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SUSCEPTIBILITY

Five-Year Survey (2014 to 2018) of Azole Resistance in Environmental *Aspergillus fumigatus* Isolates from China

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ABSTRACT A total of 191 soil samples from Hangzhou, China, were submitted to detect non-wild-type (non-WT) *Aspergillus fumigatus* and its associated mechanisms. There were 2 (4.7%), 13 (12.4%), and 31 (23.1%) isolates identified as non-WT in 2014, 2016, and 2018, respectively. The resistant mutations of TR34/L98H, TR46/Y121F/T289A, and TR34/L98H/S297T/F495I were found in 3, 5, and 5 non-WT isolates. The G448S mutation, previously only found in clinical settings, was detected in *A. fumigatus* from soil samples.

KEYWORDS *Aspergillus fumigatus*, environmental resistance, microsatellite typing, G448S

Aspergillus fumigatus, a globally distributed opportunistic pathogen, is the main cause of invasive aspergillosis (IA), which causes 40% to 90% mortality in immunocompromised patients (1). Triazole drugs (itraconazole, voriconazole, posaconazole, and isavuconazole) are the only oral drugs approved for clinical treatment and prevention of IA. Unfortunately, azole-resistant *A. fumigatus* (ARAF) has been increasingly found in patients who had received long-term antifungal treatment, azole-naive patients, and the environment (2–7). The emergence of ARAF is the primary cause of the increased numbers of treatment failures.

The mechanism of azole resistance is primarily characterized by alterations in *cyp51A*, the gene coding for sterol 14α-demethylase, which plays an important role in sterol synthesis. G54, G138, G434, M220, H147, Y121, G448, and P216 are common hot spot mutations that have been detected in ARAF from the patients under long-term antifungal treatment (8, 9). TR34/L98H and TR46/Y121F/T289A are the two most frequently identified resistance mechanisms found in environmental *A. fumigatus* strains and azole-naive patients and may result from the agricultural use of azole fungicides (9–11). Upregulation in intracellular sterol 14α-demethylase concentration because of overexpression of *cyp51A* is involved in resistance to triazole drugs. In addition, a decrease in intracellular drug concentration because of the overexpression of efflux pump genes can also result in resistance (9).

Immunocompromised patients may be infected by inhalation of airborne ARAF spores. Therefore, understanding the developmental processes and evolution of ARAF in the environment is significant for the treatment of patients with aspergillosis. Two resistant *A. fumigatus* soil isolates harboring mutations of TR34/L98H/S297T/F495I were identified in Beijing and Fuzhou, respectively (12). Ren et al. (6) reported the emergence of ARAF with the mutations TR46/Y121F/T289A and TR34/L98H/S297T/F495I in agricultural fields. We report here a 5-year survey concerning resistance in environmental *A. fumigatus* from Hangzhou in China.

A total of 191 soil samples were collected at a depth of 0 to 5 cm from the garden belt of eight hospitals during the 2014 to 2018 period from Hangzhou, Zhejiang, China (data not shown). *A. fumigatus* isolates were identified according to their microscopic

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TABLE 1 The frequency of isolation of total and non-wild-type *A. fumigatus* during 2014 to 2018[a]

PS	TS	SCDAF	Rate of SCDAF/TS (%)	NIAF	NINAF	Rate of NINAF/NIAF (%)
Jun–Sep 2014	59	27	45.8	43	2	4.7
Mar–May 2016	68	44	64.7	105	13	12.4
Mar–May 2018	64	40	62.5	134	31	23.1

[a]PS, period of sampling; TS, total samples; SCDAF, samples containing detectable *A. fumigatus*; NIAF, numbers of isolated *A. fumigatus*; NINAF, numbers of isolated non-wild-type *A. fumigatus*.

and macroscopic morphology, by their capability to grow at 48°C, and by the internal transcribed spacer ribosomal DNA and β-tubulin gene (6, 13). Susceptibility testing of *A. fumigatus* against triazole medicines (voriconazole [VRC], itraconazole [ITZ], and posaconazole [POC]) was conducted according to the CLSI M38-A2 method (14).

TABLE 2 The MICs and mutations of non-wild-type *A. fumigatus* isolates from China[a]

Strain no.	Date of sample collection (day/mo/yr)	Location of sampling sites	MIC (mg/liter)			*cyp51A* mutations
			VRC	ITZ	POC	
H-13	18/06/2014	ZPPH	1	16	0.5	TR34/L98H/S297T/F495I
H-20	12/07/2014	HCH	1	8	0.125	TR34/L98H/S297T/F495I
H-58	02/03/2016	ZPPH	16	4	1	TR46/Y121F/T289A
H-90	02/03/2016	ZPPH	2	0.5	0.125	None
H-102	02/03/2016	ZPPH	2	0.5	0.25	None
H-197	02/03/2016	ZPPH	2	0.125	0.0625	None
H-85	02/03/2016	AHFPH	8	4	0.5	G448S
H-96	02/03/2016	AHFPH	4	0.5	0.125	G448S
H-98	02/03/2016	AHFPH	8	2	1	G448S
H-103	02/03/2016	AHFPH	8	2	0.5	G448S
H-107	02/03/2016	AHFPH	8	2	0.5	G448S
H-111	02/03/2016	AHFPH	8	2	0.5	G448S
H-114	27/03/2016	ZPTCM	8	2	0.5	G448S
H-120	27/03/2016	ZPTCM	8	1	0.5	G448S
H-126	27/03/2016	ZPTCM	8	2	0.5	G448S
H-149	04/03/2018	ZPPH	2	0.5	0.0625	None
H-152	04/03/2018	ZPPH	2	0.5	0.0625	None
H-161	04/03/2018	ZPPH	2	0.5	0.0625	None
H-190	04/03/2018	ZPPH	>16	16	0.25	TR46/Y121F/T289A
H-202	04/03/2018	ZPPH	8	>16	1	TR34/L98H
H-247	04/03/2018	ZPPH	8	4	0.125	F46Y/G89G/M172V/N248T/D255E/L358L/E427K/C454C
H-234	26/03/2018	HCH	>16	1	1	TR46/Y121F/T289A
H-237	26/03/2018	HCH	4	0.25	0.0625	None
H-264	26/03/2018	HCH	4	>16	1	TR34/L98H/S297T/F495I
H-176	04/03/2018	TFHZP	2	0.25	0.0625	None
H-184	04/03/2018	TFHZP	2	0.25	0.0625	None
H-186	04/03/2018	TFHZP	2	0.125	0.0625	None
H-191	04/03/2018	TFHZP	2	0.25	0.0625	None
H-197	04/03/2018	TFHZP	2	0.125	0.0625	None
H-206	04/03/2018	TFHZP	2	0.125	0.0625	None
H-208	04/03/2018	TFHZP	4	0.5	0.0625	G170G
H-248	04/03/2018	AHFPH	2	0.5	0.0625	None
H-252	04/03/2018	AHFPH	2	0.25	0.0625	None
H-256	04/03/2018	AHFPH	2	0.5	0.25	None
H-258	04/03/2018	AHFPH	4	0.25	0.125	None
H-218	04/03/2018	AHFPH	2	0.5	0.0625	None
H-242	26/03/2018	ZPTCM	>16	0.5	0.5	TR46/Y121F/T289A
H-244	26/03/2018	ZPTCM	>16	16	0.25	TR46/Y121F/T289A
H-260	26/03/2018	ZPTCM	2	0.25	0.0625	None
H-261	26/03/2018	ZPTCM	2	0.125	0.0625	None
H-266	26/03/2018	ZPTCM	2	0.125	0.0625	None
H-259	26/03/2018	HTH	4	>16	0.5	TR34/L98H
H-263	26/03/2018	HTH	4	>16	1	TR34/L98H
H-272	01/05/2018	THZP	2	>16	1	TR34/L98H/S297T/F495I
H-277	01/05/2018	THZP	2	0.5	0.0625	None
H-280	01/05/2018	THZP	2	>16	1	TR34/L98H/S297T/F495I

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TABLE 3 The expression levels of *cyp51* and efflux transporter genes of nonmutant strains (H-237 and H-258) and susceptible strain (HT)

Strain	Relative mRNA gene expression level[a]			
	cyp51A	*cyp51B*	*AfuMDR3*	*AfuMDR4*
HT	1.00 (0.73–1.19)	1.00 (0.81–1.22)	1.00 (0.68–1.22)	1.00 (0.80–1.16)
H-237	4.12 (3.53–5.12)	1.53 (1.27–1.70)	0.81 (0.66–1.10)	3.12 (2.45–3.32)
H-258	5.55 (4.81–6.61)	1.10 (1.05–1.33)	2.51 (2.07–3.12)	0.63 (0.61–0.77)

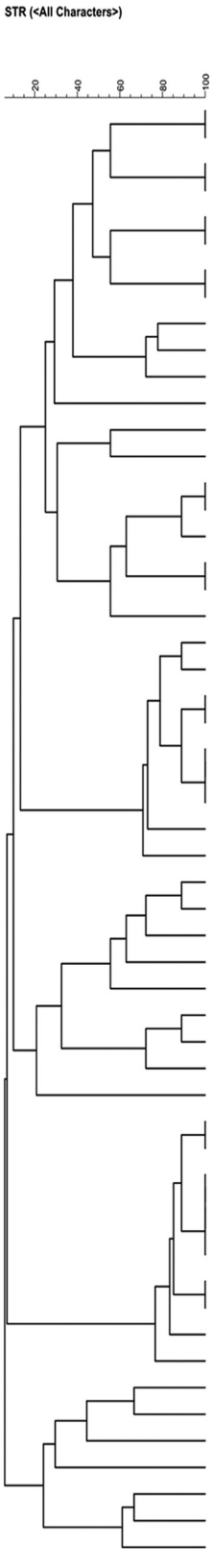
[a]HT values are averages from 5 randomly selected wild-type *A. fumigatus* isolates.

Isolates with a MIC of >1 mg/liter for ITZ and VRC and MIC of >0.25 mg/liter for POC were regarded as non-wild-type (non-WT) (15). To explore the underlying mutations of isolated non-WT *A. fumigatus*, the *cyp51A* and promoter regions were amplified and sequenced according to the method proposed by Ren et al. (6). For the non-WT strain without mutation, the mRNA expression levels of *cyp51* (*cyp51A* and *cyp51B*) and two transporters (*AfuMDR3* and *AfuMDR4*) were assessed according to the methods described by Cao et al. (16). The results were analyzed according to the $2^{-\triangle\triangle CT}$ method (17). Additionally, genotyping of non-WT *A. fumigatus* isolates was realized by microsatellite typing using a panel of nine short tandem repeats (STR) as described previously (18). To determine the genotypic relationship between environmental non-WT *A. fumigatus* and the clinical isolates, a total of 29 non-WT *A. fumigatus* isolates were used. These data originated from China (environmental, $n = 2$; clinical, $n = 15$) (12, 19–21), Netherlands (environmental, $n = 1$; clinical, $n = 6$) (19, 22), Colombia (environmental, $n = 1$) (10), India (environmental, $n = 3$) (19), and Tanzania (environmental, $n = 1$) (19).

From 2014 to 2018, a total of 282 *A. fumigatus* isolates were recovered from 191 environment samples collected from gardens around eight hospitals in Hangzhou, China (Table 1 and data not shown). The prevalence rates of *A. fumigatus* were 45.8%, 64.7%, and 62.5% in environmental samples collected in 2014, 2016, and 2018, respectively. This was consistent with previous reports showing a range of 35 to 77.8% (6, 23, 24). Out of the obtained 282 isolates, 46 strains were determined as non-WT *A. fumigatus* (Table 2). The isolate rates of non-WT *A. fumigatus* were 4.7%, 12.4%, and 23.1% in 2014, 2016, and 2018, respectively. These results show that the proportion of non-WT *A. fumigatus* in soils around hospitals in Hangzhou seemly increased during the 5 years.

Alterations in the *cyp51A* gene are usually the main causes of triazole resistance in *A. fumigatus* (9). There were six different mutations (Table 2 and data not shown) found in the isolated strains of non-WT *A. fumigatus*. The multiazole resistance mutations of TR34/L98H and TR46/Y121F/T289A were detected in 3 and 5 isolates, respectively. TR34/L98H and TR46/Y121F/T289A were first reported in Dutch patients and have been frequently found in azole-naive patients, clinics, and environmental samples worldwide (25). There is increasing evidence linking the occurrence and spread of these two resistance mechanisms to the use of triazole fungicides in agriculture (6, 26, 27). TR34/L98H/S297T/F495I, a widely reported mutation in China (12, 28), was found in 5 strains. The point mutations G448S and G170G and the mutations of F46Y/G89G/M172V/N248T/D255E/L358L/E427K/C454C were observed in 9, 1, and 1 strains, respectively.

In non-WT strains without mutations in *cyp51A*, the resistance may result from the decrease in intracellular toxin accumulation or an increase of target enzyme content. The apparent upregulation of *AfuMDR3* and *AfuMDR4* genes in the non-mutant, non-WT strains induced by ITZ was reported by Nascimento et al. (29). Recently, Cui et al. (30) reported the overexpression of *AtrF*, *AfuMDR1*, *cyp51A*, and *cyp51B* genes in non-WT strains HI-30 and HI-36 treated with tebuconazole. The expression of *cyp51A*, *cyp51B*, *AfuMDR3*, and *AfuMDR4* in strains H-237 and H-258 was assessed by quantitative reverse transcription PCR, and the results are listed in Table 3. The expression of *cyp51A* and *AfuMDR4* genes in H-237 were 4.12- and 3.12-fold greater, respectively, than those in the sensitive strains. Compared to the controls, 5.55- and 2.51- fold expression of



2A	2B	2C	3A	3B	3C	4A	4B	4C	Mutation Type	Source	Country	References
23	12	11	36	26	25	9	11	10	TR34/L98H/S297T/F495I	C	China	Chen et al., 2018
23	12	11	36	26	25	9	11	10	TR34/L98H	C	China	Deng et al., 2017
27	12	11	35	26	19	9	9	10	TR34/L98H/S297T/F495I	C	China	Chen et al., 2018
27	12	11	35	26	19	9	9	10	TR34/L98H	C	China	Deng et al., 2017
23	12	11	23	26	23	10	10	10	TR34/L98H/S297T/F495I	C	China	Chen et al., 2018
23	12	11	23	26	23	10	10	10	TR34/L98H	C	China	Deng et al., 2017
26	12	11	22	26	10	12	10	10	TR34/L98H/S297T/F495I	C	China	Chen et al., 2018
26	12	11	22	26	10	12	10	10	TR34/L98H	C	China	Deng et al., 2017
21	12	11	36	11	8	10	9	12	TR34/L98H	E	China	This study
21	12	11	36	11	11	10	9	10	TR34/L98H	E	China	This study
21	12	11	36	11	10	11	9	10	TR34/L98H	E	China	This study
27	16	14	29	22	26	10	9	10	TR34/L98H/S297T/F495I	E	China	This study
18	12	17	36	26	15	10	11	7	TR34/L98H/S297T/F495I	C	China	Chen et al., 2018
23	12	17	43	7	13	10	11	7	TR34/L98H/S297T/F495I	C	China	Chen et al., 2018
23	12	15	23	6	22	13	10	7	TR34/L98H/S297T/F495I	C	China	Chen et al., 2016
23	12	15	23	6	22	13	10	7	TR34/L98H/S297T/F495I	E	China	Chen et al., 2016
23	12	15	23	6	12	13	10	7	TR34/L98H/S297T/F495I	C	China	Chen et al., 2016
23	12	11	30	6	22	11	10	7	TR34/L98H/S297T/F495I	C	China	Chen et al., 2018
23	12	11	30	6	22	11	10	7	TR34/L98H	C	China	Deng et al., 2017
23	12	15	30	26	24	10	10	7	TR34/L98H/S297T/F495I	E	China	Chen et al., 2016
19	17	28	36	19	15	11	10	10	G448S	E	China	This study
19	17	28	36	20	15	11	10	10	G448S	E	China	This study
19	17	27	36	19	15	11	10	9	G448S	E	China	This study
19	17	27	36	19	15	11	10	9	G448S	E	China	This study
19	17	28	36	19	15	11	10	9	G448S	E	China	This study
19	17	28	36	19	15	11	10	9	G448S	E	China	This study
19	17	28	36	19	15	11	10	9	G448S	E	China	This study
18	17	28	36	20	15	11	10	9	G448S	E	China	This study
18	17	27	36	19	15	11	10	10	G448S	E	China	This study
25	19	11	25	22	9	13	8	9	TR46/Y121F/T289A	E	China	This study
25	19	11	25	20	9	13	8	9	TR46/Y121F/T289A	E	China	This study
25	19	11	30	20	9	10	8	9	TR46/Y121F/T289A	E	China	This study
25	19	11	26	24	10	13	8	9	TR46/Y121F/T289A	E	China	This study
26	19	11	25	9	19	13	8	7	TR46/Y121F/T289A	E	China	This study
26	21	12	26	20	9	13	9	9	TR46/Y121F/T289A	E	The Netherlands	Chen et al., 2015
26	21	12	26	19	9	13	9	9	TR46/Y121F/T289A	E	Colombia	Alvarezmoreno et al., 2017
26	21	12	25	19	9	14	9	9	TR46/Y121F/T289A	C	China	Chen et al., 2015
23	22	11	38	9	11	12	9	22	TR34/L98H/S297T/F495I	E	China	This study
26	21	9	33	21	11	8	14	10	TR46/Y121F/T289A	E	India	Chen et al., 2015
26	21	9	33	21	11	8	14	10	TR46/Y121F/T289A	E	India	Chen et al., 2015
26	21	9	33	22	11	8	14	10	TR46/Y121F/T289A	C	The Netherlands	Lavergne et al., 2015
26	21	9	33	22	11	8	14	10	TR46/Y121F/T289A	C	The Netherlands	Lavergne et al., 2015
26	21	9	33	22	11	8	14	10	TR46/Y121F/T289A	E	Tanzania	Chen et al., 2015
26	21	9	33	22	11	8	14	10	TR46/Y121F/T289A	C	The Netherlands	Lavergne et al., 2015
25	21	9	33	22	11	8	14	10	TR46/Y121F/T289A	C	The Netherlands	Lavergne et al., 2015
25	21	9	33	22	11	8	14	10	TR46/Y121F/T289A	C	The Netherlands	Lavergne et al., 2015
26	26	9	33	22	11	8	14	10	TR46/Y121F/T289A	C	The Netherlands	Lavergne et al., 2015
26	21	9	34	21	11	8	14	10	TR46/Y121F/T289A	E	India	Chen et al., 2015
22	19	16	11	29	20	10	10	6	F46Y/G89G/M172V/N248T/D255E/L358L/E427K/C454C	E	China	This study
22	19	16	11	29	12	10	8	11	None	E	China	This study
22	19	15	20	29	12	9	7	6	None	E	China	This study
22	18	9	21	29	16	8	9	6	G170G	E	China	This study
20	18	14	30	29	11	10	9	11	TR34/L98H/S297T/F495I	E	China	This study
20	18	18	30	29	10	10	11	11	TR34/L98H/S297T/F495I	E	China	This study
21	18	18	32	29	10	10	9	11	TR34/L98H/S297T/F495I	E	China	This study

FIG 1 Genotypic relationships of selected non-wild-type *A. fumigatus* isolates from environmental samples with non-wild-type strains from China (environmental resistant, $n = 2$; clinical resistant, $n = 15$), the Netherlands (environmental resistant, $n = 1$; clinical resistant, $n = 6$), Colombia (environmental resistant, $n = 1$), India (environmental resistant, $n = 3$), and Tanzania (environmental resistant, $n = 1$). E, environmental; C, clinical. The dendrogram is based on a categorical analysis of STR repeat numbers in combination with unweighted pair group method using average linkages clustering.

cyp51A and *AfuMDR3*, respectively, was detected in strain H-258. These results imply that the overexpression of *cyp51A* and efflux pumps are responsible for the resistance in H-237 and H-258 to triazole drugs.

STR genotyping has been widely used to study the genetic similarity of *A. fumigatus* strains due to its reproducibility and high resolution (12, 18). The genotyping data showed a large genotypic diversity in TR34/L98H (S297T/F495I), TR46/Y121F/T289A, and no-mutation genotypes, whereas most of the G448S mutations were closely clustered with similarity observed at 6 to 9 of the nine loci studied (Fig. 1). Analysis of the genetic similarity between the present environmental isolates and Chinese clinical strains ($n = 14$) with TR34/L98H (S297T/F495I) showed that the genotypes of all environmental TR34/L98H (S297T/F495I) isolates were distinct from those of the clinical TR34/L98H (S297T/F495I) strains. The genotypes of TR46/Y121F/T289A from environmental and clinical isolates showed two major clusters. The clade in the upper part of the phylogenetic tree mainly contained TR46/Y121F/T289A genotypes from China (environmental isolates, $n = 5$; clinical isolates, $n = 1$) and other Eurasian countries, the Netherlands (environmental isolates, $n = 1$), and Colombia (environmental isolates, $n = 1$). The clade in the lower part of the tree reveals a single microsatellite cluster and was mainly composed of TR46/Y121F/T289A genotypes from the Netherlands (clinical isolates, $n = 6$), Tanzania (environmental isolates, $n = 1$), and India (environmental isolates, $n = 3$). No genetic similarity was found in F46Y/G89G/M172V/N248T/D255E/L358L/E427K/C454C and G170G genotypes. Strikingly, non-WT strains containing the resistance-related mutation of G448S isolated from different hospitals in China had a nearly identical genotype. Although G448S has not been isolated from a clinic, its spread is likely to increase the azole resistance risks to immunocompromised patients. Recently, there is increasing evidence the concerning development of resistance in *A. fumigatus* through its sexual cycle (31–33). The sexual crossing experiments conducted by Camps et al. (32) demonstrated that TR34/L98H strains could outcross with azole-susceptible isolates of diverse genetic backgrounds. Zhang et al. (34) found that the new mutation of TR46^3/Y121F/T289A could arise via sexual crossing between isolates with TR46/Y121F/T289A. Sexual reproduction leads to new microsatellite genotypes. Considering the diverse STR types among non-WT isolates in this study, sexual reproduction may be involved in the development and spread of resistant *A. fumigatus*.

In conclusion, this study revealed an increasing incidence of non-WT *A. fumigatus* in Hangzhou over a 5-year survey. The G448S mutation, previously reported only in clinical settings, was recovered in environmental isolates. The high prevalence (>20%) of non-WT *A. fumigatus* in hospital environments suggests that susceptible patients will inhale spores, resulting in azole-resistant aspergillosis. These results demonstrate that regular resistance surveillance of *A. fumigatus* is necessary to obtain sufficient helpful data to avoid clinical failures.

Data availability. *cyp51A* sequences were deposited in the NCBI database under GenBank accession numbers MT418853–MT418878.

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We have no competing interests to declare.

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