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EPIDEMIOLOGY AND SURVEILLANCE

# Clinical and Microbiological Characteristics of Proven Invasive Aspergillosis Due to Rare/Cryptic Species in Allogeneic Hematopoietic Stem Cell Transplant Recipients

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**ABSTRACT** There are few reports on the clinical course of proven invasive aspergillosis (IA) due to rare/cryptic species in allogeneic hematopoietic stem cell transplant (allo-HSCT) recipients. We retrospectively reviewed the electronic medical records of patients who underwent allo-HSCT between January 2012 and December 2018. Of 934 allo-HSCT recipients, 10 were diagnosed with proven IA and 61 were diagnosed with probable IA. DNA sequencing was performed in cases of proven IA, and *Aspergillus* could be identified to the species level in 8 of the 10 cases. Three were due to *A. fumigatus*, and 5 were due to rare/cryptic *Aspergillus* species, namely, *A. turcosus*, *A. felis*, *A. viridinutans*, *A. nidulans*, and *A. calidoustus*. In these 8 patients, no patients with IA due to *A. fumigatus* died, whereas 3 of the 5 with IA due to rare/cryptic species died within 12 weeks. The 2 surviving cases of IA due to rare/cryptic species were treated with surgical resection and antifungal treatment. Susceptibility testing for cryptic species in 4 cases showed an amphotericin B MIC $>$ 1 mg/L in 3 cases, itraconazole MIC $>$ 1 mg/L in 2 cases, and voriconazole MIC $>$ 1 mg/L in 2 cases. In conclusion, more than half of the causative pathogens of proven IA were rare/cryptic species, so it is important to accurately identify the *Aspergillus* species. In addition, surgical treatment might be an important option in cases of proven IA, given the possibility that the causative organisms are azole-resistant *A. fumigatus* or rare/cryptic species.

**KEYWORDS** invasive aspergillosis, proven invasive aspergillosis, rare species, cryptic species, allogeneic hematopoietic stem cell transplant recipients

*Aspergillus* is a mold with hyaline hyphae that is ubiquitous in the environment. It causes invasive aspergillosis (IA), which is a significant cause of mortality in patients who have undergone allogeneic hematopoietic stem cell transplantation (allo-HSCT) (1). *Aspergillus* includes more than 250 species in 6 subgenera (2). The most common species causing IA is *A. fumigatus*, followed by *A. flavus*, *A. niger*, and *A. terreus* (3). However, reports of cryptic species causing IA are continually increasing as molecular identification becomes more common (4). Because some cryptic *Aspergillus* species tend to exhibit decreased triazole susceptibility (5), they are associated with a poor clinical outcome (6). Although there have been several reports of susceptibility testing for *Aspergillus* cryptic species detected in clinical specimens (4, 7), there are few reports on the frequency of rare/cryptic species in proven IA and on their clinical course in patients who have undergone allo-HSCT (8).



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The authors declare no conflict of interest.

**Received** 16 August 2021
**Returned for modification** 8 September 2021
**Accepted** 10 November 2021

**Accepted manuscript posted online** 15 November 2021
**Published** 18 January 2022

**TABLE 1** Outcomes and underlying conditions in patients with proven IA after HSCT[a]

| Species | Age | Sex | Underlying disease | Graft | GVHD | Neut < 500/$\mu$L | Diagnostic method[b] | Diagnosis | Initial therapy | Surgical debridement | 12-wk mortality |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *A. turcosus* | 20 | F | AML | BMT | − | − | Brain biopsy Day 115 | Brain abscess | L-AMB 5 mg/kg | − | Died |
| *A. felis* | 52 | F | AML | CBT | − | + | Pleural fluid culture Day 7 | IPA | L-AMB 5 mg/kg | − | Died |
| *A. viridinutans* | 57 | M | AML | CBT | − | − | Autopsy Day 46 | IPA | L-AMB 3 mg/kg | − | Died |
| *A. nidulans* | 67 | F | AML | CBT | − | + | VATS Day 39 | IPA | L-AMB 5 mg/kg | + VATS | Survived |
| *A. calidoustus* | 54 | M | AML | CBT | + | − | Brain biopsy Day 199 | Brain abscess | L-AMB 3 mg/kg | + Open craniotomy | Survived |
| *A. fumigatus* | 60 | F | AA, LGL | CBT | − | − | TBLB Day 111 | IPA | VRC + L-AMB 3 mg/kg | − | Survived |
| *A. fumigatus* | 74 | F | MDS | CBT | + | − | Sinus biopsy Day 382 | Rhinosinusitis | L-AMB 5 mg/kg | − | Survived |
| *A. fumigatus* | 68 | M | AML | CBT | − | − | Brain biopsy Day 106 | Brain abscess | VRC | + Open craniotomy | Survived |
| Unknown (pathological diagnosis) | 64 | M | MDS | CBT | + | − | TBLB Day 33 | IPA | L-AMB 5 mg/kg | − | Survived |
| Unknown (pathological diagnosis) | 55 | M | AML | CBT | − | − | TBLB Day 53 | IPA | L-AMB 3 mg/kg | − | Survived |

[a]GVHD, graft-versus-host disease; Neut, neutrophils; AML, acute myeloid leukemia; AA, aplastic anemia; LGL, large granular lymphocytic leukemia; MDS, myelodysplastic syndromes; BMT, bone marrow transplantation; CBT, cord blood transplantation; VATS, video-assisted thoracic surgery; TBLB, transbronchial lung biopsy; IPA, invasive pulmonary aspergillosis.
[b]Days since transplantation.

In this study of allo-HSCT recipients, we aimed to determine the clinical characteristics of proven IA, particularly those of IA caused by rare/cryptic species.

## RESULTS

**Overview of invasive aspergillosis cases.** During the study period, there were 934 cases of allo-HSCT (729 cases of umbilical cord blood transplantation, 122 of bone marrow transplantation, and 82 of peripheral blood stem cell transplantation); 10 (1.1%) were diagnosed with proven IA and 61 (6.5%) were diagnosed with probable IA. Of the 10 cases of proven IA, we could identify the causative *Aspergillus* species in 8 cases. In addition, 5 of the 8 cases were diagnosed with proven IA due to rare/cryptic *Aspergillus* species, namely, *A. turcosus*, *A. felis*, *A. viridinutans*, *A. nidulans*, and *A. calidoustus*. The remaining 3 cases were caused by *A. fumigatus*. In 2 proven cases with a pathological diagnosis based on transbronchial lung biopsy, DNA was extracted from paraffin sections, and the amplified products were sequenced and compared against the database. Both were negative for any fungi.

**Characteristics of the cases.** Table 1 summarizes the clinical characteristics of the 10 patients with proven IA. No patients died of proven IA caused by *A. fumigatus*, but 3 of the 5 patients with proven IA due to rare/cryptic species died. The 2 surviving patients underwent surgical resection and antifungal treatment. Surgical treatment was performed in 3 of the 10 cases: open craniotomy for brain abscess in 2 cases and video-assisted thoracic surgery for invasive pulmonary aspergillosis in 1 case. Five of the 10 patients developed IA without GVHD or neutropenia, and 2 of these 5 patients were on corticosteroids for post-transplant pulmonary complications. Five patients with GVHD and/or neutropenia developed IA in the protective environment; 5 of these patients developed IA outside the protective environment.

Table 2 shows the results of antifungal susceptibility testing of the rare/cryptic *Aspergillus* species. Susceptibility testing for cryptic species in 4 cases and major species in 2 cases revealed an AMB MIC $>$ 1 mg/L, an ITC MIC $>$ 1 mg/L, and a VRC MIC $>$ 1 mg/L in

**TABLE 2** Antifungal susceptibility testing of *Aspergillus* species isolated from cultures[a]

| | | | MIC | | | | IC50 | IC50 | MEC |
|---|---|---|---|---|---|---|---|---|---|
| Case no. | Species | Prophylaxis[b] | AMB | ITC | VRC | POS | FLC | 5FC | MFG |
| Case 1 | *A. turcosus* | MFG | 0.5 | 0.25 | 0.5 | 0.25 | 16 | >64 | <0.015 |
| Case 2 | *A. felis* | MFG | 2 | 8 | 4 | NA | >64 | >64 | 0.03 |
| Case 3 | *A. viridinutans* | ITC | 4 | 2 | 2 | 1 | >64 | >64 | 0.015 |
| Case 4 | *A. nidulans* | FLC | 2 | 0.12 | 0.12 | NA | 64 | >64 | <0.015 |
| Case 5 | *A. calidoustus* | ITC | NA | NA | NA | NA | NA | NA | NA |
| Case 6 | *A. fumigatus* | VRC | NA | NA | NA | NA | NA | NA | NA |
| Case 7 | *A. fumigatus* | VRC | 2 | 8 | 2 | NA | >64 | >32 | 0.015 |
| Case 8 | *A. fumigatus* | L-AMB 3 mg/kg | 0.25 | 0.25 | 1 | NA | >64 | >64 | <0.015 |

[a]MIC, MIC; IC50, half-maximal inhibitory concentration; MEC, minimum effective concentration; AMB, amphotericin B; ITC, itraconazole; VRC, voriconazole; POS, posaconazole; FLC, fluconazole; 5FC, 5-fluorocytosine; MFG, micafungin; L-AMB, liposomal amphotericin B; NA, not available.
[b]Antifungal agents administered on the day of IA onset.

3, 2, and 2 of the 4 cryptic cases, respectively, versus 1 of the 2 major cases for each these antifungal agents.

## DISCUSSION

Here, we determined the clinical and microbiological features of proven IA in allo-HSCT recipients. We obtained 3 major findings. First, rare/cryptic species accounted for more than half of the causative organisms of proven IA. Second, in proven IA, *A. fumigatus* and rare/cryptic species showed similarly poor susceptibility to antifungal drugs (particularly azoles). Third, although 3 of 5 patients with proven IA due to rare/cryptic species died within 12 weeks, the 2 surviving patients were treated with surgical resection and antifungal agents.

In our study, we were able to identify the species in 8 of the 10 cases, and 5 of these 8 cases were IA due to rare/cryptic species. Few studies have described the proportion of causative *Aspergillus* species for proven IA, although a study in Spain described 9 cases of proven IA but did not mention the species (12). In another study, cryptic species considered to be the causative organism of probable or proven IA accounted for 30.5% of cases (13). In addition, epidemiological surveys in Spain (12), Korea (6), and the United States (14) reported that cryptic species accounted for 7.5%–23.1% of *Aspergillus* species detected in clinical specimens, including colonizing *Aspergillus* species. Because these studies examined large numbers of cases and included the clinical pathogens of probable IA or colonization, it is difficult to compare them with our study. The difference in proportion of cryptic species in our study may be due to the very small number of cases, making it difficult to give an accurate percentage. It is also possible that the indication for PCR testing may be differed, given that our study included only cases with proven IA, many of which were cases of surgical biopsy. Probable IA is comprehensively diagnosed based on clinical features, mycological evidence, and patient background (9). Although this diagnosis is an important indicator for determining patients' treatment plan, its accuracy is not exactly clear. Therefore, it is clinically important to describe the proportion of causative species in cases of proven IA.

Interestingly, *Aspergillus* species responsible for proven IA, even *A. fumigatus*, tended to have higher MIC values than the rare/cryptic species (particularly azole). Recently, azole resistance of *A. fumigatus* has been reported in many regions (15–17). In Japan, the percentages of non-wild-type isolates with MICs of ITC, POS, and VRC above the epidemiological cutoff value have been reported as 7.1%, 2.6%, and 4.1%, respectively (18). One study reported that cryptic species tended to be more resistant to multiple antifungal agents than the usual isolated species (*A. fumigatus*, *A. flavus*, *A. terreus*, *A. tubingensis*, and *A. niger*) (13). Because probable IA cases can often be treated without biopsy, the causative organism is likely to be sensitive to antifungal agents. In contrast, proven IA is more likely to be associated with a species that is less susceptible to antifungal drugs because the treatment course is worse, requiring biopsy or surgery. Such a selection bias

may have influenced the high proportion of rare/cryptic species and the poor susceptibility in the present study.

In previous work, the mortality rate of IA after HSCT was high, with a reported 12-week mortality rate of 42.2% (19). Factors that contribute to mortality include the neutrophil count and control of the underlying malignancy (19). In our study, 3 of the 10 patients with proven IA died within 12 weeks. Three patients underwent surgical removal of the infection site and were still alive at 12 weeks. Surgical resection to debride necrotic tissue has been shown to be effective in some cases of IA (20). However, the patients that benefit most from a surgical approach are still uncertain (21). Azole resistance has been associated with overall mortality in patients with IA, even if *A. fumigatus* is the causative fungal species (22). In recent years, strategies to prevent infection with antifungal prophylaxis have been implemented (23). In such situations, with IA patients who respond poorly to antifungal treatment, early surgical intervention might be an important treatment option, given the possibility that azole-resistant *A. fumigatus* or rare/cryptic species are the causative organisms.

Several limitations of our study need to be considered. First, our study is a retrospective single-center study and statistical analysis could not be used to compare cryptic species and major species owing to the small number of cases. However, there have been few reports on the clinical course and epidemiology of the causative *Aspergillus* species of proven IA following allo-HSCT in hospitals with a large number of allo-HSCT cases (934 cases during the study period) (8), and we believe that this report is thus clinically important. Second, we could identify causative *Aspergillus* species in 8 of the 10 cases by PCR analysis. In addition, *Aspergillus* species were detected in the culture in 6 of the 8 cases. Therefore, antifungal susceptibility testing was performed in these 6 cases. Third, susceptibility testing of posaconazole was not available for all patients. However, a previous study showed correlations among triazole MICs for common *Aspergillus* species (*A. fumigatus* and *A. flavus*) (24). Therefore, the MICs for posaconazole of causative *Aspergillus* strains were expected to be high in the causative *Aspergillus* strains that showed higher MICs for the other azoles. Lastly, MIC values were collected throughout the study in accordance with CLSI M38-A2. Although a new version, M38-A3, has recently been published and we need to be careful in interpreting the MIC values, items such as determination time did not change from the previous version, so the MIC values themselves should not be greatly affected.

Further prospective studies with larger numbers of patients and PCR analysis of all cases are needed to elucidate the definitive causative *Aspergillus* species and their clinical course.

More than half of the causative pathogens of proven IA were found to be rare/cryptic species, so it is important to accurately identify the *Aspergillus* species. In addition, surgical treatment might be an important option in cases of proven IA, given the possibility that the causative organisms are azole-resistant *A. fumigatus* or rare/cryptic species.

## MATERIALS AND METHODS

We retrospectively reviewed the electronic medical records, including microbiology records, of patients who underwent allo-HSCT in Toranomon Hospital (Tokyo, Japan) between January 2012 and December 2018. We then identified patients who experienced IA within 12 months after the day of allo-HSCT and reviewed cases with proven IA. The diagnosis of proven or probable IA was based on the criteria of the European Organization for Research and Treatment of Cancer and the Mycoses Study Group Education and Research Consortium (EORTC-MSG) (9). At Toranomon Hospital, the collected specimens were cultured on Sabouraud dextrose agar (Nippon Becton, Dickinson Co., Ltd., Japan), Sabouraud Gentamicin Chloramphenicol 2 agar (bioMérieux Japan, Ltd., Japan) at 26°C, or on Potato Dextrose Agar (Eiken Chemical Co., Ltd., Japan) at 35°C.

Identification was performed through sequencing of the internal transcribed spacer region, the D1/D2 region of the rRNA gene, and the $\beta$-tubulin genes of the isolated *Aspergillus* species. The primers used in this study are shown in the supplemental table (10). The sequenced bases of the isolated *Aspergillus* species were identified by comparison with data in MycoBank (https://www.mycobank.org/) or GenBank (https://www.ncbi.nlm.nih.gov/genbank/). When the sequence bases of the isolated strain had >99% similarity to the reference sequence, this strain was considered to have been identified. The sequence analysis and antifungal susceptibility testing were performed in the National Institute of Infectious Diseases. The *in vitro* susceptibility tests with fluconazole (FLC), voriconazole (VRC), posaconazole (POS), itraconazole (ITC), amphotericin B (AMB), micafungin (MFG), and 5-fluorocytosine (5FC) were

performed according to the Clinical and Laboratory Standards (CLSI) M38-A2 document standard (11). *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 were used as the quality control strains in every susceptibility testing run in this study.

Requests for DNA sequencing were made to the National Institute of Infectious Diseases for all patients diagnosed with proven IA who attended the Department of Infectious Diseases. In cases where PCR could not be performed prospectively because the only pathological diagnosis was for transbronchial lung biopsy, DNA was extracted from paraffin sections and PCR was performed at a later date.

This study was approved by the Human Ethics Review Committee of Toranomon Hospital (approval number 2049).

## SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**, XLSX file, 0.01 MB.

## ACKNOWLEDGMENTS

The identification and drug susceptibility tests were performed by staff of the microbiology laboratory of Toranomon Hospital and the staff of the Department of Fungal Infection, National Institute of Infectious Diseases. We thank Yurika Dantsuji, Nobuko Nakayama, and Yuki Hashimoto for their technical assistance. We also thank Yutaka Takazawa, Department of Pathology, Toranomon Hospital, for the pathological assistance. This research is supported by AMED under grant number JP21fk0108094.

We declare that we have no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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