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1 Refractory invasive pulmonary aspergillosis due to *Aspergillus flavus* detected with the com-
2 bination of two in-house *Aspergillus* qPCR.

3

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16

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

18 **Competing interest statement:** The authors declare no conflict of interest.

19

20

21 **Abstract**

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

36

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

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41

42 **Introduction**

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

53 For the past several years, our center has been applying a strategy of systematic IA screening
54 combining the detection of galactomannan antigen (GM) detection and two in-house *Asper-*
55 *gillus* qPCRs [4,5]. The strategical choice of combining a mitochondrial and a ribosomal *As-*
56 *pergillus* qPCR aimed at optimizing information on the *Aspergillus* species involved, while
57 using a minimum quantity of DNA. Thus, instead of duplicating the same assay, we made the
58 choice to perform two different complementary qPCRs [4-6]. The ribosomal *Aspergillus*
59 qPCR targets a 18S area specific of *Aspergillus fumigatus*, which is a very repeated target
60 (estimated from 38 to 91 copies per genome [7]), thus, this qPCR is very sensitive. On the
61 flipside, the mitochondrial *Aspergillus* qPCR targets a less repeated area (estimated at be-
62 tween 9 and 10 mitochondrial copies per single-copy gene [8]), but the targeted area is com-
63 mon to other *Aspergillus* thermophilic species (*A. flavus*, *A. terreus*, *A. nidulans* and *A. ni-*
64 *ger*), thus this qPCR is less sensitive but allow the detection of other thermophilic *Aspergillus*
65 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

70 **Case report**

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

78 On D14 the patient presented fever and thoracic pain. On D15, the chest CT showed lobar
79 condensations and the galactomannan antigen (GM) was positive in serum (index 3.3). Both
80 in-house *Aspergillus* qPCRs were negative. An antifungal treatment by isavuconazole was
81 initiated, with 200 mg every 8 h during 2 days and then 200 mg per day (Figure 1). On D18,
82 GM was still positive and the mitochondrial *Aspergillus* qPCR (mqPCR) turned positive (GM
83 >3.5, Cycle quantification (Cq) 34; Figure 1). A broncholaveolar lavage fluid (BALF) was
84 performed on D18: direct examination was negative, the fungal culture remained sterile, the
85 *Aspergillus* qPCRs were negative and the galactomannane was positive (>3.5). The patient
86 didn't have any fever at that time. The patient needed oxygen therapy from D20. Two tracheal
87 aspiration were send to the laboratory for fungal culture on D22 and D24 but the cultures
88 remained sterile.

89 On D24, the antifungal treatment was changed for liposomal amphotericin B (3 mg/kg/day)
90 because of fever recurrence and worsening of the lesions on chest CT: GM and mqPCR were
91 still positives in serum (GM >3.5, Cq 36; Figure 1). On D35 the patient was still in aplasia
92 and was transferred in Intensive Care Unit (ICU) for acute respiratory symptoms.

93 Unfortunately, the patient was refractory to allogenic HSCT and relapsed from the leukemia.
94 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 Environmental Pro-
101 tection Agency (EPA) of Unites States (available on [https://irp-](https://irp-cdn.multiscreensite.com/c4e267ab/files/uploaded/gCQnkBNWQuSD96fPIikY_EPA_Technology%20for%20Mold%20Identification%20and%20Enumeration.pdf)
102 [cdn.multiscreensite.com/c4e267ab/files/uploaded/gCQnkBNWQuSD96fPIikY_EPA_Technol-](https://irp-cdn.multiscreensite.com/c4e267ab/files/uploaded/gCQnkBNWQuSD96fPIikY_EPA_Technology%20for%20Mold%20Identification%20and%20Enumeration.pdf)
103 [ogy%20for%20Mold%20Identification%20and%20Enumeration.pdf](https://irp-cdn.multiscreensite.com/c4e267ab/files/uploaded/gCQnkBNWQuSD96fPIikY_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

114 **Discussion**

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

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

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

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

134 The patient showed a lack of response while treated by either isavuconazole or amphotericin
135 B: the fungal biomarkers remained positive and aggravation of the lesions was observed by
136 imagery. A recent expert viewpoint recommend to wait until after ≥ 8 or ≥ 15 days of therapy
137 before changing the antifungal therapy, even if rising galactomannan antigen or radiological
138 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

143 **Conclusion**

144 This case report illustrate the interest of combining two in-house *Aspergillus* qPCRs. The mi-
145 tochondrial target, while less sensitive, allowed suspecting the involvement of another *Asper-*
146 *gillus* opportunistic species than *A. fumigatus*. Non *fumigatus* species, such as *A. terreus* and
147 *A. flavus*, are associated with particular susceptibility profiles (low sensitivity to amphotericin
148 B). Current diagnostic strategies focusing specifically on *A. fumigatus* present the risk to un-
149 der 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

