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<sub>34</sub>/L98H, TR<sub>34</sub>/L98H/S297T/F495I, and TR<sub>34</sub>/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<sub>34</sub>/L98H and TR<sub>34</sub>/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* 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% per patient and 5.1% per isolate analyses (Appendix Tables 2, 3). Ten (83.3%) patients harbored azole-resistant *A. fumigatus* that had environmental mutations, including TR<sub>34</sub>/L98H (5 isolates, 5 patients), TR<sub>34</sub>/L98H/S297T/F495I (7 isolates, 4 patients), and TR<sub>34</sub>/Y121F/T289A (1 isolate) mutations. This observation

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**References**


**Address for correspondence:** Yuanzhi Wang or Hai Jiang, School of Medicine, Shihezi University, Shihezi, Xinjiang Uygur Autonomous Region 832002, China; email: wangyuanzhi621@126.com or jianghai@icdc.com

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

Chi-Jung Wu, Wei-Lun Liu, Chih-Cheng Lai, Chien-Ming Chao, Wen-Chien Ko, Hsuan-Chen Wang, Ching-Tzu Dai, Ming-I Hsieh, Pui-Ching Choi, Jia-Ling Yang, Yee-Chun Chen

Results from this study were presented in part at the 30th International Congress of Chemotherapy and Infection, November 24–27, 2017, Taipei, Taiwan.
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 pre-
dominance of environmental resistance mechanisms
in azole-resistant A. fumigatus (1,5).

Phylogenetic analysis showed that TR\textsubscript{a}/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
close has locally evolved and adapted to the envi-
ronment. The TR\textsubscript{a}/L98H isolates were genetically
clustered with local environmental isolates or clinical
isolates from China and Europe (Appendix Table 4).
The TR\textsubscript{e}/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 con-
cern of the intercountry transfer of resistant isolates.

All TR\textsubscript{a}/L98H/S297T/F495I, TR\textsubscript{e}/L98H, and
TR\textsubscript{e}/Y121F/T289A isolates exhibited cross-resis-
tance to difenoconazole and tebuconazole (both tri-
azole fungicides) without fitness cost, demonstrated
by normal growth rates (Appendix Figure 2). The
TR\textsubscript{a}/L98H/S297T/F495I isolates and TR\textsubscript{e}/Y121F/
T289A isolates were also resistant to prochloraz (an
imidazole fungicide) (Appendix Table 2). The preva-
ience of TR\textsubscript{a}/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\textsubscript{a}/
L98H/S297T/F495I mutations (7,8).

In Taiwan, the annual consumption of difeno-
conazole and tebuconazole has exceeded that of pro-
chloraz since 2012 (Appendix Figure 3), further cre-
ating a favorable environment for maintenance and
spread of TR\textsubscript{a}/L98H, TR\textsubscript{e}/L98H/S297T/F495I, and
TR\textsubscript{e}/Y121F/T289A isolates. Thus, the One Health
approach to implement environmental antifungal
stewardship is warranted to minimize ongoing resis-
tance 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 re-
nal abscesses who was receiving voriconazole for >3
months in whom an initial urine isolate was suscep-
tible to azole; all 5 isolates were genetically identical.

Overexpression of cdr1B (a drug efflux trans-
porter) and an S269P mutation in hmg1 (a hydroxy-
methylglutaryl-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). An-
other 2 pan-azole-resistant respiratory isolates were
recovered from a patient (no. 12) who had pulmo-
nary 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\textsuperscript{F262,del}) were identified in these 2 resistant
isolates. Although hmg1\textsuperscript{F261,del} was recently reported
in azole-resistant A. fumigatus from a voriconazole-
exposed patient (4), whether cyp51A overexpression
and hmg1\textsuperscript{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 suscepti-
bility testing from patients with azole exposure.

Overall, 4 patients harboring azole-resistant A.
fumigatus with environmental mutations and 2 pa-
tients 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 pre-
vious 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; envi-
ronmental 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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Liouying, Chung Shan Medical University Hospital,
Ditmanson Medical Foundation Chia-Yi Christian Hospital,
Far Eastern Memorial Hospital, Hualien Tzu Chi Hospital,
Kaohsiung Chang Gung Memorial Hospital, Kaohsiung
Medical University Chung-Ho Memorial Hospital, Kaohsi-
ung Veterans General Hospital, Mennonite Christian Hos-
pital, National Cheng Kung University Hospital, National
Taiwan University Hospital, Show Chwan Memorial Hos-
pital, Taichung Veterans General Hospital, Tainan Sin Lau
Hospital, and Taipei City Hospital Heping Fuyou Branch.
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About the Author

Dr. Wu is an assistant investigator and attending physician at National Health Research Institutes, Zhunan, Taiwan. Her research interests include molecular epidemiology of infectious diseases and antimicrobial drug resistance in bacterial and fungal pathogens.

References


Address for correspondence: Yee-Chun Chen, Department of Internal Medicine, National Taiwan University Hospital, No. 7 Chung-Shan South Rd, Taipei 10002, Taiwan; email: yeechunchen@gmail.com

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

Mitsuru Toda, Susan E. Beekmann, Philip M. Polgreen, Tom M. Chiller, Brendan R. Jackson, Karlyn D. Beer

Author affiliations: Centers for Disease Control and Prevention, Atlanta, Georgia, USA (M. Toda, T.M. Chiller, B.R. Jackson, K.D. Beer); University of Iowa, Iowa City, Iowa, USA (S.E. Beekmann, P.M. Polgreen)

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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...
### 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*

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

*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*

<table>
<thead>
<tr>
<th>Patient</th>
<th>Strain no.</th>
<th>Year (d0)</th>
<th>Cyp51A mutation</th>
<th>MIC or MIC&lt;sub&gt;50&lt;/sub&gt;/MIC&lt;sub&gt;90&lt;/sub&gt; (range), µg/mL by CLSI M38-A2†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azole-resistant isolates§ except YL1, g054L, and g057L</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>B44</td>
<td>2012</td>
<td>TR&lt;sub&gt;r&lt;/sub&gt;/L98H/S297T/F495I</td>
<td>0.5</td>
</tr>
<tr>
<td>2</td>
<td>B51</td>
<td>2012</td>
<td>TR&lt;sub&gt;r&lt;/sub&gt;/L98H/S297T/F495I</td>
<td>0.5</td>
</tr>
<tr>
<td>3</td>
<td>D007</td>
<td>2014</td>
<td>TR&lt;sub&gt;r&lt;/sub&gt;/L98H/S297T/F495I</td>
<td>0.5</td>
</tr>
<tr>
<td>4</td>
<td>E071</td>
<td>2015</td>
<td>TR&lt;sub&gt;r&lt;/sub&gt;/L98H/S297T/F495I</td>
<td>0.5</td>
</tr>
<tr>
<td>5</td>
<td>E073</td>
<td>2015</td>
<td>TR&lt;sub&gt;r&lt;/sub&gt;/L98H/S297T/F495I</td>
<td>0.5</td>
</tr>
<tr>
<td>6</td>
<td>E074</td>
<td>2015</td>
<td>TR&lt;sub&gt;r&lt;/sub&gt;/L98H/S297T/F495I</td>
<td>0.5</td>
</tr>
<tr>
<td>7</td>
<td>S05–31</td>
<td>2016</td>
<td>TR&lt;sub&gt;r&lt;/sub&gt;/L98H/S297T/F495I</td>
<td>0.5</td>
</tr>
<tr>
<td>8</td>
<td>A31</td>
<td>2013</td>
<td>TR&lt;sub&gt;r&lt;/sub&gt;/L98H</td>
<td>0.5</td>
</tr>
<tr>
<td>9</td>
<td>S05–12</td>
<td>2016</td>
<td>TR&lt;sub&gt;r&lt;/sub&gt;/L98H</td>
<td>0.5</td>
</tr>
<tr>
<td>10</td>
<td>C03–04</td>
<td>2016</td>
<td>TR&lt;sub&gt;r&lt;/sub&gt;/L98H</td>
<td>0.5</td>
</tr>
<tr>
<td>11</td>
<td>S07–00</td>
<td>2016</td>
<td>TR&lt;sub&gt;r&lt;/sub&gt;/L98H</td>
<td>0.5</td>
</tr>
<tr>
<td>12</td>
<td>S05–20</td>
<td>2017</td>
<td>TR&lt;sub&gt;r&lt;/sub&gt;/L98H</td>
<td>0.5</td>
</tr>
<tr>
<td>13</td>
<td>S05–32</td>
<td>2018</td>
<td>TR&lt;sub&gt;r&lt;/sub&gt;/Y121F/T289A</td>
<td>0.5</td>
</tr>
<tr>
<td>14</td>
<td>YL1</td>
<td>2014</td>
<td>Polymorphisms¶</td>
<td>0.5</td>
</tr>
<tr>
<td>15</td>
<td>YL3</td>
<td>2014</td>
<td>Polymorphisms¶</td>
<td>0.5</td>
</tr>
<tr>
<td>16</td>
<td>YL4</td>
<td>2015</td>
<td>Polymorphisms¶</td>
<td>0.5</td>
</tr>
<tr>
<td>17</td>
<td>YL5</td>
<td>2015</td>
<td>Polymorphisms¶</td>
<td>0.5</td>
</tr>
<tr>
<td>18</td>
<td>YL6</td>
<td>2015</td>
<td>Polymorphisms¶</td>
<td>0.5</td>
</tr>
<tr>
<td>19</td>
<td>g054m</td>
<td>2016</td>
<td>Wild-type</td>
<td>0.12</td>
</tr>
<tr>
<td>20</td>
<td>g054L</td>
<td>2016</td>
<td>Wild-type</td>
<td>0.5</td>
</tr>
<tr>
<td>21</td>
<td>g057m</td>
<td>2016</td>
<td>Wild-type</td>
<td>0.12</td>
</tr>
<tr>
<td>22</td>
<td>g057L</td>
<td>2016</td>
<td>Wild-type</td>
<td>0.5</td>
</tr>
</tbody>
</table>

‡ *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.tr-canada.com) (ISA), and Chem Service (https://www.chemservice.com) (TBC).

¶ These isolates have F46Y/G890M172V/I224L/T225E/L358L/E427K/C454C polymorphisms.

# The MICs of 200 azole-susceptible isolates were used. Only 62 isolates were tested for MICs for DFC and TBC.

*AMB, amphotericin B; ATCC, American Type Culture Collection; DFC, difenoconazole; ISA, isavuconazole; ITR, itraconazole; ND, not done; POS, posaconazole; PRC, prochloraz; TBC, tefuconazole; VRC, voriconazole.*

† d0 and dn indicate day of and n days after the first isolation of *A. fumigatus*, respectively.

*This study was conducted at Kaohsiung Medical University Hospital (Taiwan). The study was approved by the institutional ethics committee (IRB105-0092F). The sz of 200 isolates was assayed for growth under agar dilution (0.015–0.5 µg/mL) in the presence of amphotericin B (4/1) and reference strains*
Appendix Table 3. Clinical characteristics of 12 patients harboring azole-resistant *Aspergillus fumigatus* isolates, Taiwan*

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Strain no.</th>
<th>Age, y/sex</th>
<th>Concurrent condition</th>
<th>Sample</th>
<th>Aspergillus disease†</th>
<th>Previous azole exposure</th>
<th>Sequential antifungal treatment (d)</th>
<th>Outcome</th>
<th>IA-related death</th>
</tr>
</thead>
<tbody>
<tr>
<td>1‡</td>
<td>B44, B51</td>
<td>66/F</td>
<td>DM, HCV/cirrhosis, adrenal insufficiency</td>
<td>Sputum</td>
<td>IPA, unclassified</td>
<td>No</td>
<td>VRC (1), AMB (3)</td>
<td>Died</td>
<td>Yes</td>
</tr>
<tr>
<td>2</td>
<td>D007</td>
<td>49/M</td>
<td>SLE, ESRD, bacterial septicemia, meningocencephalitis</td>
<td>Nasal swab</td>
<td>Colonization</td>
<td>POS/VR C (2 wk)</td>
<td>MCF (33)</td>
<td>Died</td>
<td>No</td>
</tr>
<tr>
<td>3</td>
<td>E071, E074; E073</td>
<td>64/F</td>
<td>Pleural effusion; sputum</td>
<td>Pleural effusion; sputum</td>
<td>Proven IPA with empyema</td>
<td>No</td>
<td>VRC (1), LAMB (3)</td>
<td>Died</td>
<td>Yes</td>
</tr>
<tr>
<td>4‡</td>
<td>S05–319 A31</td>
<td>88/F</td>
<td>Lung cancer, COPD, bronchiectasis</td>
<td>Sputum</td>
<td>Colonization</td>
<td>No</td>
<td>AMB (3), POS (10), VRC (11)</td>
<td>Alive</td>
<td>No</td>
</tr>
<tr>
<td>5‡</td>
<td>S05–122</td>
<td>90/F</td>
<td>COPD, steroid use, bronchiectasis</td>
<td>Sputum</td>
<td>Probable IPA</td>
<td>No</td>
<td>Died</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>S05–008</td>
<td>76/F</td>
<td>COPD, steroid use, bronchiectasis, DM</td>
<td>Sputum</td>
<td>Colonization</td>
<td>No</td>
<td>No</td>
<td>Alive</td>
<td>No</td>
</tr>
<tr>
<td>7</td>
<td>C03–004</td>
<td>74/M</td>
<td>B cell lymphoma, COPD, HCV, CAD</td>
<td>Sputum</td>
<td>Colonization</td>
<td>No</td>
<td>No</td>
<td>Alive</td>
<td>No</td>
</tr>
<tr>
<td>8</td>
<td>S05–222</td>
<td>36/M</td>
<td>Urine; PCN</td>
<td>Proven IA, (renal abscess)</td>
<td>VRC (3 mo)</td>
<td>VRC (93), VRC/CAS (43), LAMB (44), LAMB/5FC (25), LAMB/AND (24)</td>
<td>Died</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>YL1; YL3, YL4, YL5, YL6</td>
<td>39/M</td>
<td>MDS with RAEB-T, status post-HSCT with GVHD, bacterial septicemia</td>
<td>Sputum; BAL</td>
<td>Probable IPA</td>
<td>VRC (4 mo)</td>
<td>Died</td>
<td>Yes</td>
<td></td>
</tr>
</tbody>
</table>

*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, fluocytosine; 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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C485</td>
<td>(6)</td>
</tr>
<tr>
<td>C96</td>
<td>(6)</td>
</tr>
<tr>
<td>E2619</td>
<td>(6)</td>
</tr>
<tr>
<td>20643.023</td>
<td>(7)</td>
</tr>
<tr>
<td>20684.007</td>
<td>(7)</td>
</tr>
<tr>
<td>E1001</td>
<td>(6)</td>
</tr>
<tr>
<td>20677.079</td>
<td>(7)</td>
</tr>
<tr>
<td>2087 m1341.17–06–2012</td>
<td>(8)</td>
</tr>
<tr>
<td>2091 m1428.01–07–2012</td>
<td>(8)</td>
</tr>
<tr>
<td>C94</td>
<td>(6)</td>
</tr>
<tr>
<td>094411/7/50</td>
<td>(8)</td>
</tr>
<tr>
<td>Case 2–90d</td>
<td>(9)</td>
</tr>
<tr>
<td>The Netherlands 7</td>
<td>(10,11)</td>
</tr>
<tr>
<td>20643.017</td>
<td>(7)</td>
</tr>
<tr>
<td>2005–456307L</td>
<td>(6)</td>
</tr>
<tr>
<td>OKH50</td>
<td>(12)</td>
</tr>
<tr>
<td>04–202165</td>
<td>(13)</td>
</tr>
<tr>
<td>F2126</td>
<td>(6)</td>
</tr>
<tr>
<td>1042/09</td>
<td>(14)</td>
</tr>
<tr>
<td>14–148–2460</td>
<td>(6)</td>
</tr>
<tr>
<td>2107m1974.23–09–2012</td>
<td>(8)</td>
</tr>
<tr>
<td>A12519</td>
<td>(8)</td>
</tr>
<tr>
<td>R2–07–1_R</td>
<td>(6)</td>
</tr>
<tr>
<td>E454</td>
<td>(15)</td>
</tr>
<tr>
<td>Case 1–7d</td>
<td>(9)</td>
</tr>
<tr>
<td>Myc-2008–002 nr.42</td>
<td>(14)</td>
</tr>
<tr>
<td>Case 3–6d</td>
<td>(9)</td>
</tr>
<tr>
<td>CF/NL2992</td>
<td>(8)</td>
</tr>
<tr>
<td>Tanzania</td>
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</tr>
<tr>
<td>CF/NL0682</td>
<td>(8)</td>
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<tr>
<td>VPCI851/E/12/2/a/3</td>
<td>(8)</td>
</tr>
<tr>
<td>CF/NL0645</td>
<td>(11)</td>
</tr>
<tr>
<td>The Netherlands 2</td>
<td>(10,11)</td>
</tr>
<tr>
<td>The Netherlands 3</td>
<td>(10,11)</td>
</tr>
<tr>
<td>C195</td>
<td>(7)</td>
</tr>
<tr>
<td>12–90032258</td>
<td>(13)</td>
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<tr>
<td>Case 4–36d</td>
<td>(9)</td>
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</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Azole susceptibility (mechanism)</th>
<th>hapE</th>
<th>srbA</th>
<th>hmg1</th>
<th>erg6</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC MYA-3626</td>
<td>S (mechanism)</td>
<td>V37D</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC 16903</td>
<td>S (mechanism)</td>
<td>V37D, S82P</td>
<td>H564Y</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F2509</td>
<td>S (mechanism)</td>
<td>V37D</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F02411</td>
<td>S (mechanism)</td>
<td>V37D</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YL1</td>
<td>S (mechanism)</td>
<td>E957D</td>
<td>P212S, H564Y</td>
<td></td>
<td></td>
</tr>
<tr>
<td>YL3</td>
<td>R (mechanism)</td>
<td>E957D</td>
<td>P212S, S269P, H564Y</td>
<td>F186V</td>
<td></td>
</tr>
<tr>
<td>YL4</td>
<td>R (mechanism)</td>
<td>E957D</td>
<td>P212S, S269P, H564Y</td>
<td>F186V</td>
<td></td>
</tr>
<tr>
<td>YL5</td>
<td>R (mechanism)</td>
<td>E957D</td>
<td>P212S, S269P, H564Y</td>
<td></td>
<td></td>
</tr>
<tr>
<td>YL6</td>
<td>R (mechanism)</td>
<td>E957D</td>
<td>P212S, S269P, H564Y</td>
<td>F186V</td>
<td></td>
</tr>
<tr>
<td>g054m</td>
<td>R (mechanism)</td>
<td>E957D</td>
<td>P212S, S269P, H564Y</td>
<td></td>
<td></td>
</tr>
<tr>
<td>g057m</td>
<td>R (mechanism)</td>
<td>E957D</td>
<td>P212S, S269P, H564Y</td>
<td></td>
<td></td>
</tr>
<tr>
<td>g054L</td>
<td>S (mechanism)</td>
<td>V37D</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>g057L</td>
<td>S (mechanism)</td>
<td>V37D</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B44</td>
<td>R (TRw/L98H/S297T/F495I)</td>
<td>V37D</td>
<td>H564Y</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A31</td>
<td>R (TRw/L98H)</td>
<td>V37D</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*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.
<table>
<thead>
<tr>
<th>Category</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Academic society</td>
<td>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.</td>
</tr>
<tr>
<td>Laboratory personnel</td>
<td>Identify clinically relevant <em>Aspergillus</em> isolates at the species complex level and confirm <em>A. fumigatus</em> by thermotolerance test (growth at 50°C) (19); screen for azole resistance with azole agar plates for clinically relevant <em>A. fumigatus</em> isolates and screen multiple colonies (&lt;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 <em>A. fumigatus</em> is suspected and identified.</td>
</tr>
<tr>
<td>Physicians</td>
<td>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 <em>A. fumigatus</em> 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).</td>
</tr>
<tr>
<td>Hospital environment</td>
<td>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).</td>
</tr>
<tr>
<td>Reference mycology laboratory</td>
<td>Identify <em>Aspergillus</em> isolates to the species level by molecular methods; confirm antifungal susceptibility of <em>Aspergillus</em> isolates with reference CLSI or EUCAST methods; perform periodic reference MIC testing of isolates of <em>A. fumigatus</em> complex (&gt;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.</td>
</tr>
<tr>
<td>Scientists and plant pathologists</td>
<td>Identify the key azole fungicides that select azole-resistant <em>Aspergillus</em>; propose better fungicide application strategies to minimize resistance development (24).</td>
</tr>
<tr>
<td>Agricultural authority</td>
<td>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.</td>
</tr>
<tr>
<td>Governance</td>
<td>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).</td>
</tr>
</tbody>
</table>

*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.
References


