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Multicenter Study of Azole-Resistant *Aspergillus fumigatus* Clinical Isolates, Taiwan[1]

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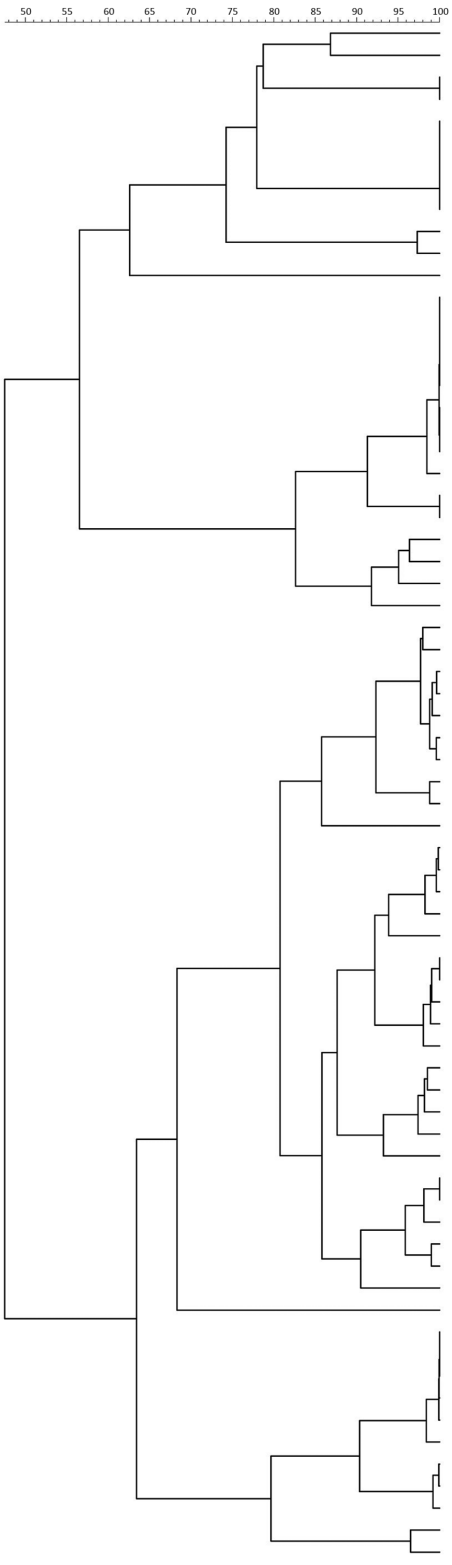
In a multicenter study, we determined a prevalence rate of 4% for azole-resistant *Aspergillus fumigatus* in Taiwan. Resistance emerged mainly from the environment (TR_{34}/L98H, TR_{34}/L98H/S297T/F495I, and TR_{46}/Y121F/T289A mutations) but occasionally during azole treatment. A high mortality rate observed for azole-resistant aspergillosis necessitates diagnostic stewardship in healthcare and antifungal stewardship in the environment.

Worldwide emergence of azole-resistant *Aspergillus fumigatus* since the late 2000s threatens human health (*1*). Azole resistance in *A. fumigatus* might develop during patient therapy with medical azoles or through exposure to azole fungicides in the environment; environmental exposure predominantly involves TR_{34}/L98H and TR_{46}/Y121F/T289A mutations in *cyp51A* (*1*).

Taiwan is an island country in eastern Asia that is geographically separated from mainland Eurasia and has a long history of azole fungicide use. To delineate the influence of clinical and environmental use of azoles on resistance, we conducted a multicenter study that investigated 375 *A. fumigatus* sensu stricto isolates collected during August 2011–March 2018 from 297 patients at 11 hospitals in Taiwan (Appendix Table 1, Figure 1, https://wwwnc.cdc.gov/EID/article/26/4/19-0840-App1.pdf).

We confirmed the presence of azole resistance by using the Clinical Laboratory Standard Institute method (Appendix Table 1) (*2*). Isolates resistant to ≥1 medical azoles (itraconazole, voriconazole, posaconazole, and isavuconazole) were defined as azole-resistant *A. fumigatus* and examined for resistance mechanisms, microsatellite-based phylogenetic relatedness, and growth rates following previously described methods (*3*,*4*).

Overall, 19 isolates from 12 patients were azole-resistant *A. fumigatus*. These isolates had resistance rates of 4.0%/patient and 5.1%/isolate analyses (Appendix Tables 2, 3). Ten (83.3%) patients harbored azole-resistant *A. fumigatus* that had environmental mutations, including TR_{34}/L98H (5 isolates, 5 patients), TR_{34}/L98H/S297T/F495I (7 isolates, 4 patients), and TR_{46}/Y121F/T289A (1 isolate) mutations. This observation



Microsatellite profile (STR *AF*)									Strain	Country	Source	Phenotype (mechanism)
2A	2B	2C	3A	3B	3C	4A	4B	4C				
23	12	15	23	22	6	13	10	7	C485	China	C	R (TR34/L98H/S297T/F495I)
23	12	15	23	12	6	13	10	7	C96	China	C	R (TR34/L98H/S297T/F495I)
19	18	22	25	20	7	10	8	11	g054L	Northern TW	C	S
19	18	22	25	20	7	10	8	11	g057L	Northern TW	C	S
21	18	18	23	18	15	12	10	5	YL1	Northern TW	C	S
21	18	18	23	18	15	12	10	5	YL3	Northern TW	C	R (cdr1B overexpression)
21	18	18	23	18	15	12	10	5	YL4	Northern TW	C	R (cdr1B overexpression)
21	18	18	23	18	15	12	10	5	YL5	Northern TW	C	R
21	18	18	23	18	15	12	10	5	YL6	Northern TW	C	R (cdr1B overexpression)
19	16	13	30	30	6	6	9	5	AN0106-6	Southern TW	E	R (TR34/L98H/S297T/F495I)
19	16	12	29	36	10	8	10	5	D007	Northern TW	C	R (TR34/L98H/S297T/F495I)
23	21	16	20	12	7	16	10	11	E2619	The Netherlands	C	R (TR34/L98H)
19	23	14	30	28	26	12	9	15	B44	Northern TW	C	R (TR34/L98H/S297T/F495I)
19	23	14	30	28	26	12	9	15	B51	Northern TW	C	R (TR34/L98H/S297T/F495I)
19	23	14	30	28	26	12	9	15	HL148-2	Eastern TW	E	R (TR34/L98H/S297T/F495I)
19	23	14	30	28	26	12	9	15	PE10A1	Southern TW	E	R (TR34/L98H/S297T/F495I)
19	23	14	30	28	26	12	9	15	TC244-2	Central TW	E	R (TR34/L98H/S297T/F495I)
19	23	14	30	29	26	12	9	15	E071	Northern TW	C	R (TR34/L98H/S297T/F495I)
19	23	14	30	29	26	12	9	15	E073	Northern TW	C	R (TR34/L98H/S297T/F495I)
19	23	14	30	29	26	12	9	15	E074	Northern TW	C	R (TR34/L98H/S297T/F495I)
19	23	14	35	28	26	12	9	15	SD090A1	Southern TW	E	R (TR34/L98H/S297T/F495I)
19	16	12	29	29	21	6	9	5	g054m	Northern TW	C	R (cyp51A overexpression)
19	16	12	29	29	21	6	9	5	g057m	Northern TW	C	R
23	12	11	36	25	26	9	11	10	20643.023	China	C	R (TR34/L98H/S297T/F495I)
27	12	11	35	19	26	9	9	10	20684.007	China	C	R (TR34/L98H/S297T/F495I)
23	12	15	30	24	26	10	10	7	E1001	China	E	R (TR34/L98H/S297T/F495I)
23	12	11	23	23	26	10	10	10	20677.079	China	C	R (TR34/L98H/S297T/F495I)
23	22	13	68	9	8	8	9	11	2087 m1341.17-06-2012	Germany	C	R (TR34/L98H)
20	21	12	93	9	10	8	11	22	2091 m1428.01-07-2012	Germany	C	R (TR34/L98H)
13	10	9	38	9	10	8	10	8	C94	China	C	R (TR34/L98H)
13	10	9	30	8	10	8	9	8	S05-122	Southern TW	C	R (TR34/L98H)
13	10	8	72	9	9	8	9	9	094411/7/50	The Netherlands	C	R (TR34/L98H)
25	10	12	79	9	9	8	10	11	Case 2-90d	Denmark	C	R (TR34/L98H)
13	9	10	30	8	7	8	9	8	HL102-1	Eastern TW	E	R (TR34/L98H)
10	20	17	42	8	11	12	10	7	Japan Dec 2013	Japan	C	R (TR46/Y121F/T289A)
9	20	13	43	9	10	12	9	9		The Netherlands	E	R (TR46/Y121F/T289A)
18	20	15	42	24	6	9	10	10	20643.017	China	C	R (TR34/L98H)
14	23	8	30	9	6	8	10	20	2005-456307L	The Netherlands	C	R (TR34/L98H)
14	21	8	28	9	6	8	10	18	OKH50	Japan	C	R (TR34/L98H)
14	21	8	31	9	6	8	10	20	04-202165	Australia	C	R (TR34/L98H)
10	20	8	30	9	6	8	10	20	F2126	The Netherlands	C	R (TR34/L98H/S297T/F495I)
14	20	9	31	9	10	8	10	28	1042/09	India	C	R (TR34/L98H)
10	20	8	42	9	10	12	10	20	14-148-2460	France	C	R (TR46/Y121F/T289A)
10	20	8	42	9	10	12	10	20	2107 m1974.23-09-2012	Germany	C	R (TR46/Y121F/T289A)
10	20	12	42	8	11	12	9	20	A12519	Germany	C	R (TR46/Y121F/T289A)
10	23	9	45	9	10	8	9	20	C03-004	Central TW	C	R (TR34/L98H)
14	20	8	40	9	11	8	10	20	R2-07-1_R	Denmark	C	R (TR34/L98H/S297T/F495I)
25	22	9	40	9	6	8	10	21	S07-008	Southern TW	C	R (TR34/L98H)
26	19	11	38	9	10	8	9	20	S16B1-1	Southern TW	E	R (TR34/L98H)
25	22	9	38	9	10	8	9	16	SD202A3	Southern TW	E	R (TR34/L98H)
20	21	10	31	11	10	8	10	20	A31	Southern TW	C	R (TR34/L98H)
25	23	10	31	10	6	8	10	26	S05-319	Southern TW	C	R (TR34/L98H/S297T/F495I)
25	10	10	38	9	10	8	10	21	S14B1C3	Southern TW	E	R (TR34/L98H)
25	10	10	38	9	10	8	10	21	TY366B-3	Northern TW	E	R (TR34/L98H)
20	10	8	36	9	11	8	10	23	E454	Kuwait	E	R (TR34/L98H)
14	10	9	30	9	6	8	10	20	Case 1-7d	Denmark	C	R (TR34/L98H/S297T/F495I)
18	12	9	38	9	6	8	10	21	S05-205	Southern TW	C	R (TR34/L98H)
10	10	8	31	9	7	8	11	24	Myc-2008-002 nr.42	The Netherlands	E	R (TR34/L98H)
20	20	28	32	9	6	8	10	20	Case 3-6d	Denmark	C	R (TR34/L98H)
26	21	9	33	11	22	8	14	10	CF/NL2992	The Netherlands	C	R (TR46/Y121F/T289A)
26	21	9	33	11	22	8	14	10	S05-322	Southern TW	C	R (TR46/Y121F/T289A)
26	21	9	33	11	22	8	14	10		Tanzania	E	R (TR46/Y121F/T289A)
25	21	9	33	11	22	8	14	10	CF/NL0682	The Netherlands	C	R (TR46/Y121F/T289A)
26	21	9	33	11	21	8	14	10	VPCI651/Ei/12/2/a/3	India	E	R (TR46/Y121F/T289A)
26	26	9	33	11	22	8	14	10	CF/NL0645	The Netherlands	C	R (TR46/Y121F/T289A)
26	21	12	26	9	22	13	9	9		The Netherlands	C	R (TR46/Y121F/T289A)
26	21	12	26	9	21	13	9	9		The Netherlands	C	R (TR46/Y121F/T289A)
26	21	12	25	9	19	14	9	9	C195	China	C	R (TR46/Y121F/T289A)
25	20	19	31	9	10	10	14	5	12-90032258	Australia	C	R (TR34/L98H)
26	21	16	32	9	10	8	14	10	Case 4-36d	Denmark	C	R (TR46/Y121F/T289A)

Figure. Genetic relatedness among *Aspergillus fumigatus* isolates based on microsatellite genotyping, Taiwan. Scale bar indicates percentage relatedness. *AF*, *A. fumigatus*; C, clinical; E, environmental; R, azole-resistant; S, azole-susceptible; STR, short tandem repeat; TW, Taiwan.

is consistent with the estimated global prevalence of azole resistance in *Aspergillus* (3%–6%) and the predominance of environmental resistance mechanisms in azole-resistant *A. fumigatus* (*1*,*5*).

Phylogenetic analysis showed that TR_{34}/L98H/S297T/F495I isolates from 2 patients with pulmonary aspergillosis (isolates B44 and B51 in 2012, isolates E071, E073, and E074 in 2015) (Figure) belonged to a local microsatellite genotype widely distributed in the environment of Taiwan (*3*), indicating that this clone has locally evolved and adapted to the environment. The TR_{34}/L98H isolates were genetically clustered with local environmental isolates or clinical isolates from China and Europe (Appendix Table 4). The TR_{46}/Y121F/T289A isolate (S05–322) recovered in 2018, which colonized a patient without overseas travel, was genetically identical to a clone prevalent in the Netherlands and Tanzania (*6*), raising the concern of the intercountry transfer of resistant isolates.

All TR_{34}/L98H/S297T/F495I, TR_{34}/L98H, and TR_{46}/Y121F/T289A isolates exhibited cross-resistance to difenoconazole and tebuconazole (both triazole fungicides) without fitness cost, demonstrated by normal growth rates (Appendix Figure 2). The TR_{34}/L98H/S297T/F495I isolates and TR_{46}/Y121F/T289A isolates were also resistant to prochloraz (an imidazole fungicide) (Appendix Table 2). The prevalence of TR_{34}/L98H/S297T/F495I isolates in Taiwan might be attributed to widespread use of prochloraz over the past 3 decades. Studies have suggested an association between use of imidazole fungicides and emergence of azole-resistant *A. fumigatus* with TR_{34}/L98H/S297T/F495I mutations (*7*,*8*).

In Taiwan, the annual consumption of difenoconazole and tebuconazole has exceeded that of prochloraz since 2012 (Appendix Figure 3), further creating a favorable environment for maintenance and spread of TR_{34}/L98H, TR_{34}/L98H/S297T/F495I, and TR_{46}/Y121F/T289A isolates. Thus, the One Health approach to implement environmental antifungal stewardship is warranted to minimize ongoing resistance selection in the fields.

Six azole-resistant *A. fumigatus* isolates with wild-type *cyp51A* were obtained from 2 patients. Four pan–azole-resistant urinary isolates were sequentially recovered from a patient (no. 11) with *A. fumigatus* renal abscesses who was receiving voriconazole for >3 months in whom an initial urine isolate was susceptible to azole; all 5 isolates were genetically identical.

Overexpression of *cdr1B* (a drug efflux transporter) and an S269P mutation in *hmg1* (a hydroxymethylglutaryl-CoA reductase) were identified in 4 resistant isolates but not in the initial susceptible isolate (Appendix Table 5, Figure 4), suggesting their roles involved in azole resistance (*4*,*9*). Another 2 pan–azole-resistant respiratory isolates were recovered from a patient (no. 12) who had pulmonary aspergillosis and was receiving voriconazole for 4 months. Azole-susceptible and azole-resistant isolates co-existed in this patient, which echoes the international recommendation suggesting testing multiple colonies (≥5) from a single culture (*1*). *Cyp51A* overexpression and an F262 deletion in *hmg1*(*hmg1*^{F262_del}) were identified in these 2 resistant isolates. Although *hmg1*^{F261_del} was recently reported in azole-resistant *A. fumigatus* from a voriconazole-exposed patient (*4*), whether *cyp51A* overexpression and *hmg1*^{F262_del} act synergistically to cause resistance warrants further studies.

Finally, reduced colony sizes were observed in all 6 azole-resistant *A. fumigatus* isolates with wild-type *cyp51A* (Appendix Figure 2). Thus, attention should be paid to select colonies of various sizes for susceptibility testing from patients with azole exposure.

Overall, 4 patients harboring azole-resistant *A. fumigatus* with environmental mutations and 2 patients harboring azole-resistant *A. fumigatus* with wild-type *cyp51A* showed development of invasive aspergillosis, and all had aspergillosis-related deaths. High mortality rates for azole-resistant aspergillosis we observed (6/6, 100%) and for those from a previous report (*10*) emphasize the need for a proposed integrated algorithm for management and control of azole-resistant aspergillosis (Appendix Table 6).

In conclusion, we report a health threat that arose from clinical and environmental use of azoles; environmental use contributed at a larger and global scale. These data necessitate diagnostic stewardship in the clinic and antifungal stewardship in the environment.

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Knowledge of Infectious Disease Specialists Regarding Aspergillosis Complicating Influenza, United States

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In an online survey, we found that nearly one fifth of physicians in the United States who responded had seen or heard about a case of invasive pulmonary aspergillosis after severe influenza at their institution. However, <10% routinely used galactomannan testing to test for this fungus in patients with severe influenza.

Invasive pulmonary aspergillosis (IPA) occurs primarily among immunocompromised patients with a history of organ or stem cell transplantation, chemotherapy, or immunosuppressive medications. However, a multicenter retrospective study in the Netherlands and Belgium suggested that patients

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Multicenter Study of Azole-Resistant *Aspergillus fumigatus* Clinical Isolates, Taiwan

Appendix

Appendix Table 1. Details of participating hospitals, antifungal susceptibility testing, and isolate collection for analysis of azole-resistant *Aspergillus fumigatus* clinical isolates, Taiwan*

Hospital	Location	Period of collection	Susceptibility testing method†	
			Isolates before June 2017	Isolates during June 2017–March 2018
Chi-Mei Medical Center, Luiying (CMMC)	Southern	2015 Feb–2018 Mar	CLSI M38-A2	Screening azole agar plates; confirmed by CLSI M38-A2
National Cheng-Kung University Hospital (NCKUH)	Southern	2011 Aug–2018 Mar	CLSI M38-A2	Screening azole agar plates; confirmed by CLSI M38-A2
National Taiwan University Hospital (NTUH)	Northern	2012 Feb–2018 Mar	YeastOne	Screening azole agar plates; confirmed by CLSI M38-A2
TSARM hospitals				
Changhua Christian Hospital	Central	2016 Jul–Sep	CLSI M38-A2	NI
Ditmanson Medical Foundation Chia-Yi Christian Hospital	Southern	2016 Jul–Sep	CLSI M38-A2	NI
Hualien Tzu Chi Hospital	Eastern	2016 Jul–Sep	CLSI M38-A2	NI
Kaohsiung Medical University Chung-Ho Memorial Hospital	Southern	2016 Jul–Sep	CLSI M38-A2	NI
Kaohsiung Veterans General Hospital	Southern	2016 Jul–Sep	CLSI M38-A2	NI
Show Chwan Memorial Hospital	Central	2016 Jul–Sep	CLSI M38-A2	NI
Taichung Veterans General Hospital	Central	2016 Jul–Sep	CLSI M38-A2	NI
Tainan Sin Lau Hospital	Southern	2016 Jul–Sep	CLSI M38-A2	NI

*This study was approved by the Institutional Review Boards of the National Health Research Institutes (no. EC1040502-E and EC1050307) and participating hospitals: CMMC (10607-L01 and 10711-L03), NCKUH (B-ER-101–342), and NTUH (201605098RIPA). NI, no isolate; TSARM, Taiwan Surveillance of Antimicrobial Resistance of Molds.

†*A. fumigatus* sensu stricto was identified on the basis of morphologic characteristics, growth at 50°C, and sequence analyses of the internal transcribed spacer region and calmodulin gene (*1*). For isolates from CMMC, NCKUH, and TSARM hospitals, MICs of antifungal agents were determined by using the Clinical and Laboratory Standards Institute (CLSI) M38-A2 method. The MICs for the isolates from NTUH were determined by using the Sensititer YeastOne method (Trek Diagnostic Systems, http://www.trekds.com); isolates with any of the following MIC values (i.e., ≥2, ≥2, and ≥0.25 μg/mL of itraconazole, voriconazole, and posaconazole, respectively) were reexamined by using the CLSI method. For isolates collected during June 2017–March 2018, azole resistance was screened by using azole-containing agar plates. In brief, the conidia of these isolates were directly inoculated onto 3 Sabouraud dextrose agar plates supplemented with itraconazole (2 μg/mL), voriconazole (1 μg/mL), or posaconazole (0.25 μg/mL), and incubated at 37°C. Colonies that grew after 2–4 d on any of the azole-containing agar plates were selected for the MIC determination by using the CLSI method.

Appendix Table 2. Laboratory characteristics of *Aspergillus fumigatus* clinical isolates from 12 patients with aspergillosis, Taiwan*

Patient no.	Strain no.	Year (day)†	*Cyp51A* mutation	MIC or MIC_{50}/MIC_{90} (range), µg/mL, by CLSI M38-A2‡							
				AMB	ITR	VRC	POS	ISA	DFC	TBC	PRC
Azole-resistant isolates§ except YL1, g054L, and g057L											
1	B44	2012 (d0)	TR_{34}/L98H/S297T/F495I	0.5	>16	2	1	>16	>32	16	>32
1	B51	2012 (d2)	TR_{34}/L98H/S297T/F495I	0.5	>16	2	1	>16	>32	16	>32
2	D007	2014	TR_{34}/L98H/S297T/F495I	0.5	>16	2	1	>16	>32	>32	>32
3	E071	2015 (d0)	TR_{34}/L98H/S297T/F495I	0.5	>16	2	1	>16	>32	>32	>32
3	E073	2015 (d3)	TR_{34}/L98H/S297T/F495I	0.5	>16	2	1	>16	>32	>32	>32
3	E074	2015 (d4)	TR_{34}/L98H/S297T/F495I	0.5	>16	2	1	>16	>32	>32	>32
4	S05–319	2018	TR_{34}/L98H/S297T/F495I	0.5	>16	2	1	>16	>32	>32	>32
5	A31	2013	TR_{34}/L98H	0.5	>16	4	1	16	>32	32	4
6	S05–122	2016	TR_{34}/L98H	0.5	>16	8	1	8	>32	>32	2
7	C03–004	2016	TR_{34}/L98H	0.5	>16	4	1	8	32	>32	2
8	S07–008	2016	TR_{34}/L98H	0.5	>16	4	1	8	32	>32	2
9	S05–205	2017	TR_{34}/L98H	0.5	>16	4	1	8	32	>32	2
10	S05–322	2018	TR_{46}/Y121F/T289A	0.5	>16	>16	1	>16	>32	>32	>32
11	YL1	2014 (d0)	Polymorphisms¶	0.5	0.25	0.5	0.25	1	4	4	0.25
11	YL3	2014 (d100)	Polymorphisms¶	0.5	>16	8	1	8	32	>32	2
11	YL4	2015 (d134)	Polymorphisms¶	0.5	>16	8	1	8	32	>32	2
11	YL5	2015 (d165)	Polymorphisms¶	0.5	>16	2–4	0.5	2	16	16	1
11	YL6	2015 (d185)	Polymorphisms¶	0.5	>16	8	1	8	32	>32	2
12	g054m	2016 (d0)	Wild-type	0.12	>16	8	1	8	32	>32	2
12	g054L	2016 (d0)	Wild-type	0.5	0.5	0.5	0.06	0.5	1	4	0.25
12	g057m	2016 (d2)	Wild-type	0.12	>16	4	1	8	32	>32	2
12	g057L	2016 (d2)	Wild-type	0.5	0.5	0.5	0.06	0.5	1	4	0.25
Azole-susceptible isolates, n = 200#				0.5/1 (0.12–1)	0.5/0.5 (0.03–1)	0.5/1 (0.12–1)	0.12/0.25 (0.015–0.25)	ND	2/4 (1–8)	4/8 (2–8)	ND

*AMB, amphotericin B; ATCC, American Type Culture Collection; DFC, difenoconazole; ISA, isavuconazole; ITR, itraconazole; ND, not done; POS, posaconazole; PRC, prochloraz; TBC, tebuconazole; VRC, voriconazole.
†d0 and dn indicate day of and n days after the first isolation of *A. fumigatus*, respectively.
‡*Candida parapsilosis* ATCC 22019 and *A. fumigatus* ATCC MYA 3626 were used as quality control and reference strains.
§Because of good essential agreement in the azole MIC values between the CLSI and European Committee on Antimicrobial Susceptibility Testing (EUCAST) methods (*2*) and the lack of established CLSI clinical breakpoints for *A. fumigatus*, the MIC interpretive criteria for resistance in this study followed the EUCAST clinical breakpoints (i.e., >2, >2, >0.25, and >1 µg/mL) for itraconazole, voriconazole, posaconazole, and isavuconazole, respectively (*3*).The drugs for susceptibility testing were obtained from Sigma-Aldrich (https://www.sigmaaldrich.com) (AMB, ITR, VRC, POS, DFC, and PRC), Toronto Research Chemicals (https://www.trc-canada.com) (ISA), and Chem Service (https://www.chemservice.com) (TBC).
¶These isolates have F46Y/G89G/M172V/N248T/D255E/L358L/E427K/C454C polymorphisms.
#The MICs of 200 azole-susceptible isolates were used. Only 62 isolates were tested for MICs for DFC and TBC.

Appendix Table 3. Clinical characteristics of 12 patients harboring azole-resistant *Aspergillus fumigatus* isolates, Taiwan*

Patient no.	Strain no.	Age, y/ sex	Concurrent condition	Sample	*Aspergillus* disease†	Previous azole exposure	Sequential antifungal treatment (d)	Outcome	IA-related death
1‡	B44, B51	66/F	DM, HCV/cirrhosis, adrenal insufficiency	Sputum	IPA, unclassified	No	VRC (1), AMB (3)	Died	Yes
2	D007	49/M	AML, peritonitis	Nasal swab	Colonization	POS/VRC (2 wk)	MCF (33)	Died	No
3	E071, E074; E073	64/F	SLE, ESRD, bacterial septicemia, meningoencephalitis	Pleural effusion; sputum	Proven IPA with empyema	No	VRC (1), LAMB (3)	Died	Yes
4	S05–319	88/F	DM, influenza B	Sputum	Colonization	No	No	Alive	No
5‡	A31	59/M	Lung cancer, COPD, bronchiectasis	BAL	Proven IPA	No	AMB (3), POS (10), VRC (11)	Died	Yes
6	S05–122	90/F	COPD, steroid use, bronchiectasis	Sputum	Probable IPA	No	No	Died	Yes
7	C03–004	–	–	Sputum	–	–	–	–	–
8	S07–008	–	–	Ear	–	–	–	–	–
9	S05–205	76/F	COPD, steroid use, bronchiectasis, DM	Sputum	Colonization	No	No	Alive	No
10	S05–322	74/M	B cell lymphoma, COPD, HCV, CAD	Sputum	Colonization	No	No	Alive	No
11	YL1;§ YL3, YL4, YL5, YL6	36/M	HIV/AIDS	Urine; PCN	Proven IA, (renal abscess)	VRC (3 mo)	VRC (93), VRC/CAS (43), LAMB (44), LAMB/5FC (25), LAMB/AND (24)	Died	Yes
12	g054m, g054L,§ g057m, g057L§	39/M	MDS with RAEB-T, status post-HSCT with GVHD, bacterial septicemia	Sputum; BAL	Probable IPA	VRC (4 mo)	AMB (18), POS (21), AMB (13), AMB/AND (8), AND (5), AMB/AND (1), AND (4), AMB/AND (2)	Died	Yes

*AMB, conventional amphotericin B; AML, acute myeloid leukemia; AND, anidulafungin; BAL, bronchoalveolar lavage; CAD, coronary artery disease; CAS, caspofungin; COPD, chronic obstructive pulmonary disease; DM, diabetes mellitus; ESRD, end-stage renal disease; 5FC, flucytosine; GVHD, graft versus host disease; HCV, hepatitis C virus infection; HSCT, allogeneic hematopoietic stem cell transplantation; IA, invasive aspergillosis; IPA, invasive pulmonary aspergillosis; LAmB, liposomal amphotericin B; MCF, micafungin; MDS with RAEB-T, myelodysplastic syndrome with refractory anemia and excess blast in transformation; PCN, percutaneous nephrostomy; POS, posaconazole; SLE, systemic lupus erythematosus; VRC, voriconazole; –, data not available.

†Clinical data for patients harboring azole-resistant *A. fumigatus* were reviewed, and IA was classified according to the EORTC/MSG definition (*4*).

‡Three isolates (A31, B44, and B51) from 2 patients have been described in our previous report (*5*).

§YL1, g054L, and g057L were azole susceptible.

Appendix Table 4. References for 38 oversea *Aspergillus fumigatus* strains included in microsatellite-based phylogenetic analysis during a multicenter study of azole-resistant *Aspergillus fumigatus* clinical isolates, Taiwan

Strain	Reference
C485	(*6*)
C96	(*6*)
E2619	(*6*)
20643.023	(*7*)
20684.007	(*7*)
E1001	(*6*)
20677.079	(*7*)
2087 m1341.17–06–2012	(*8*)
2091 m1428.01–07–2012	(*8*)
C94	(*6*)
094411/7/50	(*8*)
Case 2–90d	(*9*)
Japan Dec 2013	(*10*)
The Netherlands 7	(*10,11*)
20643.017	(*7*)
2005–456307L	(*6*)
OKH50	(*12*)
04–202165	(*13*)
F2126	(*6*)
1042/09	(*14*)
14–148–2460	(*6*)
2107m1974.23–09–2012	(*8*)
A12519	(*8*)
R2–07–1_R	(*6*)
E454	(*15*)
Case 1–7d	(*9*)
Myc-2008–002 nr.42	(*14*)
Case 3–6d	(*9*)
CF/NL2992	(*8*)
Tanzania	(*11*)
CF/NL0682	(*8*)
VPCI651/Ei/12/2/a/3	(*8*)
CF/NL0645	(*11*)
The Netherlands 2	(*10,11*)
The Netherlands 3	(*10,11*)
C195	(*7*)
12–90032258	(*13*)
Case 4–36d	(*9*)

Appendix Table 5. Gene substitutions in azole-resistant *Aspergillus fumigatus* isolates and control strains, Taiwan*

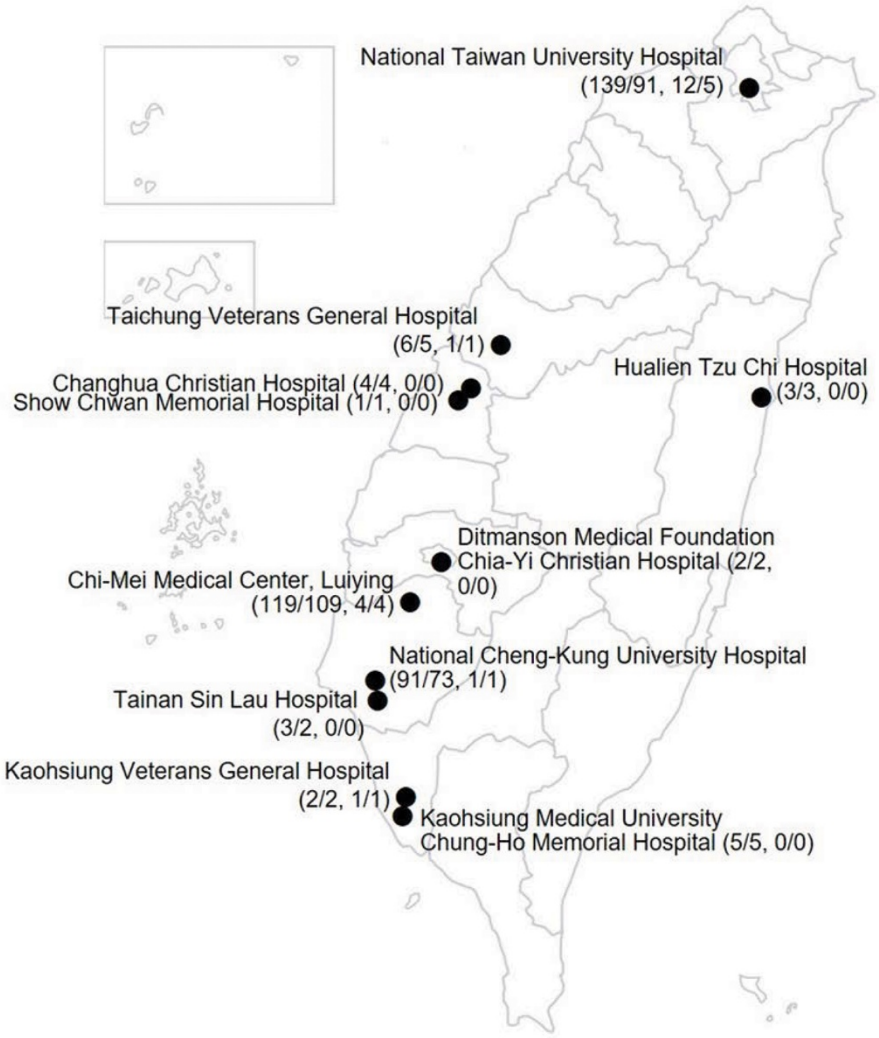
Strain no.	Azole susceptibility (mechanism)	Gene substitutions†			
		hapE	*srbA*	*hmg1*	*erg6*
ATCC MYA-3626	S	–	V37D	–	–
ATCC 16903	S	–	V37D, S82P	H564Y	–
F2509	S	–	V37D	–	–
F02411	S	–	A865V	P212S, H564Y	–
YL1	S	–	E957D	P212S, H564Y	–
YL3	R	–	E957D	P212S, S269P, H564Y	F186V
YL4	R	–	E957D	P212S, S269P, H564Y	F186V
YL5	R	–	E957D	P212S, S269P, H564Y	–
YL6	R	–	E957D	P212S, S269P, H564Y	F186V
g054m	R	–	A865V	F262_del, H564Y	–
g057m	R	–	A865V	F262_del, H564Y	–
g054L	S	–	V37D	–	–
g057L	S	–	V37D	–	–
B44	R (TR_{34}/L98H/S297T/F495I)	–	V37D	H564Y	–
A31	R (TR_{34}/L98H)	–	V37D	–	–

*ATCC, American Type Culture Collection; del, deletion; R, resistant; S, susceptible; –, wild type.
†The *hapE*, *hmg1*, *erg6* and *srbA* genes were sequenced as described (*16–18*). The *hapE* and *srbA* sequences were compared with those of *A. fumigatus* f293; the *hmg1* and *erg6* sequences were compared with those of *A. fumigatus* A1163.

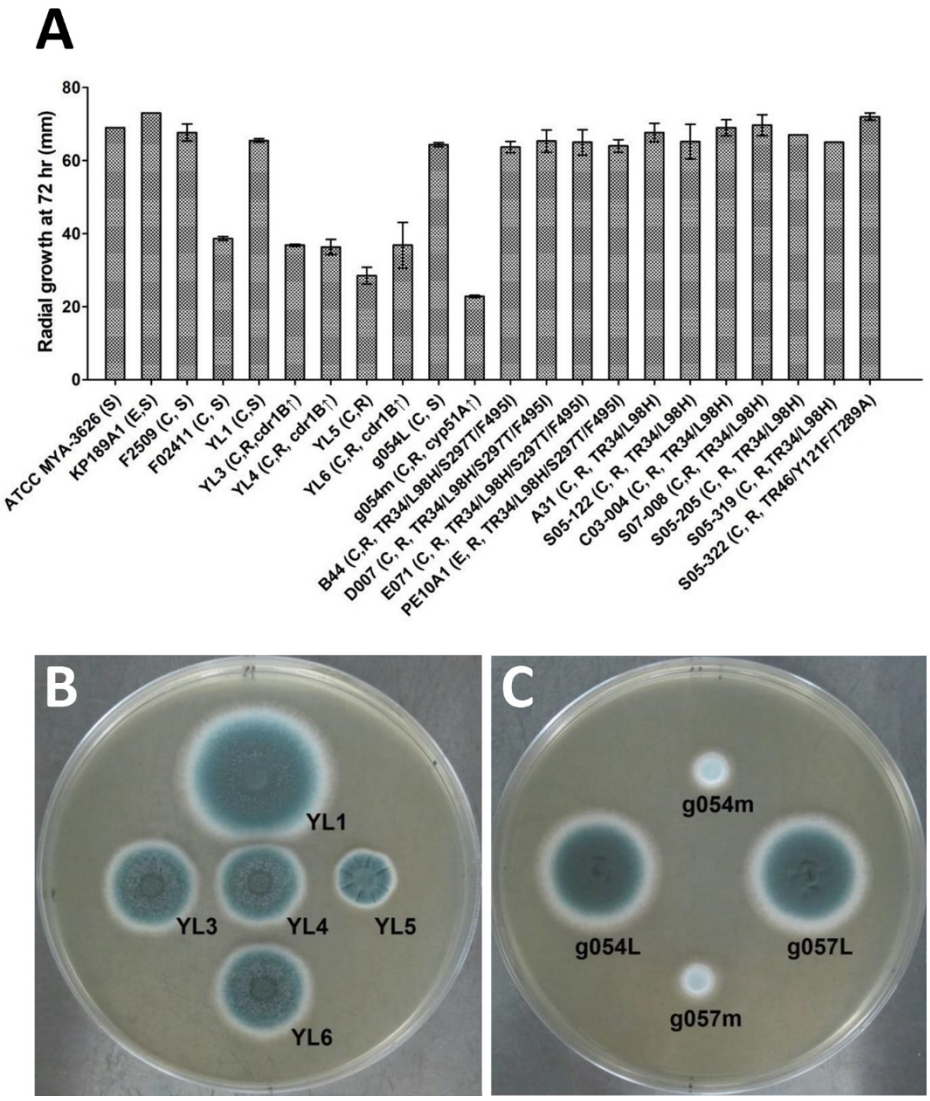
Appendix Table 6. Proposed integrated algorithm for management and control of azole-resistant aspergillosis, Taiwan*

Category	Response
Academic society	Incorporate the issue of azole resistance into national guidelines for management of aspergillosis; hold educational programs to improve diagnosis and treatment for azole-resistant aspergillosis.
Laboratory personnel	Identify clinically relevant *Aspergillus* isolates at the species complex level and confirm *A. fumigatus* by thermotolerance test (growth at 50°C) (*19*); screen for azole resistance with azole agar plates for clinically relevant *A. fumigatus* isolates and screen multiple colonies (≤5 colonies) from a single specimen (*19*); for colonies grown on any azole agar plate, perform azole MIC testing by using reference CLSI or EUCAST methods or an alternative Sensititer YeastOne assay (*19,20*); If MIC testing is not available, refer isolates to a mycology reference laboratory (*19*); prompt notification of the clinical team if azole-resistant *A. fumigatus* is suspected and identified.
Physicians	Be familiar with patient risk factors for invasive aspergillosis; obtain clinical specimens for fungal culture as possible; select empirical antifungal agents according to the updated local prevalence rate of azole resistance (*21*); antifungal susceptibility testing is recommended for *A. fumigatus* isolates from invasive diseases and should be repeated on later isolates if infection persists despite treatment (*21*); be aware of the possibility of azole-resistance in patients unresponsive to azole treatment; consider amphotericin B–based or azole/echinocandin combination therapy for azole-resistant aspergillosis (*19,21*).
Hospital environment	Segregate patients from construction or renovation, potted plants, and flowers in wards and patients' room (*22*); control the airborne dissemination of fungal spores, (e.g., barriers, containment, air handling, HEPA filters, sealed windows, sealing the area of construction or renovation activities if possible) (*23*).
Reference mycology laboratory	Identify *Aspergillus* isolates to the species level by molecular methods; confirm antifungal susceptibility of *Aspergillus* isolates with reference CLSI or EUCAST methods; perform periodic reference MIC testing of isolates of *A. fumigatus* complex (≥100 isolates) (*19*); sequence *cyp51A* genes in resistant isolates to determine the nature and trends in *cyp51A* mutation distribution (*19*); establish molecular typing methods; collect strains.
Scientists and plant pathologists	Identify the key azole fungicides that select azole-resistant *Aspergillus*; propose better fungicide application strategies to minimize resistance development (*24*).
Agricultural authority	Include azole fungicides that select azole-resistant isolates into national pesticide risk reduction programs; advise farmers to reduce culprit azole fungicide use by in rotation with alternative fungicides with different modes of action.
Governance	Update and evaluate the global situation; accredit national mycology reference laboratories; implement antifungal stewardship programs in agriculture in addition to hospitals and animal husbandry to achieve the One Health goal (*24*).

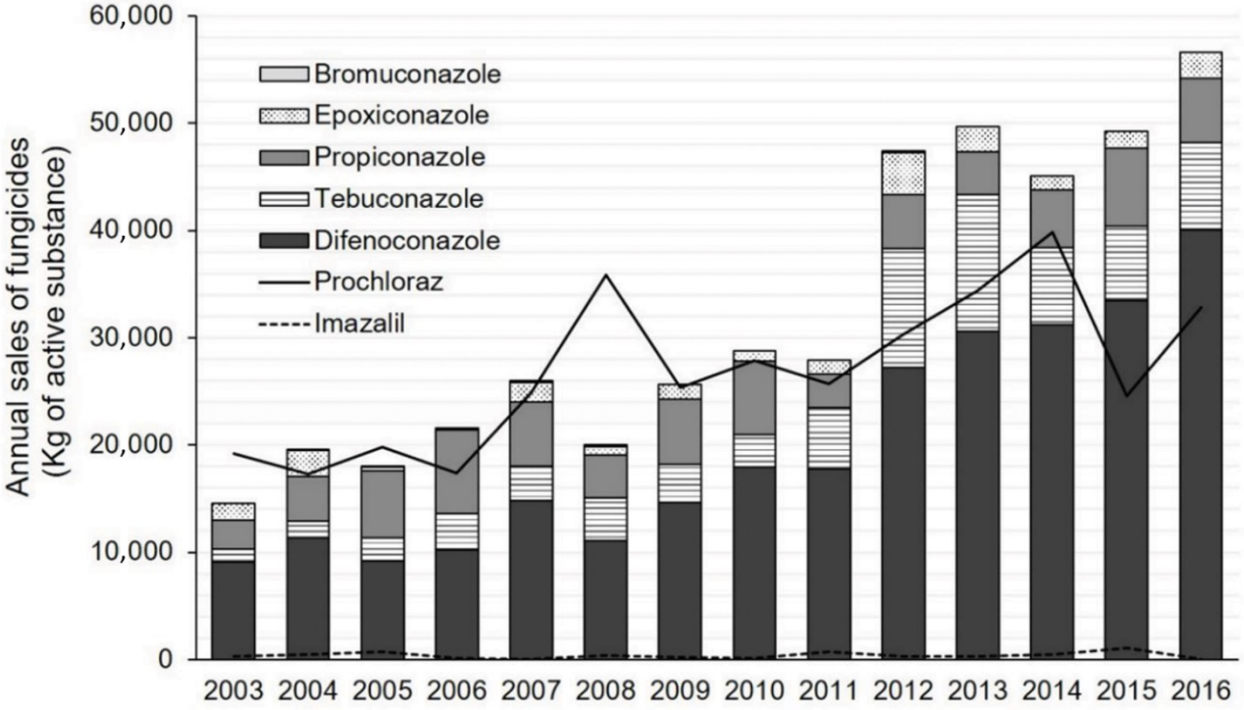
*CLSI, Clinical and Laboratory Standards Institute; EUCAST, European Committee on Antimicrobial Susceptibility Testing.



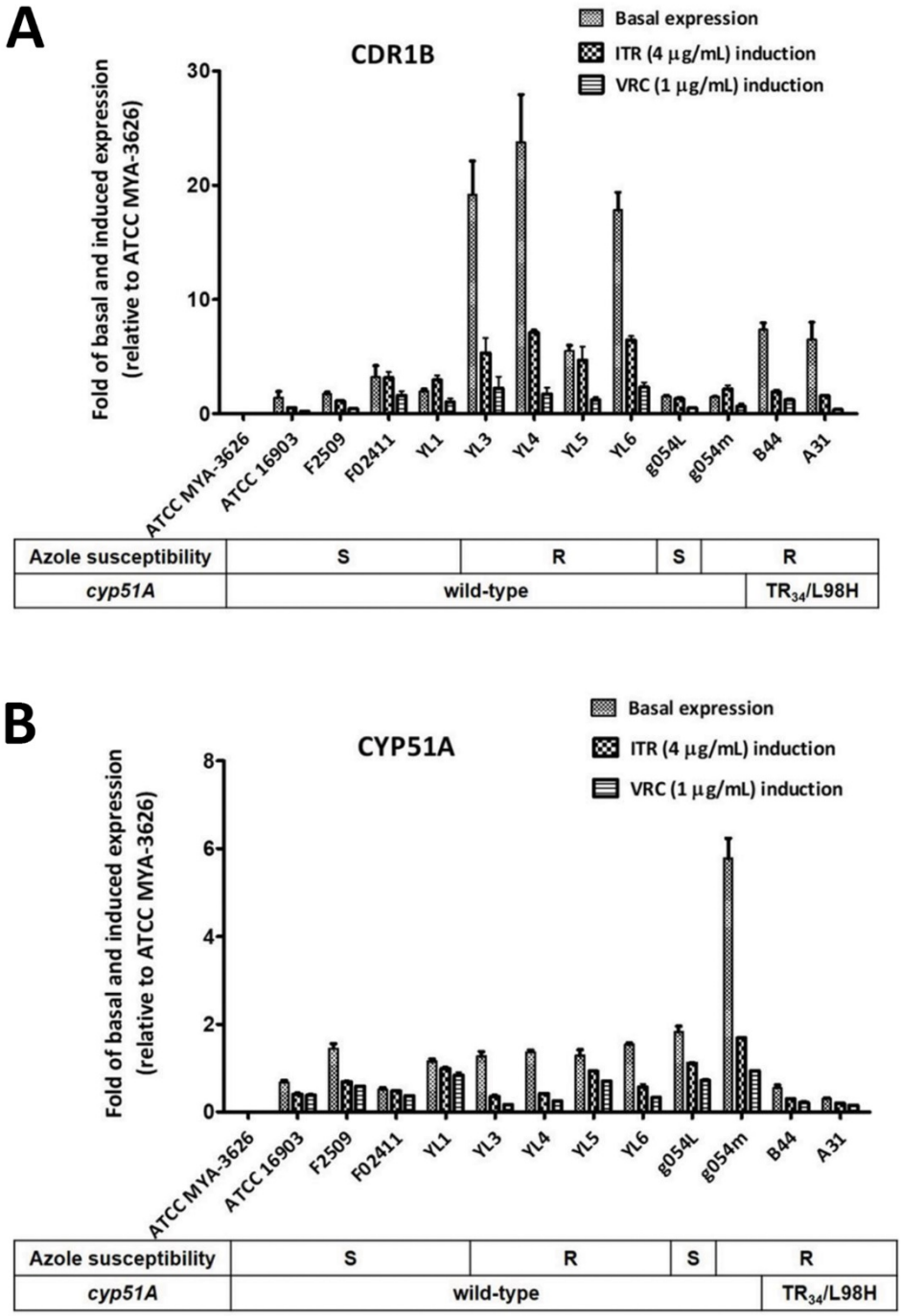
Appendix Figure 1. Distribution of participating hospitals and isolate collection areas for *Aspergillus fumigatus*, Taiwan. Values in parentheses indicate no. *A. fumigatus* isolates/no. patients and no. azole-resistant *A. fumigatus* isolates/no. patients.



Appendix Figure 2. A) Radial growth of *Aspergillus fumigatus* isolates on Sabouraud dextrose agar plates at 35°C, Taiwan. The radius of the growing colony was measured after 72 hours of incubation. Values are the mean diameter of triplicate samples. Error bars indicate SD. Colonies of B) YL1, YL3, YL4, YL5, and YL6 from patient 11 and C) g054m, g054L, g057m, and g057L from patient 12 observed at 72 hours. C, clinical isolate; E, environmental isolate; R, azole-resistant; S, azole-susceptible; ↑, overexpression.



Appendix Figure 3. Annual sales of azole fungicides in Taiwan, 2003–2016. Annual sales of imidazole fungicides (imazalil and prochloraz) and triazole fungicides (bromuconazole, difenoconazole, epoxiconazole, propiconazole, and tebuconazole) are shown according to data derived from Domestic Manufacturers Production and Sale of Pesticides published by the Taiwan Crop Protection Industry Association (*25*).



Appendix Figure 4. mRNA expression levels of A) a drug efflux transporter gene, *cdr1B*, and B) *cyp51A* in *Aspergillus fumigatus* isolates, Taiwan. Expression levels were normalized to β-tubulin levels and compared with those in *A. fumigatus* ATCC MYA-3626. Error bars indicate SD. Results for the *cyp51B* gene and other transporter genes (*AfuMDR1*, *AfuMDR2*, *AfuMDR3*, *AfuMDR4*, *atrF*, and *MFS56*) were inconclusive and are not shown. ATCC, American Type Culture Collection; ITR, itraconazole; VRC, voriconazole.

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