

Epidemiology and Antifungal Susceptibilities of Clinically Isolated *Aspergillus* Species in Tertiary Hospital of Southeast China

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Background and Aim: Infection caused by *Aspergillus* species poses a growing global concern, yet their prevalence in Southeast China lacks comprehensive documentation. This retrospective analysis aims to elucidate the epidemiological role and antifungal susceptibilities of *Aspergillus* species at Huashan Hospital of Fudan University, Shanghai, China.

Methods: Data spanning from 2018 to 2022, encompassing demographic, clinical, and laboratory information on *Aspergillus* species isolates were analyzed. The isolates were subjected to susceptibility testing using YeastOne™ broth microdilution system.

Results: A total of 253 *Aspergillus* isolates were identified, with *A. fumigatus* (57.71%) being the predominant species, followed by *A. niger* (26.88%), *A. flavus* (10.67%), and *A. terreus* (3.95%). Notably, the highest number of isolates originated from the Department of Infectious Disease (28.06%), with sputum (54.94%) being the primary source of isolation, where *A. fumigatus* was the dominant species. Gastrointestinal disorder (23.90%), hepatic disorder (9.09%), and diabetes (8.30%) were identified as the most prevalent underlying conditions, with *A. fumigatus* being the most abundant species in each case, accounting for 65.08%, 82.60%, and 73.91%, respectively, followed by *A. flavus*. Non-wild-type (NWT) *Aspergillus* isolates exhibited higher resistance against amphotericin B (AMB) compared to triazoles. Specifically, *A. fumigatus* showed greater resistance to AMB, with only 23.28% of isolates being susceptible, while the majority of isolates were susceptible to triazoles like itraconazole (ITR) and posaconazole (POS). POS demonstrated the highest efficacy against all species. Sequencing revealed mutations in the promoter region of the *cyp51A* gene and at positions Y121F and E247K in *A. fumigatus* which confer resistance to ITR, voriconazole (VRC), and POS.

Conclusion: These findings contribute to a better understanding of the epidemiology and antifungal resistance pattern of *Aspergillus* species in the region, providing valuable insights for the management of *Aspergillus*-related infections.

Keywords: *Aspergillus*, antifungal susceptibility, epidemiology

Introduction

Aspergillus species are commonly found in soil, decaying biological materials, and restrained places, which cause a serious fungal infection known as aspergillosis.¹ Infections caused by these ubiquitous species often challenging to diagnose, and the mortality rate can be as high as 30%–95%.² In recent years, the incidence of invasive aspergillosis has increased significantly due to the use of various immunosuppressive agents, the development of organ transplantation, and the increase of AIDS. Among these, *Aspergillus fumigatus* is the most common species responsible for about 70–80% of human aspergillosis cases. Infections from other species such as *A. niger*, *A. flavus*, and *A. terreus* are also increasing, particularly in individuals with weakened immune systems.³

The problem of aspergillosis is increasing, provoking a significant risk to global healthcare system.⁴ However, the incidence of aspergillosis in China is not well documented, with only a few studies conducted in specific regions, such as the southeast.⁵ A 2017 study estimated that there are 3 million global cases of chronic pulmonary aspergillosis and

2.5 million of invasive aspergillosis.⁶ Aspergillosis is more likely to affect people with weakened immune systems, such as those with blood cancers, organ transplants, and chronic lung conditions, as well as those who come into contact with environmental factors like dust and mold.⁷ One study showed that mortality increased by 60% in COVID-19-infected patients and by 20% in patients with solid organ transplants.^{8,9}

The common treatment approach for aspergillosis involves the use of triazole antifungal drugs, such as isavuconazole (ICA), itraconazole (ITR), posaconazole (POS), and voriconazole (VRC).¹⁰ In cases where isolates are resistant to azoles, significant antifungal agents like amphotericin B (AMB) and echinocandins come into play.¹¹ Echinocandins are typically administered as part of a multi-drug antifungal treatment in conjunction with azoles, rather than as a standalone treatment therapy.¹²

Many factors, such as the type, severity, and susceptibility profile of the infecting *Aspergillus* isolate, influence the selection of a specific antifungal agent.¹³ Anti-fungal susceptibility testing is crucial for selecting suitable antifungal treatment, as it helps to identify wild-type and non-wild-type *Aspergillus* isolates with respect to specific antifungal agents.¹⁴

The susceptibility of *Aspergillus* isolates to antifungal drugs shows variation depending on the species, geographical location, and local patterns of antifungal agent consumption.¹⁰ There has been a recent rise in non-wild-type *Aspergillus* isolates, particularly in relation to azole drugs, which are the mainstay of therapy for aspergillosis.¹⁴ Recent studies have identified several mutations in the *cyp51A* gene that are strongly associated with azole resistance in *A. fumigatus*. These mutations are reported to be linked to overexpression of the *mdr* gene, which may contribute to drug resistance in *A. fumigatus*.^{15,16} Another study reported that genes responsible for encoding proteins involved in cell wall remodeling, oxidative stress response, and energy metabolism are key factors contributing to antifungal drug resistance in *Aspergillus* species.¹⁷ Surveillance studies that examine antifungal susceptibility patterns in *Aspergillus* species play a crucial role in detecting these emergent non-wild-type pathogens.¹⁸ Such data is essential for directing the appropriate selection of antifungal agents and has a significant impact on the management and outcomes of aspergillosis.¹⁵

This study, which is being conducted as a retrospective analysis, aims to look into the epidemiology, risk factors, and antifungal susceptibilities of *Aspergillus* species within Huashan hospital in Shanghai, China. The results of this study are expected to provide valuable insights for healthcare officials, aiding in the effective management of aspergillosis caused by *Aspergillus* species. Furthermore, the findings may have suggestions for deciding suitable antifungal drugs and developing therapeutic plans specific to the region.

Materials and Methods

Patient Data Collection

The current retrospective study from the five years (2018–2022) was conducted at Huashan Hospital, which is one of the top tertiary hospitals in China located in Southeast region. Demographic and clinical information of the patient host to clinical isolates of *Aspergillus* species was gathered from the hospital's digital record. The collected data encompassed various patient/host characteristics, including gender, age, reported department and sample source according to recent similar study.⁵ *Aspergillus* species isolates from hospital record were subjected to analysis using appropriate statistical tests.

Isolation and Identification of *Aspergillus* Species

Standard clinical mycological measures were followed for the processing of the samples collected to isolate, purify and identify *Aspergillus* species.¹⁹ Briefly, the samples were processed for culturing through inoculating onto Sabouraud Dextrose Agar (Crmicrobio, China). The plates were then incubated at 35°C for at least 7 days. The Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-ToF-MS) developed by Zybion in China was utilized to identify the *Aspergillus* species.²⁰ Resistant isolates were further confirmed through molecular identification.²¹

In vitro Antifungal Susceptibility Testing

The susceptibility testing for antifungal drugs (AMB; Amphotericin B, CAS; Caspofungin, ITR; Itraconazole, POS; Posaconazole, VRC; Voriconazole) was conducted using the Sensitizer YeastOne™ system (Thermo Fisher Scientific, USA). In brief, purified colonies of the *Aspergillus* species were added to a sterilized saline solution. The conidial suspension was adjusted to 0.5 McFarland ($0.6\sim5\times10^6$ CFU/mL), and 100 µL of this was added to 11 mL of YeastOne™ inoculum broth and mixed well. Afterwards, 100 µL of the mixture was introduced into each well of the YeastOne™ micro-dilutions 96-well plate (Thermo Fisher Scientific, USA). *A. fumigatus* ATCC 204305 and *Candida parapsilosis* ATCC 22019 strains were used as the control reference. The broth microdilution method, following the standards set by the Clinical and Laboratory Standards Institute (CLSI). The susceptibility test findings were compared to the MIC breakpoints and epidemiological cut-off values (ECVs) established by the CLSI.

CLSI breakpoints were not available for the mentioned drugs. However, CLSI (M57S-4th ed.) provides ECVs for these drugs, which were used to classify the strains as wild-type or non-wild-type. Briefly, *A. fumigatus* strains having MIC values greater than 1 µg/mL were classified as non-wild type for ITR. In contrast, *A. fumigatus* isolates were categorized as resistant, intermediate, and susceptible when the MIC for VRC was ≥ 2 µg/mL, 1 µg/mL, and ≤ 0.5 µg/mL, respectively. For *A. niger*, strains were categorized as non-wild type if their ITR, POS, and VRC MIC values exceeded 4 µg/mL, 2 µg/mL, and 2 µg/mL, respectively. *A. flavus* strains were classified as non-wild type if their ITR, POS, and VRC MIC values exceeded 1 µg/mL, 0.5 µg/mL, and 2 µg/mL, respectively. Regarding *A. terreus*, strains were classified as non-wild type if their MIC values for ITR, POS, and VRC exceeded 2 µg/mL, 1 µg/mL, and 2 µg/mL, respectively.^{22,23}

Identification of cyp51A Anomaly

The cyp51A gene is well-documented as a key target in antifungal resistance, particularly against azole drugs. Mutations in this gene are directly linked to resistance mechanisms in *A. fumigatus* and other species.¹⁶ To uncover possible genomic mechanisms associated with antifungal resistance in the *Aspergillus* species that were non-wild type and non-susceptible, we sequenced both the promoter region and the coding region of the cyp51A gene. Briefly, the selected isolates were inoculated into 15 mL of GYEP medium (containing 2% glucose, 0.3% yeast extract, 1% peptone) and cultured for 48 hours at 37°C.²⁴

DNA extraction was carried out using a commercial kit (Tiangen, China) following the provided instructions. Three sets of primers were designed in total: one primer for sequencing the promoter region, and two primers for sequencing the cyp51A gene, targeting amplicons of 1050 bp and 500 bp, respectively (Table 1). Primers for polymerase chain reaction (PCR) and sequencing were designed based on the promoter and coding region of cyp51A (Table 1). The cycling conditions included an initial step at 95°C for 5 minutes, followed by 40 cycles of 94°C for 30 seconds, 50–57°C (as per Table 1) for 45 seconds, and 72°C for 2 minutes. The process concluded with a final extension at 72°C for 7 minutes. Purification of the PCR products was performed using the Omega E.Z.N.A.® Cycle Pure (CP) Kit (Omega Bio-Tek, Norcross GA/USA) according to the manufacturer's instructions. Subsequently, DNA sequences were determined using a Dye Terminator Cycle Sequencing [DTCS] Quick Start Kit (Beckman-Coulter Inc, Brea CA/USA) and CEQ™ 8000

Table 1 Primers, Annealing Temperatures, Target Sequence and Annealing Positions

Region	Annealing temperature	Primers		Target sequence (bp)	Annealing position
Promoter region	53°C	Fp	5'-GGAGATACTATGGCTTTCAT-3	200	1-20
		Rp	5'-GTATGCTGGAACACACCT-3	200	181-200
cyp51A	57°C	F1	5'-ATGGTGCCGATGCTATGG-3'	1040	357-375
Primer 1		R1	5'-TACTGGAGCGGAGGAAGA-3'	1040	1389-1407
cyp51A	50°C	F3	5'-CTGCGTTAGAGTCTCATGT-3'	500	859-879
Primer 2		R3	5'-TGGCCAGCTGTTCTCTGAT-3'	500	1340-1359

capillary electrophoresis DNA sequencer (Beckman Coulter). The obtained sequences were aligned with the sequence from an azole-susceptible strain (GenBank accession no. AF338659), and mismatches were identified through ClustalW analysis.²⁵ Along with sequencing of the *cyp51A* gene, identification of the *Aspergillus* species was reconfirmed through sequencing of the β -tubulin gene.

Statistical Analysis

The data is presented in Mean $\pm$ Standard Deviation where the prevalence of *Aspergillus* species between different hospital wards and departments was compared using the Fisher's exact test, chi-square tests, and ANOVA. p-values of <0.05 were considered statistically significant.

Results

Aspergillus Species Distribution

In the current study a total number of 253 *Aspergillus* species were retrospectively collected from 2018 to 2022. It was observed that six species of the *Aspergillus* are involved in the cause of aspergillosis. The highest number of isolates was *A. fumigatus* (146, 57.71%), followed by *A. niger* (68, 26.88%), *A. flavus* (27, 10.67%) and *A. terreus* (10, 3.95%). Only one isolate of *A. clavatus* and *A. nidulans* was reported respectively. A detailed depiction of the reported species is given in (Figure 1A). In 2018 and 2019, there were 82 recorded *Aspergillus* species. The number of reported cases decreased to 30 in the year 2020, 31 in the year 2021, and 28 in the year 2022. In comparison to the total number of cases recorded in 2018 and 2019, the number of reported cases in the subsequent years decreased by approximately 0.36-fold which could be attributed to the rise in cases of severe acute respiratory syndrome coronavirus 2 (SARS CoV 2) and challenges with sample collection. *A. fumigatus* was responsible for the highest portion in each recorded year, followed by *A. niger*. Detailed results are presented in (Figure 1B).

Among the various hospital departments, the department of infectious diseases reported the highest number of cases (71, 28.06%), followed by the Respiratory and Critical Care Medicine Department with (n = 60, 23.72%) cases. The department of Dermatology reported the third-highest number of cases (16, 8.70%), while the departments of Cardiology and Endocrinology each reported just one case. Additionally, *A. fumigatus* was the predominant cause in the highest number of cases in each department of the hospital. Detailed results of *Aspergillus* species with number and percentage of cases are given in (Table 2).

The majority of isolates (204, 80.63%) among all sample types were isolated from Respiratory samples. The second highest number of isolates was recovered from Body discharges (13, 5.13%), followed by CSF (12, 4.74%) isolates. From the bodily fluids, the minimum number of isolates obtained was (5, 1.97%). *A. fumigatus* accounted for the greatest number of isolates in each instance. Detailed results are presented in (Figure 2).

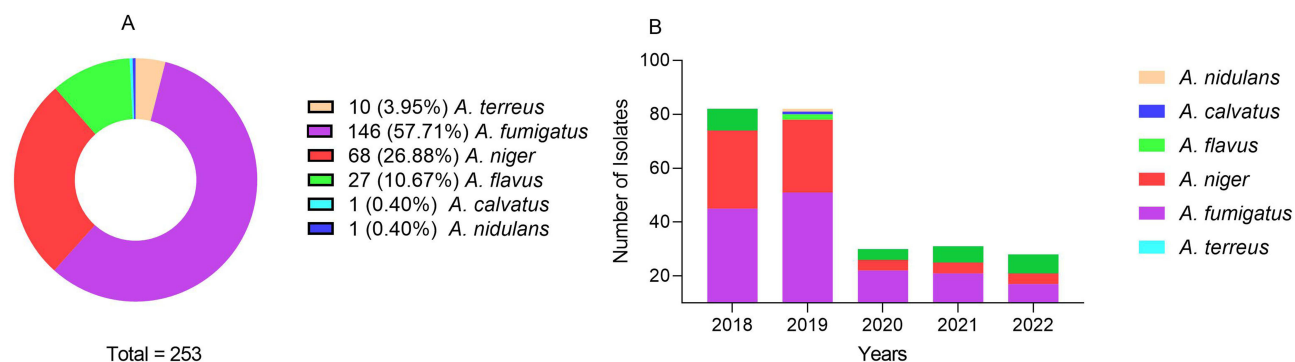


Figure 1 (A) *Aspergillus* species distribution from 2018–2022. (B) *Aspergillus* species distribution each year.

Table 2 *Aspergillus* Species Isolates Were Reported from Different Departments of the Hospital Presenting the Occurrence of *Aspergillus* Species from Each Sample

Department	Total (253,100%)	<i>A. fumigatus</i>	<i>A. niger</i>	<i>A. flavus</i>	<i>A. terreus</i>	<i>A. clavatus</i>	<i>A. nidulans</i>
Antibiotics	13 (5.14)	9 (69.23)	3 (23.07)	1 (7.69)	0 (0)	0 (0)	0 (0)
Cardiology	1 (0.40)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Dermatology	22 (8.70)	12 (54.54)	4 (18.18)	5 (22.72)	1 (4.54)	0 (0)	0 (0)
Emergency ward	3 (1.19)	1 (33.33)	1 (33.33)	1 (33.33)	0 (0)	0 (0)	0 (0)
General surgery	15 (5.93)	9 (60)	4 (26.66)	1 (6.66)	1 (6.67)	0 (0)	0 (0)
Geriatrics	16 (6.32)	10 (62.5)	5 (31.25)	1 (6.25)	0 (0)	0 (0)	0 (0)
Hematology	3 (1.19)	3 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Infectious disease	71 (28.06)	41 (57.74)	24 (33.8)	4 (5.63)	2 (2.81)	0 (0)	0 (0)
Intensive care unit	13 (5.14)	7 (53.84)	1 (7.69)	4 (30.76)	1 (7.69)	0 (0)	0 (0)
Internal medicine	5 (1.98)	2 (40)	2 (40)	1 (20)	0 (0)	0 (0)	0 (0)
Nephrology	10 (3.95)	8 (80)	1 (10)	0 (0)	1 (10)	0 (0)	0 (0)
Neurosurgery	14 (5.53)	6 (42.85)	4 (28.57)	2 (14.28)	2 (14.28)	0 (0)	0 (0)
Otolaryngology	5 (1.98)	2 (40)	3 (60)	0 (0)	0 (0)	0 (0)	0 (0)
Rehabilitation	2 (0.79)	2 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Respiratory& Critical care	60 (23.72)	33 (50)	16 (26.66)	7 (11.66)	2 (3.34)	1 (1.66)	1 (1.67)

Characteristics of *Aspergillus* Species Hosts

Table 3 shows the demographic and clinical characteristics of the *Aspergillus* species isolated from the hosts. A significant number of cases (73.12%) were reported in the male population (185 isolates), while the female population had only 68 isolates (26.88%). This pattern was consistent across all the species. Majority of cases occurred in senior adults, with a median age of 64 years and an interquartile range of 51~74 years. Statistically significant differences ($p < 0.05$) were observed in the distribution of *Aspergillus* species among various age groups. *A. niger* showed higher

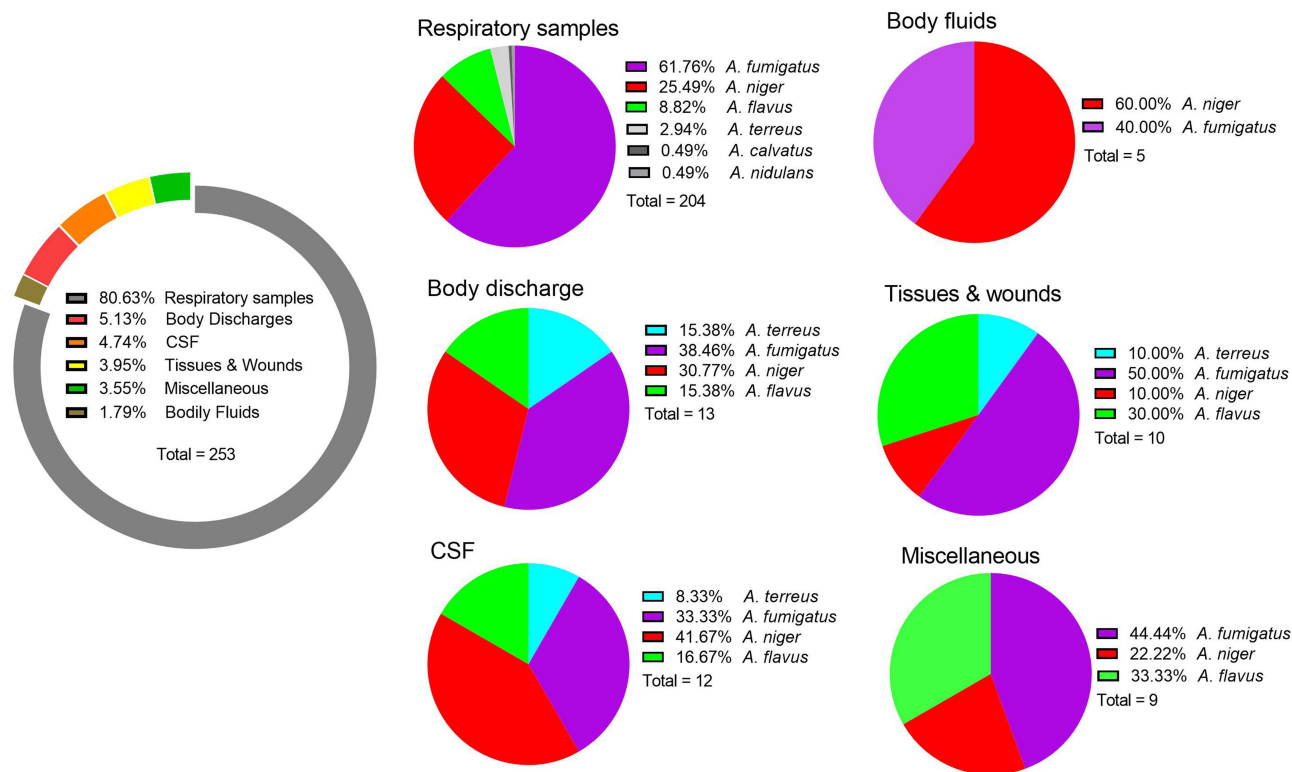


Figure 2 *Aspergillus* species isolated from different biological samples. Each pie chart next to the donut chart.

Table 3 Baseline Characteristics of *Aspergillus* Species

Variables	Total (253,100%)	<i>A. fumigatus</i> (146, 57.70%)	<i>A. niger</i> (68,28.87%)	<i>A. flavus</i> (27,10.67%)	<i>A. terreus</i> (10, 3.95%)	X ² , df	P value
Gender							
Male	185 (73.12)	105 (57.83)	52 (28.11)	18 (5.94)	6 (3.24)	283, 3	<0.0001
Female	68 (26.88)	39 (75.35)	16 (23.53)	9 (13.23)	4 (5.88)	42.2, 3	<0.0001
Age groups							
2 to 17 Years	5 (2.37)	1 (20.00)	3 (60.00)	1 (20.00)	0 (0.00)	6, 3	0.1991
18 to 60 Years	106 (41.90)	60 (56.60)	25 (23.58)	14 (13.21)	7 (6.60)	142.3, 3	<0.0001
61 to 80 Years	110 (43.48)	62 (56.36)	34 (30.91)	10 (9.10)	2 (1.81)	174, 3	<0.0001
>80 Years	32 (12.65)	23 (71.87)	6 (18.75)	2 (6.25)	1 (3.12)	82, 3	<0.0001
Median age (IQR)	64 (51–74)	65 (51–77)	63 (52–70)	58 (45–69)	60 (55–74)	0.548, 3	0.9646
Underlying pulmonary status							
COPD	51 (20.15)	42 (82.35)	0 (0.00)	5 (9.80)	4 (7.84)	90.6, 3	<0.0001
Bronchiectasis	34 (13.44)	26 (76.47)	2 (5.88)	4 (11.76)	2 (5.88)	48.4, 3	<0.0001
Asthma	37 (14.62)	27 (73.97)	0 (0.00)	6 (16.21)	4 (10.81)	47.4, 3	<0.0001
Pneumonia	18 (7.11)	16 (88.89)	0 (0.00)	2 (11.11)	0 (0.00)	39.8, 3	<0.0001
Respiratory failure	13 (5.14)	9 (69.23)	1 (7.69)	3 (23.07)	0 (0.00)	15, 3	0.0018
Underlying systematic disorder							
Gastrointestinal disorders	63 (23.90)	41 (65.08)	7 (11.11)	15 (23.81)	0 (0.00)	62.1, 3	<0.0001
Renal failure	7 (2.76)	6 (85.71)	0 (0.00)	0 (0.00)	1 (14.28)	14.1, 3	0.0027
Hepatic disorders	23 (9.09)	19 (82.60)	0 (0.00)	2 (8.69)	2 (8.69)	41.2, 3	<0.0001
Diabetes	21 (8.30)	17 (73.91)	2 (8.69)	2 (8.69)	2 (8.69)	29.3, 3	<0.001
Cardiovascular disease	18 (7.11)	10 (55.55)	4 (22.22)	4 (22.22)	0 (0.00)	11.3, 3	0.0101
Hypertension	18 (7.11)	8 (44.44)	3 (16.67)	2 (11.11)	5 (27.78)	4.67, 3	0.1979
Osteopathy	2 (0.79)	1 (50.00)	0 (0.00)	0 (0.00)	1 (50.00)	2, 3	0.5724
No underlying	101 (39.92)	71 (70.29)	21 (20.79)	6 (5.94)	3 (2.97)	118, 3	<0.0001

Abbreviations: X², chi-square; df, degree of freedom; p-value, probability value; IQR, Inter quartile range; COPD, Chronic Obstructive Pulmonary Disease.

prevalence in the younger age group (under 18 years), while *A. fumigatus* was more commonly found in adults and older age groups (over 18 years). Since our hospital predominantly treats adult patients, only five cases were reported in the 2–17 age group, consisting of three cases of *A. niger*, one of *A. fumigatus*, and one of *A. flavus*. *A. terreus* consistently ranked as the least prevalent across all age groups, followed by *A. flavus*.

It was observed that 60.46% of patients had underlying pulmonary issues of various types, with chronic obstructive pulmonary disease (COPD) being the most prevalent (20.15%), followed by asthma (14.62%). Additionally, 60.08% of patients had other underlying conditions, among which gastrointestinal disorders were the most common (23.90%), followed by hepatic disorders (9.09%) and diabetes (8.30%). In all cases, *A. fumigatus* was the dominant infecting species. However, the hospital's electronic records did not clarify whether patients with underlying pulmonary conditions also had other systemic disorders. Statistically significant difference ($p < 0.05$) was also observed among the species based on, underlying pulmonary status and underlying systemic disorder. Detailed results are provided in Table 3.

Antifungal Susceptibility Profiles

Antifungal susceptibility assays were conducted on *Aspergillus* isolates, with detailed MIC/MEC values, range, and geometric mean (GM) provided in Table 4. The profile of Wild type (WT) species is visually presented in Figure 3. *Aspergillus spp.* exhibited remarkably low MECs for CAS (≤ 0.008 to $0.50 \mu\text{g/mL}$). Regarding azoles, the majority of isolates demonstrated MICs of $\leq 0.5 \mu\text{g/mL}$ for POS, ITR, and VRC. All *Aspergillus* species (100%) were susceptible to

Table 4 Antifungal Susceptibility Patterns and Characteristics in the Five Common *Aspergillus* Species

MIC or MEC $\mu\text{g/mL}$						
Species (n = 253)		AMB	CAS	ITR	POS	VRC
<i>A. fumigatus</i> (n = 146)	GM	2.61	0.03	0.22	0.09	0.28
	MIC50	2	0.03	0.25	0.12	0.25
	MIC90	4	0.06	0.50	0.12	0.50
	Mode	2	0.03	0.25	0.06	0.25
	Range	1.00–16.00	<0.008–0.50	0.12–1.00	0.06–0.25	0.12 - >16.00
<i>A. niger</i> (n = 68)	GM	1.66	0.02	0.32	0.13	0.59
	MIC50	2	0.03	0.25	0.12	0.50
	MIC90	4	0.12	0.25	0.12	1.00
	Mode	2	0.03	0.25	0.25	0.50
	Range	0.50–4.00	<0.008–0.25	0.12 - >16.00	0.06–2.00	0.25 - >16.00
<i>A. flavus</i> (n = 27)	GM	2.91	0.03	0.22	0.14	0.50
	MIC50	4	0.03	0.25	0.12	0.25
	MIC90	8	0.06	0.25	0.25	1.00
	Mode	4	0.12	0.25	0.12	0.25
	Range	1.00–16.00	<0.008–0.12	0.12–1.00	0.03–0.25	0.03 - >16.00
<i>A. terreus</i> (n = 10)	I	4	<0.008	0.12	0.12	0.50
<i>A. clavatus</i> (n = 1)	I	4	0.03	0.12	0.06	0.25
<i>A. nidulans</i> (n = 1)	I	4	0.03	0.12	0.06	0.25

POS. In the case of *A. fumigatus*, ITR emerged as the most effective antifungal drug following POS, with 139 (95%) isolates showing susceptibility to ITR. A minimal number of *A. fumigatus* isolates (34, 23.28%) were susceptible to AMB. For *A. niger*, AMB was the second most effective drug, with 52 isolates (76.47%) displaying susceptibility, while VRC was the least effective, with only 12 (17.64%) isolates showing susceptibility. In the case of *A. flavus*, ITR proved as effective as POS, with all isolates being susceptible to ITR, while AMB was the least effective antifungal drug, with 23 (85.11%) isolates being susceptible. As in *A. terreus*, all tested drugs exhibited equal effectiveness except for ITR.

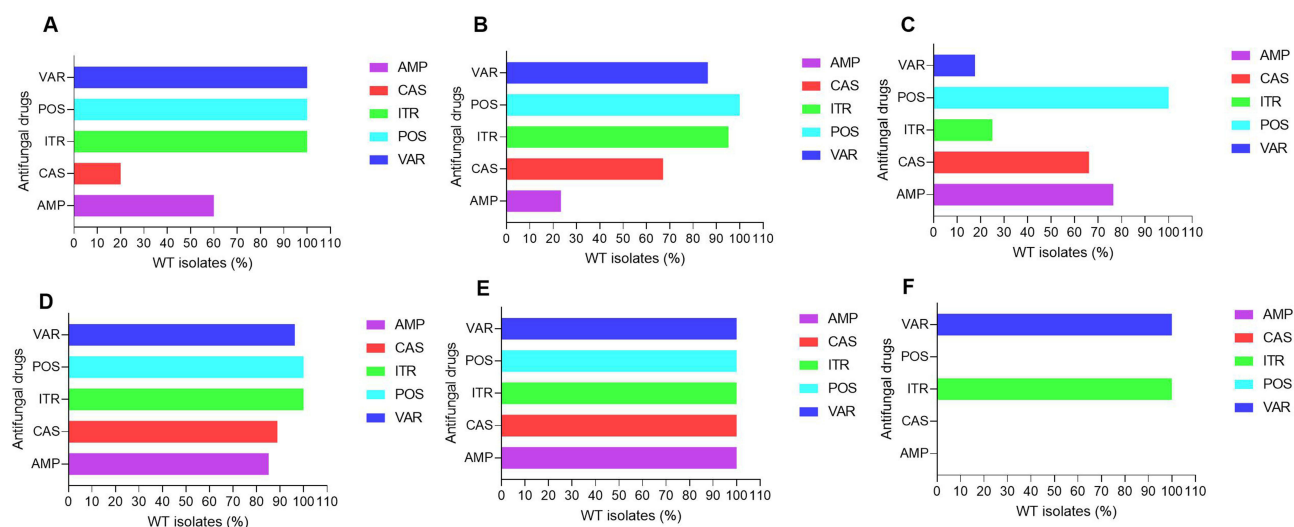
**Figure 3** The percentage of susceptible and Wild-type *Aspergillus* species isolated in the current study. AMB; Amphotericin B, CAS; Caspofungin, ITR; Itraconazole, POS; Posaconazole, VRC; Voriconazole. (A) *A. terreus* (B) *A. fumigatus* (C) *A. niger* (D) *A. flavus* (E) *A. calactus* (F) *A. nidulans*.

Table 5 Detail of the *cyp51A* Gene Mutation Along with Minimum Inhibitory Concentration (MIC) of *Aspergillus* Isolates Studied in the Current Study

Isolate	MIC (µg/mL)					Gene Mutation							
	AMB	CAS	ITR	POS	VRC	TR46	F46Y	Y121F	M172V	N248T	D255E	T289A	E427K
S18	2.61	0.03	0.22	0.09	0.28	Y	N	N	Y	N	N	N	Y
S19	2	0.03	0.25	0.12	0.25	N	N	N	N	N	N	N	N
S20	4	0.06	0.50	0.12	0.50	N	N	Y	N	N	N	N	Y

Notes: S18-S20 = *A. fumigatus*, Y = mutation is present, N = mutation is not present, P = point mutation, AMB = Amphotericin B, CAS = Caspofungin, ITR = Itraconazole, POS = Posaconazole, VRC = Voriconazole.

Gene Mutations of *cyp51A* Linked with Azole Resistance

In order to delve deeper into the association between azole resistance in *Aspergillus* species and mutations in the *cyp51A* gene, the gene was subjected to sequencing and subsequently compared with the wild-type gene. It was observed that mutation is present in the promoter region of isolate S18, while it is absent in isolates S19 and S20. Point mutations were observed at M172V and E427K in isolate S18. No mutations were observed in S19 while in S20 mutations were observed at Y121F and E427Y (Table 5).

Discussion

The present retrospective work examines the occurrence of *Aspergillus* species, along with the demographic and clinical characteristics linked to these species, within a tertiary care hospital located in Southeast China. Our analysis reveals a notable surge in *Aspergillus* cases during 2018 and 2019, yet a subsequent decline in the following three years. This is in contrast to earlier research that showed an increase in the incidence of aspergillosis.²⁶ The decrease observed in our investigation raises questions about potential shifts in epidemiological factors influencing the prevalence of *Aspergillus* species infections.²⁷ Furthermore, these findings underscore the importance of continuous surveillance studies to monitor and understand the evolving dynamics of aspergillosis, providing valuable insights for public health interventions and medical management. Our study indicates that *A. fumigatus* is the predominant species identified, followed by *A. niger* and *A. flavus*. These results align with previous studies that reported similar patterns, confirming consistency in the prevalence of these *Aspergillus* species across different studies.²⁸ The occurrence of *A. fumigatus* is attributed to its ubiquity, rapid growth, and opportunistic nature as a pathogen.²⁹ Other studies have also documented *A. flavus* and *A. terreus* as commonly occurring species. The diverse prevalence of dominant *Aspergillus* species may be influenced by geographical and environmental factors.³⁰ The prevalence of *A. fumigatus* in our study (57.71%) aligns with reports from Europe and North America, where it is the dominant species in *Aspergillus* infections.³¹ Similarly, resistance patterns, such as reduced susceptibility to amphotericin B, are consistent with global trends.³² However, our study also highlights unique regional aspects, such as higher susceptibility to posaconazole compared to studies from South Asia, which report emerging resistance.³² These differences may be attributed to variations in antifungal usage or environmental factors.³³

Upon analyzing the incidence of *Aspergillus* species based on hospital departments, it was noted that over one-third of the total cases (28.03%) originated from the Department of Infectious Disease. The justification for increased reporting from this department lies in its dedicated role in monitoring and managing of infectious diseases.³⁴ The department with the second-highest incidence of reported aspergillosis isolates, particularly *A. fumigatus*, in the current study is Respiratory and Critical Care Medicine, contributing to nearly one-third (23.4%) of the total cases. This may be attributed to the pulmonary infective nature of *Aspergillus* species, coupled with the weakened immune systems of patients in critical care unit, prolonged usage of corticosteroids, and immune-suppressive drugs.¹⁰ To address this issue, it is crucial to implement timely detection, quick anti-fungal therapy, rigorous infection surveillance measures, regulation of immunity, and continuous education and surveillance.³⁵ According to a recent study, prophylaxis is needed in patients who are at risk of invasive aspergillosis due to changing medical conditions.²³ In our study, we do not have specific information on these groups, but patients in the departments of ICU, respiratory and critical care medicine, and nephrology are likely to be immunocompromised and may need antifungal prophylaxis.

In the analysis of *Aspergillus* species incidence based on samples, it was observed that most of the isolates (80.63%) come from Respiratory samples sputum, followed by Body discharge fluids and CSF with 5.13% and 4.74% of total cases, respectively, with *A. fumigatus* being the most prominent species. These findings align with a prior study covering a 20-year period, which reported the incidence of *Aspergillus* in China. The study noted that *A. fumigatus* is an endemic respiratory pathogen frequently isolated from sputum and associated fluids.³⁶ The isolation of *Aspergillus* species from Biliary secretion through T-Tube, Fibroscope, and Catheter indicates contamination of these equipments and suggests a systemic nature of the infection.³⁷

Upon examining the incidence of *Aspergillus* species based on gender, it was discovered that the majority of cases (73.12%) are documented in the male population. This pattern persists across each reported species. Our findings align with a recent study that also observed a higher prevalence of *Aspergillus* species in the male population. The study additionally proposed that smoking and occupational exposure could be contributing factors to the elevated incidence among males.⁵ However, further investigation is required to pinpoint the underlying causes. In terms of age, the majority of infections were documented in the senior adult age group, with a median age of 64 years (IQR; 51–74). This study reveals that each documented species shows a heightened prevalence among individuals aged above 50 years. Furthermore, these species are mostly associated with cases of pulmonary aspergillosis, mainly in individuals within the respiratory and critical care unit who have substantial pulmonary and systematic disorders. The increased susceptibility of older individuals to aspergillosis could be linked to their compromised immune systems and the presence of substantial associated complications.³⁸ Amongst children (age group 2–17 years), only five cases were reported where three cases were of *A. niger*, one was of *A. flavus* and one was of *A. fumigatus*. The high prevalence of *A. niger* is similar to a recent published study, where *A. niger* was highly prevalent in the children.⁵ However, it is yet to be explored what causes the incidence of *Aspergillus* in children and the prevalence of any one species in particular.

When the cases were analyzed on the basis on underlying pulmonary status and underlying systemic disorders, it was observed that 51 (20.15%) patients are suffering from chronic obstructive pulmonary disease, 37 (14.62%) are suffering from Asthma and 34 (13.44%) are suffering from Bronchiectasis. A recent investigation has revealed that obstructive pulmonary aspergillosis occurs as a result of a relapse of acute respiratory aspergillosis, with asthma and bronchiectasis identified as associated factors.³⁹ This underscores the critical significance of early detection and appropriate treatment of aspergillosis. It was noted that most patients did not exhibit underlying disorders. However, among those with disorders, gastrointestinal issues were the most prevalent, observed in 23.90% of the cases. Research has indicated that the use of antibiotics and medications may contribute to the development of gastric problems.⁴⁰

We analyzed the isolates for susceptibility to amphotericin B, caspofungin, itraconazole, posaconazole, and voriconazole. The observed 100% susceptibility of *Aspergillus* species is consistent with earlier research findings that have documented the effectiveness of POS against aspergillosis.^{41,42} POS is recognized as a broad-spectrum antifungal drug and is frequently recommended as the initial choice for fungal treatment.⁴³ Our research revealed that ITR ranks as the second most effective drug against the identified fungal pathogens, particularly *A. fumigatus*, with 95% of the isolates demonstrating susceptibility to ITR. These results align with recent publications that identified ITR as highly effective against *A. fumigatus* isolates.¹⁴ Nevertheless, the limited efficacy of AMB underscores the necessity for alternative antifungal agents when dealing with pathogens resistant to AMB.⁴⁴ Regarding *A. niger*, our findings suggest that AMB emerges as the most effective drug, with 74.47% of *A. niger* isolates showing susceptibility to AMB. These results align with the well-established understanding that AMB is the most efficacious antifungal drug against *A. niger*.⁴⁵ Additionally, our study reveals that *A. niger* exhibits the least susceptibility to VRC, a trend consistent with recent reports indicating resistance of *A. niger* to VAR.⁴⁶ For *A. flavus*, our investigation indicates that 85% of the isolates exhibited susceptibility to AMB, while for other drugs, this percentage ranged from 96% to 100%. These findings align with recently published data, which also reported the susceptibility of *A. flavus* to several tested antifungal drugs.³⁶ In our investigation of *A. terreus*, it was observed that ITR displayed the lowest effectiveness among the tested drugs. Given *A. terreus*'s inherent resistance to amphotericin B, these results underscore the significance of exploring alternative antifungal agents in the management of *A. terreus* infections.⁴³

We also sequenced *cyp51A* gene and its promoter, for the azole resistant *A. fumigatus* strains. Mutations were observed in the gene as well as in the promoter region. Recent studies have also reported mutation in the promotor region

of the *cyp51A* gene.⁴⁷ Our finding of mutations at Y121F and E247K aligns with a recently published study that identified similar point mutations in azole-resistant *Aspergillus* isolates at these specific positions.¹⁰ Studies have shown that these mutations may alter the binding site of the CYP51 enzyme, impairing the efficacy of azoles. These observations underscore the need for continuous surveillance of such mutations to guide antifungal therapy effectively.^{48,49}

The retrospective nature of this study introduces certain limitations, as some data may be unavailable for analysis. The present study is confined to a single center, with a comparatively low sample size, thereby restricting the generalizability of the results to other regions in China. Additionally, information on antifungal treatment earlier the isolation of *Aspergillus* species for the included patients was not accessible, impeding an investigation into the impact of early antifungal therapy on resistance occurrence. Despite these limitations, the findings of this study may provide valuable insights for health officials managing aspergillosis in Southeast China. To address these limitations and enhance our understanding, future studies should focus on epidemiology and antifungal resistance approaches across multiple centers at the molecular level. Furthermore, it is essential to establish the association between previous antifungal treatment and the progression of resistance in fungal isolates.

Conclusion

The current investigation documented 253 isolates of *Aspergillus* species, with a significant majority attributed to *A. fumigatus*. Among non-*fumigatus* cases, *A. niger* featured prominently, followed by *A. flavus*. The Department of Infectious Disease reported a substantial number of isolates, underscoring its pivotal role in infection management. POS emerged as the most potent antifungal agent against all *Aspergillus* species. The prevalence of *A. fumigatus* in our study consistently aligns with reports from Europe and North America. Similarly, resistance patterns, such as reduced susceptibility to AMB, are consistent with global trends. However, our study shows higher susceptibility to POS compared to studies from South Asia, which report emerging resistance. It is essential to conduct extensive molecular-based surveillance studies with large and diverse populations, within the framework of One Health approaches, to continuously monitor *Aspergillus* species and facilitate effective management of aspergillosis.

Data Sharing Statement

Data used in this paper is included in the paper.

Ethics Approval and Informed Consent

The study was authorized by the Ethics Committee of Huashan Hospital, Fudan University (permission number: KY2014-219 R) and carried out in accordance with the Declaration of Helsinki's principles. Every participant in the research gave their informed permission.

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Author Contributions

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

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Disclosure

The authors declare no conflicts of interest.

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