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Original Article

Characteristics of azole-resistant *Aspergillus fumigatus* attached to agricultural products imported to Japan☆

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ABSTRACT

Due to the increase in the number of azole-resistant *Aspergillus fumigatus*, there is an urgent need of data to predict future trends and prevent further spreading. The intercountry transfer of resistant *A. fumigatus* on plant bulbs have been reported. We investigated existence and characteristics of resistant isolates attached to agricultural products imported to Japan.

We purchased 292 samples in Japan. All samples were screened for the existence of azole-resistant *A. fumigatus*. For positive isolates, minimum inhibitory concentrations of the drugs were determined. We also analyzed Cyp51A, Hmg1, and Erg6 mutations of these isolates and conducted microsatellite genotyping.

Fourteen azole-resistant isolates were detected, of which 13 were cultured from flower bulbs imported from the Netherlands. Among them 5 were from 11 bulbs of *Hippeastrum* (45.5%), 5 were from 24 bulbs of *Gladiolus* (20.8%), 2 were from 4 bulbs of *Ixia* (50.0%), and 1 was from 22 bulbs of *Tulipa* (4.5%). Only 1 resistant isolate was cultured from the 10 bulbs of *Narcissus* (10.0%) originating in Japan. Various novel mutations including Y121F/T289A in Cyp51A with no tandem repeat in promoter region were discovered from imported strains.

Our study provides important data showing that agricultural imports provide a possible route for their intercontinental spread and raises the concern that strains harboring highly diverse Cyp51A mutations might increase in clinical settings in the future.

1. Introduction

Aspergillus fumigatus, one of the most common infection-causing fungal pathogen in humans, has a broad clinical spectrum, from invasive aspergillosis (IA) to allergic and chronic aspergillosis [1]. Azole antifungal drugs play crucial roles against all types of aspergillosis. They include voriconazole, the primary drug used against IA, and an oral formulation of azole, which is used to treat outpatients with chronic pulmonary aspergillosis (CPA) [2–4].

Gradually, the spread of azole-resistant *A. fumigatus* isolates has become a global public health concern [5]. An increase in azole resistance rates could worsen prognoses for patients with IA because voriconazole monotherapy is now used as a first-line therapy for IA treatment, making it impossible to treat patients with CPA on an outpatient basis because azole is the only class having an oral formulation [6]. Thus, increase in azole resistance will challenge our current primary treatment strategies, and increase mortality and patient hospital stay duration and costs [5,6]. Due to the increase in the number of azole-resistant *A. fumigatus*, there is an urgent need of data to predict future trends and prevent further spreading [7].

Two confirmed routes of azole resistance selection are the patient route, for which resistance develops in the lungs of patients with CPA after long-term triazole treatment [8], and the

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environmental route, in which resistance occurs after environmental exposure of *A. fumigatus* to fungicides used routinely for crop protection and preservation of materials [5]. Resistant isolates originating from the environment may be more important for the global spread of azole-resistant *A. fumigatus* than those from the patient route. Indeed, global population genetics analysis of *A. fumigatus* revealed that the same genotypes were isolated from intercontinental sources, such as the air in Belgium and patients in the United States [9].

Recently, Zhang et al. reported that composting of organic matter containing azole residues might be important for resistance development [10]. Additionally, the recovery of resistant isolates from flower fields and intercountry transfer of resistant *A. fumigatus* on plant bulbs have also been reported [11,12]. Therefore, we investigated existence and characteristics of azole-resistant *A. fumigatus* attached to agricultural products imported to Japan.

2. Material and methods

2.1. Samples

We purchased 292 samples, consisting of 248 flower bulbs and 44 root crops, in Japan. Among them, 167 products were imported from the Netherlands, 18 products from France, 18 products from China, and 3 products from India (Table 1). All of these countries were known to contain azole-resistant *A. fumigatus*. The remaining 86 products originated from Japan and were used as a comparison with the imported products.

2.2. Screening of A. fumigatus

All samples were screened for the existence of *A. fumigatus* by direct culture in potato dextrose broth (PDB) supplemented with 100 μg/mL chloramphenicol (Wako Pure Chemical Industries, Japan) and incubated at 48 °C. Because multiple isolates were sometimes recovered from one sample in the liquid media, we picked each fungus mass up individually by aseptic operation and isolation culture with potato dextrose agar was performed for all positive isolates. Isolates were identified as *A. fumigatus* due to macroscopic colony morphology, micromorphological characteristics, and the ability to grow at 48 °C.

2.3. Screening and antifungal susceptibility testing of azole-resistant isolates

Azole resistance was screened by culture of each conidia from *A. fumigatus* isolates in either 4 μg/mL itraconazole- or voriconazole-supplemented PDB at 30 °C for 7 days. For positive isolates, minimum inhibitory concentrations (MICs) of the drugs were determined using the Clinical and Laboratory Standards Institute (CLSI) M38-A2 broth microdilution method [13]. Susceptibility tests of isolates that screened positive for resistance were performed at least three times per isolate; each test was performed on different days. All positive isolates were subjected to additional molecular identification by ribosomal internal transcribed spacer (ITS) and ribosomal large-subunit D1-D2 amplification, and β-tubulin sequencing as described previously [14,15].

2.4. Sequencing cyp51A, hmg1, and erg6 genes and microsatellite genotyping

Mutations in the azole target protein Cyp51A, a lanosterol 14α-demethylase found in *A. fumigatus*, are a major mechanism responsible for azole resistance [5]. In addition, Hagiwara et al. recently found mutations in Hmg1 and Erg6 involved in ergosterol biosynthesis and multiple isolates with increased resistance to azole possessed a mutation in Hmg1 [16]. To analyze these genes of resistant isolates, genomic DNA was extracted from resistant isolates using the MasterPure yeast DNA purification kit (Epicentre Biotechnologies, Madison, WI). The full coding region and the promoter region of *cyp51A*, *hmg1*, and *erg6* genes were amplified as previously described [16,17]. Sequence alignments were performed against the sequence from an azole-susceptible strain (GenBank accession no. AF338659 for Cyp51A, Fungi DB accession nos. AFUB_020,770 for Hmg1, and AFUB_099,400 for Erg6). Mutations were confirmed by repeating the PCR and sequencing of the

Table 1
Samples used in this study.

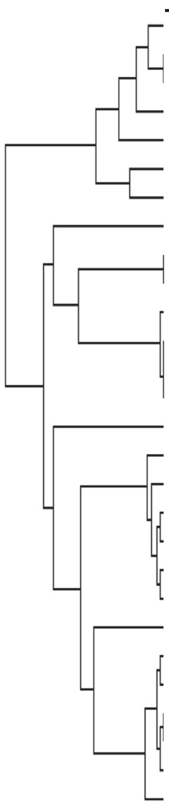
Country of origin	Genus	Application	Number of samples, n	Number of azole-resistant *Aspergillus fumigatus* isolates n (%)
Agricultural imports				
The Netherlands	*Chionodoxa*	Flower bulb	3	
The Netherlands	*Gladiolus*	Flower bulb	24	5 (20.8)
The Netherlands	*Hippeastrum*	Flower bulb	11	5 (45.5)
The Netherlands	*Hyacinthus*	Flower bulb	3	
The Netherlands	*Ixia*	Flower bulb	4	2 (50.0)
The Netherlands	*Muscari*	Flower bulb	5	
The Netherlands	*Oxalis*	Flower bulb	72	
The Netherlands	*Scilla*	Flower bulb	5	
The Netherlands	*Sprekelia*	Flower bulb	1	
The Netherlands	*Triteleia*	Flower bulb	12	
The Netherlands	*Tulipa*	Flower bulb	22	1 (4.5)
The Netherlands	*Zantedeschia*	Flower bulb	5	
France	*Allium*	Root crop	18	
China	*Arctium*	Root crop	18	
India	*Zephyranthes*	Flower bulb	3	
Japanese products				
Japan	*Colocasia*	Root crop	8	
Japan	*Hessea*	Flower bulb	1	
Japan	*Ipheion*	Flower bulb	10	
Japan	*Narcissus*	Flower bulb	10	1 (10.0)
Japan	*Nerine*	Flower bulb	2	
Japan	*Tulipa*	Flower bulb	55	

All samples were obtained in Japan.

Table 2
Characteristics of azole-resistant *Aspergillus fumigatus* isolates.

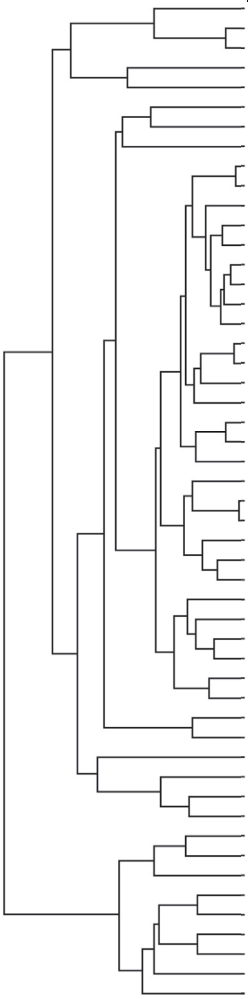
Isolate ID	Source	MIC, mg/L		Cyp51A mutations	Hmg1 mutations	Erg6 mutations
		ITR	VOR			
Agricultural imports						
NGS-ER6	*Gladiolus*	1	>8	Y121F/T289A	E105K/S212P/Y564H	No mutation
NGS-ER1	*Gladiolus*	0.5	>8	TR_{46}/Y121F/T289A	S212P/Y564H	No mutation
NGS-ER5	*Gladiolus*	1	>8	TR_{46}/Y121F/T289A	S212P/Y564H	No mutation
NGS-ER7	*Gladiolus*	2	>8	TR_{46}/Y121F/T289A	E105K/S212P/Y564H	No mutation
NGS-ER2	*Gladiolus*	1	>8	TR_{46}^3/Y121F/M172I/T289A/G448S	S212P/Y564H	A291T
NGS-ER14	*Hippeastrum*	1	>8	No mutation	S212P	No mutation
NGS-ER3	*Hippeastrum*	0.5	>8	TR_{46}/Y121F/M172I/T289A/G448S	S212P/S541G/Y564H	No mutation
NGS-ER10	*Hippeastrum*	2	>8	TR_{46}^3/Y121F/M172I/T289A/G448S	S212P/Y564H	A291T
NGS-ER11	*Hippeastrum*	2	>8	TR_{46}^3/Y121F/M172I/T289A/G448S	S212P/Y564H	A291T
NGS-ER12	*Hippeastrum*	2	>8	TR_{46}^3/Y121F/M172I/T289A/G448S	S212P/Y564H	A291T
NGS-ER15	*Ixia*	1	>8	TR_{34}/L98H/T289A/I364V/G448S	E105K/S212P/Y564H	A291T
NGS-ER16	*Ixia*	2	>8	TR_{46}/Y121F/T289A/S363P/I364V/G448S	S212P/Y564H	No mutation
NGS-ER4	*Tulipa*	1	>8	TR_{46}/Y121F/T289A	S212P/Y564H	No mutation
Japanese products						
NGS-ER8	*Narcissus*	>8	8	TR_{34}/L98H	E105K/S212P/Y564H	No mutation

All samples were obtained in Japan. The origin country for all of the agricultural imports was the Netherlands. NGS-ER6 and NGS-ER7 were isolated from same sample. ITR: itraconazole, VOR: voriconazole.



STR*Af* 2A	2B	2C	3A	3B	3C	4A	4B	4C	Strain ID	Cyp51A mutations	Country	Type of sample
10	20	8	39	9	10	12	10	18	NGS-ER1●	TR_{46}/Y121F/T289A	Japan	Imports from the Netherlands
10	20	8	42	9	10	12	10	20	No name	TR_{46}/Y121F/T289A	France	Clinical
10	20	8	42	9	10	12	10	20	No name	TR_{46}/Y121F/T289A	Germany	Clinical
10	20	12	42	8	11	12	9	20	No name	TR_{46}/Y121F/T289A	Germany	Clinical
15	20	8	43	8	11	15	9	18	NGS-ER16●	TR_{46}/Y121F/T289A/S363P/I364V/G448S	Japan	Imports from the Netherlands
10	20	17	42	8	11	12	10	7	No name	TR_{46}/Y121F/T289A	Japan	Clinical
9	20	13	43	9	10	12	9	9	No name	TR_{46}/Y121F/T289A	The Netherlands	Environmental
25	20	8	30	7	9	14	9	18	NGS-ER3●	TR_{46}/Y121F/M172I/T289A/G448S	Japan	Imports from the Netherlands
25	20	12	42	9	6	12	10	7	NGS-ER6●	Y121F/T289A	Japan	Imports from the Netherlands
25	20	12	42	9	6	12	10	7	NGS-ER7●	TR_{46}/Y121F/T289A	Japan	Imports from the Netherlands
24	23	18	26	9	6	12	9	7	NGS-ER2●	TR_{46}^3/Y121F/M172I/T289A/G448S	Japan	Imports from the Netherlands
24	23	19	26	9	6	12	9	7	NGS-ER10●	TR_{46}^3/Y121F/M172I/T289A/G448S	Japan	Imports from the Netherlands
24	23	19	26	9	6	12	9	7	NGS-ER11●	TR_{46}^3/Y121F/M172I/T289A/G448S	Japan	Imports from the Netherlands
24	23	19	26	9	6	12	9	7	NGS-ER12●	TR_{46}^3/Y121F/M172I/T289A/G448S	Japan	Imports from the Netherlands
18	20	14	43	10	22	8	10	7	NGS-ER5●	TR_{46}/Y121F/T289A	Japan	Imports from the Netherlands
25	21	12	27	9	20	14	9	7	NGS-ER4●	TR_{46}/Y121F/T289A	Japan	Imports from the Netherlands
26	21	12	25	9	19	14	9	9	No name	TR_{46}/Y121F/T289A	China	Clinical
26	21	12	26	9	22	13.3	9	9	No name	TR_{46}/Y121F/T289A	The Netherlands	Clinical
26	21	12	26	9	21	13.3	9	9	No name	TR_{46}/Y121F/T289A	The Netherlands	Clinical
26	21	12	26	9	20	13.3	9	9	No name	TR_{46}/Y121F/T289A	The Netherlands	Clinical
26	21	12	27	9	20	13.3	9	9	No name	TR_{46}/Y121F/T289A	The Netherlands	Environmental
26	21	16	32	9	10	8	14	10	No name	TR_{46}/Y121F/T289A	Denmark	Clinical
26	21	9	33	11	21	8	14	10	No name	TR_{46}/Y121F/T289A	India	Environmental
26	21	9	34	11	21	8	14	10	No name	TR_{46}/Y121F/T289A	India	Environmental
26	21	9	33	11	22	8	14	10	No name	TR_{46}/Y121F/T289A	Tanzania	Environmental
26	21	9	33	11	22	8	14	10	No name	TR_{46}/Y121F/T289A	The Netherlands	Clinical
25	21	9	33	11	22	8	14	10	No name	TR_{46}/Y121F/T289A	The Netherlands	Clinical
26	26	9	33	11	22	8	14	10	No name	TR_{46}/Y121F/T289A	The Netherlands	Clinical

Fig. 1. Dendrogram of 11 *A. fumigatus* isolates of the Y121F/T289A group found in this study based on the profiles of nine numbers of short tandem repeat markers, including 17 reference strains with TR_{46}/Y121F/T289A. Strains found in this study are indicated by circular dots.



STRAf 2A	2B	2C	3A	3B	3C	4A	4B	4C	Strain ID	Cyp51A mutations	Country	Type of sample
18	23	15	38	11	48	10	9	8	18512R	TR_{34}/L98H	France	Clinical
14	21	12	31	9	38	8	10	5	T11_R	TR_{34}/L98H	Denmark	Clinical
14	21	14	31	12	37	8	9	5	SSI-54	TR_{34}/L98H	Denmark	Clinical
18	24	14	11	19	29	16	9	20	C821	TR_{34}/L98H	China	Clinical
19	23	14	30	28	26	12	9	15	B44	TR_{34}/L98H	Taiwan	Clinical
18	20	15	42	24	6	9	10	10	20643.017	TR_{34}/L98H	China	Clinical
14	24	14	39	10	7	8	11	5	T22_R	TR_{34}/L98H	Denmark	Clinical
25	20	19	31	9	10	10	14	5	12-90032258	TR_{34}/L98H	Australia	Clinical
14	20	9	31	9	10	8	10	28	1042/09	TR_{34}/L98H	India	Clinical
14	20	9	32	9	10	8	11	29	E218	TR_{34}/L98H	Kuwait	Environmental
14	20	8	21	9	6	8	10	20	Hamid02	TR_{34}/L98H	Iran	Environmental
10	20	10	28	9	7	8	11	19	E1489	TR_{34}/L98H	The Netherlands	Clinical
10	20	8	32	9	7	8	9	19	T18_R	TR_{34}/L98H	Denmark	Clinical
14	21	8	31	9	6	8	10	20	04-202165	TR_{34}/L98H	Australlia	Clinical
14	21	8	28	9	6	8	10	18	OKH50	TR_{34}/L98H	Japan	Clinical
14	21	8	32	9	10	8	10	20	G3236	TR_{34}/L98H	The Netherlands	Clinical
14	20	8	34	9	6	8	12	20	F0622	TR_{34}/L98H	The Netherlands	Clinical
14	21	17	32	11	10	8	14	18	KUFF250	TR_{34}/L98H	Japan	Clinical
14	20	17	31	11	10	8	14	20	No name	TR_{34}/L98H	France	Environmental
14	24	14	31	9	10	10	10	20	0817960/39	TR_{34}/L98H	The Netherlands	Clinical
20	21	10	31	11	10	8	10	20	A31	TR_{34}/L98H	Taiwan	Clinical
14	23	8	40	9	11	8	10	20	2005-456307L	TR_{34}/L98H	The Netherlands	Clinical
14	23	10	37	9	10	8	9	20	E1299	TR_{34}/L98H	The Netherlands	Clinical
13	20	10	47	10	11	8	11	19	E1344	TR_{34}/L98H	The Netherlands	Clinical
22	10	9	33	10	6	8	12	18	NGS-ER15●	TR_{34}/L98H/T289A/I364V/G448S	Japan	Imports from the Netherlands
20	10	8	36	9	11	8	10	23	E454	TR_{34}/L98H	Kuwait	Environmental
20	10	8	35	9	11	8	10	24	Kw2772-coin8	TR_{34}/L98H	Kuwait	Clinical
13	10	11	31	12	11	8	9	20	TY366B-2	TR_{34}/L98H	Taiwan	Environmental
10	10	8	31	9	7	8	11	24	Myc-2008-002/42	TR_{34}/L98H	The Netherlands	Environmental
10	10	8	31	9	11	8	10	19	Myc-2008-002/60	TR_{34}/L98H	The Netherlands	Environmental
10	20	9	29	15	11	14	9	10	NGS-ER8●	TR_{34}/L98H	Japan	Environmental
10	21	9	32	11	11	8	14	10	NAN089	TR_{34}/L98H	Colombia	Environmental
14	21	8	31	9	7	8	10	10	E3198	TR_{34}/L98H	The Netherlands	Clinical
14	20	9	31	9	10	12	10	8	VPCI236/14	TR_{34}/L98H	India	Clinical
13	10	9	38	9	10	8	10	8	C94	TR_{34}/L98H	China	Clinical
10	10	8	31	9	11	8	10	9	Myc-2008-002/46	TR_{34}/L98H	The Netherlands	Environmental
20	10	8	50	10	11	8	7	11	G1319	TR_{34}/L98H	The Netherlands	Clinical
25	10	13	45	10	9	10	7	11	1978	TR_{34}/L98H	Germany	Clinical
23	19	12	9	10	34	10	8	12	TD054-1	TR_{34}/L98H	Taiwan	Environmental
13	10	9	10	9	9	8	9	9	SSI-39	TR_{34}/L98H	Denmark	Clinical
23	10	9	10	9	6	8	9	19	SSI-48	TR_{34}/L98H	Denmark	Clinical
23	10	17	10	11	10	12	10	20	1976	TR_{34}/L98H	Germany	Clinical
13	10	8	72	9	9	8	9	9	094411/7/50	TR_{34}/L98H	The Netherlands	Clinical
20	10	8	73	9	9	8	10	21	E3145	TR_{34}/L98H	The Netherlands	Clinical
20	10	8	91	9	13	8	9	11	F0646	TR_{34}/L98H	The Netherlands	Clinical
23	22	13	68	9	8	8	9	11	2087	TR_{34}/L98H	Germany	Clinical
20	21	12	83	10	7	8	9	10	SSI-27	TR_{34}/L98H	Denmark	Clinical
18	21	8	87	11	7	9	9	5	R044	TR_{34}/L98H	Kuwait	Environmental
23	22	16	97	12	7	16	9	10	1962	TR_{34}/L98H	Germany	Clinical
23	22	13	98	9	8	8	9	11	1899	TR_{34}/L98H	Germany	Clinical
20	21	12	93	9	10	8	11	22	2091	TR_{34}/L98H	Germany	Clinical

Fig. 2. Dendrogram of 2 *A. fumigatus* isolates of the TR_{34}/L98H group found in this study based on the profiles of nine numbers of short tandem repeat markers, including 49 reference strains with TR_{34}/L98H. Strains found in this study are indicated by circular dots.

relevant region three times using the closest primer. Microsatellite genotyping of resistant isolates was also performed as previously described [8,18].

3. Results and discussion

We obtained 203 *A. fumigatus* isolates from all samples. Fourteen isolates were found to be resistant by growing in voriconazole-supplemented PDB; among them, only one isolate grew in itraconazole-supplemented PDB. We confirmed that all isolates showed higher voriconazole MIC than the 1 μg/mL epidemiological cut-off value [5]. Among the 14 resistant isolates, 13 were cultured from flower bulbs imported from the Netherlands, of which 5 were from 11 bulbs of *Hippeastrum* (45.5%), 5 were from 24 bulbs of *Gladiolus* (20.8%), 2 were from 4 bulbs of *Ixia* (50.0%), and 1 was from 22 bulbs of *Tulipa* (4.5%) (Table 1). Only 1 resistant isolate was cultured from the 10 bulbs of *Narcissus* (10.0%) originating in Japan.

Among the 13 resistant *A. fumigatus* isolates imported from the Netherlands through flower bulbs, 12 isolates had various kinds of novel mutations and 1 isolates had no mutations in Cyp51A (Table 2). The patterns of mutations in Cyp51A were divided into two groups, a group including Y121F/T289A and a group including TR_{34}/L98H. Here, the Y121F/T289A group consisted of Y121F/T289A (no tandem repeat in the promoter region), TR_{46}/Y121F/T289A, TR_{46}/Y121F/M172I/T289A/G448S, TR^3_{46}/Y121F/M172I/T289A/G448S (triple 46-bp promoter repeat), and TR_{46}/Y121F/T289A/S363P/I364V/G448S. The mutation TR_{46}/Y121F/T289A was already well-known globally, and TR_{46}/Y121F/M172I/T289A/G448S and TR^3_{46}/Y121F/M172I/T289A/G448S were recently reported from the Netherlands [10]. Surprisingly, an isolate containing the Y121F/T289 mutation but without a 46-bp promoter repeat showed azole resistance (MIC of voriconazole was >8 μg/mL). A group including TR_{34}/L98H had TR_{34}/L98H/T289A/I364V/G448S in one isolate, in which some of the hotspot mutations, such as T289A, I364V, and G448S, were the same as in the Y121F/T289A group. One resistant isolate cultured from a flower bulb originating in Japan contained the TR_{34}/L98H mutation, which was previously detected in Japan among clinical and environment isolates [17,19].

We found some mutations in Hmg1 and Erg6, which differed from hot spots associated with azole resistance [16]. In addition, strains with mutations in the hotspot of these genes should show high MIC of itraconazole, but strains we found showed not so high MICs (Table 2). Therefore we considered that mutations we found are not associated with azole resistance.

Microsatellite genotyping of 14 resistant isolates showed genotypes that were distinct from each other except for isolates harboring TR^{3}_{46}/Y121F/M172I/T289A/G448S (Figs. 1 and 2).

Here, we identified the occurrence of intercontinental spreading of azole-resistant *A. fumigatus* harboring several kinds of Cyp51A mutations through agricultural imports such as plant bulbs. Some novel mutations in the Cyp51A were also discovered—the diversity of which can be potentially explained by sexual reproduction in compost heaps [10]. Resistant isolates with TR_{46}/Y121F/T289A and TR_{34}/L98H were previously detected in the environment and in clinical samples in Japan [17,19,20]. Therefore, careful surveillance of agricultural imports is essential, especially of flower bulbs. Additionally, for effective surveillance, it should be noted that both itraconazole and voriconazole were used at least for azole resistance screening tests, as the majority of azole-resistant isolates, especially those in the Y121F/T289A group, cannot be detected by screening with itraconazole only.

4. Funding and transparency declarations

All authors declare that they have no conflicts of interest for this work. KI received a honorarium and research grant from Pfizer, Co. Inc.

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