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Refractory invasive pulmonary aspergillosis due to *Aspergillus flavus* detected with the combination of two in-house *Aspergillus* qPCR.

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Running head: invasive pulmonary aspergillosis due to *A. flavus*

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Abstract

We present a case of probable invasive pulmonary aspergillosis due to *Aspergillus flavus*, in a female patient treated for an acute myeloid leukemia. Two weeks after an allogenic stem cell transplantation a probable invasive pulmonary aspergillosis was diagnosed based on thoracic imaging combined with positive galactomannan antigen and positive in-house mitochondrial *Aspergillus* qPCR in serum. Although an antifungal treatment was initiated, *Aspergillus* qPCR and galactomannan antigen remained positive in serum and worsening of the thoracic lesions was observed. The discordance between the negativity of the in-house ribosomal *Aspergillus* qPCR (specific to *A. fumigatus*) and the positivity of the in-house mitochondrial *Aspergillus* qPCR (targeting *A. fumigatus* and some other *Aspergillus*) allowed the suspicion of a thermophilic *Aspergillus* species that was not *A. fumigatus*. No strain was obtained in culture but the involvement of *A. flavus* was confirmed using a specific *A. flavus* qPCR. This case illustrated the usefulness of our original strategy combining two different in-house *Aspergillus* qPCRs, in addition to galactomannan assay, to diagnose invasive aspergillosis in hematology patients.

Key words: *Aspergillus flavus*, invasive pulmonary aspergillosis, *Aspergillus* qPCR, galactomannan antigen, amphotericin B

Introduction

Invasive aspergillosis (IA) remains a clinically challenging opportunistic invasive fungal disease (IFD) in heavily immunocompromised patients. *Aspergillus fumigatus* is in cause of approximately 80% of IA; *A. flavus* being the second most frequent pathogenic species (approximately 15-20%) [1]. Allogenic hematopoietic stem cell transplantation (HSCT) is the only curative option for several hematological malignancies but is associated with prolonged periods of neutropenia. More than 1660 cases of IA were reported to the French National Referent Center of Invasive Fungal Disease from 2012 to 2018 [2]. The global mortality associated with IA remains high in France: 42.5% at 3 months reported by the French National Referent Center of Invasive Fungal Disease [2]. The early diagnosis of IA and the prompt initiation of antifungal therapy have been shown to reduce mortality in patients with IA [3].

For the past several years, our center has been applying a strategy of systematic IA screening combining the detection of galactomannan antigen (GM) detection and two in-house *Aspergillus* qPCRs [4,5]. The strategical choice of combining a mitochondrial and a ribosomal *Aspergillus* qPCR aimed at optimizing information on the *Aspergillus* species involved, while using a minimum quantity of DNA. Thus, instead of duplicating the same assay, we made the choice to perform two different complementary qPCRs [4-6]. The ribosomal *Aspergillus* qPCR targets a 18S area specific of *Aspergillus fumigatus*, which is a very repeated target (estimated from 38 to 91 copies per genome [7]), thus, this qPCR is very sensitive. On the flipside, the mitochondrial *Aspergillus* qPCR targets a less repeated area (estimated at between 9 and 10 mitochondrial copies per single-copy gene [8]), but the targeted area is common to other *Aspergillus* thermophilic species (*A. flavus*, *A. terreus*, *A. nidulans* and *A. niger*), thus this qPCR is less sensitive but allow the detection of other thermophilic *Aspergillus* species. This strategy has proven useful for early detection of IA [4-6].

66 We report here a case of probable invasive pulmonary aspergillosis due to *Aspergillus flavus*
67 that was diagnosed thanks to our local strategy of combining two in-house *Aspergillus*
68 qPCRs.

69

Case report

A 58-year-old female patient was diagnosed with acute myeloid leukemia (AML) with complex karyotype and received an allogenic HSCT (D0) five months after the AML diagnosis. The last myelogram, after induction chemotherapy with idarubicin and aracytin, salvage and consolidation chemotherapy with amsacrine and etoposide, showed 6% of myeloblasts and the thoracic computed tomography (CT) scan before the allogenic HSCT was normal. The antifungal prevention was fluconazole from the day the conditioning regimen was begun.

On D14 the patient presented fever and thoracic pain. On D15, the chest CT showed lobar condensations and the galactomannan antigen (GM) was positive in serum (index 3.3). Both in-house *Aspergillus* qPCRs were negative. An antifungal treatment by isavuconazole was initiated, with 200 mg every 8 h during 2 days and then 200 mg per day (Figure 1). On D18, GM was still positive and the mitochondrial *Aspergillus* qPCR (mqPCR) turned positive (GM >3.5, Cycle quantification (Cq) 34; Figure 1). A bronchoalveolar lavage fluid (BALF) was performed on D18: direct examination was negative, the fungal culture remained sterile, the *Aspergillus* qPCRs were negative and the galactomannane was positive (>3.5). The patient didn't have any fever at that time. The patient needed oxygen therapy from D20. Two tracheal aspiration were send to the laboratory for fungal culture on D22 and D24 but the cultures remained sterile.

On D24, the antifungal treatment was changed for liposomal amphotericin B (3 mg/kg/day) because of fever recurrence and worsening of the lesions on chest CT: GM and mqPCR were still positives in serum (GM >3.5, Cq 36; Figure 1). On D35 the patient was still in aplasia and was transferred in Intensive Care Unit (ICU) for acute respiratory symptoms. Unfortunately, the patient was refractory to allogenic HSCT and relapsed from the leukemia. She died on D40.

95

96 ***Aspergillus flavus* qPCR**

97 DNA extraction from 1 mL of serum (D21) was performed using a Large Volume MagNa
98 Pure Nucleic Acid Isolation Kit on a MagNa Pure Compact apparatus (Roche Diagnostics,
99 Meylan, France).

100 The different sets of primers and probe targeting *A. flavus* designed by Environnemental Pro-
101 tection Agency (EPA) of Unites States (available on [https://irp-](https://irp-cdn.multiscreensite.com/c4e267ab/files/uploaded/gCQnkBNWQuSD96fPlIkY_EPA_Technology%20for%20Mold%20Identification%20and%20Enumeration.pdf)
102 [cdn.multiscreensite.com/c4e267ab/files/uploaded/gCQnkBNWQuSD96fPlIkY_EPA_Technol-](https://irp-cdn.multiscreensite.com/c4e267ab/files/uploaded/gCQnkBNWQuSD96fPlIkY_EPA_Technology%20for%20Mold%20Identification%20and%20Enumeration.pdf)
103 [ogy%20for%20Mold%20Identification%20and%20Enumeration.pdf](https://irp-cdn.multiscreensite.com/c4e267ab/files/uploaded/gCQnkBNWQuSD96fPlIkY_EPA_Technology%20for%20Mold%20Identification%20and%20Enumeration.pdf)) were used as a specific
104 qPCR assay [9]. The qPCR was performed in duplicate in a 20 µL final volume (10 µL of
105 master mix Gene Expression from Applied, 5 µL DNA, 1000 nM primers, 500 nM probe and
106 DNA-free water). Reactions were run using the Applied Biosystems 7500 Fast (Life Technol-
107 ogies Carlsbad, CA, USA) as follows: 3 min at 95°C and 45 cycles (15s at 95°C and 1 min at
108 60°C). The specificity of the qPCR method was verified using 30 strains of microorganisms.
109 Quantitative results were expressed using the quantification cycle (Cq) that marked the cycle
110 at which fluorescence of the sample became significantly different from the baseline signal.
111 The sample collected on D21 was positive with a Cq of 36 (both duplicates were positive).

112

113

Discussion

In the present clinical case, the patient was diagnosed with a probable IA due to *A. flavus* that evolved unfavorably. The probable IA diagnosis was based on host factors, chest CT and positive biomarkers in serum (galactomannan antigen and mitochondrial in-house *Aspergillus* qPCR). Positive *Aspergillus* qPCR in serum has been included to the microbial criteria allowing the EORTC/MSG classification of IA as probable since 2020 [10].

We showed previously that the combination of mitochondrial and ribosomal targets increased the sensitivity of qPCR for the diagnosis of invasive aspergilliosis [4,5].

The discordance between the negativity of the in-house ribosomal *Aspergillus* qPCR, specific to *A. fumigatus*, and the positivity of the in-house mitochondrial *Aspergillus* qPCR (targeting *A. fumigatus* and other *Aspergillus* species) allowed us to suspect that another thermophilic *Aspergillus* species than *A. fumigatus* was involved. The involvement of *A. flavus* was confirmed using a specific *A. flavus* qPCR. Unfortunately, as no strain was obtained in culture, antifungal susceptibility could not be tested.

Aspergillus flavus is the second most common opportunistic pathogen isolated in case of invasive aspergillosis [11]. Experimental data suggest that *A. flavus* may be more virulent than *A. fumigatus* [12]. Several studies investigated the susceptibility to antifungals for *A. flavus* strains and reported that the *A. flavus* strains tested in their work displayed reduced susceptibility to amphotericin B, with MIC values ≥ 2 mg/L [11, 13-17]; higher MIC values to isavuconazole for *A. flavus* strains were also reported [13].

The patient showed a lack of response while treated by either isavuconazole or amphotericin B: the fungal biomarkers remained positive and aggravation of the lesions was observed by imagery. A recent expert viewpoint recommend to wait until after ≥ 8 or ≥ 15 days of therapy before changing the antifungal therapy, even if rising galactomannan antigen or radiological increase in size were observed [17]. In our case, the patient received 9 days of isavuconazole

139 before switching by amphotericin B. The persistence of positive galactomannan antigen and
140 of positive *Aspergillus* qPCR in serum were poor prognostic signs and argued in favor of a
141 refractory disease [17].

142

Conclusion

This case report illustrates the interest of combining two in-house *Aspergillus* qPCRs. The mitochondrial target, while less sensitive, allowed suspecting the involvement of another *Aspergillus* opportunistic species than *A. fumigatus*. Non *fumigatus* species, such as *A. terreus* and *A. flavus*, are associated with particular susceptibility profiles (low sensitivity to amphotericin B). Current diagnostic strategies focusing specifically on *A. fumigatus* present the risk to under consider non *fumigatus* species, which could lead to non-optimal care.

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218 **Figure legends**

219 **Figure 1.** Evolution of events for the patient

Figure 1

