First case of breakthrough pneumonia due to *Aspergillus nomius* in a patient with acute myeloid leukemia

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We report the first known case of a breakthrough pulmonary infection caused by *Aspergillus nomius* in an acute myeloid leukemia patient receiving caspofungin therapy. The isolate was identified using matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) and sequencing-based methods. The organism was found to be fully susceptible, *in vitro*, to echinocandin antifungal agents.

**Keywords** Aspergillosis, breakthrough, acute myeloid leukemia, *Aspergillus nomius*, antifungal susceptibility

**Introduction**

Recently, *Aspergillus* species other than *Aspergillus fumigatus* have been reported as etiological agents of invasive aspergillosis (IA) [1]. The latter is a major cause of morbidity and mortality in patients with hematological malignancies, particularly those with acute myeloid leukemia [2]. *Aspergillus flavus* is second only to *A. fumigatus* as a cause of invasive pulmonary aspergillosis [3] and appears more virulent and more resistant to antifungal drugs than other *Aspergillus* species [4].

*Aspergillus nomius* is the anamorphic species belonging to *Aspergillus* section *Flavi*, which currently comprises 22 species that can be grouped into seven clades (*A. flavus, Aspergillus tamarii, A. nomius, Petromyces alliaceus, Aspergillus togoensis, Aspergillus leporis, and Aspergillus avenaceus*) based on morphological characters, sequence data, and extrolite profiles [5]. Aflatoxins have been shown to be produced by several species in this section, including the newly described *Aspergillus pseudonomius* sp. nov. and *Aspergillus pseudocaelatus* sp. nov. [5]. Except for *A. flavus*, members of the *Aspergillus* section *Flavi* are rarely reported to cause severe systemic infections in hematological and other patients [6–9]. *A. nomius* has thus far only been isolated from a single case of keratitis after ocular injury [10].

**Case report**

In July 2009, a 66-year-old man was admitted to our hospital for fatigue and dyspnea, with a previously negative clinical history. A diagnosis of *de novo* acute myeloid leukemia (AML) was made after examination of peripheral blood and bone marrow. Routinely performed fungal surveillance cultures at admission did not reveal the presence of fungi. After diagnosis, the patient was started on induction chemotherapy (mitoxantrone, etoposide, and cytarabine) and antifungal prophylaxis with oral non-absorbable polyenes. The day after the last course of chemotherapy (day 1), the patient became febrile, was deeply neutropenic (neutrophil count of 50/mm$^3$), and developed a persisting diarrhea. A routine chest X-ray was negative. The first set of blood, sputum, and stool samples were collected for culture and ceftazidime ($2 \times 4.5$ g/day) and amikacin (15 mg/kg/day) were started empirically. Since cultures were negative on day 5 but the patients symptoms persisted, therapy with liposomal amphotericin B (3 mg/kg/day) was started. While a chest computed tomography (CT) was negative, an abdomen CT showed thickening of the colon and inflammatory stranding of the adjacent mesenteric fat, which were compatible with an infectious enterocolitis.
On day 10, a culture of a second stool sample yielded *Candida albicans* and the antifungal was switched to intravenous caspofungin (loading dose 70 mg/day, followed by 50 mg/day). On day 20, the neutrophil count rose to 1,310/mm³ and the abdominal symptoms disappeared, but he developed cough and dyspnea. Serum specimens, obtained on day 20 and thereafter, tested positive for (1→3) β-D-glucan (BDG; Fungitell, Associates of Cape Cod Inc, Falmouth, MA, USA with cut-off of 80 pg/ml) (Fig. 1).

On day 24, a new chest CT scan revealed a nodule with a halo sign in the right upper lobe and a concomitant serum galactomannan (GM) assay was positive (Platelia Aspergillus; Bio-Rad, Marnes-la-Coquette, France; optical density index cut-off of 0.5) (Fig. 1). At this point the patient was started on antifungal therapy with intravenous voriconazole (loading dose 2 × 6 mg/kg/day, followed by 2 × 4 mg/kg/day). In the meantime, second and subsequent sputum specimens were collected. Microscopic examination of these samples revealed numerous hyphae, and portions of these samples inoculated on Sabouraud dextrose agar (SDA) yielded *Aspergillus nomius* after 3–5 days incubation at 30°C. On day 38, after 14 days of voriconazole treatment, a chest CT scan showed initial resolution of the pulmonary lesion and BDG and GM serum levels had gradually decreased (Fig. 1). On day 40, the patient was discharged on oral voriconazole therapy (2 × 200 mg/day). The patient was followed by monitoring GM and BDG serum levels which eventually became negative on day 59 or the 35th day of voriconazole therapy. Two months after discharge, the patient’s conditions deteriorated and he died for chemotherapy-refractory AML. Post-mortem examination documented no foci of fungal pneumonia.

For morphological studies [11], the clinical isolate was subcultured onto Czapek Dox yeast extract and malt extract agar plates. The colonies were velvety to floccose, consisting of white or light orange-brown aerial hyphae with sparse to moderately abundant conidia. The reverse of the colonies were light yellow or orange-brown. No sclerotia were observed. Conidiophores were echinulate and hyaline, with globose to subglobose vesicles and biseriate phialides (Fig. 2). Conidia were globose to subglobose, and echinulate (Fig. 2). On the basis of these microscopic and macroscopic characteristics, the fungus was identified as member of the *Aspergillus* section *Flavi*.

MALDI-TOF-based species identification of the clinical isolate was performed as previously described [12]. Briefly, the surface of the fungal colony grown on SDA was scraped and the resulting mixture of hyphae and conidia was directly spotted onto a polished steel target plate (Bruker Daltonics, Bremen, Germany). After mixing with absolute ethanol, a saturated solution of α-cyano-4-hydroxy cinnamic acid in 50% acetoni trile-2.5% trifluoroacetic acid (Bruker Daltonics) was added and allowed to co-crystallize with the sample at room temperature. Measurements were performed with a microflex LT mass spectrometer (Bruker Daltonics) [12]. The spectrum obtained matched the expected reference spectrum included in a inhouse-upgraded MALDI-TOF MS database, resulting in a log(score) value > 2.0, which was used for identification at the species level. Cluster analysis based on a matrix of pairwise correlation values [12] for spectra of selected reference strains belonging to section *Flavi* and our clinical isolate resulted in separate clusters at the species level (Fig. 3). Interestingly, the patient’s isolate sub-clustered with the *Aspergillus oryzae* strains and this cluster was distinct from that of *A. flavus* and *Aspergillus alliaceus*.

For molecular studies, the isolate was sub-cultured on SDA at 37°C for 5 days. DNA from fungal cultures was extracted using the DNeasy plant DNA extraction minikit (Qiagen, Milan, Italy) according to the manufacturer’s instructions. PCR amplification was performed using fungus-specific primers for the internal transcribed spacer 1 and 2 regions flanking 5.8S ribosomal DNA (rDNA) (ITS1-5.8S-ITS2) [11] and portions of the β-tubulin and calmodulin genes [13,14]. PCR products were sequenced as previously described [15], and species identification was performed by searching databases using the BLAST sequence analysis tool (http://www.ncbi.nlm.nih.gov/BLAST/). The isolate was assigned to *A. nomius* based on the complete identity with the corresponding sequences of the *A. nomius* type strain CBS 260.88. Antifungal susceptibility testing was performed using the reference broth microdilution method of the Clinical and Laboratory Standards Institute document M38-A2 [16]. *Aspergillus fumigatus* ATCC 2004305 and *Aspergillus flavus* ATCC 2004304 were employed as quality control strains. Minimum inhibitory concentrations (MIC) at 48 h were obtained for...
amphotericin B, fluconazole, itraconazole, voriconazole, and posaconazole, whereas minimum effective concentrations (MECs) at 48 h were collected for caspofungin, micafungin, and anidulafungin. Antifungal susceptibility results were as follows (MIC and MEC values in mg/l): amphotericin B 0.5, fluconazole 128, itraconazole 0.016, voriconazole 0.06, posaconazole 0.016, caspofungin 0.008, micafungin 0.008, and anidulafungin 0.016.

Discussion

Given that IA remains one of major clinical problems in hematological patients, rapid discrimination among the increasing diversity of filamentous fungal etiologic species is very important for the physician in a decision-making algorithm that can guide patient management [14]. The genus *Aspergillus* is divided into several sections or complexes that encompass nearly 200 species, of which approximately 40 have been reported in cases of human aspergillosis [17]. Thus, identifying individual *Aspergillus* species is sometimes clinically important, as for example *A. flavus*-like species which can demonstrate variable antifungal susceptibility, and the subsequent treatment may be challenged by drug resistance [6,7]. Since some *Aspergillus* species have overlapping morphological features, it is quite difficult to establish their
identification on the basis of phenotypic features [13]. In this regard, while comparative sequence analyses of one or several gene regions are required for molecular species-level identification within Aspergillus complexes, MALDI-TOF is sufficiently robust to support species-level identification of clinical isolates in a timely and straightforward manner [12].

During the last decades, the availability of less toxic antifungal drugs (i.e., liposomal amphotericin B and caspofungin) have facilitated their use in empirical treatment of patients with acute leukemia and prolonged neutropenia or HSCT recipients [18] which may result in the selection of resistant fungal strains [19]. While a high rate of breakthrough IAs was observed among patients receiving empirical antifungal therapy for persistent fever and neutropenia, recent data suggest that not all antifungal agents may have the same efficacy in preventing such breakthrough invasive fungal infections [20]. In the latter investigation, all breakthrough IA cases occurred with patients being empirically treated with caspofungin, as opposed to none of the patients treated with amphotericin B [20]. In our case, we initially instituted empirical therapy with liposomal amphotericin B when the patient’s fever persisted despite administration of broad spectrum antibacterials. However, because of the intervening abdominal Candida infection, antifungal therapy was changed to caspofungin, a drug widely used due to its excellent safety profile [21]. Our therapeutic choice was supported by the absence in our patient, prior to the diagnosis of IA, of evidence of a fungal infection obtained with chest X-ray and the repeatedly negative results in the surveillance Aspergillus GM tests. It is unclear how the in vitro MEC correlates with echinocandin treatment outcome, although less susceptible isolates have been recovered from patients with treatment failure [22]. Nonetheless, in vitro antifungal susceptibility testing of our A. nomius isolate revealed an organism fully susceptible not only to caspofungin but also to micafungin and anidulafungin. This is consistent with a previous study showing that among the Aspergillus strains isolated from nine hematopoietic stem cell transplant (HSCT) recipients treated empirically with caspofungin only two exhibited high MICs to this antifungal [23]. In this context, it should be recalled that caspofungin is the only echinocandin to be licensed in Europe for the treatment of IA in adult or pediatric patients whose infections are refractory to or intolerant of other therapies (i.e., amphotericin B, lipid formulations of amphotericin B and/or itraconazole). Although experience with caspofungin as first-line therapy for IA has been reported in just two prospective trials, the antifungal contributed to 33% complete response rate among patients with acute leukemia and allogeneic HSCT recipients [24,25].

Caspofungin is only fungistatic against Aspergillus in vitro, whereas voriconazole and liposomal amphotericin B are fungicidal, and this might translate into lower clinical efficacy [20]. Therefore, we are not surprised to see in our patient a progressive improvement, until resolution of clinical and radiological findings attributable to IA after the initiation of voriconazole therapy. Notably, the therapeutic choice of voriconazole was also dictated by positive GM and BDG test results that enabled us to promptly start antifungal therapy before the recovery of the etiologic agent in culture. In addition, the gradual decrease of GM and BDG levels observed in our patient during voriconazole therapy adds further support to the role of fungal antigens as surrogate markers for both diagnosis and treatment monitoring of IA [26–28].

In conclusion, our case shows that clinicians should be aware of a possible breakthrough Aspergillus species infection in neutropenic patients, especially those with acute myeloid leukemia treated with chemotherapy who develop invasive fungal infections while receiving an echinocandin. Further investigations are needed to better define the types of fungal pathogens and their frequency causing breakthrough mycoses.

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References

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