Azole susceptibility in clinical and environmental isolates of *Aspergillus fumigatus* from eastern Hokkaido, Japan

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**ABSTRACT**

Azole antifungals are used not only clinically for fungal infections but also used as agricultural fungicides. Recently, azole-resistant *Aspergillus fumigatus* containing a tandem repeat in the promoter region of *cyp51A* combined with amino acid substitution(s) appears in the environment in Eurasia, especially in several European countries. Although azole fungicides have been used in Japan, especially in Hokkaido, surveillance and characterization of *A. fumigatus* in Hokkaido have not been reported. In this study, we collected soil samples from farms that used an azole fungicide in the Tokachi area of eastern Hokkaido, isolated 91 *A. fumigatus* strains, and determined the minimal inhibitory concentrations of medical azoles required for these strains. Moreover, because causative agent *A. fumigatus* is ubiquitous in the air and acquired from the environment, we collected 22 clinical isolates of *A. fumigatus* to measure their susceptibility to medical azoles in a hospital in the Tokachi area. Our data show that almost all *A. fumigatus* isolates retained susceptibility to medical azoles. Clinical isolates OKH34 and OKH6 showed 8 and 2 μg/mL of voriconazole, respectively, as the minimal inhibitory concentration. Both isolates did not contain tandem repeat in *cyp51A* promoter region. An isolate contained G448S mutation in *cyp51A* conferring voriconazole resistance, which is the first report from Japan. Our data shows the existence of azole-resistant and low azole-susceptible clinical isolates and highlight the necessity for continuous surveillance in Japan because resistant *A. fumigatus* strains can arise through clinical or environmental selection or could be introduced from overseas.

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2012 (2012 agricultural chemical year [5]), Snelders and colleagues reported that epoxiconazole, difenoconazole,-propiconazole, bro-muconazole, and tebuconazole exhibited similar docking poses to medical triazoles [6]. Of these five fungicides, tebuconazole, propiconazole, and difenoconazole were placed first, second, and fourth, respectively, in terms of their usage in the 2012 agricultural—chemical year in Japan. These three fungicides were mainly shipped to Hokkaido [5]. Recently, Kano and colleagues examined the azole susceptibility of *A. fumigatus* isolates from a farm in Japan that used tetraconazole twice a year for over 15 years [7]. The study showed that the isolated strains did not exhibit resistance to tetraconazole or itraconazole (ITCZ). In this study, we determined the MIC of medical antifungals at various concentrations (Dry Plate Eiken; Osaka, Japan)-buffered RPMI 1640 medium (Sigma–Aldrich Co., St. Louis, MO) adjusted to pH 7.0 at 1 × 10⁶ cells/mL, and then were inoculated to each well of 96-well microplates, containing medical antifungals at various concentrations (Dry Plate Eiken; Eiken Chemical Co., Ltd., Tokyo, Japan). After 48 h of culture at 35 °C, the growth inhibition of mold was determined with visual observation. MIC and MEC of antifungals against isolates from soil samples was shown in Table 1. All of 91 isolates from these soil samples were susceptible to medical azoles. These results suggest that TR34/L98H–or TR46/Y121F/T289A-type were not prevalent in Tokachi area of eastern Hokkaido.

**Table 1**

<table>
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<tr>
<th>Antifungals</th>
<th>No. of isolates</th>
<th>With MIC or MEC (µg/mL) of:</th>
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<tr>
<td></td>
<td>Total</td>
<td>≤0.015</td>
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| Itraconazole     | 91              | 62     | 26   | 3   | 3   | 4   | 3   | 3   | 3
| Voriconazole     | 91              | 2      | 40   | 46  | 3   | 3   | 3   | 3   | 3
| Amphotericin B   | 91              | 5      | 60   | 23  | 3   | 3   | 3   | 3   | 3
| Micafungin       | 91              | 91     | 91   | 91  | 91  | 91  | 91  | 91  | 91

To compare cyp51A and the upstream regions of *A. fumigatus* OKH6, OKH34 and otherazole susceptible *A. fumigatus* strains (AF293, OKH1 and OKH2), we determined these nucleotide sequences. To amplify and determine the nucleotide sequences of cyp51A with the upstream region, primers listed in Supplementary Table 3 were used. As shown in Supplementary Fig. 1a, the coding sequence of cyp51A in OKH6 strain was identical to those in OKH1 and OKH2 strains. On the other hand, OKH34 strain has a unique amino acid substitution from glycine to serine in the residue 448 of cyp51A. In the coding sequence of cyp51B, OKH6, but not OKH1 and OKH2, has P327S mutation (Supplementary Fig. 1b). The 1 kb upstream region of cyp51A of OKH6 did not contain tandem repeats. We examined the expression level of cyp51A and cyp51B in OKH6 and compared with the expression levels in OKH1 and OKH2. After statically culturing for 48 h at 35 °C in MOPS-RPMI medium, total RNA were recovered from mycelia with Direct-zol RNA MiniPrep kit with TRI-Reagent (Zymo Research Irvine, CA). Real-time quantitative PCR was performed using THUNDERBIRD SYBR qPCR Mix (Toyobo Co., Ltd., Osaka, Japan) and LightCycle 480 II (Roche Diagnostics, Basel, Switzerland). Primers were listed in Supplementary Table 3. The expression levels of cyp51A and cyp51B in OKH6 were comparable to those of OKH1 and OKH2 (Fig. 1). These results indicate that the G448S mutation in cyp51A contributed to resistance of OKH34 to VRCZ.

With the exception of cyp51A of *A. fumigatus* OKH34 possessed a mutation at glycine residue 448 to serine. The point mutation has been described in several manuscripts [8–12], but the mutant has not been reported from Japan. Clinical isolates including G448S mutation described in several manuscripts were resistant to VRCZ and ITCZ [9–11]. In Japan, to the best of our knowledge, the mutant has not been reported. On the other hand, G448S mutants obtained by in vitro—selection were resistance to voriconazole [8]. Our data also indicated that the G448S mutant conferred resistance to voriconazole. The patient had received treatment with VRCZ (200 mg daily), and on day 85 after starting VRCZ treatment *A. fumigatus* OKH34 was isolated from a sputum specimen. Before the antifungal treatment, an *A. fumigatus* strain OKH31 was isolated from the patient and was susceptible to VRCZ, as well as ITCZ. This mutation, therefore, might have been induced during this treatment. Another strain, OKH6, showed low sensitivity to VRCZ and the cyp51A sequence did not contain any mutation, contributing to azole resistance, and the...
expression levels of cyp51A and cyp51B did not changed in OKH6. In cyp51B coding sequence of OKH6, we found P327S mutation. Since the mutation has not been reported, the importance remains unknown. Recently, the structure of A. fumigatus Cyp51B was resolved [13], indicating that P327-residue located far from the active site of Cyp51B. Therefore, the mutation might not be related the VRCZ low susceptibility. Further investigation is required to elucidate a role of the P327S mutation to azole resistance.

In conclusion, we isolated A. fumigatus from soil samples obtained from the Tokachi area, and all of the isolates were susceptible to medical azoles. These results suggest that TR34/L98H- or TR46/Y121F/T289A-type resistant strain was clinically isolated from a patient with cerebral aspergillosis. J Clin Microbiol 2012;50:2531-7.


Table 2
Mic and MEC against clinical isolates of A. fumigatus.

| Antifungals      | No. of isolates | Total | With MIC or MEC (µg/mL) of: | <0.015 | 0.03 | 0.06 | 0.12 | 0.25 | 0.5 | 1 | 2 | 4 | 8 | >8 |
|------------------|----------------|-------|-----------------------------|--------|-----|-----|-----|-----|-----|---|---|---|---|----|---|
| Itraconazole     | 22             |       |                             | 7      | 12  | 3   |     |     |     |   |   |   |   |     |
| Voriconazole     | 22             |       |                             | 6      | 13  | 1   | 1   | 1   |     |   |   |   |   |     |
| Amphotericin B   | 22             |       |                             | 2      | 10  | 9   | 1   |     |     |   |   |   |   |     |
| Micafungin       | 22             | 21    | 1                           |        |     |     |     |     |     |   |   |   |   |     |

* MEC are shown for micafungin.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jiac.2016.03.002.

References


