographic Data and Clinical Characteristics of the Patients

Characteristic	All Patients (n=105)	IA (n=55)	CPA (n=37)	ABPA (n=12)	*Aspergillus fumigatus* (n=80)*	Non-*fumigatus Aspergillus* (n=30)*
Age, y, median (IQR)	65 (52–72)	67 (53–73)	66 (54–70)	54 (35–63)	66 (52–72)	62 (52–71)
Male sex	79 (75.2)	43 (78.2)	29 (78.4)	7 (58.3)	61 (76.3)	22 (73.3)
Underlying diseases						
Chronic lung diseases						
COPD	15 (14.3%)	2 (3.6%)	10 (27.0%)	3 (25.0%)	13 (16.3%)	3 (30.0%)
Bronchiectasis	19 (18.1%)	1 (1.8%)	14 (37.8%)	4 (33.3%)	16 (20.0%)	4 (13.3%)
Asthma	4 (3.8%)	1 (1.8%)	0 (0.0%)	3 (25.0%)	4 (5.0%)	1 (3.3%)
Prior pulmonary tuberculosis	11 (10.5%)	1 (1.8%)	9 (24.3%)	1 (8.3%)	10 (12.5%)	2 (6.7%)
Malignancies						
Lung cancer	15 (14.3%)	11 (20.0%)	3 (8.1%)	1 (8.3%)	10 (12.5%)	6 (20.0%)
Other solid tumors	6 (5.7%)	4 (7.3%)	2 (5.4%)	0 (0.0%)	4 (5.0%)	2 (6.7%)
Hematologic malignancies	6 (5.7%)	5 (9.1%)	1 (2.7%)	0 (0.0%)	4 (5.0%)	2 (6.7%)
Transplantation						
Liver transplantation	14 (13.3%)	13 (23.6%)	0 (0.0%)	0 (0.0%)	12 (15.0%)	2 (6.7%)
Kidney transplantation	1 (1.0%)	1 (1.8%)	0 (0.0%)	0 (0.0%)	1 (1.3%)	0 (0.0%)
Heart transplantation	5 (4.8%)	5 (9.1%)	0 (0.0%)	0 (0.0%)	5 (6.3%)	0 (0.0%)
Hematopoietic cell transplantation	2 (1.9%)	2 (3.6%)	0 (0.0%)	0 (0.0%)	2 (2.5%)	0 (0.0%)
Diabetes	13 (2.9%)	5 (9.1%)	7 (18.9%)	0 (0.0%)	13 (16.3%)	1 (3.3%)
Autoimmune diseases	15 (14.3%)	10 (18.2%)	5 (13.5%)	0 (0.0%)	9 (11.3%)	6 (20.0%)
Chronic renal failure	10 (9.5%)	9 (16.4%)	1 (2.7%)	0 (0.0%)	9 (11.3%)	2 (6.7%)
Multiple organ dysfunction syndrome	7 (6.7%)	7 (12.7%)	0 (0.0%)	0 (0.0%)	6 (7.5%)	2 (6.7%)
Prior azole therapy	15 (14.3%)	3 (5.5%)	9 (24.3%)	3 (25.0%)	12 (15.0%)	4 (13.3%)
Hospital admission	99 (94.3%)	55 (100.0%)	33 (89.2%)	10 (83.3%)	77 (96.3%)	27 (90.0%)
ICU admission	40 (38.1%)	38 (69.1%)	1 (2.7%)	0 (0.0%)	31 (38.8%)	11 (36.7%)
Antifungal therapy	102 (97.1%)	54 (98.2%)	37 (100.0%)	10 (83.3%)	77 (96.3%)	30 (100.0%)
Mortality	37 (35.2%)	34 (61.8%)	3 (8.1%)	0 (0.0%)	30 (37.5%)	9 (30.0%)

Notes: *Five patients were coinfected by both *A. fumigatus* and non-*fumigatus Aspergillus*.

Abbreviations: ABPA, allergic bronchopulmonary aspergillosis; COPD, chronic obstructive pulmonary disease; CPA, chronic pulmonary aspergillosis; IA, invasive aspergillosis; ICU: intensive care unit; IQR, interquartile range.

with post-operative cutaneous aspergillosis. The mortality rate of patients with IA was 7.6 folds higher than those with CPA. Among patients who had hematopoietic cell or solid organ transplantation, the rate of mortality within the observation period was 65.0%.

Distribution of Aspergillus Isolates

Using MALDI-ToF-MS, we have identified 9 different *Aspergillus* species. The most frequent species was *A. fumigatus sensu stricto* (72.3%), followed by *A. flavus* (15.2%), *A. niger* (4.5%), and *A. terreus* (1.8%). For azole non-susceptible or non-wild type *A. fumigatus* isolates, we further used *β-tubulin* sequencing to confirm the species identification. Our sequencing results showed 100% consistency and all species were identified as *A. fumigatus sensu stricto*. These *β-tubulin* gene sequence data have been submitted to the GenBank databases under accession number OR136835 to OR136841. Several cryptic species were identified, including two isolates of *A. lentulus*, and one *A. sydowii*. In addition, two *A. oryzae*, one *A. nidulans* and one *A. versicolor* were identified. Co-infection with multiple *Aspergillus* species was not very often observed in our hospital. Only five patients were observed coinfecting with *A. fumigatus* and *A. flavus*, whereas one patient was coinfected by *A. niger* and *A. flavus*. Two *A. fumigatus* strains (P13-27328-1 and P13-27328-2) with different morphological appearance were isolated from the same patient.

Susceptibility to Azoles

The antifungal susceptibility testing results of 112 *Aspergillus* species for medical azoles are represented in the MIC range, MIC_{50}, and MIC_{90} (Table 2). The overall prevalence of azole non-susceptible or non-wild type isolates was 7.1% (8 out of 112). Seven patients (6.7%) were infected with azole non-susceptible or non-wild type isolates. Among *A. fumigatus* isolates, four were found resistant to voriconazole and non-wild type for itraconazole. Isolate P81-27727 was resistant to voriconazole only, whereas isolate P3-27224 was non-wild type for itraconazole and intermediate to voriconazole (Figure 1). For isolate P53-27660, the MIC values of posaconazole, voriconazole and itraconazole were determined as 0.5 ug/mL, 2 ug/mL and 1 ug/mL using YeastOne™ broth microdilution system, respectively. The MIC value of voriconazole by the CLSI method was one dilution lower than this from YeastOne™ (1 ug/mL, intermediate). For posaconazole and itraconazole, the results from YeastOne™ and CLSI methods were identical. We also detected non-wild type isolate in the non-*fumigatus* species. One *A. niger* isolate had voriconazole and itraconazole MIC values above the ECVs. The azole non-wild type rate of *A. niger* was

Table 2 Susceptibility Profiles of *Aspergillus* Species Isolated from Aspergillosis Patients

Species (Number)		MIC (ug/mL) for		
		Posaconazole	Voriconazole	Itraconazole
A. fumigatus sensu stricto (81)	MIC range MIC_{50} MIC_{90}	0.03–2 0.12 0.25	0.12->8 0.25 0.5	0.06->16 0.25 0.5
A. flavus (17)	MIC range MIC_{50} MIC_{90}	0.25–0.5 0.25 0.5	0.5–1 0.5 1	0.25–0.5 0.25 0.5
A. niger (5)	MIC range	0.5–1	0.5–8	0.5->16
A. terreus (2)	MIC range	0.03–0.25	0.12–0.5	0.03–0.25
A. lentulus (2)	MIC range	0.5	1	0.5
A. oryzae (2)	MIC range	0.25–0.5	0.25–4	0.25–0.5
A. nidulans (1)	MIC range	0.12	0.12	0.12
A. versicolor (1)	MIC range	0.25	0.25	0.5
A. sydowii (1)	MIC range	0.25	0.5	0.5

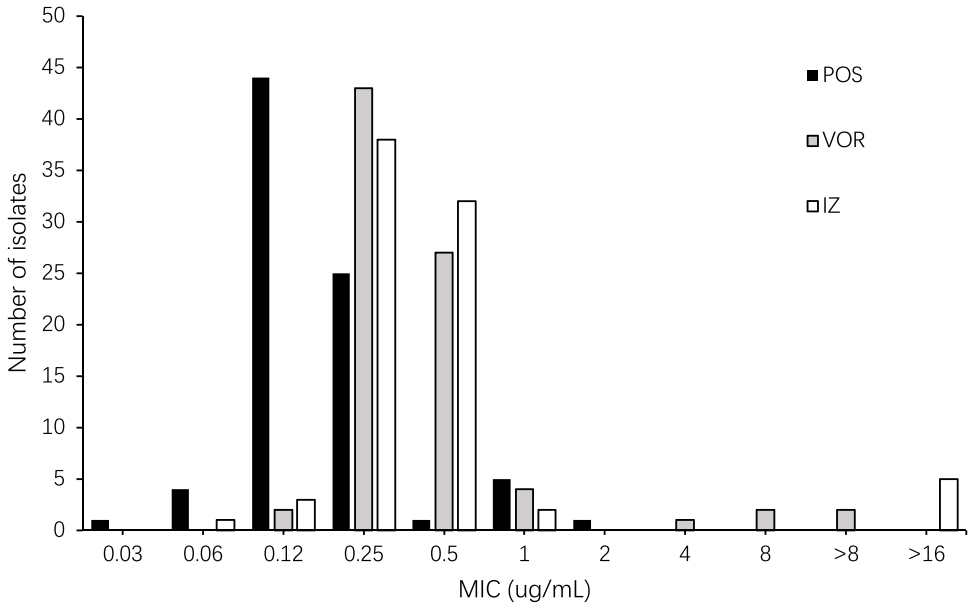

Figure 1 Distribution of posaconazole (POS), voriconazole (VOR) and itraconazole (IZ) MICs for 81 clinical *A. fumigatus* isolates.

20%. For the other *Aspergillus* sections species identified in our study, since ECVs or clinical breakpoints have not been established by CLSIs or European Committee on Antimicrobial Susceptibility Testing (EUCAST), we only reported measured MICs without determining whether they were resistant to the three azoles. One isolate of *A. oryzae* (O20-27329) recovered from a CPA patient had MIC of 4 ug/mL towards voriconazole. Two *A. lentulus* isolates had MICs of 1 ug/mL towards voriconazole.

Molecular Determination of Azole-Resistance Mechanism, ARAf Prevalence and Patient Outcomes

Five different mutations were detected in the *cyp51A* gene in six AR*Af* isolates. Four of them harbored TR34/L98H mutations, one carried TR46/Y121F/T289A mutations, and one with N248K mutation. In addition, in voriconazole-intermediate isolate P53-27660, we did not detect any mutations on the *cyp51A* gene. These *cyp51A* gene sequence data have been submitted to the GenBank databases under accession number OQ679715 to OQ679721. According to the number of patients included in this study, the overall prevalence of AR*Af* in all patients was 6.7%, which is lower than the prevalence in ICU patients (9.7%) and in the patients who had received prior azole therapy (16.7%) (Supplement Table 1). The mortality rate of patients infected with AR*Af* was 50% which is higher than the mortality rate of patients infected with azole-susceptible *A. fumigatus* (36.5%). For IA patients, patients with non-susceptible/non-wild type isolates had mortality rate of 75%, whereas patients with azole susceptible isolates had mortality rate of 60.8%. However, all CPA and ABPA patients with non-susceptible/non-wild isolates survived over the observation period. Hence, AR*Af* were not associated with a higher mortality than azole susceptible isolates in CPA and ABPA patients.

Molecular Characterization of *A. fumigatus*

The 81 *A. fumigatus* isolates that recovered in our study were categorized into 8 CSP types, t01 (53.1%), t02 (6.2%), t03 (6.2%), t04A (28.4%), t10 (1.2%), t11 (1.2%), t16 (2.5%), and t21 (1.2%). The Simpson's index of diversity was calculated at 0.64. Besides that, we observed no significant differences in the prevalence of CSP types between different hospital wards and departments (Table 3). We also compared the distribution of resistant alleles in different CSP types. We showed that isolates carrying TR34/L98H allele were mainly distributed over CSP types t02 and t11. Remarkably, the t01 type contained both azole-susceptible and azole-resistant isolates that harbored TR46/Y121F/T289A or N248K mutations. Isolate P53-27660 (intermediate to voriconazole without mutation in *cyp51A* gene) belonged to t04A (Figure 2).

Table 3 Prevalence of CSP Types Between Different Hospital Wards and Departments

CSP1 Types	Medicine General	Respiratory Wards and ICU	Surgical General and ICU	Outpatients and Emergency Room	p-value
t01	16	10	11	6	0.124
t02	2	0	2	1	0.3617
t03	1	4	0	0	0.1519
t04A	2	9	8	4	0.1007
t10	1	0	0	0	0.679
t11	0	0	0	1	0.1481
t16	0	2	0	0	0.5123
t21	0	1	0	0	> 0.9
Total	22	26	21	12	

Discussion

In this study, we reported findings from the survey on 112 *Aspergillus* species isolated from patients diagnosed with aspergillosis in Zhongshan Hospital Fudan University in Shanghai, China. We characterized the species distribution as well as their susceptibility to three medical azoles. For the major species in our survey, *A. fumigatus*, we further determined its azole non-susceptible or non-wild type mechanisms by focusing on the mutations in the *cyp51A* gene and characterized the genetic diversity using CSP-typing. Our results provided a regional perspective on the management of aspergillosis patients as well as pathogen characterization in China.

The Second Abundant Aspergillus Species Differs Across Geographical Regions

Similar to previous epidemiological reports in China and other countries, the most frequently isolated species from aspergillosis patients is *A. fumigatus*.[18–21] However, the second abundant *Aspergillus* species differed across geographical regions. In our study, *A. flavus* was the most common non-*fumigatus Aspergillus* species. This is consistent with several other reports in Asia, Africa, and the Middle East.[12,35,36] By contrast, data from Switzerland and Korea described *A. niger* as the most common non-f*umigatus Aspergillus* species.[14,37] This difference could be caused by many factors such as climatic conditions and characteristics of patient populations. For example, in tropical and subtropical regions such as Northeastern Iran, *A. flavus* has been reported to be the leading cause of invasive pulmonary aspergillosis.[38] It has higher fitness than the other non-*fumigatus Aspergillus* species because it is able to survive in hot and dry conditions.[35] This would lead to a higher prevalence of *A. flavus* in these regions, which is supported by aspergillosis surveys in these countries.[35,37–40] Besides, the second dominant *Aspergillus* species also depends on patient populations. In a Danish cystic fibrosis patient study, *A. terreus* was reported as the second most common species.[41] While 60% of non-*fumigatus Aspergillus* causing invasive aspergillosis in children and adults after hematopoietic stem cell transplantation (HSCT) and chemotherapy in a Russian medical center was *A. niger*.[42] This suggests that interactions between different *Aspergillus* species and patient characteristics could be important determinants of the distribution and spreading of *Aspergillus* species.[43]

Aspergillus Susceptibilities to Azoles are Highly Geographically Variable

The prevalence of azole-resistant *Aspergillus* species is geographically variable. Our study showed that 7.1% of *Aspergillus* species were either azole non-susceptible or non-wild type. This is similar to the reports in many other Asian countries including China, Japan, Korea, Pakistan, and Taiwan, where the prevalence of azole-resistant *Aspergillus* species was lower than 10%.[18–22,43–46] However, the prevalence of azole resistance in Asian countries is much lower than that in European countries.[10] AR*Af* has been reported in almost all European countries with the highest rate described in

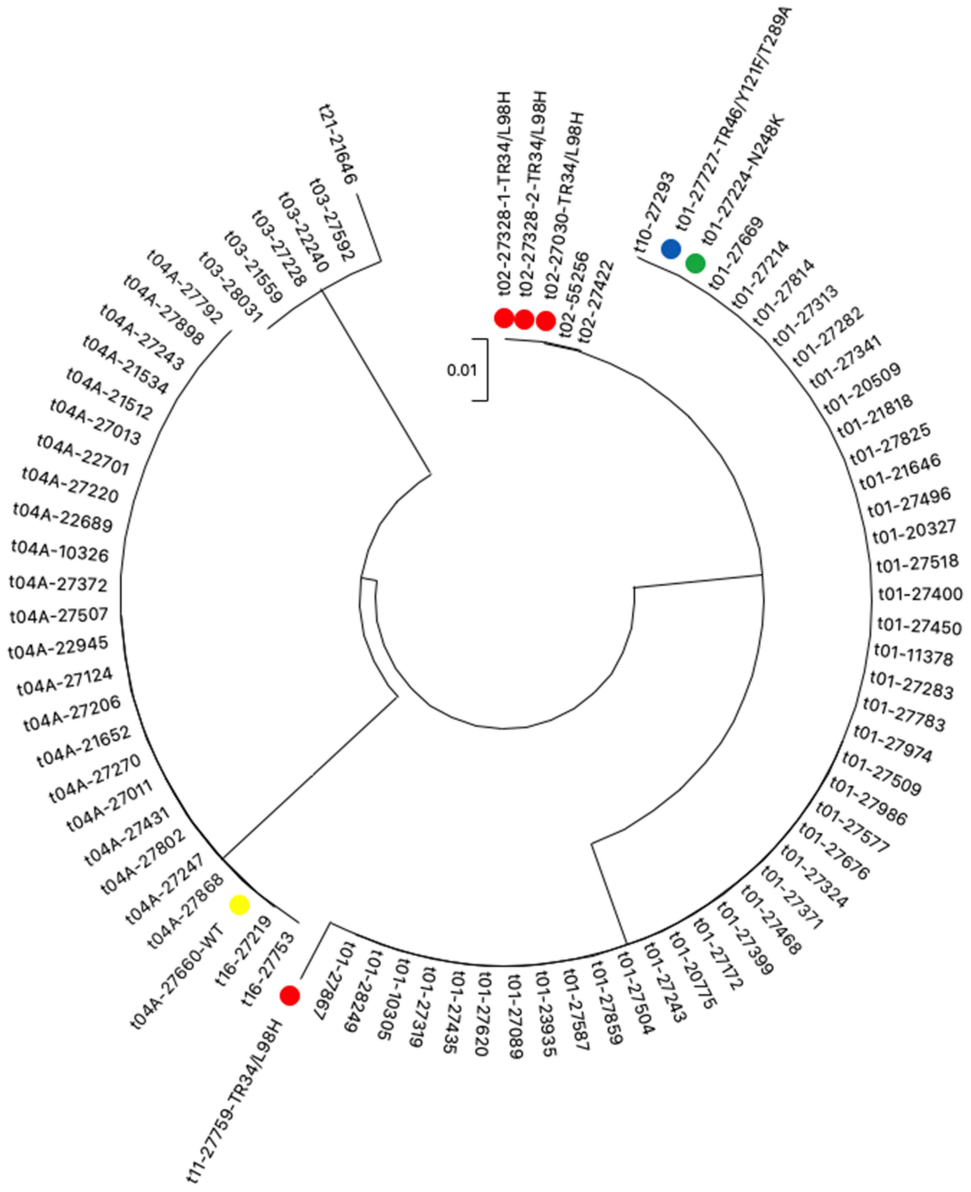

Figure 2 Phylogram based on *csp* gene sequences, showing the phylogenetic relationship between 81 *A. fumigatus* isolates. *cyp51A* mutations of azole non-susceptible or non-wild type isolates are given after CSP types. "WT" denotes voriconazole-intermediate isolate with unaltered *cyp51A* sequence.

the United Kingdom to be 28%.[15] Even within the same country, the prevalence of AR*Af* is geographically variable. A Chinese surveillance study observed AR*Af* was concentrated in the eastern and southeastern areas of China.[22]

This variation between different studies may mainly result from the patient's underlying conditions, prior azole exposure, and/or selective pressure from localized azole fungicide use. Resistance can develop after exposure to antifungal drugs either after long-term medical azole therapy (patient route) or after exposure to agriculture azole fungicides (environmental route).[9] In our study, the overall azole non-susceptible or non-wild type rate in patients who had received prior azole therapy was 20% (2 patients infected with *A. fumigatus* and 1 infected with *A. niger*). Similar high levels of azole-resistance have also been reported in chronic pulmonary aspergillosis patients and cystic fibrosis patients with long-term azole treatment.[47] Furthermore, localized resistance selection following exposures to agricultural fungicide uses may contribute to the variation in resistance against azoles. In Europe, the extensive application of agricultural azole has been associated with the emergence of AR*Af* in agricultural fields and AR*Af* detected in hospitals.[48] In China, we do have surveillance on determining sources of *Aspergillus* resistance.[49] Four patients infected with AR*Af* in our study had no prior azole exposure. Based on a recent population genomic study which

suggests azole-naïve patients acquire AR*Af* from the environment, we hypothesize that some of the clinical AR*Af* isolates in our study are environmental origin.[50]

Resistant isolates that recovered from ICU patients, hematopoietic cells or solid organ transplantation recipients usually receive more clinical attention because they often lead to severe complications and high mortality.[51,52] According to the number of *A. fumigatus* isolates recovered in this study, the rate of AR*Af* was 12.5% in ICU patients which is 2 folds higher than in those who have not been admitted to ICU. We also showed that hematopoietic cell or solid organ transplantation recipients are also more likely to harbor AR*Af* with a prevalence to be 10%. A higher prevalence of AR*Af* in similar patient populations was also observed in the Netherland and Germany, which was suggested to be due to the prophylactic and empiric prescribing of antifungals in these patients.[52,53] This also partially explains our observation of a much higher AR*Af* rate than another hospital in Shanghai (2.6%), which has a distinct patient population.[19]

Since our main *Aspergillus* species is *A. fumigatus*, we were able to further infer the genetic mechanism underlying its azole resistance surveyed mutations in both the promoter region and the protein-coding of the *cyp51A* genes in all resistant *A. fumigatus*. We found tandem repeats (TR) in the promoter region including TR34/L98H and TR46/Y121F/T289A, which were also considered to be of environmental origin.[9,50] This further supports our previous hypothesis that some AR*Af* in our survey could result from broad application of agricultural azole fungicides. In addition to the TR in promoter region, point mutations in the *cyp51A* gene are another main mechanism responsible for the increase in MICs to azoles.[9] In our study, we also noticed a special case, isolate P3-27224 harbored N248K mutation in *cyp51A* gene. It was obtained from IA patient who had severe hepatitis and liver failure with no prior azole exposure. This isolate exhibited high MIC towards itraconazole (>16 ug/mL) but relatively lower MIC to posaconazole and voriconazole (1ug/mL). N248K was found in a Belgian lung transplant patient who had prior voriconazole treatment.[54] This isolate was resistant to voriconazole only. More recently, this polymorphism was detected in AR*Af* isolated from the environment of Vietnam.[55] However, N248K amino acid substitutions have also been reported in many azole-susceptible strains, particularly in Asian countries.[44,56] These make it difficult to confirm whether N248K mutation confers azole resistance. Hence, further investigations on this polymorphism are required.

The infection with azole non-wild type non-*fumigatus* species continues to emerge in recent years and started causing high morbidity and mortality in many countries.[57] In our study, an isolate of *A. niger* (O15-27838) was found to be resistant to both voriconazole and itraconazole. It was isolated from a CPA patient with bronchiectasis. Besides that, *A. oryzae* O20-27329 recovered from another CPA patient was found resistant to voriconazole. A potential contributor to this resistance found in both patients could be their azole therapies prior to *Aspergillus* species detection. We also identified three other cryptic species which contribute to 2.7% of all isolated *Aspergillus* species. *A. lentulus* is a cryptic species of *A. fumigatus* complex. It was observed to exhibit high MICs for all triazoles in several studies and was considered to have intrinsic azole resistance.[58] In this study, two *A. lentulus* isolates had MICs of 0.5 ug/mL, 1 ug/mL and 0.5 ug/mL to posaconazole, voriconazole and itraconazole, respectively. As neither ECVs nor clinical breakpoints have not been established for *A. lentulus*, applying clinical breakpoint of voriconazole for *A. fumigatus* suggests these two *A. lentulus* isolates were intermediate voriconazole resistant. This suggests the necessity of a surveillance program on azole resistance in non-*fumigatus Aspergillus* species as well as their genetic mechanisms.[59]

Genetic Diversity Survey Suggests a Potential Clonal Expansion of *A. fumigatus*

Genetic typing of *A. fumigatus* isolates yielded from both environmental and clinical sources in different geographic regions has been done to demonstrate the clonality between these isolates.[60] Various genotyping methods were introduced, including multi-locus length polymorphism analysis (STRAf) and sequencing of the repeat region of the *csp1* gene (CSP typing). Although STRAf typing has high discriminatory, it may over interpret fast-changing loci.[61] CSP typing method is highly interlaboratory reproducible and easy handling which made it widely adopted.[33,34] Although there are a total of 30 different CSP types of *A. fumigatus* described,[60] we only observed eight types in our study, with t01 being the dominant CSP type (53.1%) and t04 (28.4%) the second. This pattern was also observed previously in China and clinical isolates in the Netherland, where t01 dominates surveyed *A. fumigatus* (37.3% and 34.5%, respectively).[33,62] The second dominant CSP type in our study, t04A, was reported to dominate in several other countries including Iran (45.6%), Argentina (41.3%), Mexico (38.8%), Australia (28.7%), Spain (22.9%) and another study conducted in Beijing,

China (31.5%), suggesting t04 could have the potential to dominate in China as well.[34,63–66] One report in a German survey showed that t03 was the most common type among clinical isolates,[60] whereas another Dutch survey showed that t04B was the more prevalent one in the environmental and clinical isolates.[67] The fact that CSP types t01, t03, and t04A and t04B of *A. fumigatus* have become the most prevalent types worldwide which suggests their ability to better survive in diverse and adverse conditions.[64,68]

We used Simpson's index of diversity to quantify the genetic diversity of *A. fumigatus* recovered in our study. We calculated Simpson's index based on the data reported in other CSP typing studies and included the results in Supplement Table 2. The Simpson's index in our study was 0.64, which is lower than the average of indices in the other studies (0.81, ranging from 0.76 to 0.86). This suggests a potential occurrence of clonal expansion in our hospital. We hypothesize that *A. fumigatus* may undergo microevolutionary processes during the infection in human hosts and further adaptation to specific niches.[69] Although our current evidence is not enough to confirm the clonal spread of CSP type t01 in Chinese patient populations or our hospital, we propose to include more environmental samplings and phylogenetic comparisons to determine the causes of limited CSP type diversity. This will allow us to understand the spread of *A. fumigatus* in the local population and healthcare facilities which may result in novel pathogenetic mechanisms.

AR*Af* isolates in this study were distributed over CSP types t01, t02, t04A and t11. AR*Af* with TR34/L98H were mainly found in CSP type t02 (3/4) which were genetically less diverse than azole-susceptible isolates. Similar observations were also noted in previous publications.[60,63] These suggest that isolates with TR34/L98H polymorphism may develop from a common ancestor and emerged independently. Similar conclusions were drawn by pan-genome analysis.[50] However, further work on global collections and analysis of *A. fumigatus* will be needed to confirm the hypothesis of TR34/L98H polymorphism origins.

CSP typing was also used to determine *A. fumigatus* nosocomial outbreaks.[70] In our study, two CSP type t16 *A. fumigatus* (P11-27753 and P17-27219) recovered from two separate Respiratory ICU patients with probable invasive aspergillosis. It was a rare CSP type that has only been reported in a Dutch clinical study.[33] P11-27753 was recovered from a patient with tracheotomy who was admitted to Respiratory ICU from January 2020 to March 2020. P17-27219 was isolated from a patient who had tracheal intubation during the hospital stay. This patient was admitted to Respiratory ICU in May 2020. Coincidentally, both patients were assigned to the same hospital bed. Although we did not detect *A. fumigatus* in the environment of our ICU wards through routine nosocomial infection monitoring, this coincidence suggests the possibility of the nosocomial spread of *A. fumigatus* in the hospital.

Conclusion

To conclude, results from this study provided us with a regional report on the antifungal resistance and related characteristics of *Aspergillus* species in Eastern China. Further comparisons of our results with those in other regions may reflect an environmental origin of resistant *Aspergillus* isolates and diverse antifungal resistance mechanisms in hospitals. Based on these data, we hypothesized that there could be a potential clonal expansion of *A. fumigatus* in Chinese patient populations as well as a potential nosocomial outbreak in our hospital, while further surveys and experiments are needed to test this hypothesis. Given that infection of azole-resistant *Aspergillus* species continues to emerge, further surveillance study is warranted to guide antifungal therapy and for epidemiological purposes.

Data Sharing Statement

The *cyp51A* gene sequence data in this study are openly available at the GenBank databases under accession number OQ679715 to OQ679721.

Ethics Approval and Informed Consent

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of Zhongshan Hospital, Fudan University (approval number B2022-376R) obtained on 1 September 2022. Informed consent was obtained from all subjects involved in the study.

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.

Funding

This work was supported by research grants from the Zhongshan Hospital, Fudan University (2021ZSQN41), to Yuyi Zhang.

Disclosure

The authors report no conflicts of interest in this work.

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