Original Article

Ubiquitous Distribution of Azole-Resistant Aspergillus fumigatus-Related Species in Outdoor Environments in Japan

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Abstract

Aspergillus fumigatus-related species are responsible for causing aspergillosis, which is a fatal infectious disease. Recently, there has been a series of reports of A. fumigatus-related species that are resistant to azole drugs used in clinical practice for the treatment of fungal infections. Some of these species have been isolated from outdoor environments. Testing the drug susceptibility of the strains from outdoor environments, therefore, is important. In this study, we isolated and cultured 72 strains of A. fumigatus-related species from the outdoor environment in Japan. The isolates identified via morphological observation and molecular phylogenetic analysis were Aspergillus felis, Aspergillus lentulus, Aspergillus pseudoviridinutans, Aspergillus udagawae, and Aspergillus wyomingensis. The results of the drug susceptibility testing revealed that A. felis (6 of 14 strains) and A. pseudoviridinutans (13 of 17 strains) were resistant to itraconazole (ITCZ), with 4 mg/L or higher minimum inhibitory concentrations (MICs). The voriconazole (VRCZ)-resistant strains with 4 mg/L or higher MICs were A. felis (14 of 14), A. lentulus (4 of 4), A. pseudoviridinutans (15 of 17), A. udagawae (23 of 34), A. wyomingensis (1 of 3), and A. pseudoviridinutans (1 of 3). Among them, A. felis (1 of 14) and A. pseudoviridinutans (7 of 17) demonstrated 8 mg/L or higher MICs for ITCZ and VRCZ. These results indicate that A. fumigatus-related species resistant to ITCZ and VRCZ are widely distributed in outdoor environments in Japan.

Key words: Aspergillus fumigatus, drug susceptibility, TUB-2, Viridinutantes, voriconazole

Introduction

The genus Aspergillus includes the causative agents of allergic diseases and invasive aspergillosis, which is a fatal infection in humans, cats, and dogs⁹⁻¹¹. In particular, Aspergillus fumigatus, which belongs to Aspergillus section Fumigati, is the most important causative agent of invasive aspergillosis. Recently, a series of species genetically related to A. fumigatus have been isolated in clinical practice to determine the importance of these species⁴⁻⁷. The following A. fumigatus-related species have been isolated in clinical settings: Aspergillus aureoles, Aspergillus felis, Aspergillus hiratsukae, Aspergillus lentulus, Aspergillus pseudoviridinutans, Aspergillus thermomutatus, Aspergillus udagawae, Aspergillus viridinutans, and Aspergillus wyomingensis⁶,⁸⁻¹⁶. These species, except A. hiratsukae, A. thermomutatus, and A. lentulus, are included in the series Viridinutantes proposed by Houbraken et al.¹⁷. In addition to these five species, ser. Viridinutantes also includes Aspergillus acrensis, Aspergillus arcoverdensis, A. aureoles, Aspergillus bezerrae, Aspergillus curviformis, Aspergillus frankstonensis, and Aspergillus siamensis and currently consists of 12 species.¹⁷ Houbraken et al.¹⁷ further proposed eight series, including ser. Fumigati to which A. lentulus belongs, ser. Unilaterales to which A. hiratsukae belongs, and ser. Thermomutati to which A. thermomutatus belongs.

Some strains of A. fumigatus have been reported to be resistant to azole antifungals.¹⁸ A. fumigatus resistant to itraconazole (ITCZ) was first discovered by Denning et al.¹⁹ and is now reported worldwide.²⁰ For A. fumigatus-related species, clinical strains of A. felis, A. lentulus, A. pseudoviridinutans, A. udagawae, and A. viridinutans have been reported to have MICs of ≥ 4 mg/L for both ITCZ and voriconazole (VRCZ)²¹⁻²⁴. Lyskova et al.²¹ and Talbot et al.²² conducted drug susceptibility testing to azole antifungals on fungal...
species belonging to ser. *Viridinutan*(es) isolated from outdoor environments and clinical sources. The results indicated that the strains of the following species were resistant to ITCZ or VRCZ, or both, with MICs of ≥ 4 mg/L: *A. acren*ensis, *A. arcov*erden*is*, *A. auro*reol*es*, *A. felis*, *A. frankto*n*en*sis*, *A. pseudovirid*in*utan*(es), *A. siamensis*, *A. udagawa*e, *A. viridi*n*tan*(es), and *A. wyoming*ensis. However, Lyskova et al.\(^{16,23}\) and Talbot et al.\(^{20}\) did not show the results of drug susceptibility testing separately for environmental and clinical strains. Thus, it remains unclear how many species are resistant toazole antifungals in outdoor environments. In Japan, there have been reports of drug susceptibility tests using clinical strains\(^{16,23}\), but few studies have been conducted on environmental strains.

From 2012 to 2017, we surveyed the diversity of species of *Aspergillus* in the outdoor environment in Japan and isolated many *A. fumigatus*-related species in the process. In this study, we conducted drug susceptibility tests and molecularphylogenetic analysis using partial nucleotide sequences of the β-tubulin gene (*TUB-2*) on these environmental strains andJapanese clinical strains deposited at the Medical Mycology Research Center (MMRC), Chiba University. Our results indicate that *A. fumigatus*-related species resistant to azole antifungal drugs may be universally distributed in outdoor environments in Japan.

### Materials and methods

#### Sampling and fungal isolation

An ecological survey was conducted from 2012 to 2017 to investigate the species diversity of the causative agents of invasive aspergillosis and the distribution pattern of each fungal species in outdoor environments throughout Japan across diverse climates and landscapes. The strains of *A. fumigatus*-related species isolated in that survey from soil and air by the following methods were used in this study. The soil-sampling sites from where the strains of *A. fumigatus*-related species were isolated are listed in Table 1. At each sampling site, 10–15 soil samples at least 5 m apart were obtained. The source of each soil was classified into four types in accordance with the landscape of the sampling site: forest (13 sites), grassland (1 site), bare land (2 sites), and farmland (2 sites) (Table 1). Approximately 100 g of soil was collected from the field after removing any plant litter accumulating on the surface and brought to the laboratory. Approximately 20–40 g of soil was placed in a sterilized culture bottle (5 cm in diameter and 10 cm in height), and then sterilized corn grains were placed on top of the soil and incubated at 35 °C for 7–10 days. After incubation, the conidia possibly identified as *A. fumigatus*-related species, which appeared on the corn grains, were isolated under a stereomicroscope (SZH10; Olympus, Tokyo, Japan) and cultured on corn meal agar (CMA, Nissui Pharmaceutical, Tokyo, Japan) supplemented with chloramphenicol (CP) at room temperature. Subsequently, the conidia that formed on the CMA were isolated and cultured on malt extract agar (MA, Nissui Pharmaceutical, Tokyo, Japan). Air sample (200 L) was collected in 2016 from the roof of a building that was approximately 40-m high in Funabashi, Chiba, Japan, and from an adjacent forest (elevation: 25 m) using an air sampler (Mas-100 Eco; Merck, Darmstadt, Germany) with CMA medium supplemented with CP. The strains cultured on MA were identified via macroscopic and microscopic morphological observations and the *TUB-2* sequences and are presented in Table 1. Isolates from the same sample were excluded when the strains were identified as the same species. The strains isolated from human and animal clinical specimens in Japan were obtained from MMRC, Chiba University, through the National Bio-Resource Project, Japan (http://www.nbrp.jp/) (Table 1).

#### DNA extraction and molecular studies

Genomic DNA was extracted from the mycelia cultured on MA overlaid with a cellophane membrane using the cetyltrimethylammonium bromide method\(^{25}\). Polymerase chain reaction (PCR) was performed using Quick Taq HS DyeMix (Toyobo, Osaka, Japan). PCR primer pair Bt2a (5´‒GGTAACCAAATCGGTGCTGCTTTC‒3´) or T10 (5´‒ACGATAGGTTCACCTCCAGAC‒3´) and BenA2 (5´‒AGTTGTCGGGACGGAAGAG‒3´) or Bt2a and Bt2b (5´‒ACCTCAGTGATGTAGACCCCTTGCGC‒3´)\(^{21}\) were used to obtain the sequences of *TUB-2*. DNA fragments were amplified using a PCR thermal cycler (DNA Engine; Bio-Rad, California, USA) with the following thermal cycling schedule: the first cycle of 5 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 54°C for annealing, and 1 min at 72°C, with a final cycle of 10 min at 72°C. PCR products were purified according to the manufacturer’s protocol using a FastGene Gel/PCR Extraction Kit (Nippon Genetics, Tokyo, Japan). Purified PCR products were sequenced by Fasmac (Kanagawa, Japan). The sequences obtained in this study were deposited in the DNA Data Bank of Japan (DDBJ) (Table 1).

#### Phylogenetic analysis

The sequences were aligned by MAFFT v.7\(^{32}\), and the dataset was utilized for phylogenetic analysis using maximum-likelihood estimation with RAxML-NG\(^{1,20}\) implemented in raxmlGUI 2.0.5\(^{50}\). The best-fitting nucleotide substitution model (K80 + I model) was selected using ModelTest-Ng v.0.1.6\(^{10}\) according to the Akaike information criterion (AICc). The bootstrap procedure of Lemoine et al.\(^{32}\) was used with 1,000 replicates to estimate clade support.

#### Antifungal susceptibility testing

Each strain was tested for susceptibility to ITCZ and VRCZ and cultured for 7–14 days to form conidia, and then conidia suspensions were prepared. The micro-liquid dilution method according to the Clinical and Laboratory Standards Institute’s M38-A2 procedure was used with the yeast-like fungi DP.
| Species | Strain No. | Source | Location | Accession No. | Reference |
|---------|------------|--------|----------|---------------|-----------|
| A. udagawae | CBS 114217 | Soil | Brazil | LT795800 | Lyskova et al (Unpublished) |
| A. aureus | CBS 13334 | Soil | Brazil | AR811845 | Matsuzawa et al 2015 |
| A. freenis | NREL 2244 | Soil | Ghana | EF69808 | Peterson et al 2008 |
| A. fumigatus | NREL 2439 | Soil | Australia | EF69012 | Peterson et al 2008 |
| A. aegerita | SRA 7291 | Grassland soil | in cattle farm | LC631859 | This study |
| A. tatenoi | CBS 407.93 | Soil from sugarcane plantation | Brazil | LC631860 | This study |
| A. spinosus | CBS 54303 | Soil | Japan | LC631861 | This study |
| A. siamensis | IFM 59564 | Soil | Japan | LC631862 | This study |
| A. wyomingensis | IFM 60053 | Soil | Japan | LC631863 | This study |
| A. acrensis | IFM 61579 | Soil | Japan | LC631864 | This study |
| A. pseudoviridinutans | IFM 62091 | Soil | Japan | LC631865 | This study |
| A. brevipes | NHVA 1 | Bare land soil | Japan | LC631866 | This study |
| A. fumigatus | NHVA 1 | Bare land soil | Japan | LC631867 | This study |
| A. felis | NHVA 1 | Bare land soil | Japan | LC631868 | This study |
| A. brevipes | NHVA 1 | Bare land soil | Japan | LC631869 | This study |
| A. aegerita | NHVA 1 | Bare land soil | Japan | LC631870 | This study |
| A. pseudoviridinutans | NHVA 1 | Bare land soil | Japan | LC631871 | This study |
| A. fumigatus | NHVA 1 | Bare land soil | Japan | LC631872 | This study |
| A. brevipes | NHVA 1 | Bare land soil | Japan | LC631873 | This study |
| A. aegerita | NHVA 1 | Bare land soil | Japan | LC631874 | This study |
| A. pyriformis | NHVA 1 | Bare land soil | Japan | LC631875 | This study |
| A. aegerita | NHVA 1 | Bare land soil | Japan | LC631876 | This study |
| A. pseudoviridinutans | NHVA 1 | Bare land soil | Japan | LC631877 | This study |
| A. pyriformis | NHVA 1 | Bare land soil | Japan | LC631878 | This study |
| A. aegerita | NHVA 1 | Bare land soil | Japan | LC631879 | This study |
| A. pseudoviridinutans | NHVA 1 | Bare land soil | Japan | LC631880 | This study |
| A. pyriformis | NHVA 1 | Bare land soil | Japan | LC631881 | This study |
| A. aegerita | NHVA 1 | Bare land soil | Japan | LC631882 | This study |
| A. pyriformis | NHVA 1 | Bare land soil | Japan | LC631883 | This study |
| A. aegerita | NHVA 1 | Bare land soil | Japan | LC631884 | This study |
| A. pyriformis | NHVA 1 | Bare land soil | Japan | LC631885 | This study |
| A. aegerita | NHVA 1 | Bare land soil | Japan | LC631886 | This study |
| A. pyriformis | NHVA 1 | Bare land soil | Japan | LC631887 | This study |
| A. aegerita | NHVA 1 | Bare land soil | Japan | LC631888 | This study |
| A. pyriformis | NHVA 1 | Bare land soil | Japan | LC631889 | This study |
| A. aegerita | NHVA 1 | Bare land soil | Japan | LC631890 | This study |
| A. pyriformis | NHVA 1 | Bare land soil | Japan | LC631891 | This study |
| A. aegerita | NHVA 1 | Bare land soil | Japan | LC631892 | This study |
| A. pyriformis | NHVA 1 | Bare land soil | Japan | LC631893 | This study |
| A. aegerita | NHVA 1 | Bare land soil | Japan | LC631894 | This study |
| A. pyriformis | NHVA 1 | Bare land soil | Japan | LC631895 | This study |
| A. aegerita | NHVA 1 | Bare land soil | Japan | LC631896 | This study |
| A. pyriformis | NHVA 1 | Bare land soil | Japan | LC631897 | This study |
| A. aegerita | NHVA 1 | Bare land soil | Japan | LC631898 | This study |
| A. pyriformis | NHVA 1 | Bare land soil | Japan | LC631899 | This study |
| A. aegerita | NHVA 1 | Bare land soil | Japan | LC631900 | This study |
| A. pyriformis | NHVA 1 | Bare land soil | Japan | LC631901 | This study |
| A. aegerita | NHVA 1 | Bare land soil | Japan | LC631902 | This study |
| A. pyriformis | NHVA 1 | Bare land soil | Japan | LC631903 | This study |
| A. aegerita | NHVA 1 | Bare land soil | Japan | LC631904 | This study |
| A. pyriformis | NHVA 1 | Bare land soil | Japan | LC631905 | This study |
| A. aegerita | NHVA 1 | Bare land soil | Japan | LC631906 | This study |
| A. pyriformis | NHVA 1 | Bare land soil | Japan | LC631907 | This study |
| A. aegerita | NHVA 1 | Bare land soil | Japan | LC631908 | This study |
| A. pyriformis | NHVA 1 | Bare land soil | Japan | LC631909 | This study |
| A. aegerita | NHVA 1 | Bare land soil | Japan | LC631910 | This study |
| A. pyriformis | NHVA 1 | Bare land soil | Japan | LC631911 | This study |
| A. aegerita | NHVA 1 | Bare land soil | Japan | LC631912 | This study |
| A. pyriformis | NHVA 1 | Bare land soil | Japan | LC631913 | This study |
| A. aegerita | NHVA 1 | Bare land soil | Japan | LC631914 | This study |
| A. pyriformis | NHVA 1 | Bare land soil | Japan | LC631915 | This study |
| A. aegerita | NHVA 1 | Bare land soil | Japan | LC631916 | This study |
| A. pyriformis | NHVA 1 | Bare land soil | Japan | LC631917 | This study |
| A. aegerita | NHVA 1 | Bare land soil | Japan | LC631918 | This study |
| A. pyriformis | NHVA 1 | Bare land soil | Japan | LC631919 | This study |
| A. aegerita | NHVA 1 | Bare land soil | Japan | LC631920 | This study |
| A. pyriformis | NHVA 1 | Bare land soil | Japan | LC631921 | This study |
| A. aegerita | NHVA 1 | Bare land soil | Japan | LC631922 | This study |
| A. pyriformis | NHVA 1 | Bare land soil | Japan | LC631923 | This study |
| A. aegerita | NHVA 1 | Bare land soil | Japan | LC631924 | This study |
| A. pyriformis | NHVA 1 | Bare land soil | Japan | LC631925 | This study |
| A. aegerita | NHVA 1 | Bare land soil | Japan | LC631926 | This study |
| A. pyriformis | NHVA 1 | Bare land soil | Japan | LC631927 | This study |
| A. aegerita | NHVA 1 | Bare land soil | Japan | LC631928 | This study |
| A. pyriformis | NHVA 1 | Bare land soil | Japan | LC631929 | This study |
| A. aegerita | NHVA 1 | Bare land soil | Japan | LC631930 | This study |
| A. pyriformis | NHVA 1 | Bare land soil | Japan | LC631931 | This study |
| A. aegerita | NHVA 1 | Bare land soil | Japan | LC631932 | This study |
| A. pyriformis | NHVA 1 | Bare land soil | Japan | LC631933 | This study |
| A. aegerita | NHVA 1 | Bare land soil | Japan | LC631934 | This study |
| A. pyriformis | NHVA 1 | Bare land soil | Japan | LC631935 | This study |
| A. aegerita | NHVA 1 | Bare land soil | Japan | LC631936 | This study |
| A. pyriformis | NHVA 1 | Bare land soil | Japan | LC631937 | This study |
| A. aegerita | NHVA 1 | Bare land soil | Japan | LC631938 | This study |
| A. pyriformis | NHVA 1 | Bare land soil | Japan | LC631939 | This study |
Results

We isolated 72 strains of *A. fumigatus*-related species from outdoor environments in Japan. Based on morphological characteristics and molecular phylogenetic analysis, the isolates were identified as *A. felis* (14), *A. lentulus* (4), *A. pseudoviridinutans* (17), *A. udagawae* (34), and *A. wyomingensis* (3) (Table 1). A comparison of the number of isolates of each *A. fumigatus*-related species among the isolation sources demonstrated their widespread distribution; namely, *A. felis*: 28.6% from bare land soil, 14.3% from grassland soil, and 57.1% from forest soil; *A. lentulus*: 50% from forest soil, 25% from farm soil, and 25% from air; *A. pseudoviridinutans*: 5.9% from bare land soil, 35.3% from grassland soil, 52.9% from forest soil, and 5.9% from farm soil; *A. udagawae*: 55.9% from forest soil, 38.2% from farm soil, and 5.9% from air; and *A. wyomingensis*: 100% from forest soil. The clinical strains newly identified in this study were *A. felis* (1), *A. lentulus* (4), *A. pseudoviridinutans* (1), and *A. udagawae* (3). Molecular phylogenetic analysis based on the TUB-2 sequences revealed three major clades: *A. felis* and *A. pseudoviridinutans* clade (Bootstrap values (BS) = 94%), *A. lentulus* clade (BS = 85%), and *A. udagawae* and *A. wyomingensis* clade (BS = 95%) (Fig. 1). *A. fumigatus*-related species formed clades with high BS (*A. felis*, BS = 92%; *A. lentulus*, BS = 92%; *A. pseudoviridinutans*, BS = 97%; *A. udagawae*, BS = 100%; and *A. wyomingensis*, BS = 93%) (Fig. 1). For all species, environmental and clinical strains were found to be genetically similar (Fig. 1).

The results of the drug susceptibility testing are summarized in Table 2 (See Table S for results of each strain). Of the 72 strains, 19 (26.4%) and 57 (79.2%) were resistant to ITCZ and VRCZ, respectively, with MICs ≥ 4 mg/L. No ITCZ-resistant strains of *A. felis* and VRCZ, respectively, with MICs ≥ 8 mg/L; these strains were also highly resistant to VRCZ. The molecular phylogenetic tree showed that azole drug-resistant strains were found in various lineages regardless of genetic relatedness (Fig. 1).

Discussion

In this study, we isolated *A. felis*, *A. lentulus*, *A. pseudoviridinutans*, *A. udagawae*, and *A. wyomingensis*, which are *A. fumigatus*-related species and cause aspergillosis, from outdoor environments in Japan. Among these, *A. fumigatus*-related species that have been reported from clinical isolates are considered to be clinically important owing to their pathogenicity. The first step in the pathogenesis of aspergillosis is the inhalation of airborne spores and their entry into the human body. Therefore, it is important to evaluate the species diversity of *A. fumigatus*-related species in outdoor environments and clinical settings since the dominant habitat of *A. fumigatus*-related species is considered to be the outdoor environment. All the *A. fumigatus*-related species identified in this study have been previously reported to be isolated from outdoor environments, such as the following: *A. felis*: soil from coal mine accumulations in the United States, forest soil in Australia, spoil banks and soil crusts in the Czech Republic, water surface in Portugal, and air in Germany and Spain; *A. lentulus*: corn and pepper fields in Korea, soil after herbicide use in Australia, *Coffea* sp. in Denmark, and cocoa beans of unknown origin; *A. pseudoviridinutans*: soils from India and Brazil (environment details unknown) and *Pinus caribaea* in Sri Lanka; *A. udagawae*: soils from prairie and soil from mine waste dump in the United States, plantations in Brazil, spoil bank and soil crust in the Czech Republic, and soils in China, Russia, Thailand, and Turkey (environment details unknown); and *A. wyomingensis*: soils from coal mine accumulations in the United States and soils in China and Russia (environment details unknown). Multiple strains of *A. felis*, *A. udagawae*, and *A. wyomingensis* have been reported in Australia, Brazil, and the United States. Conversely, only one strain each of *A. lentulus* and *A. pseudoviridinutans* has been reported in each of the aforementioned countries. Interestingly, we isolated numerous *A. lentulus* and *A. pseudoviridinutans* species in this study. Reports of isolation of *A. fumigatus*-related species in outdoor environments are incomplete; however, there are many reports of isolation from large animals and also humans. In Japan, most reports were on *A. fumigatus*-related species obtained from clinical sources; there were only few reports on these species obtained from outdoor environments. In this study, we isolated...
Fig. 1 Maximum-likelihood phylogenetic tree of *Aspergillus fumigatus*-related species isolated in this study based on the TUB-2 sequences.

The bootstrap (BS) values (> 50%) are presented at the nodes. The strain name in bold indicates environmental strains. Other strains are clinical strains. The “T” at the end of the strain name indicates the ex type.
A. fumigatus-related species in a wide area of Japan, regardless of the latitude or altitude, as presented in Table 1. So far, although the diversity of this taxon in outdoor environments has not been sufficiently investigated, there have been few reports of its isolation, and it seems to be widely distributed in outdoor environments regardless of the climatic zone.

Through drug susceptibility testing of A. fumigatus-related species from outdoor environments using ITCZ and VRCZ, we were able to identify ITCZ-resistant strains of A. felis and A. pseudoviridinutans and VRCZ-resistant strains of A. felis, A. lentulus, A. udagawae, and A. wyomingensis. In A. felis and A. pseudoviridinutans, we isolated strains that are highly resistant to ITCZ and VRCZ with MICs ≥ 8 mg/L. Studies have tested the susceptibility of these species and found resistant strains with the following MICs: A. felis (MIC against ITCZ: > 16 mg/L, MIC against VRCZ: 8 mg/L), A. lentulus (MIC against ITCZ and VRCZ: 4 mg/L), A. pseudoviridinutans (MIC against ITCZ: > 16 mg/L, MIC against VRCZ: 4 mg/L), A. udagawae (MIC against ITCZ: > 16 mg/L, MIC against VRCZ: 16 mg/L), and A. wyomingensis (MIC against ITCZ: > 16 mg/L, MIC against VRCZ: 4 mg/L)\(^5\), \(^21\), \(^22\), \(^36\).

However, although studies have described the results of the susceptibility testing of clinical and environmental strains, there was no clear genetic differentiation between the strains resistant to ITCZ or VRCZ or both and those that were not resistant in the drug susceptibility testing. The mechanism of drug resistance of A. fumigatus-related species was recently studied by Talbot et al \(^22\), and the M172A/V and D255G mutations in the cyp51A gene associated with the resistance of A. fumigatus to azole were found in the test species (A. arcoverdensis, A. aureolus, A. felis, A. frankstonensis, A. pseudoviridinutans, A. siamensis, A. udagawae, A. viridinutans, and A. wyomingensis). However, the mechanism of drug resistance of A. fumigatus-related species has not yet been elucidated. Although the cyp51A mutation was not analyzed in this study, it should be considered in future studies to clarify the evolutionary process of drug-resistant strains in outdoor environments.

This study demonstrated that A. fumigatus-related species, which adapt to various environments, are widely distributed in outdoor environments and suggested that many of these strains are resistant to ITCZ or VRCZ or both. We are currently conducting ecological studies to elucidate the distribution patterns of A. fumigatus-related species in various outdoor environments in Japan.

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Table 2. Minimum inhibitory concentrations (MICs) of Aspergillus fumigatus-related species resistant to ITCZ and VRCZ isolated in Japan

| Species                  | Number of strains | Source | MIC value (mg/L) |
|--------------------------|-------------------|--------|------------------|
|                          |                   |        | ITCZ             | VRCZ             |
|                          |                   |        | 2 > 2 ≥ 4        | 2 > 2 ≥ 4        |
| *Aspergillus felis*      | 14                | E*     | 4                | 4                |
|                          | 4                 | C**    | 3                | 1                |
| *A. lentulus*            | 4                 | E      | 4                | 4                |
|                          | 6                 | C      | 6                | 2                |
| *A. pseudoviridinutans*  | 17                | E      | 1                | 3                |
|                          | 3                 | C      | 1                | 2                |
| *A. udagawae*            | 34                | E      | 32               | 2                |
|                          | 8                 | C      | 8                | 2                |
| *A. wyomingensis*        | 3                 | E      | 3                | 2                |

*: Environmental, **: Clinical
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Conflicts of interest

The authors declare that they have no conflict of interest.

Transparency declarations

All authors: none to declare.

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