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Short Report

Most Azole-Susceptible Isolates of *Aspergillus fumigatus* in Hokkaido, Japan are Clustered with Worldwide Strains That Do Not Have Tandem Repeats in *Cyp51A* Promoter

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ABSTRACT

In this study, we analyzed *Aspergillus fumigatus* short tandem repeat patterns of 106 strains isolated from the outdoor air, clinical specimens, and king penguins (*Aptenodytes patagonicus*) with aspergillosis in Japan, and compared them with those of 668 strains from AfumID (including six isolates from Japan). The results showed that the isolates were classified into three major groups. Group II contained most of the azole-resistant strains with 34- and 46-bp tandem repeats in *cyp51A* promoter. As in our previous study, OKH50 and Env1 strains were classified in Group II. Most of the azole-susceptible strains obtained in Japan were classified in Group III.

Key words : *Aspergillus fumigatus*, azole-resistant, short tandem repeat

Our previous studies demonstrated that *Aspergillus fumigatus* strains OKH50 and Env1 with 34-bp tandem repeats (TR_{34}) in the *cyp51A* promoter isolated respectively from a patient and an environmental sample in Japan had identical short tandem repeat (STR) patterns o[1, 2]. The analysis of the STR pattern of 34 strains in the previous study[1] showed that OKH50, Env1, and azole-resistant strains with TR_{34} in the *cyp51A* promoter isolated in Europe, Middle East, India, and Australia were closely related[1, 2]. However, only OKH50 and nine strains isolated in Japan were examined in our previous study[1]. Therefore, the relation between OKH50 and Env1 with other *A. fumigatus* strains is not fully understood. In this study, we analyzed *A. fumigatus* STR patterns of 89 strains isolated from clinical specimens and one strain, Env1, isolated from the environment in Obihiro, Japan. The clinical isolates included several sets of strains obtained from the same patients. Fourteen strains isolated from the captive environment of king penguins (*Aptenodytes patagonicus*) in Noboribetsu of Japan and two strains isolated from king penguins with aspergillosis in Noboribetsu of Japan were also included in the analysis. We then compared them with STR patterns of 668 strains isolated around the world.

The strains used in this study are shown in Supplementary Table 1. Fungal species of these strains were identified with the sequences of internal transcribed spacer regions (ITS1 and 2) and partial nucleotide sequences of β-tubulin, *rodA,* and calmodulin genes as described previously[2]. For this study, we examined 89 clinical isolates, which had been isolated at a hospital in Obihiro City, including 67 isolates from our earlier research[3]. At Noboribetsu City, Hokkaido in Japan, 3 strains from the air and 11 strains isolated from swabs swiped at the surface of the aquarium facility for captive king penguins were

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collected[4]. Two strains from king penguins with aspergillosis used for this study were isolated as described in our earlier studies[5, 6]. Genomic DNA from *A. fumigatus* strains was prepared as described elsewhere[7]. To amplify STR loci, we used primers described by de Valk[8] for this study using the usual techniques. Fragment analysis and sizing analysis were performed with a 3730xl DNA Analyzer (Thermo Fisher Scientific Inc., Waltham, MA) by Fasmac Co., Ltd. (Kanagawa, Japan). For genetic analysis using the STR pattern, the poppr ver. 2.9.3[9, 10] package of R software[11] was used to calculate the average Bruvo's distance over all loci in a population; UPGMA was used for the clustering. In addition to the 104 strains analyzed for STR in this study, we included STR patterns of OKH50 and Env1 as previously reported[1, 2] and 668 strains from AfumID (https: //afumid. shinyapps. io/afumID/)[12] in the analysis (Supplementary Table 2).

The 106 strains obtained in Japan and 668 strains obtained from AfumID were classified into three major groups on the basis of the STR patterns (Supplementary Fig. 1). Group III contained only 11 of 180 TR_{34}-type azole-resistant strains (Table 1). From Japan, 28 and 84 isolates were included in Groups II and III, respectively (Supplementary Fig. 1). The *A. fumigatus* strains OKH50 and Env1, which were TR_{34}-type azole-resistant isolates reported previously[1, 2], were included in Group II, which is consistent with our earlier result. The other 26 Japanese isolates were azole-susceptible strains. As shown by Hagiwara[13] and Nakano[14], TR_{34}- and TR_{46}-type azole-resistant strains were isolated from imported plant bulbs such as *Tulipa* spp. and *Gladiolus* spp. Various ornamental flowering plants have been imported from Europe to Japan for over 100 years[15]. The Japanese isolates included in Group II might have originated from the Eurasian Continent before the global spread of TR_{34}- and TR_{46}-type strains and might have colonized the domestic environment. Similar to the global expansion of pathogenic chytrid fungi[16], international trade of agricultural crops planted in the soil might be the cause of the disturbance of native species or strains and the environment.

Minimum inhibitory concentrations (MICs) of four antifungals against isolates from the same patients with identical STR patterns were determined with a broth microdilution method described earlier[2]. The data are shown in Supplementary Table 3. OKH186 showed quite slow growth. The strain was isolated 28 months after OKH131 was isolated. The MIC values of voriconazole and itraconazole determined at 48 hrs after inoculation were reduced to 1/8 or less than MICs against OKH131 (Supplementary Table 3), suggesting that the strain had changed qualitatively to survive in the host during chronic infection. OKH185 showed four- and eight-times higher MICs for itraconazole and voriconazole, respectively, compared to OKH141, which was isolated 18 months before OKH185 (Supplementary Table 3). The sequence of *cyp51A* gene and the promoter showed no mutation or tandem repeat. However, the comparison of *hmg1* gene between OKH141 strain and OKH185 strain showed a point mutation resulting in an amino acid substitution at the 305th position from serine to proline. The mutation reported in earlier publications[17, 18] contributed to the decrease of azole susceptibility[18].

Table 1. Numbers of *Aspergillus fumigatus* strains in each group shown in Supplementary Fig. 1.

Group	Non-TR strains	TR_{34}-type	TR_{46}-type
I	2 (0)	2 (0)	0 (0)
II	260 (26)	168 (2)	83 (0)
III	248 (84)	11 (0)	0 (0)
Total	510 (110)	181 (2)	83 (0)

The numbers of Japanese isolates are shown in parentheses.
TR: tandem repeats

Of the 16 isolates from the environment and king penguins with aspergillosis in an aquarium, 9 were clustered in a branch (Supplementary Fig. 1), suggesting that they are domestic STR types and that the king penguins had acquired the causative strains from the environment. However, the cluster also contains many strains isolated from Obihiro City. Further research to clarify geographical differences is warranted.

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Conflicts of interest

None.

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