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EPIDEMIOLOGY AND SURVEILLANCE

Elevated MIC Values of Imidazole Drugs against *Aspergillus fumigatus* Isolates with TR_{34}/L98H/S297T/F495I Mutation

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ABSTRACT The use of azole fungicides in agriculture is believed to be one of the main reasons for the emergence of azole resistance in *Aspergillus fumigatus*. Though widely used in agriculture, imidazole fungicides have not been linked to resistance in *A. fumigatus*. This study showed that elevated MIC values of imidazole drugs were observed against *A. fumigatus* isolates with TR_{34}/L98H/S297T/F495I mutation, but not among isolates with TR_{34}/L98H mutation. Short-tandem-repeat (STR) typing analysis of 580 *A. fumigatus* isolates from 20 countries suggested that the majority of TR_{34}/L98H/S297T/F495I strains from China were genetically different from the predominant major clade comprising most of the azole-resistant strains and the strains with the same mutation from the Netherlands and Denmark. Alignments of sterol 14α-demethylase sequences suggested that F495I in *A. fumigatus* was orthologous to F506I in *Penicillium digitatum* and F489L in *Pyrenophora teres*, which have been reported to be associated with imidazole resistance. *In vitro* antifungal susceptibility testing of different recombinants with *cyp51A* mutations further confirmed the association of the F495I mutation with imidazole resistance. In conclusion, this study suggested that environmental use of imidazole fungicides might confer selection pressure for the emergence of azole resistance in *A. fumigatus*.

KEYWORDS *Aspergillus fumigatus*, imidazole drugs, drug resistance, Cyp51A, evolution

Aspergillus fumigatus is an opportunistic fungal pathogen. In recent years, with the increase in the number of susceptible patients, such as those with malignant tumors and hematopoietic stem cell transplantation, the incidence of invasive pulmonary aspergillosis (IPA) has increased, and *A. fumigatus* has now become one of the main infectious pathogens associated with the death of hospitalized patients (1, 2). Azoles are the main drug class used in the management of *Aspergillus* diseases. However, since the emergence of itraconazole (ITC)-resistant *A. fumigatus* in 1997, reports of azole-resistant *A. fumigatus* strains from clinical and environmental sources have been increasing (3). The prevalence of azole resistance in *A. fumigatus* isolates from some clinical care centers was as high as 10%, posing a great challenge for clinical treatment of *Aspergillus* disease (4). It has been believed that there are two distinct routes of resistance development (5–11). One is long-term azole therapy for patients, which leads to point mutations in the sterol 14α-demethylase gene *cyp51A*, including

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TABLE 1 *In vitro* testing of the antifungal susceptibilities of 24 *Aspergillus fumigatus* isolates to ten azole compounds[a] according to the EUCAST method

Strain ID	Source	Azole resistant	*cyp51A* mutation(s)	EUCAST MIC (mg/liter)									
				ITC	VRC	POS	EPO	BRO	TEB	DIF	PRO	IMA	PRC
C94	Clinical	Yes	TR_{34}/L98H	≥16	2–4	1	≥32	8	8	8	≥32	2	0.5–1
C116	Clinical	Yes	TR_{34}/L98H	≥16	4	0.5	≥32	8	16	8	≥32	4	0.5–1
C135	Clinical	Yes	TR_{34}/L98H	≥16	2	1	≥32	16	8	16	≥32	2	0.5–1
C136	Clinical	Yes	TR_{34}/L98H	≥16	2	0.5	≥32	8	8	8	≥32	2	0.5–1
C821	Clinical	Yes	TR_{34}/L98H	≥16	4	1	≥32	16	16	≥32	≥32	2	1
C1664	Clinical	Yes	TR_{34}/L98H	≥16	8	1	≥32	16	≥32	≥32	≥32	2	2
STJ0105	Clinical	Yes	TR_{34}/L98H	≥16	8	2	≥32	8	16	≥32	≥32	2	1
XJ138	Clinical	Yes	TR_{34}/L98H	≥16	2–4	0.5	≥32	8	16	≥32	≥32	1	0.5
C96	Clinical	Yes	TR_{34}/L98H/S297T/F495I	≥16	1	0.5	≥32	≥32	16	≥32	≥32	8	≥32
C485	Clinical	Yes	TR_{34}/L98H/S297T/F495I	≥16	2	1	≥32	≥32	16	≥32	≥32	8	≥32
E739	Environmental	Yes	TR_{34}/L98H/S297T/F495I	≥16	2	0.5	≥32	≥32	16	≥32	≥32	8	≥32
E1001	Environmental	Yes	TR_{34}/L98H/S297T/F495I	≥16	1	0.5	≥32	≥32	16	16	≥32	8	≥32
C195	Clinical	Yes	TR_{46}/Y121F/T289A	1	≥16	0.5–1	≥32	≥32	8–32	≥32	≥32	≥32	16–32
C02810	Clinical	Yes	G54R	≥16	0.5	≥8	1	0.25–0.5	1–2	0.25	1–2	0.06	0.25
STJ0119	Clinical	Yes	G54V	≥16	0.25	1	0.5–1	0.5–1	1	0.5	1–2	0.06–0.125	0.25
C79	Clinical	No	D262Y	0.25	0.5	0.06	2	1	2	2	4	0.125	0.125
C98	Clinical	No	F46Y/M172V/N248T/D255E/E427K	1	1	0.25	4	4	2	2	8–16	0.25	0.25
C490	Clinical	No	N248K	1	0.25–0.5	0.125	1	0.5	0.5	0.5	2	0.125	0.125
C68	Clinical	No	None	0.5	0.5–1	0.25	4	4	4	2–4	8	0.25	1
C239	Clinical	No	None	0.5	0.5	0.125	4	2–4	2	2	8	0.25	0.5
E509	Environmental	No	N248K	0.5–1	0.25	0.06–0.125	2	0.5–1	1	1	1–2	0.125	0.125
E631	Environmental	No	None	0.25	0.25–0.5	0.125	2	1	2	1–2	1–2	0.125	0.25
E1069	Environmental	No	A9T	0.25–0.5	0.5	0.125	2	2	2	2	8	0.25	0.25
E1109	Environmental	No	None	0.25	0.5	0.06	2	1–2	2	2	4	0.125	0.25

[a]ITC, itraconazole; VRC, voriconazole; POS, posaconazole; EPO, epoxiconazole; BRO, bromuconazole; TEB, tebuconazole; DIF, difenoconazole; PRO, propiconazole; IMA, imazalil; PRC, prochloraz.

substitutions at G54, P216, M220, G138, and G448. The other is the application of azole fungicides in the environment, which leads to mutations in the *cyp51A* gene in combination with a tandem repeat (TR) in the promoter region of the gene, including the TR_{34}/L98H and TR_{46}/Y121F/T289A mutations.

The emergence of *A. fumigatus* strains with different *cyp51A* mutations might be associated with selection pressure from different azole drugs and the ability of *A. fumigatus* to adapt to the human host and the natural environment. Therefore, it has been suggested that starting, switching, and stopping azole therapy involves a risk of selecting for highly resistant strains with wild-type fitness (12). TR_{34}/L98H was the predominant type of mutation for azole resistance in *A. fumigatus* strains from the Netherlands, Germany, France, India, and many other countries (13–16). However, our previous study and other studies from China (7, 17, 18) showed that the prevalence of the TR_{34}/L98H/S297T/F495I mutation was comparable to, or even higher than, that of the TR_{34}/L98H mutation among azole-resistant *A. fumigatus* isolates in China, while the former mutation was less frequently identified in other countries. The genotypic and phenotypic characterizations of *A. fumigatus* strains with different *cyp51A* mutation types and their association with the use of agricultural azole compounds merit further studies.

This study aims to demonstrate the *in vitro* susceptibility of *A. fumigatus* to major azole fungicides and its genetic relationship with different *cyp51A* mutations, as well as to verify the association of elevated MIC values with imidazole fungicides and TR_{34}/L98H/S297T/F495I mutation by site-directed mutagenesis.

RESULTS

The results of *in vitro* susceptibility testing showed that five triazole fungicides—epoxiconazole, bromuconazole, tebuconazole, difenoconazole, and propiconazole—exhibited elevated MIC values (MICs, ≥8 mg/liter) for all the *A. fumigatus* isolates harboring the TR_{34}/L98H, TR_{34}/L98H/S297T/F495I, or TR_{46}/Y121F/T289A mutation. Prochloraz and imazalil had higher MIC values for all four TR_{34}/L98H/S297T/F495I isolates than for the eight TR_{34}/L98H isolates. The MIC values of the seven azole fungicides tested against the two azole-resistant *A. fumigatus* isolates with G54R or G54V mutation were similar to those for the azole-susceptible isolates (Table 1). The growth phenotypes of these isolates on drug-containing minimal medium (MM) plates are shown in

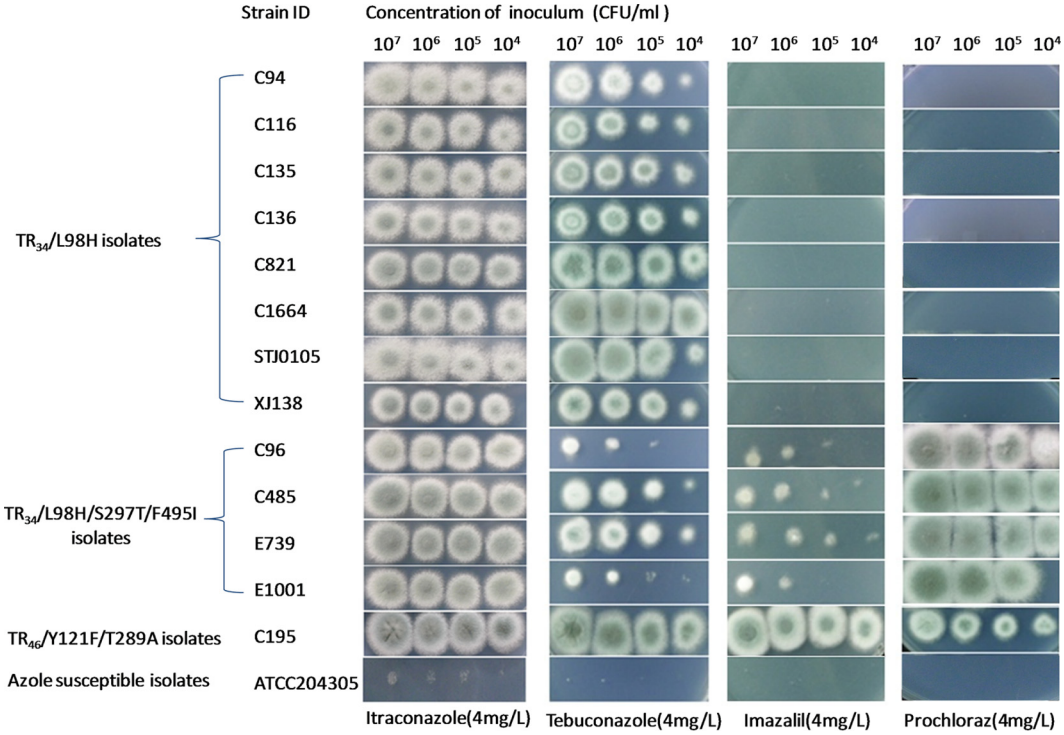


FIG 1 Growth of 14 *Aspergillus fumigatus* isolates on minimal medium plates containing four different azole drugs (35°C, 72 h).

Fig. 1. All the TR_{34}/L98H/S297T/F495I isolates could grow on MM plates containing 4 mg/liter of prochloraz and imazalil, while the eight TR_{34}/L98H isolates could not.

The population structure of 580 *A. fumigatus* isolates based on short-tandem-repeat (STR) typing analysis is shown in Fig. 2. Most of the TR_{34}/L98H strains were distributed in the lower right clade of the minimum spanning tree, which represents the major clone complex of the azole-resistant *A. fumigatus* strains disseminating all around the world. Eight of 12 TR_{34}/L98H/S297T/F495I strains originated from China were distributed in the upper left clade of the tree. This distribution suggested that these strains might have different evolutionary sources than the major TR_{34}/L98H clone complex. The genotypic relationships of 17 *A. fumigatus* isolates with TR_{34}/L98H/S297T/F495I mutation are shown in Fig. 3, which indicates that the TR_{34}/L98H/S297T/F495I strains from China are genetically unrelated to the TR_{34}/L98H/S297T/F495I strains from the Netherlands and Denmark.

An amino acid sequence alignment based on the Cyp51A sequence from *A. fumigatus* and orthologous protein sequences from 10 other fungal pathogens is shown in Fig. S1 in the supplemental material. The alignments have been summarized, and fungicide resistance-associated mutations are given in Table 2. The F495I mutation in *A. fumigatus* is demonstrated to be orthologous to F506I in *Penicillium digitatum* and F489L in *Pyrenophora teres*, while no orthologous mutation was found for the L98H or S297T mutation in *A. fumigatus*. The Y121F mutation in *A. fumigatus* is also demonstrated to be orthologous to numerous mutations in agricultural fungal pathogens, including Y136H in *P. digitatum*, Y136F in *Erysiphe necator*, *Mycosphaerella fijiensis*, and *Mycosphaerella graminicola*, and Y136H in *Blumeria graminis*.

Successful construction of recombined *A. fumigatus* strains containing S297T, F495I, TR_{34}/L98H, TR_{34}/L98H/S297T, TR_{34}/L98H/F495I, and TR_{34}/L98H/S297T/F495I was confirmed by diagnostic PCR testing and sequencing of the *cyp51A* genes of the recombinants. The MICs for recombinant *A. fumigatus* strains with different *cyp51A* mutations are shown in Table 3. The recombinants with an S297T or F495I mutation produced MICs similar to those for the wild-type *A. fumigatus* CEA Δ*ku80* recipient strain. The MICs of all three antifungal drugs and the seven fungicides for TR_{34}/L98H mutants showed

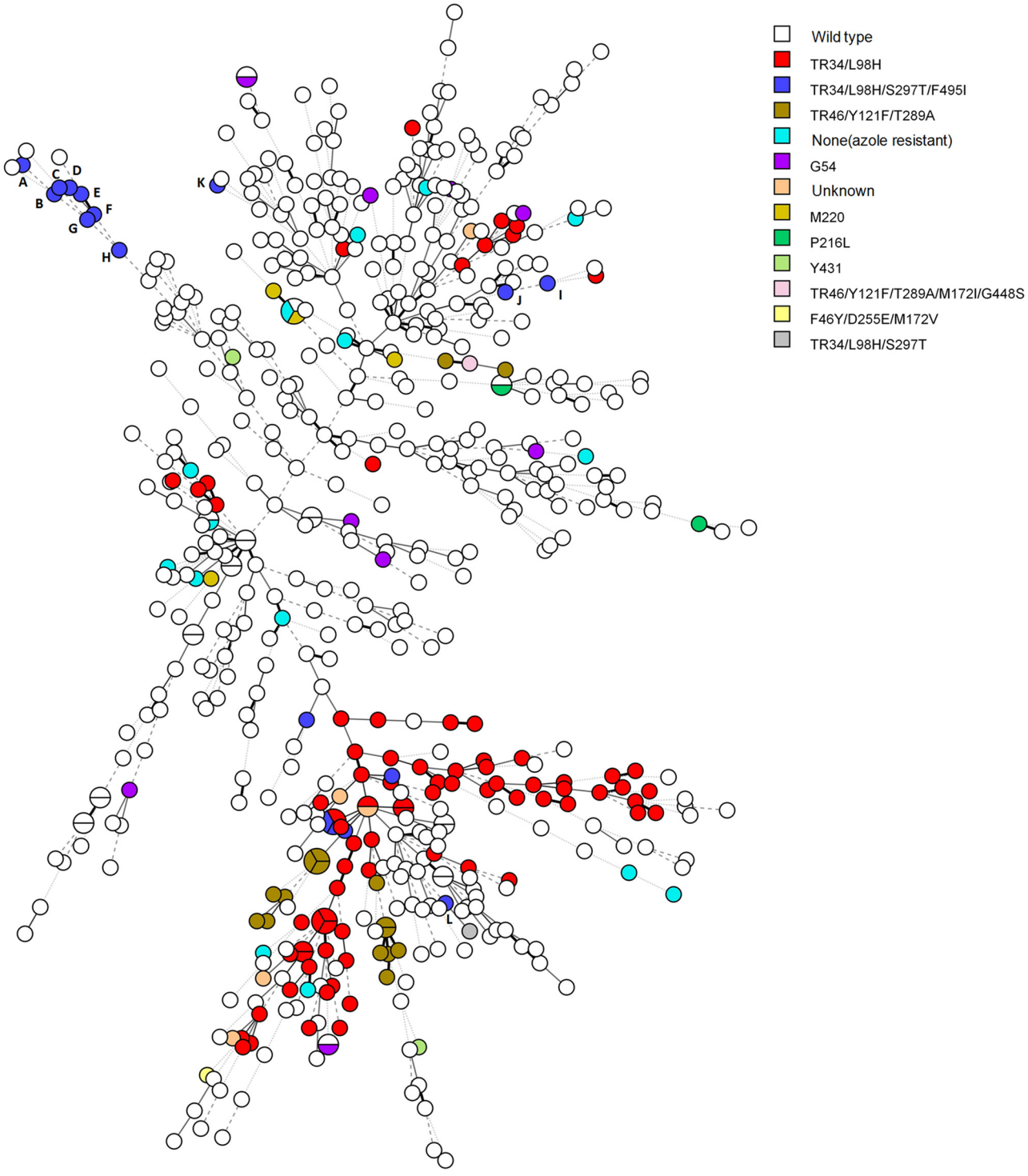
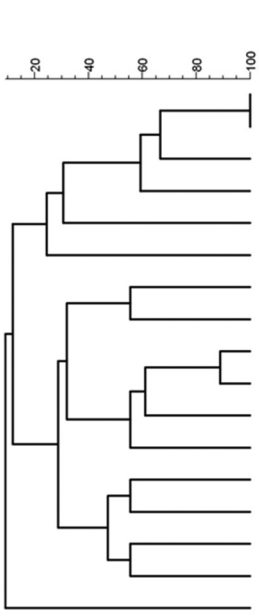


FIG 2 Minimum spanning tree of 580 *Aspergillus fumigatus* isolates based on all nine microsatellite markers of STR typing. Each circle represents one unique genotype. Different colors indicate different *cyp51A* mutations as shown in the key. Circles labeled A to L correspond to the 12 Chinese isolates with the TR_{34}/L98H/S297T/F495I mutation (A, 20684.007; B, C96; C, 20684.022; D, 20677.086; E, 20643.023; F, E739; G, 20677.079; H, E1001; I, 20677.089; J, 20684.002; K, B44; L, 20643.017).

increases over those for CEA Δ*ku80*. When S297T was introduced into the TR_{34}/L98H strain, the MICs of the two imidazoles remained similar. When F495I alone or both F495I and S297T were introduced in addition to the TR_{34}/L98H mutation, a higher prochloraz MIC value (≥32 mg/liter) was observed. The MIC values of itraconazole, posaconazole,



STR												
2A	2B	2C	3A	3B	3C	4A	4B	4C	Strain ID	Country	Source
14.0	20.0	8.0	40.0	9.0	11.0	8.0	10.0	20.0	KLM-R2	Denmark	Clinical
14.0	20.0	8.0	40.0	9.0	11.0	8.0	10.0	20.0	08-36-03-25	Netherlands	Clinical
10.0	20.0	8.0	30.0	9.0	6.0	8.0	10.0	20.0	F2126	Netherlands	Clinical
14.0	10.0	9.0	30.0	9.0	6.0	8.0	10.0	20.0	Afu_key29	Denmark	Clinical
15.0	23.0	9.0	28.0	10.0	6.0	8.0	10.0	14.0	08-12-12-13	Netherlands	Clinical
18.0	20.0	15.0	42.0	24.0	6.0	9.0	10.0	10.0	20643.017	China	Clinical
18.0	12.0	17.0	36.0	15.0	26.0	10.0	11.0	7.0	20677.089	China	Clinical
23.0	12.0	17.0	43.0	13.0	7.0	10.0	11.0	7.0	20684.002	China	Clinical
23.0	12.0	15.0	23.0	22.0	6.0	13.0	10.0	7.0	E739	China	Environmental
23.0	12.0	15.0	23.0	12.0	6.0	13.0	10.0	7.0	C96	China	Clinical
23.0	12.0	11.0	30.0	22.0	6.0	11.0	10.0	7.0	20677.086	China	Clinical
23.0	12.0	15.0	30.0	24.0	26.0	10.0	10.0	7.0	E1001	China	Environmental
23.0	12.0	11.0	23.0	23.0	26.0	10.0	10.0	10.0	20677.079	China	Clinical
26.0	12.0	11.0	22.0	10.0	26.0	12.0	10.0	10.0	20684.022	China	Clinical
23.0	12.0	11.0	36.0	25.0	26.0	9.0	11.0	10.0	20643.023	China	Clinical
27.0	12.0	11.0	35.0	19.0	26.0	9.0	9.0	10.0	20684.007	China	Clinical
19.0	23.0	14.0	30.0	28.0	26.0	12.0	9.0	15.0	B44	China	Clinical

FIG 3 Genotypic relationships among 17 *Aspergillus fumigatus* isolates with TR_{34}/L98H/S297T/F495I mutation from China, the Netherlands, and Denmark. The dendrogram is based on a categorical analysis of nine microsatellite markers in combination with UPGMA clustering. The bar indicates the percentage of identity.

and the seven fungicides for the recombinants with TR_{34}/L98H/S297T/F495I mutation were at least 2-fold higher than those for CEA Δ*ku80*. The differences in growth ability on azole-containing MM plates between CEA Δ*ku80* and the six constructed strains are clearly shown in Fig. 4 and are consistent with the results of *in vitro* susceptibility testing.

DISCUSSION

Azoles are an important class of drugs widely used in human and animal health, as well as in agriculture and horticulture. Over the past few years, evidence for an

TABLE 2 Corresponding mutation sites in agricultural pathogens based on alignment to the reference sequence from *Aspergillus fumigatus cyp51A*[a]

Amino acid substitution(s) in *Aspergillus fumigatus*	Homologous position in other species[b]
N22D	NA
G54E/K/R/V/W	NA
L98H	NA
V101F	NA
Y121F	Y136H (*Penicillium digitatum*), Y136F (*Erysiphe necator*), Y136F (*Mycosphaerella fijiensis*), Y136H (*Blumeria graminis*), Y137F (*Mycosphaerella graminicola*)
G138C/R/S	NA
P216L	NA
F219C	NA
M220K/I/T/V	NA
T289A	NA
S297T	NA
Y431C	Y461D (*Mycosphaerella fijiensis*), Y459C/D/P/S (*Mycosphaerella graminicola*)
G432A/S	G460D (*Mycosphaerella graminicola*)
G434C	NA
G448S	G459S (*Penicillium digitatum*)
T470N	NA
F495I	F506I (*Penicillium digitatum*), F489L (*Pyrenophora teres*)

[a]GenBank accession number AF338659.
[b]NA, not available.

TABLE 3 MICs for recombinant *Aspergillus fumigatus* strains with different Cyp51A amino acid substitutions according to the EUCAST method

Isolate	Cyp51A substitution(s)		EUCAST MIC (mg/liter)[a]									
	TR[b]	Coding gene	ITC	VRC	POS	EPO	BRO	TEB	DIF	PRO	IMA	PRC
CEA Δ*ku80* recipient strain	−	−	0.5	0.5	0.125	2–4	2	2	1–2	4	0.125	0.25
CEA Δ*ku80*_S297T	−	S297T	0.5–1	1	0.25	4	4	2	2	4	0.25	0.5
CEA Δ*ku80*_F495I	−	F495I	0.5	0.5–2	0.03–0.125	2–4	1	1–2	2	8	0.5	0.5–1
CEA Δ*ku80*_TR_{34}/L98H	+	L98H	≥16	4	1	≥32	16	16–32	16	≥32	4–8	2
CEA Δ*ku80*_TR_{34}/L98H/S297T	+	L98H/S297T	8–16	2	0.5–1	≥32	32	16	16	≥32	2–4	4
CEA Δ*ku80*_TR_{34}/L98H/F495I	+	L98H/F495I	≥16	2	0.5	≥32	8	16–32	≥32	≥32	8	>32
CEA Δ*ku80*_TR_{34}/L98H/S297T/F495I	+	L98H/S297T/F495I	≥16	1–2	1	≥32	≥32	16	≥32	≥32	16	≥32

[a]ITC, itraconazole; VRC, voriconazole; POS, posaconazole; EPO, epoxiconazole; BRO, bromuconazole; TEB, tebuconazole; DIF, difenoconazole; PRO, propiconazole; IMA, imazalil; PRC, prochloraz.
[b]TR, 34-bp tandem repeat in the promoter region. −, absent; +, present.

environmental route of resistance development in *A. fumigatus* due to applications of azole fungicides in agriculture has been accumulating (9); however, this link has not been proven (19). One of the main arguments has arisen from the lack of evidence of an association between two imidazoles, imazalil and prochloraz, and resistance in patients, although these imidazoles have been widely used since the 1970s (20). Our study clearly demonstrates an association of elevated MIC values of imidazole fungicides with TR_{34}/L98H/S297T/F495I mutation in *A. fumigatus*. Although MICs have been used to evaluate the *in vitro* activities of fungicides against *A. fumigatus* (9, 21), there is currently no agreement on breakpoints to define resistance in the fungicides tested. Therefore, the significance of a 1- or 2-fold difference in the MICs of azole fungicides could not be determined (e.g., it is not known whether the clinical isolates C116 and XJ138 harbor different adaptive abilities in a natural soil environment sprayed with imazalil fungicides). This is one major limitation of this study. More studies are needed in the future to determine the significance of minor differences in MIC values of azole fungicides for *A. fumigatus*.

The results of STR typing analysis (Fig. 2) showed that 8 of the 12 Chinese TR_{34}/L98H/S297T/F495I strains clustered in the upper left clade seem to belong to the same clone complex, while the other 4 strains were dispersed in the tree and possibly have different evolutionary origins. Both Fig. 2 and Fig. 3 reveal that the Chinese TR_{34}/L98H/S297T/F495I strains are genetically different from the strains with the same mutation from the Netherlands and Denmark. These findings suggested that most of the *A.*

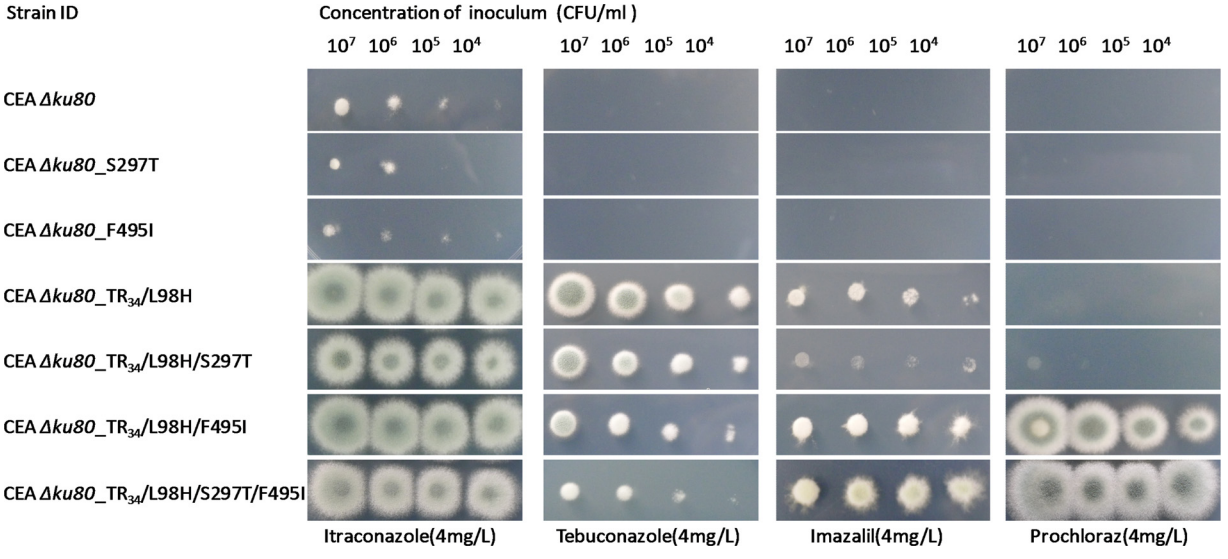


FIG 4 Growth of *Aspergillus fumigatus* CEA Δ*ku80* and six constructed strains on minimal medium plates containing four different azole drugs (35°C, 72 h).

fumigatus strains with TR_{34}/L98H/S297T/F495I mutation from China possibly evolved from an extremely adaptive recombinant event under the selection pressure of imidazole fungicides within China, in a manner similar to the emergence of clonal TR_{34}/L98H strains in India (16). The recent report of agricultural TR_{34}/L98H/S297T/F495I isolates in China further supports our hypothesis (8).

Another piece of evidence comes from alignments of Cyp51 sequences from *A. fumigatus* and agricultural pathogens. These showed that the F495I mutation in *A. fumigatus* corresponds to F506I in *P. digitatum* and F489L in *P. teres*. It has been suggested that the F506I mutation plays an important role in the structure and function of Cyp51 and contributes to prochloraz resistance in *P. digitatum* strains from China (22). This mutation was found in combination with G459S mutation (22). F489L mutation in *P. teres* has also been shown to be associated with a high level of prochloraz resistance, and further structural *in silico* modeling analysis showed that interaction of F489L with the heme cavity produced a localized constriction of the region adjacent to the docking site that is predicted to result in lower binding affinities (22). In *P. teres*, the F495L mutation was the sole polymorphism of Cyp51A to be found only in resistant and not in sensitive isolates (23). The insertion of TR in the promoter region also represents an important mechanism of fungicide resistance and could contribute to overexpression of the *cyp51* gene in many agricultural pathogens (11, 24, 25). More importantly, an investigation of 25 prochloraz-resistant *P. digitatum* isolates from China showed that F506I mutations in the *cyp51* gene arose in combination with an insertion of 199 bp in the promoter region (22), suggesting that azole-resistant agricultural and clinical fungal pathogens harbor similar resistance mechanisms and probably have originated from the same evolutionary routes.

Repeated *in vitro* susceptibility testing showed that there were one or two 2-fold MIC differences for some strains when the same isolate was tested several times, suggesting that the results of susceptibility testing were generally stable and reliable. The susceptibility testing of different recombinants with *cyp51A* mutations further confirms the association of the F495I mutation with imidazole resistance. Although the single F495I mutation causes only a slight increase in the MIC values of imidazole fungicides, it could lead to high levels of prochloraz and imazalil resistance together with the introduction of the 34-bp tandem repeat and the L98H mutation. It has been shown that the 34-bp TR in the *cyp51A* gene of *A. fumigatus* is by itself not sufficient for the multi-azole resistance phenotype, and the substitution at codon 98 is a key alteration in this resistance mechanism (26). Our study suggested that the introduction of the F495I mutation was important for the survival of TR_{34}/L98H/S297T/F495I strains under selection pressure from imidazole fungicides. The exact role of the S297T mutation is unknown. One hypothesis is that it is required to compensate for the harmful effect of F945I on normal protein function, just as T289A does in TR_{46}/Y121F/T289A strains (27). The emergence of these special combinations of mutations possibly represent evolutionary consequences of the genotype–fitness maps (28). TR_{34}/L98H/S297T/F495I and TR_{46}/Y121F/T289A strains could be regarded as two of the multiple fitness peaks in the fitness landscape of *A. fumigatus*. Imazalil showed lower MIC values for clinical isolates than for the laboratory mutants in our study. This difference was possibly due to the absence of selection pressure from imidazole drugs on clinical isolates, which might have evolved to compensate for the adverse effect of *cyp51A* mutations.

Our findings raise many questions for future studies. First, could the TR_{34}/L98H/S297T/F495I mutation be induced under selection pressure from imidazole fungicides under experimental conditions? Second, why do the TR_{34}/L98H/S297T/F495I isolates from different countries or regions have different evolutionary sources? Third, is there any dose-effect relationship between amounts of imidazole consumption and the prevalence of imidazole resistance in agriculture or other environments? Could the problem of TR_{34}/L98H/S297T/F495I dissemination be contained by reducing the application of imidazole fungicides in the environment?

In conclusion, our study is the first to demonstrate the association of elevated MIC

values of imidazole fungicides with TR_{34}/L98H/S297T/F495I mutation in *A. fumigatus*, which deepens our understanding of environmental routes of azole resistance development. Additional epidemiological and experimental evidence is needed for guiding the rational use of environmental fungicides and containing the problem of increasing antifungal resistance.

MATERIALS AND METHODS

***In vitro* antifungal susceptibility testing.** A collection of 13 azole-resistant *A. fumigatus* clinical isolates, 5 azole-susceptible clinical isolates, 2 azole-resistant environmental isolates, and 4 azole-susceptible environmental isolates was selected for investigation of the *in vitro* activities of seven azole fungicides and three clinical azole drugs. Among the 24 isolates tested, 5 azole-resistant clinical isolates (C1664, STJ0105, XJ138, C02810, and STJ0119) were newly collected through a surveillance program, while the remaining 19 isolates were from a previous study (7). All data for the 5 newly collected isolates, as well as *in vitro* activity data of the seven fungicides with the 19 previously collected isolates, were generated in this study.

In vitro testing of the susceptibilities of 24 *A. fumigatus* isolates to seven azole fungicides was conducted according to the EUCAST broth microdilution E.DEF 9.3 reference method (29). The seven fungicides were epoxiconazole, bromuconazole, tebuconazole, difenoconazole, propiconazole, imazalil, and prochloraz. The concentrations tested for all seven fungicides ranged from 0.06 to 32 mg/liter. Identification of strain species and *cyp51A* mutations, as well as *in vitro* testing of the susceptibilities of the five newly collected isolates to ITC (0.03 to 16 mg/liter), voriconazole (VRC) (0.03 to 16 mg/liter), and posaconazole (POS) (0.0156 to 8 mg/liter), were conducted as reported previously (7). *A. fumigatus* ATCC 204305 was included as a reference for quality control. The MICs for each strain were determined through at least three repeats of testing. The ranges of MICs are provided for those strains with different results after repeated testing. The growth of 14 *A. fumigatus* isolates on minimal medium (MM) plates containing 4 mg/liter of ITC, tebuconazole, imazalil, or prochloraz was also assessed. A series of freshly harvested spores were spotted onto the antifungal-drug-containing medium, and then the inoculated plate was cultured at 35°C for 72 h for observation.

STR typing analysis. A search of published papers giving the results of STR typing for *A. fumigatus* was conducted. Among isolates harboring the same *cyp51A* mutation and STR type from each country, only the first isolate was included for analysis. As a result, STR typing data of 424 isolates from 20 countries (16, 30–54) were obtained. A total of 151 isolates with distinct STR types from our previous study (7) and 5 newly collected isolates in this study were also included for final analysis. The nine microsatellite markers (STRAf 2A, 2B, 2C, 3A, 3B, 3C, 4A, 4B, and 4C) for the five newly collected isolates were determined as described previously (41). The STR typing data were analyzed by Bionumerics 7.5 and are presented as a minimum spanning tree for categorical data with default settings. The genetic relationships of 19 *A. fumigatus* isolates with TR_{34}/L98H/S297T/F495I mutation were analyzed by a categorical analysis of nine microsatellite markers using UPGMA (unweighted pair group method with arithmetic means) clustering.

Alignments of sterol 14α-demethylase sequences. Amino acid sequences of sterol 14α-demethylase (Cyp51) from *A. fumigatus*, *Aspergillus flavus*, and nine agricultural fungal pathogens (*Blumeria graminis*, *Monilinia fructicola*, *Mycosphaerella fijiensis*, *Penicillium digitatum*, *Oculimacula yallundae*, *Pyrenophora teres*, *Zymoseptoria tritici*, *Erysiphe necator*, and *Venturia inaequalis*) were downloaded from NCBI GenBank. Reported amino acid substitutions associated with azole resistance were annotated. Alignments of sequences were generated using ClustalW (http://www.genome.jp/tools/clustalw/). The alignments are available as .pdf files in the supplemental material.

***cyp51A* site-directed mutagenesis.** In order to explore the potential roles of S297T and F495I mutations in fungicide resistance, amino acid substitutions were introduced into the *cyp51A* gene of an *A. fumigatus* strain through fusion PCR methods. The 5′ flank and 3′ flank fragments of the *cyp51A* gene were amplified from *A. fumigatus* CEA Δ*ku80* genomic DNA using the primers listed in Table S1 in the supplemental material, and the pyrithiamine or hygromycin resistance gene was also amplified from plasmids constructed in our laboratory. The four PCR products were then fused with the nested primer pair cyp51A-S297T-up1S/cyp51A-S297T-dwA or cyp51A-F495I-up1S/cyp51A-F495I-dwA (Table S1). The fusion PCR product was transformed into the recipient strain A1160 to generate the S297T or F495I mutation. For the construction of *A. fumigatus* with a TR_{34}/L98H, TR_{34}/L98H/S297T, TR_{34}/L98H/F495I, or TR_{34}/L98H/S297T/F495I mutation, the genomic DNA of a previously identified TR_{34}/L98H or TR_{34}/L98H/S297T/F495I isolate was used as the template for the amplification of corresponding *cyp51A* fragments. The transformants were confirmed by sequencing of the *cyp51A* coding region (7) as well as by diagnostic PCR with two pairs of primers (cyp51A-T960A-yS/cyp51A-T960A-yA and cyp51A-T1554A-yS/cyp51A-T1554A-yA [Table S1]) targeting *cyp51A* and the adjacent regions of the constructed strains. *In vitro* susceptibility testing of constructed strains and the growth experiment with these strains on drug-containing MM plates were conducted using the same methods as those for the 24 clinical and environmental *A. fumigatus* isolates.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.01549-17.

SUPPLEMENTAL FILE 1, PDF file, 1.1 MB.

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We have no conflicts of interest to declare.

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