

The robust and rapid role of molecular testing in precision fungal diagnostics: A case report



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ABSTRACT

Diagnosis of invasive fungal disease remains an ongoing challenge for clinicians, while continuously evolving treatment regimens increase patient risk for invasive infection. This case highlights how molecular testing led to the diagnosis of co-infection with two fungal pathogens producing invasive disease in a hematopoietic stem cell transplant recipient with graft-versus-host disease (GVHD).

1. Introduction

We present the case of a hematopoietic stem cell transplant recipient with graft-versus-host disease (GVHD) who, in the presence of augmented immunosuppression, developed evidence of breakthrough invasive fungal infection. In this report, we highlight novel diagnostic tools that were integral to accurate identification of the two fungal organisms responsible for this presentation, as well as the current and future role for molecular testing in diagnosis of invasive fungal disease.

2. Case

A 62-year-old male was diagnosed with acute myeloid leukemia, transformed from chronic myelomonocytic leukemia, and underwent induction chemotherapy with cytarabine and daunorubicin (day – 60). During this time, he received acyclovir and posaconazole prophylaxis. Two months later he underwent busulfan/fludarabine conditioning and matched sibling hematopoietic stem cell transplantation (day 0). At that time, antimicrobial prophylaxis was switched to acyclovir, voriconazole, and dapsone. One month after transplant, his tacrolimus was transitioned to cyclosporine due to patient headaches and concerns for toxicity (day +30).

Six months after transplant, due to liver function test (LFT) de-regulations, antifungal prophylaxis was changed from voriconazole to fluconazole (day +180). Still, lab abnormalities persisted and he

subsequently demonstrated evidence of GVHD of the skin. He was started on high-dose prednisone 2 mg/kilogram (mg/kg) with a gradual taper and continued on cyclosporine. Due to progressive GVHD involving the skin, liver, mouth, and eye, ruxolitinib was added.

One year after transplant, his LFTs and skin rash had improved, but he developed worsening exertional dyspnea (day +400). Pulmonary function testing showed a decrease in forced expiratory volume and chest computed tomography (CT) scan demonstrated subtle ground glass opacities without cavitation, mild air trapping on expiratory images, and bronchiectasis. Due to high clinical suspicion for bronchiolitis obliterans syndrome and concern for risks of the procedure, bronchoscopy was deferred. Prednisone and ruxolitinib doses were both increased and he was started on scheduled azithromycin and extracorporeal photopheresis twice weekly.

However, two months later (day +460), due to worsening dyspnea and a new oxygen requirement, a chest CT scan was repeated and showed progressive upper lung groundglass opacities and centrilobular nodules. He was started on intravenous (IV) methylprednisolone and empiric broad-spectrum antibiotics and fluconazole was changed to voriconazole for mold prophylaxis in the setting of augmented immunosuppression. A noninvasive infectious workup, including respiratory viral polymerase chain reaction (PCR) and serum galactomannan antigen testing, was otherwise unrevealing. Serum beta-D-glucan (BDG) was elevated to 149 pg/mL (reference: > 80 pg/mL). He received an empiric course of antibacterials and was continued on

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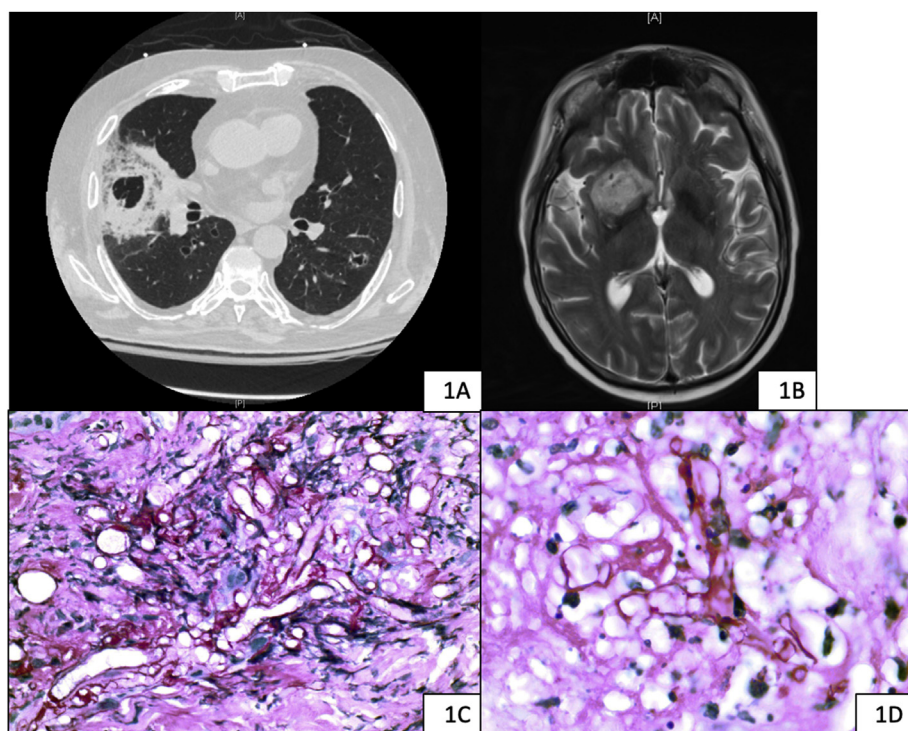


Fig. 1. 1A. Chest CT with a thick-walled cavitary lesion in the right middle lobe with substantial surrounding consolidative and groundglass opacities. 1B. MR Brain demonstrating a 3.3 cm hyperintense lesion in the right frontal lobe. 1C, 1D. Skin biopsy histopathology: fungal hyphae, wide with varying morphology from ribbon-like to round with no definitive septa identified (PAS/D 40X, 100X).

prednisone 60mg twice daily with a gradual taper, along with cyclosporine and ruxolitinib for presumed GVHD flare. His breathing initially improved, but he remained oxygen dependent, requiring 2 L per minute via nasal cannula at rest. Three weeks later, he was tapered off ruxolitinib and started ibrutinib (day +490).

One week after starting ibrutinib, he returned with progressive dyspnea, myalgias, and weakness (day +500). He was hypothermic and tachycardic on presentation with evidence of acute kidney injury (creatinine increased to 2.5 mg/dL from a baseline from 0.8) and mild rhabdomyolysis (creatinine kinase increased to 1200 U/L). Transaminases remained stably mildly elevated and voriconazole trough level was elevated at 6.1 µg/mL. Repeat chest CT scan demonstrated interval development of thick-walled cavitary lesions, with additional consolidative and groundglass opacities, predominantly in the bilateral upper lobes (Fig. 1A).

He was commenced on IV meropenem, tedizolid, and isavuconazole empirically. Within 24 hours, his renal function had improved, and IV liposomal amphotericin was added (day +501). Blood cultures were negative. Repeat serum BDG was increased to 359 pg/mL (from 149 pg/mL one month prior), while serum galactomannan antigen, urine histoplasma antigen, and serum cryptococcal antigen testing was negative. Bronchoscopy was performed (day +501) and notable for brown, thick secretions throughout the lungs, worse in the right middle and lower lobes. Bronchoalveolar lavage (BAL) galactomannan antigen was positive at > 6.3. BAL cytology demonstrated rare forms suggestive of fungal hyphae on Grocott's methenamine silver (GMS) stain and no evidence of malignancy. Fungal cultures grew a mold identified as *Aspergillus fumigatus*.

Two days later (day +503), he was noted to have a fixed and dilated left pupil. Magnetic resonance imaging (MRI) of the brain showed a 3.3 cm hyperintense lesion in the right frontal lobe and a 1.5 cm additional lesion in the right occipital lobe (Fig. 1B). A necrotic skin lesion was noted on the right inner thigh and biopsied. Pathology of the lesion was consistent with angioinvasive fungal infection. Wide hyphal forms were identified on Periodic Acid-Schiff (PAS/D) stains, with varying morphology from ribbon-like to round with no septa, suggestive of Mucorales (Fig. 1C and D). Skin biopsy bacterial, mycobacterial, and fungal stains – specifically GMS – and cultures were unrevealing.

After his skin biopsy and BAL culture results were known, combination antifungal therapy with IV micafungin 150 mg every 24 hours, IV liposomal amphotericin 5 mg/kg every 24 hours, and IV isavuconazole 372mg every 24 hours was continued (day +504). However, he became progressively more lethargic, and required increasing amounts of vasopressor support. His care was transitioned to focus on comfort measures and he passed away rapidly (day +505).

Subsequent results returned post-mortem. Antifungal susceptibility testing (AST) of the *Aspergillus* isolate (performed by broth dilution at a referral laboratory) [1], demonstrated minimum inhibitory concentrations (MICs) of 0.06 µg/mL for posaconazole, 2 µg/mL for voriconazole (epidemiologic cutoff value [ECV] 1 µg/mL), and 2 µg/mL for isavuconazole (ECV 1 µg/mL), suggestive of a non-wild-type isolate [2]. No susceptibility testing was performed for amphotericin, as resistance to this drug was felt to be low, given lack of prior exposure. Additionally, before death, microbial cell-free DNA next-generation sequencing (NGS) had been sent from plasma (Karius Test, Redwood City, CA) [3]. This returned within 48 hours (day +507) and demonstrated the presence of *Cunninghamella* at 222,057 DNA molecules per microliter (reference < 10 molecules per microliter), as well as *Aspergillus lentulus* in lesser amounts. Given the discrepancy of these findings with available microbiology, additional testing was performed to clarify the diagnosis. Due to the unusual antifungal susceptibility profile, the BAL isolate identified as *A. fumigatus* was sent for DNA sequencing (using beta-tubulin and calmodulin partial gene sequence targets) and confirmed to be *Aspergillus lentulus*, a cryptic species within the *A. fumigatus* complex that exhibits decreased susceptibility to azoles, amphotericin B, and echinocandins and has been associated with refractory cases of invasive aspergillosis (IA) [4]. In addition, the skin tissue specimen was sent for broad-range fungal PCR [5], which confirmed co-infection due to *Cunninghamella bertholletiae* or *polymorpha* by 18S ribosomal DNA sequencing.

3. Discussion

This case highlights several important points involved in the care of severe and chronically immunocompromised populations. The first is that the combination of multiple immunosuppressive agents,

particularly ibrutinib, significantly increases the risk for invasive fungal infection (IFI). Ibrutinib is an irreversible inhibitor of Bruton tyrosine kinase (BTK), a component of B-cell receptor signaling and important for B-cell proliferation. It is also expressed by macrophages and involved in phagocytosis and neutrophil recruitment [6–8]. Ibrutinib was approved in 2013 by the FDA for use in B-cell malignancies [9] and in 2017, it was the first FDA-approved therapy for second-line chronic GVHD [10]. Several authors have reported on the emergence of opportunistic IFIs among patients treated with ibrutinib, particularly when used in combination with high doses of corticosteroids [11–13]. These infections often occur early in the course of treatment initiation and present with extra-pulmonary manifestations, particularly with rapid central nervous involvement, similar to that seen in our patient.

Additionally, this case significantly emphasizes the potential role for novel fungal diagnostics, specifically molecular testing, in securing more precise pathogen identification in a timely manner. The current gold standard for diagnosis of proven IFI is identification of pathogen on histopathology or culture. However, in many instances, due to patient- and procedure-specific factors, tissue samples are difficult to obtain, and there may be a delay to positivity or lack of growth on culture. Furthermore, the presence of a fungus (or fungal elements) on culture from a non-sterile site does not always represent invasive disease. Serum fungal markers, such as the galactomannan and BDG, can aid in diagnosis but have variable sensitivity and specificity, and are prone to both false positive and negative results [14–16]. Our patient repeatedly had negative serum galactomannan testing, likely due to the fact that he was on prophylactic antifungal therapy throughout his clinical course.

In instances where cultures and serologic markers may be unreliable, supplemental molecular testing can be valuable, particularly NGS. We utilized Karius testing, a non-invasive plasma-based NGS platform that examines circulating microbial cell-free DNA present in the setting of deep-seated and disseminated infections [17]. This proved to be a useful aid in identifying a Mucorales, even in the presence of negative cultures. This testing is one of an increasing number of NGS options that offer the ability to rapidly diagnose a variety of different pathogens. Furthermore, in our case, advanced PCR methods with 18S sequencing of tissue confirmed the presence of this additional mold (*Cunninghamella*) on skin biopsy when cultures were negative. While our patient did have histopathologic evidence of a Mucorales on tissue biopsy, had his skin lesion not been detected on thorough physical exam, tissue would not have been obtained, leading to a potential delay in diagnosis. Had peripheral blood NGS been performed earlier, our patient may have been able to avoid biopsy altogether. Thus, this testing may be an opportunity to reduce invasive procedures in future patients, particularly in high-risk critically ill populations where such procedures carry significant risk.

Finally, this case highlights how molecular testing can clarify diagnostic and treatment dilemmas that arise when traditional microbiologic data does not clearly fit with the clinical context, as well as the recognition of mixed infections in severely immunocompromised populations. In our case, with standard microbiologic phenotypic techniques, respiratory cultures originally identified the mold as *A. fumigatus*. However, this was later confirmed to be *A. lentulus* based on Karius testing and targeted fungal DNA sequencing. This was similarly described previously in a case series of patients with IFIs identified by cell-free DNA NGS [18]. Sequencing can be an added tool to other rapid identification platforms such as time-of-flight mass spectrometry (MALDI-TOF) [19]. *A. fumigatus* and *A. lentulus* share greater than 90% nucleotide homology and appear morphologically similar on culture. However, differentiation between species is important as *A. lentulus* shows decreased *in vitro* susceptibility to many triazoles, as reflected in our case, which can significantly impact clinical management [20]. Our patient's isolate demonstrated MICs higher than the ECV for *A. fumigatus* across multiple antifungals, which suggested acquired or intrinsic resistance. Although AST was available for this patient, species

identification allowed for additional clarity in identifying the accurate pathogen and ensuring appropriate therapy. This level of clarity can enhance our understanding of the epidemiology of invasive fungal disease and can be especially useful in outbreak settings. While it is not currently feasible to identify all *A. fumigatus* isolates to the species level due to limitations in cost and time expenditure, this testing should be a strong consideration in certain cases, particularly if there is a concern for poor response to antifungal therapy or demonstration of unusual antifungal resistance patterns.

Two main barriers exist to full diagnostic application of such tools. One is the financial cost of novel sequencing methods, though this should decrease as analytic methods improve. It is also important to understand the need for cautious interpretation of NGS and other metagenomics results in the context of the overall clinical picture of the patient. Such tests have the potential to pick up all identified microbial pathogens (including colonizing or host organisms), not just those producing disease in the patient, increasing the potential for false positives in testing. Thus (as with traditional microbiologic data), providers should not solely treat a positive test before taking into consideration if the listed pathogen(s) is consistent with the patient's presentation.

In conclusion, patients with hematologic malignancies and particularly those on ibrutinib remain at high risk for a number of IFIs that can present rapidly and as co-infections requiring different management strategies. This case highlights a need for improved fungal diagnostics and highlights the potential role for molecular testing to aid in real-time clinical decision-making and better precision medicine.

Ethical Form

Please note that this journal requires full disclosure of all sources of funding and potential conflicts of interest. The journal also requires a declaration that the author(s) have obtained written and signed consent to publish the case report from the patient or legal guardian(s).

The statements on funding, conflict of interest and consent need to be submitted via our Ethical Form that can be downloaded from the submission site www.ees.elsevier.com/mmcr. **Please note that your manuscript will not be considered for publication until the signed Ethical Form has been received.**

Declaration of competing interest

D.K.H was a previous employee of Karius.

S.P.B. received salary support as principle investigator and site investigator in studies of the Karius test in immunocompromised pneumonia. He also is a consultant for pneumonia therapeutics program for Armata Pharmaceuticals.

E.K.M. is serving on the Clinical Adjudication Committee for the Karius PICKUP study.

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References

- [1] CLSI, Reference method for broth dilution antifungal susceptibility testing of filamentous fungi, Approved Standard - Second Edition. CLSI Document M38–A2, Clinical and Laboratory Standards Institute, Wayne, PA, 2008.
- [2] CLSI, Epidemiological cutoff values for antifungal susceptibility testing, CLSI Supplement M59, second ed., Clinical and Laboratory Standards Institute, Wayne, PA, 2018.
- [3] T.A. Blauwkamp, S. Thair, M.J. Rosen, et al., Analytical and clinical validation of a microbial cell-free DNA sequencing test for infectious disease, *Nat Microbiol.* 4 (4) (2019) 663–674.
- [4] F. Lamoth, *Aspergillus fumigatus*-related species in clinical practice, *Front. Microbiol.* 7 (2016) 683.

- [5] D. Marshall, D.J. SenGupta, D.R. Hoogestraat, et al., Multilocus sequencing for rapid identification of molds, CAP Today Feature Story (February 2013).
- [6] S. Fiorcari, R. Maffei, V. Audrito, et al., Ibrutinib modifies the function of monocyte/macrophage population in chronic lymphocytic leukemia, *Oncotarget* 7 (40) (2016) 65968–65981.
- [7] J.A. Woyach, Ibrutinib and Aspergillus: a Btk-targeted risk, *Blood* 132 (18) (2018) 1869–1870.
- [8] A. Bercusson, T. Colley, A. Shah, A. Warris, D. Armstrong-James, Ibrutinib blocks Btk-dependent NF- κ B and NFAT responses in human macrophages during *Aspergillus fumigatus* phagocytosis, *Blood* 132 (18) (2018) 1985–1988.
- [9] E.D. Deeks, Ibrutinib: a review in chronic lymphocytic leukaemia, *Drugs* 77 (2) (2017) 225–236.
- [10] S.M. Jaglowski, B.R. Blazar, How ibrutinib, a B-cell malignancy drug, became an FDA-approved second-line therapy for steroid-resistant chronic GVHD, *Blood Adv.* 2 (15) (2018) 2012–2019.
- [11] T. Varughese, Y. Taur, N. Cohen, et al., Serious infections in patients receiving ibrutinib for treatment of lymphoid cancer, *Clin. Infect. Dis: Off. Publ. Infect. Dis. Soc. Am.* 67 (5) (2018) 687–692.
- [12] M. Baron, J.M. Zini, T. Challan Belval, et al., Fungal infections in patients treated with ibrutinib: two unusual cases of invasive aspergillosis and cryptococcal meningoencephalitis, *Leuk. Lymphoma* 58 (12) (2017) 2981–2982.
- [13] G. Chamilos, M.S. Lionakis, D.P. Kontoyiannis, Call for action: invasive fungal infections associated with ibrutinib and other small molecule kinase inhibitors targeting immune signaling pathways, *Clin. Infect. Dis: Off. Publ. Infect. Dis. Soc. Am.* 66 (1) (2018) 140–148.
- [14] M.M. Leeflang, Y.J. Debets-Ossenkopp, J. Wang, et al., Galactomannan detection for invasive aspergillosis in immunocompromised patients, *Cochrane Database Syst. Rev.* 12 (2015) Cd007394.
- [15] K.A. Marr, S.A. Balajee, L. McLaughlin, et al., Detection of galactomannan antigenemia by enzyme immunoassay for the diagnosis of invasive aspergillosis: variables that affect performance, *J. Infect. Dis.* 190 (3) (2004) 641–649.
- [16] F.M. Marty, S. Koo, Role of (1- \rightarrow 3)-beta-D-glucan in the diagnosis of invasive aspergillosis, *Med. Mycol.* 47 (Suppl 1) (2009) S233–S240.
- [17] Armstrong AE, Rossoff J, Holleman D, et al. Cell-free DNA next-generation sequencing successfully detects infectious pathogens in pediatric oncology and hematopoietic stem cell transplant patients at risk for invasive fungal disease. *Pediatric Blood & Cancer.*0(0):e27734.
- [18] D.K. Hong, T.A. Blauwkamp, M. Kertesz, et al., Liquid biopsy for infectious diseases: sequencing of cell-free plasma to detect pathogen DNA in patients with invasive fungal disease, *Diagn. Microbiol. Infect. Dis.* 92 (3) (2018) 210–213.
- [19] P.E. Verwer, W.B. van Leeuwen, V. Girard, et al., Discrimination of *Aspergillus lentulus* from *Aspergillus fumigatus* by Raman spectroscopy and MALDI-TOF MS, *Eur. J. Clin. Microbiol. Infect. Dis: Off. Publ. Eur. Soc. Clin. Microbiol.* 33 (2) (2014) 245–251.
- [20] S.A. Balajee, J.L. Gribskov, E. Hanley, D. Nickle, K.A. Marr, *Aspergillus lentulus* sp. nov., a new sibling species of *A. fumigatus*, *Eukaryot. Cell* 4 (3) (2005) 625–632.