Infection and Drug Resistance

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ORIGINAL RESEARCH

Aspergillus Species in Lower Respiratory Tract of Hospitalized Patients from Shanghai, China: Species Diversity and Emerging Azole Resistance

This article was published in the following Dove Press journal:
Infection and Drug Resistance

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Purpose: To investigate species diversity and prevalence of antifungal resistance among clinical isolates of *Aspergillus* spp. in Shanghai, China.

Patients and Methods: In this study, the *Aspergillus* spp. isolates were analyzed by multilocus sequence typing (MLST) targeting the internal transcribed spacer (ITS) regions, and partial β-tubulin (*BenA*) and calmodulin (*CaM*) genes. The susceptibilities of these isolates to nine antifungal agents were determined according to the protocol in document M38-A3 established by the Clinical and Laboratory Standards Institute (CLSI).

Results: The most common *Aspergillus* spp. was *A. fumigatus* (58.2%), followed by the *A. flavus* complex (23.5%), and *A. niger* complex (15.3%). Isolates belonging to *A. tamarii* and *A. effusus* of the *A. flavus* complex and *A. tubingensis* and *A. awamori* of the *A. niger* complex were identified. Moreover, several mutations were found in the azole target *cyp51A* gene (TR_{46}/Y121F/T289A and F46Y, G89G, M172V, N248T and D255E) in azole-resistant isolates of *A. fumigatus*.

Conclusion: The results of our study revealed a diversity of species in the lower respiratory tract of inpatients in Shanghai and approximately 9% of our isolates were resistant to at least one of the triazole antifungals. Formulation of local treatment strategies to combat emerging azole resistance and species diversity in clinically relevant *Aspergillus* spp. is needed.

Keywords: *Aspergillus*, antifungal susceptibility, identification, molecular typing, China

Introduction

Aspergillus is a diverse genus with a high economic, social and health impact, in which about 40 *Aspergillus* spp. are clinically relevant.[1–3] The *A. fumigatus* complex is the major cause of aspergillosis worldwide, followed by the *A. flavus* complex, the *A. niger* complex, and *A. terreus*.[1,2,4] *Aspergillus* spp. can cause a broad spectrum of pulmonary aspergillosis (PA), ranging from an allergic reaction (allergic broncho-pulmonary aspergillosis; ABPA) to various infections such as chronic pulmonary aspergillosis (CPA) or invasive pulmonary aspergillosis (IPA).[5–7] *Aspergillus* spp. colonization is an important prerequisite to subsequent infections,[8] particularly in the lower respiratory airways.[8,9]

The differentiation of *Aspergillus* spp. at the species level has been shown to correlate with specific patterns of antifungal susceptibility.[7,10,11] However, morphological features are frequently insufficient to differentiate *Aspergillus* spp. to species level.[1,4,12] Over the last decade, molecular identification methods, such as multilocus sequence typing (MLST) targeting the internal transcribed spacer (ITS), partial

β-tubulin (*BenA*) and calmodulin (*CaM*) gene regions, have shown capable of discriminating cryptic species among morphologically similar *Aspergillus* spp.[1,13] Consequently, epidemiological characteristics of clinical isolates of *Aspergillus* spp. have become increasingly accurate.[7,14,15] For example, several non-*A. fumigatus* spp. and cryptic species within the genus *Aspergillus*, have been reported as causative agents of aspergillosis worldwide, including China.[16–18]

The recommended first-line drugs to treat aspergillosis are triazoles.[19] However, triazole-resistant isolates of *Aspergillus* spp. have been increasingly detected across the globe since the early 2000s, likely due to the widespread use of azoles in agriculture and clinics.[15,20,21] Several resistance mechanisms have been reported in *A. fumigatus* isolates,[21] particularly mutations in the *cyp51A* gene.[22] The main resistance mechanism, TR_{34}/L98H, has been documented since 2007.[23] More recently, an emerging mutation, TR_{46}/Y121F/T289A, has been identified in many European countries.[22,24] In China, while the majority of mutations are the TR_{34}/L98H, new mutations, such as TR_{46}/Y121F/T289A, have been identified frequently since 2010.[18,25]

Geographical differences in the prevalence of different *Aspergillus* spp., especially in azole-resistant *A. fumigatus* have been observed among national and regional populations in the world, including China.[26,27] Although several studies on *A. fumigatus* in China have been reported,[26,28] studies focused on isolates of non-*A. fumigatus* spp. are limited, especially on cryptic *Aspergillus* species. Moreover, antifungal resistance in these reported isolates is rare.[8,18,29]

Shanghai, the largest and a medically well-developed city located in Eastern China, is currently faced with an increasing number of hospitalized patients coming from not only Shanghai but also other parts of China. Thus, *Aspergillus* infections of the hospitalized patients in Shanghai could provide a snapshot of the national profile on aspergillosis. The aim of this study was to investigate the diversity of *Aspergillus* spp. isolated from the lower respiratory airways of hospitalized patients in Shanghai via an MLST method and to determine their in vitro susceptibility to currently available antifungal drugs.

Patients and Methods

Collection of Clinical Isolates

From January 2016 to March 2018, a total of 98 isolates of *Aspergillus* spp. were collected from the bronchoalveolar lavage fluid (BALF) belonging to 98 hospitalized patients. The patients were identified to have proven or probable *Aspergillus* spp. infections or colonization prior to their admissions to two tertiary teaching hospitals: Shanghai Changzheng Hospital (1280-beds) and Shanghai Huashan Hospital (1216-beds). Ethical approval was obtained, and all patients involved understood and agreed to the usage of the clinical isolates in the present study. The details of the isolates are listed in Supplementary Table S1.

Isolation and Phenotypic Identification

The putative isolates of *Aspergillus* spp. were first grown on Sabouraud dextrose agar (SDA; Oxoid, UK) for three days at 35°C in aerobic conditions. These isolates were then morphologically identified as *Aspergillus* spp. using Czapek-dox agar (CDA; Oxoid, UK) and potato dextrose agar (PDA; Oxoid, UK) according to their macro-morphological and microscopic features of the colonies.[30]

Molecular Identification

Genomic DNA of each isolate was extracted following the CTAB protocol described previously.[31] Fragments of the three genes *BenA, CaM*, and ITS regions were amplified directly from the genomic DNA as described previously.[1,32,33] PCR products were sequenced on an ABI 3770XL capillary sequencer (Applied Biosystems, Lennik, The Netherlands). Sequence reads were assembled and edited using SeqMan v.7.0.0 (DNASTAR, Madison, WI, USA). The details of the primer sequence and PCR amplification conditions are listed in Supplementary Table S2.

The sequences were aligned using the server version of the MAFFTv. 7.0 (www.ebi.ac.uk/Tools/msa/mafft/), followed by the manual checking in BIOEDIT v. 7.0.5.2. All sequences of the isolates have been deposited in GenBank, and the accession numbers are listed in Supplementary Table S1. Representative sequences from known species of the *Aspergillus* genus were retrieved from GenBank and included in our alignment. Corresponding gene sequences of *Penicillium chrysogenum* strain CBS 306.48 were chosen as outgroup in phylogenetic analysis. The best-fit model of sequence evolution was determined by MEGA version 7.0.14 (Center for Evolutionary Medicine and Informatics, Tempe, AZ). After verifying the best models, phylogenetic trees were inferred using the maximum likelihood (ML) method with 1000 rounds of re-sampling, and bootstrap branch support >80% was regarded as robust for species identification. Phylogenetic trees were viewed and edited with FIGTREE v. 1.1.2 software.

In vitro Antifungal Susceptibility

According to the criteria of M38-A3 in document established by Clinical and Laboratory Standards Institute (CLSI),[34] we determined in vitro susceptibility of all isolates to itraconazole (ITZ, Sigma-Aldrich, Basingstoke, UK), voriconazole (VRZ, Sigma-Aldrich, Basingstoke, UK), posaconazole (PSZ, Sigma-Aldrich, Basingstoke, UK), ravuconazole (RVZ, Toronto Research Chemicals Inc, Toronto, Canada), isavuconazole (ISZ, Toronto Research Chemicals Inc, Toronto, Canada), anidulafungin (AFG, Toronto Research Chemicals Inc, Toronto, Canada), caspofungin (CFG, Sigma-Aldrich, Basingstoke, UK), micafungin (MFG, Toronto Research Chemicals Inc, Toronto, Canada), and amphotericin B (AmB, Sigma-Aldrich, Basingstoke, UK). Final concentrations of antifungal agents ranged from 0.03 to 16 μg/mL for ITZ, VRZ, PSZ, RVZ, ISZ and AmB, and 0.015 to 8 μg/mL for AFG, CFG and MFG. Stock solutions of drugs were prepared in dimethyl sulfoxide, and stored at –80°C until used. The reference isolate used here for comparison was *Candida parapsilosis* strain ATCC 22,019. The in vitro susceptibility testing was performed in triplicates for each isolate. The Minimal inhibitory concentration (MIC) data obtained were reported as the ranges, MIC_{50} and MIC_{90}. The proposed epidemiological cutoff values (ECVs) of *Aspergillus* spp. for ITZ, VRZ, PSZ, ISZ, CFG and AmB followed those by the CLSI for antifungal agents (Supplementary Table S3).[35–38] There are no ECVs currently available for RVZ, AFG or MFG.

Sequencing of *A. fumigatus cyp51A* Gene

The full sequences of the *cyp51A* gene, including the promoter region, of all triazole-resistant *A. fumigatus* isolates were amplified and sequenced following the protocols described previously.[39] The sequences obtained were aligned with the sequence of a triazole-susceptible isolate (GenBank accession AF338659) using ClustalW software.[40] Specifically, we followed the steps described by Deng et al[28] and scanned the predicted *cyp51A* amino-acid sequence for substitutions, particularly those linked to triazole resistance as identified previously.

Statistical Analysis

The data collected during the study period were analyzed using IBM SPSS Statistics 23.0 software. Percentages of the species diversity among different patient populations were compared using χ^2 test. A P value less than 0.05 was considered statistically significant.

Results

Demographic Data of the Patients

In our study, the mean age of the patients was 61.9 (± standard deviation: 16.6 years; range: 18–94 years), with the largest group in the age range of 61–80 years (48.0%; 47/98). The male/female gender ratio was 3.1 (74/24). According to the recently defined standardized criteria,[7] the clinical profiles of the patients were divided into colonization (80.6%; 79/98), CPA (14.3%; 14/98), IPA (3.1%; 3/98), and ABPA (2.0%; 2/98) groups. No significant differences (P value = 0.642) were observed among the age group of the patients about the species diversity (Figure 1). Remarkably, the proportion of *A. fumigatus* among the colonization patients (53.2%; 42/79) was significantly lower (P value = 0.034) than that among the diseased patients with clinical aspergillosis (CPA, IPA and ABPA) (78.9%; 15/19). All the patients were HIV-negative, whereas half of them had underlying conditions. The most prevalent underlying condition was type 2 diabetes mellitus (15.3%; 15/98), followed by solid tumor (12.2%; 12/98), chronic lung disease (10.2%; 10/98), and chronic liver disease (5.1%; 5/98), etc. About 15% (15/98) of the patients had more than one kind of underlying condition. The detailed clinical profiles of each patient are presented in Supplementary Table S1.

Identification of *Aspergillus* spp

A total of six species complex were unambiguously identified among the 98 isolates based on sequences of three DNA markers, including *A. fumigatus* (58.2%; 57/98), the *A. flavus* complex (23.5%; 23/98), the *A. niger* complex (15.3%; 15/98), *A. sydowii* (1.0%; 1/98), *A. terreus* (1.0%; 1/98), and *A. nidulans* (1.0%; 1/98). Interestingly, our phylogenetic analysis based on the concatenated sequences (*BenA, CaM* and ITS) identified four rarely observed species in these patients, *A. tamarii* and *A. effusus* in the *A. flavus* complex; and *A. tubingensis* and *A. awamori* in the *A. niger* complex (Figure 2 and Supplementary Table S1).

In vitro Antifungal Drug Susceptibility

With the exception of azoles and AmB in some cases, most tested antifungal compounds demonstrated potent activity against the 98 *Aspergillus* isolates (Table 1). The antifungal drug echinocandins exhibited the MIC values ranging from 0.015 to 1 μg/mL. AmB also showed potent activity, with MICs ranging from 1 to 4.0 μg/mL, and with geometric means (GM) closer to the lowest MIC value, which was

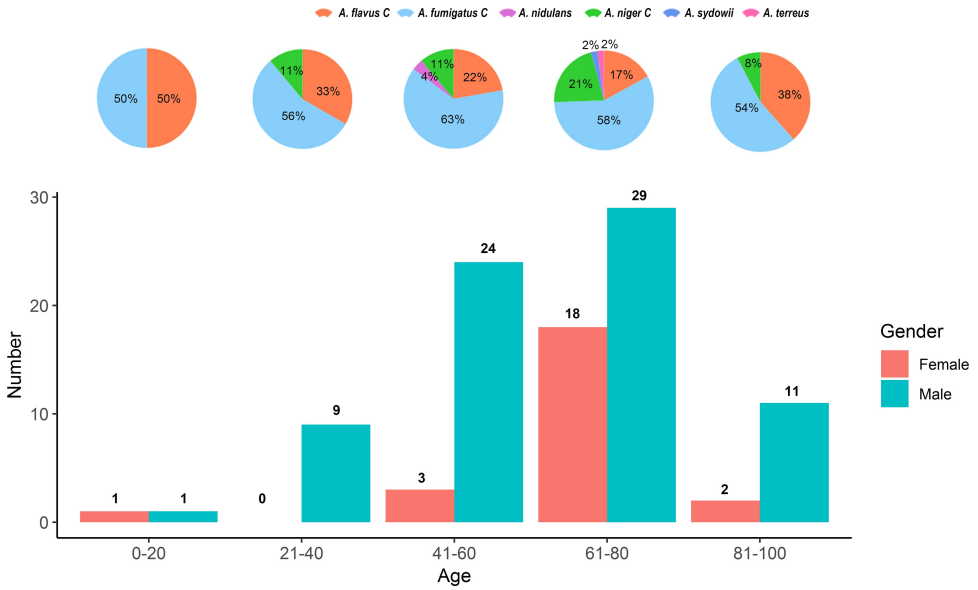

Figure 1 Distribution of *Aspergillus* spp. in the lower respiratory tract of hospitalized patients of different ages and sexes in Shanghai, China. (The top pie chart shows *Aspergillus* species distribution within each of the five 20-year age groups, 0–20; 21–40; 41–60; 61–80; and 81–100. The bottom bar graph shows the number of *Aspergillus* isolates from the two sexes for each of the five age groups. Aside from the 0–20 age group where only two isolates were obtained, *A. fumigatus* was the most common species across all age groups and that more isolates were obtained from males than females).

observed against *A. fumigatus* (1.245 μg/mL), the *A. flavus* complex (1.620 μg/mL) and the *A. niger* complex (1.05μg/mL). For azoles, more variable results were observed with MIC values ranging from 0.06 to 16.0 μg/mL. The lowest azole GM MIC value was PSZ with GM MIC (0.187 μg/mL) against *A. fumigatus*, and the highest was ISZ against *A. niger*, with GM MIC value (2.297 μg/mL). Of the 98 isolates, 11 showed resistance to one or several of the tested antifungal drugs (Table 2). Among the 57 isolates of *A. fumigatus*, four were resistant to the azoles, with one isolate (in particular CMXY 13,113) being resistant to several azoles ITZ, VRZ, RVZ and ISZ. Two *A. fumigatus* isolates (CMXY 14,287 and CMXY 10,234) were resistant to AmB. Among the 23 isolates of the *A. flavus* complex, four were resistant to azoles, while two isolates (CMXY 22,879 and CMXY 27,481) were resistant to selected azoles (PSZ and ISZ) and AmB. One *A. tubingensis* isolate (CMXY 27,207) was resistant to multiple antifungals ITZ, VRZ, RVZ, PSZ and ISZ.

Sequence Variation Among Triazole-Resistant Isolates at the *cyp51A* Gene

Among the azole-resistant strains of *A. fumigatus*, one (CMXY13,113) had two mutations in the *cyp51A* gene and a 46-bp tandem repeat in the gene promoter (TR_{46}/Y121F/T289A). One ITZ resistant strain (CMXY15837) and one PSZ and ISA double resistance strain (CMXY28940) of *A. fumigatus* showed several polymorphisms in the *cyp51A* gene (F46Y, G89G, M172V, N248T, D255E). However, no *cyp51A* promoter or amino-acid sequence variation was observed for the azole-resistant isolate CMXY25241 (Table 2).

Discussion

Aspergillus spp. cause a wide spectrum of human diseases and affect more than 14 million people worldwide.[7,41] Diverse *Aspergillus* spp. frequently differ in their susceptibilities to antifungal drugs and geographical differences exist in the prevalence of different *Aspergillus* spp.[42,43] The results of our study expand the knowledge on species diversity and antifungal susceptibility of *Aspergillus* spp. from hospitalized patients in Shanghai, China.

In our study, colonization was the dominant (80.6%) clinical profile of *Aspergillus* spp. among the hospitalized patients, which should be taken seriously in China. Several publications indicate colonization to be an important risk factor for the development of IPA,[8,44] with approximately 18% of colonized patients developing IPA, especially patients with COPD.[45] The proportion of the patients colonized by non-*A. fumigatus* is significantly higher than that among the patients with CPA, IPA or ABPA, which was not observed in a similar study reported from Japan.[9] The significance of non-*A. fumigatus*, especially

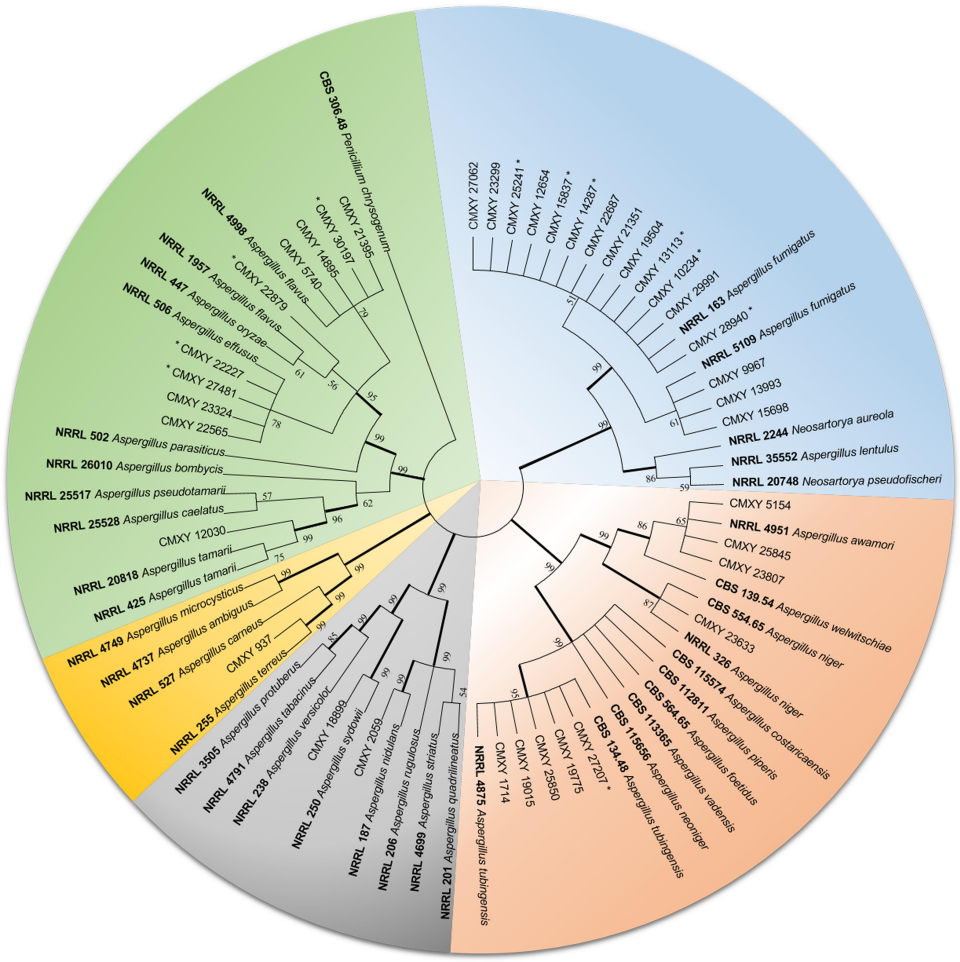

Figure 2 A phylogenetic tree for the representative *Aspergillus* spp. isolates from our study using the maximum likelihood method based on the combined sequences of ITS, *BenA* and *CaM* loci. (Type strains for all species found in our samples were included as references for confirmation of species identification. "*" means antifungal resistant isolates of *Aspergillus* spp. in our study; "bold font" indicates type strain for that species).

the *A. niger* complex, colonizing the lower respiratory airways among immunocompromised patients, needs further research.[8] CPA is the second frequent profile of *Aspergillus* spp. in our study, though the exact incidence is unclear in other regions.[7] The prevalence of IPA (3.1%) in our study was within the range reported previously in Europe (0.2–6.9%).[46,47] Similarly, the prevalence of ABPA (2%) in our sample was similar to the recent data from a global survey (2.5%).[48,49]

In the present study, *A. fumigatus* was the most frequently isolated (58.2%). This species is known to be prevalent in indoor environments in China[50,51] and has a superior ability to survive in the lower respiratory tracts of humans.[7] Similar prevalence has also been reported from Beijing, China (59.3%)[18] and Madrid, Spain (54.7%).[52] The *A. flavus* complex is the second (23.5%) most common group of *Aspergillus* spp. in our study, which is also the second most prevalent etiological agent of IA worldwide.[18,53] It should be noted that the *A. flavus* complex has been reported to cause outbreaks in hospital, especially after surgery in high-risk patients.[53] In India, the *A. flavus* species complex was also the most common *Aspergillus* spp. isolated from air-conditioned areas.[54] Although the prevalence of IA caused by the *A. niger* complex has increased recently,[55] all isolates of the *A. niger* complex analyzed in this study were collected from colonized patients but without active aspergillosis. However, one patient had diabetes and the prognosis of pulmonary colonization caused by the *A. niger* complex is generally poor among patients with diabetes.[56] In addition, we identified one *A. sydowii* isolate from lower respiratory tract of a female patient. This species also is an important etiologic agent of superficial infections.[57]

Table 1 Summary Distribution of Susceptibilities of the 98 Clinical Isolates of *Aspergillus* spp. to Nine Antifungal Drugs as Determined Based on the M38-3A Protocol Established by the Clinical and Laboratory Standards Institute (CLSI)

Species/Number		MIC or MEC (μg/mL) for								
		AFG	CFG	MFG	ITZ	VRZ	RVZ	PSZ	ISZ	AmB
A. fumigatus complex (57)	MIC range	≤0.015–0.03	0.06–1	≤0.015–0.12	0.25–8	0.12–16	0.25–16	0.06–1	0.25–16	1–4
	MIC_{50}	≤0.015	0.25	0.03	0.5	0.5	1	0.25	0.5	1
	MIC_{90}	0.03	0.25	0.12	1	0.5	2	0.25	1	2
	GM	0.021	0.221	0.034	0.615	0.443	0.765	0.187	0.711	1.245
A. flavus complex (23)	MIC range	≤0.015–0.25	0.12–0.25	≤0.015–0.25	0.12–0.5	0.5–2	1–2	0.12–1	0.5–2	1–4
	MIC_{50}	0.03	0.12	0.06	0.25	1	1	0.25	1	2
	MIC_{90}	0.06	0.25	0.12	0.5	2	2	1	2	2
	GM	0.034	0.169	0.057	0.291	0.970	1.128	0.328	1.031	1.620
A. niger complex (15)	MIC range	≤0.015–0.03	0.06–0.12	0.03–0.25	0.5–16	0.5–16	0.5–16	0.25–2	0.5–16	0.5–2
	MIC_{50}	≤0.015	0.12	0.12	1	1	2	0.5	2	1
	MIC_{90}	0.03	0.12	0.25	1	2	4	0.5	4	2
	GM	0.020	0.109	0.090	0.912	1.260	1.910	0.416	2.297	1.05
A. sydowii (1)	MIC range	0.06	0.03	0.03	1	2	2	1	2	2
A. terreus (1)	MIC range	≤0.015	0.12	≤0.015	0.25	2	2	0.12	1	2
A. nidulans (1)	MIC range	0.06	0.06	0.03	0.5	0.25	0.25	0.06	0.25	2

Abbreviations: AFG, anidulafungin; CFG, caspofungin; MFG, micafungin; ITZ, itraconazole; VRZ, voriconazole; RVZ, ravuconazole; PSZ, posaconazole; ISZ, isavuconazole; AmB, amphotericin B; GM, geometric means; MIC, Minimal inhibitory concentration; MEC, Minimal effective concentration; MIC_{50}, MIC/MEC at which 50% of the isolates tested were inhibited; MIC_{90}, MIC/MEC at which 90% of the isolates tested were inhibited.

Table 2 Minimal Inhibitory Concentrations of Antifungal Drug-Resistant Isolates of *Aspergillus* spp. in Our Study as Determined Based on the M38-3A Protocol Established by the Clinical and Laboratory Standards Institute (CLSI)

Species	Number	MIC (μg/mL) for						*cyp51A*
		ITZ	VRZ	RVZ	PSZ	ISZ	AmB	
A. fumigatus	CMXY 13,113	8	16	16	1	16	†	TR_{46}/Y121F/T289A
A. fumigatus	CMXY 15,837	4	†	†	†	†	†	F46Y, G89G, M172V, N248T, D255E
A. fumigatus	CMXY 28,940	†	†	†	0.5	2	†	F46Y, G89G, M172V, N248T, D255E
A. fumigatus	CMXY 25,241	†	†	†	1	†	†	#
A. fumigatus	CMXY 10,234	†	†	†	†	†	4	#
A. fumigatus	CMXY 14,287	†	†	†	†	†	4	#
A. effusus	CMXY 22,227	†	†	†	1	†	†	##
A. flavus	CMXY 22,879	†	†	†	1	2	4	##
A. effusus	CMXY 27,481	†	†	†	1	2	4	##
A. flavus	CMXY 30,197	†	†	†	1	†	†	##
A. tubingensis	CMXY 27,207	16	16	16	2	16	†	##

Notes: "†" means the MIC ≤ epidemiological cutoff values; "#" means the mutant site of *cyp51A* gene was not be found; "##" means the *cyp51A* gene were not analyzed.
Abbreviations: ITZ, itraconazole; VRZ, voriconazole; RVZ, ravuconazole; PSZ, posaconazole; ISZ, isavuconazole; AmB, amphotericin B; MIC, minimal inhibitory concentration.

Owing to the application of MLST,[1,58] approximately 8–19% of isolates of *Aspergillus* spp. were recently identified as cryptic *Aspergillus* spp. in Spain and Portugal.[27,59] Among those newly defined "cryptic" species, our samples contained four in two of the species complexes: *A. tamarii* and *A. effusus* in the *A. flavus* complex and *A. tubingensis* and *A. awamori* in the *A. niger* species complex. The frequencies of the cryptic species in our samples (15.3%) are similar to those reported earlier. Interestingly, no cryptic species was identified in the *A. fumigatus* complex, which might be due to the limited number of clinical isolates.

Currently, azoles, particularly VRZ, remain the preferred agents for treatment of IA.[19,60] Our results suggested that most azoles (in particular PSZ) were highly active against *Aspergillus* spp. However, the reduced susceptibility to novel triazoles, such as RVZ and ISZ, was found in the *A. niger* complex. PSZ is the most potent drug against strains of *A. fumigatus* (GM MIC 0.187 μg/mL) and the *A. niger* complex (GM MIC 0.416 μg/mL), which was consistent with previous studies from China and Italy.[17,30] The novel azoles ISZ and RVZ demonstrated a strong inhibitory activity against both *A. fumigatus* and *A. flavus*, similar to the results of previous studies from India and China.[28,61] However, the reduced susceptibility (MIC≥1μg/mL) of ISZ and RVZ among the majority isolates of the *A. niger* complex in our study was not consistent with the results of previous reports from Spain and the USA.[36,62] In addition, our results also suggest that echinocandins (in particular AFG) are the potent drugs against clinical isolates of *Aspergillus* spp., which was similar to the previous studies from the USA, India and China.[28,61,63] Therefore, our results support the current recommendation issued by the Infectious Diseases Society of America in using azoles as the primary treatment against *Aspergillus* spp. infections,[19] in conjunction with echinocandins and/or polyenes if needed.

Since the first reported ITZ-resistant *A. fumigatus* isolate in 1997,[64] the isolates of azole-resistant *A. fumigatus* have been increasingly reported worldwide.[7,65,66] The amount of azole fungicide used in China is much higher than those used in European countries,[26] which suggests a high selective pressure for environmental *A. fumigatus*. Several studies have indicated that the TR_{34}/L98H and TR_{34}/L98H/S297T/F495I mutations were the predominant mutations in China.[26,65,67] In 2015, three clinical isolates of *A. fumigatus* were found to harbor either the TR_{34}/L98H/S297T/F495I or the TR_{34}/L98H mutations in Fuzhou, Nanjing, and Shanghai.[65] One study reported a worldwide clonal expansion of triazole-resistant isolates with the TR34/L98H mutations, while triazole-resistant isolates with the TR34/L98H/S297T/F495I mutation from China were genetically distinct from resistant isolates in other countries.[26] Notably, the TR_{46}/Y121F/T289A mutation was found in one isolate of *A. fumigatus* (CMXY 13113). This isolate showed a high level of multi-azole resistance, similar to the result from the Netherlands in 2015.[68] Studies so far have shown that isolates with the TR_{46}/Y121F/T289A mutation are typically resistant to ITZ and VRZ but have variable susceptibility to PSZ. Such a result is different from that observed for strains with the TR_{34}/L98H mutation, which typically show ITZ resistance, but with variable susceptibility to VRZ and PSZ.[69,70] To our knowledge, this is the first time the mutation has been identified within isolates of *A. fumigatus* in Shanghai, China. In addition, the F46Y, G89G, M172V, N248T and D255E mutations were found in two other isolates in our study, which also was observed in Australia and Netherlands.[71,72] However, no *cyp51A* promoter or amino-acid sequence mutation was observed for the azole-resistant isolate CMXY25241. This result suggests that mutation(s) in gene(s) other than *cyp51A* was likely responsible. For example, a recent study reported that mutations in ATP-binding cassette (ABC) or major facilitator superfamily (MFS) efflux pumps were associated with triazole resistance in *A. fumigatus*.[21] Our findings suggest diverse genetic backgrounds among the isolates of azole-resistant *A. fumigatus* from Shanghai, China. In addition, *A. fumigatus* isolates harboring the TR_{34}/L98H or TR_{46}/Y121F/T289A mutations were not only found in azole-naïve patients, but also found in soil and/or woody debris samples in New Zealand, India, Tanzania, and Africa,[73] consistent with these mutations being originated from azole fungicide usage in the environment and agriculture.

Conclusions

Our study revealed a considerable diversity of *Aspergillus* spp. in lower respiratory tract of hospitalized patients in Shanghai, China. Although the frequency of azole resistance is relatively low (approximately 9%), a number of strains showed resistance to multiple triazoles and even to multiple classes of antifungal drugs. The profiles of antifungal drug susceptibility provide important information for clinicians and local public health officials in determining the best treatment and prevention strategies. Our study emphasizes the need to not only identify all isolates of *Aspergillus* spp. at the species level, but also to perform antifungal susceptibility tests in clinical laboratories worldwide.

Ethics Approval and Consent to Participate

The protocol has been reviewed by the human research ethics committee of the Institutional Review Board (IRB) of Huashan Hospital Affiliated to Fudan University and since the project falls under the category observational study and all fungal strains were from residual samples used in clinical diagnosis or were strains from their subcultures, it has been

determined they meet the criteria for exemption. The study was conducted in accordance with the Helsinki Declaration. Physicians working in the hospitals were consulted for the study to ensure the protections of life, health, dignity, privacy, and confidentiality of personal information of all study participants. After consultation with the IRB, formal ethical approval application was reviewed and waivered and written patient consent was deemed not required (Ethics Approval Number: KY2020-874).

Acknowledgments

We thank the Department of Dermatology, Shanghai Changzheng Hospital and Shanghai Huashan Hospital, for supporting this research. This work was supported by the National Natural Science Foundation of China (No. 81271762 and 81720108026), and Shanghai Science and Technology counsel projects of 16411961600. The sponsor had no involvement in any of the stages from the study design to submission of the paper for publication.

Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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