Antifungal Susceptibilities of *Aspergillus fumigatus* Clinical Isolates Obtained in Nagasaki, Japan


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We investigated the triazole, amphoterin B, and micafungin susceptibilities of 196 *A. fumigatus* clinical isolates in Nagasaki, Japan. The percentages of non-wild-type (non-WT) isolates for which MICs of itraconazole, posaconazole, and voriconazole were above the ECV were 7.1%, 2.6%, and 4.1%, respectively. A G54 mutation in *cyp51A* was detected in 64.2% (9/14 isolates) and 100% (5/5 isolates) of non-WT isolates for itraconazole and posaconazole, respectively. Amphoterin B MICs of ≥2 μg/ml and micafungin minimum effective concentrations (MECs) of ≥16 μg/ml were recorded for two and one isolates, respectively.

The clinical importance of *Aspergillus* infection has increased as the number of immunocompromised patients has risen (16). Antifungals are recommended for treatment of patients with invasive pulmonary aspergillosis (IPA) or chronic pulmonary aspergillosis (CPA) are triazoles, amphoterin B, and echinocandins (13, 15, 37). Patients with CPA often need years of treatment (13, 37). Although oral therapy is important for carrying out long courses of treatment, azoles (with the exception of fluconazole) are the only class of oral drugs licensed for the treatment of aspergillosis (14, 37).

*Aspergillus fumigatus* is the most common and pathogenic species of *Aspergillus* (34, 37). Antifungal resistance of *A. fumigatus*, especially to azoles, is one of the concerns in treatment of aspergillosis. During the last decade, many cases of treatment failure due to azole-resistant *Aspergillus* infection have been reported, and in the past few years a growing body of papers about antifungal susceptibilities of *A. fumigatus* has been accumulating (1, 3–6, 9, 10, 12, 18, 23–27, 31–33, 35, 36). Even though an increased rate of azole resistance has been reported recently in the Netherlands and the United Kingdom, the prevalence of azole resistance reportedly remains low in other countries (1, 3, 6, 9, 12, 23, 25, 33).

The azole target protein lanosterol 14α-demethylase of *Aspergillus* is encoded by the *cyp51A* gene, and mutations of *cyp51A* are a major mechanism of azole resistance (8, 17, 19, 20, 22, 32). Some mutational hotspots, such as G54, M220, and TR/L98H, have been identified as causes of azole resistance (2, 21, 22). Of these mutations, TR/L98H is especially prevalent in the Netherlands. An environmental origin (resulting from agricultural antifungal drug usage) is suspected, in spite of the fact that the mechanism(s) of mutation induction has not been shown definitively (24, 31, 32).

We studied the antifungal susceptibility of 196 *A. fumigatus* clinical isolates obtained in the Pneumology Department of Nagasaki University Hospital, Nagasaki, Japan. The isolates were collected between February 1994 and April 2010. All of the isolates were subjected to susceptibility testing and *cyp51A* sequence analysis. All isolates were identified as *A. fumigatus* by macroscopic colony morphology, micromorphological characteristics, and the ability to grow at 48°C. Non-wild-type (non-WT) isolates were subjected to additional molecular identification by amplification of ribosomal internal transcribed spacers (ITSs) and ribosomal large-subunit D1/D2 sequencing as described previously (11). MICs of itraconazole, posaconazole, voriconazole, and amphoterin B and minimum effective concentrations (MECs) of micafungin were determined using the Clinical and Laboratory Standards Institute (CLSI) M38-A2 broth microdilution method. Assays were performed using RPMI 1640 broth (0.2% dextrose), final inoculum concentrations ranging from 0.4 × 10⁴ to 5 × 10⁴ CFU/ml, and 48 h of incubation at 35°C (7). The MIC was defined as the lowest drug concentration that produced complete growth inhibition; the MEC was read as the lowest concentration of drug that led to the growth of small, rounded, compact hyphal forms compared to the hyphal growth seen in the control well. Susceptibility tests of non-WT isolates were performed at least three times for each isolate; each test was performed on different days. Because clinical breakpoints have not been established yet, we used epidemiological cutoff values (ECVs) to evaluate azole susceptibility (9, 25, 29). Wild-type (WT) isolates of *A. fumigatus* (MIC ≤ ECV) were distinguished from non-WT isolates (MIC > ECV), which may exhibit acquired low-susceptibility mechanisms. ECVs used in this study were as follows: itraconazole, 1 μg/ml; posaconazole, 0.5 μg/ml; voriconazole, 1 μg/ml, all as previously suggested (9, 25).

For sequence analyses, genomic DNA was extracted from non-WT isolates using a MasterPure yeast DNA purification kit (Epicentre Biotechnologies, Madison, WI). The full coding region of the *cyp51A* gene was amplified as previously described (32). DNA sequences were determined using a BigDye Terminator ver-

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Table 1: MIC and MEC distributions of five antifungals

<table>
<thead>
<tr>
<th>Antifungal agent</th>
<th>No. of isolates</th>
<th>With a MIC or MEC (µg/ml)</th>
<th>% of non-WT isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>≤0.03</td>
<td>0.06</td>
</tr>
<tr>
<td><strong>Triazoles</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Itraconazole</td>
<td>182 (14)</td>
<td>1</td>
<td>28</td>
</tr>
<tr>
<td>Posaconazole</td>
<td>182 (14)</td>
<td>14</td>
<td>108</td>
</tr>
<tr>
<td>Voriconazole</td>
<td>182 (14)</td>
<td>1</td>
<td>35</td>
</tr>
<tr>
<td><strong>Polyene</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>182 (14)</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td><strong>Echinocandin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Micafungin</td>
<td>182 (14)</td>
<td>177 (14)</td>
<td>2</td>
</tr>
</tbody>
</table>

* MIC distributions for each agent were obtained by subtracting from the total isolates tested the 14 non-WT isolates resistant to itraconazole. The MIC distribution for the 14 non-WT isolates is in parentheses.

* MICs are shown for amphotericin B, itraconazole, posaconazole, and voriconazole; MECs are shown for micafungin.

Table 2: MICs and Cyp51A substitutions in 22 non-WT Aspergillus fumigatus isolates

<table>
<thead>
<tr>
<th>Isolate</th>
<th>MIC (µg/ml)</th>
<th>Itraconazole</th>
<th>Posaconazole</th>
<th>Voriconazole</th>
<th>Cyp51A substitution</th>
</tr>
</thead>
<tbody>
<tr>
<td>MF-452</td>
<td>&gt;8</td>
<td>0.5</td>
<td>0.5</td>
<td>I266N</td>
<td></td>
</tr>
<tr>
<td>MF-469</td>
<td>8</td>
<td>1</td>
<td>0.25</td>
<td>G54E, I266N</td>
<td></td>
</tr>
<tr>
<td>MF-460</td>
<td>4</td>
<td>2</td>
<td>0.25</td>
<td>G54E, I266N</td>
<td></td>
</tr>
<tr>
<td>MF-357</td>
<td>4</td>
<td>0.5</td>
<td>I266N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MF-468</td>
<td>4</td>
<td>0.5</td>
<td>0.25</td>
<td>G54E, I266N</td>
<td></td>
</tr>
<tr>
<td>MF-329</td>
<td>4</td>
<td>0.5</td>
<td>0.25</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>MF-331</td>
<td>2</td>
<td>&gt;16</td>
<td>0.25</td>
<td>G54W</td>
<td></td>
</tr>
<tr>
<td>MF-327</td>
<td>2</td>
<td>2</td>
<td>0.12</td>
<td>G54R</td>
<td></td>
</tr>
<tr>
<td>MF-439</td>
<td>2</td>
<td>0.5</td>
<td>0.25</td>
<td>G54E, I266N</td>
<td></td>
</tr>
<tr>
<td>MF-473</td>
<td>2</td>
<td>0.5</td>
<td>0.25</td>
<td>G54E, I266N</td>
<td></td>
</tr>
<tr>
<td>MF-454</td>
<td>2</td>
<td>0.5</td>
<td>0.12</td>
<td>G54E, I266N</td>
<td></td>
</tr>
<tr>
<td>MF-472</td>
<td>2</td>
<td>0.5</td>
<td>0.12</td>
<td>G54E, I266N</td>
<td></td>
</tr>
<tr>
<td>MF-843</td>
<td>2</td>
<td>0.25</td>
<td>2</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>MF-748</td>
<td>2</td>
<td>0.25</td>
<td>1</td>
<td>ND*</td>
<td></td>
</tr>
<tr>
<td>MF-1011</td>
<td>1</td>
<td>2</td>
<td>0.12</td>
<td>G54W</td>
<td></td>
</tr>
<tr>
<td>MF-855</td>
<td>1</td>
<td>0.25</td>
<td>2</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>MF-336</td>
<td>1</td>
<td>0.25</td>
<td>2</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>MF-486</td>
<td>1</td>
<td>0.25</td>
<td>2</td>
<td>None</td>
<td></td>
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<tr>
<td>MF-520</td>
<td>1</td>
<td>0.25</td>
<td>2</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>MF-1091</td>
<td>0.5</td>
<td>0.25</td>
<td>2</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>MF-474</td>
<td>0.5</td>
<td>0.25</td>
<td>2</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>MF-303</td>
<td>0.5</td>
<td>0.12</td>
<td>2</td>
<td>None</td>
<td></td>
</tr>
</tbody>
</table>

* ND, not determined.
seven isolates (Table 2). No TR/L98H-bearing isolates were
detected. The I266N mutation, which has (to our knowledge) not
been reported previously, was also seen in otherazole-susceptible
isolates; therefore, it might not be directly related to azole resist-
tance. Of 21 non-WT isolates, 9 had no CYP51A substitution (Ta-
ble 2). Interestingly, most non-WT isolates for voriconazole
did not possess a cyp51A mutation. Although Bueid et al. reported an
increase of frequency of azole-resistant isolates without cyp51A
mutations, other possible resistant mechanisms (e.g., upregula-
tion of efflux pump) have not yet been fully identified (6, 28, 30),
and further analysis is warranted.

Only a few previous analyses have examined changes in suscep-
tibility over time; therefore, it is not clear that the frequency of
azole-resistant A. fumigatus is increasing worldwide (12, 25, 33).
Nevertheless, mechanisms of resistance induction in clinical set-
tings or the environment (e.g., selection following agricultural
antifungal exposure) remain poorly understood. Given that azole
usage varies from one country to another, the mechanism of azole
resistance may differ between regions.

In this study, we found a low prevalence of resistance to tria-
zoles in Japanese isolates of A. fumigatus, a clinically important
fungus of increasing concern in respiratory medicine. The pro-
portions of non-WT isolates were similar to those previously re-
ported for other countries. In the future, Japanese A. fumigatus
isolates may develop azole resistance by different mechanisms
(such as TR/L98H); therefore, we urge the continued monitoring
of azole susceptibility in this species.

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