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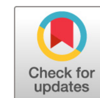
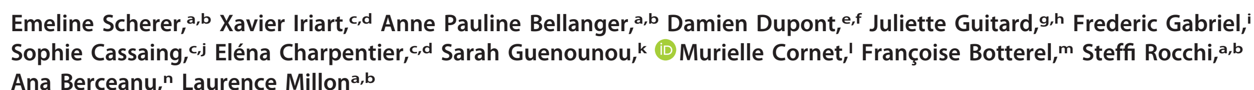


Quantitative PCR (qPCR) Detection of Mucorales DNA in Bronchoalveolar Lavage Fluid To Diagnose Pulmonary Mucormycosis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ABSTRACT Early diagnosis and treatment are essential to improving the outcome of mucormycosis. The aim of this retrospective study was to assess the contribution of quantitative PCR detection of Mucorales DNA in bronchoalveolar lavage fluids for early diagnosis of pulmonary mucormycosis. Bronchoalveolar lavage fluid samples ($n = 450$) from 374 patients with pneumonia and immunosuppressive conditions were analyzed using a combination of 3 quantitative PCR assays targeting the main genera involved in mucormycosis in France (*Rhizomucor*, *Mucor/Rhizopus*, and *Lichtheimia*). Among these 374 patients, 24 patients had at least one bronchoalveolar lavage fluid sample with a positive PCR; 23/24 patients had radiological criteria for invasive fungal infections according to consensual criteria; 10 patients had probable or proven mucormycosis, and 13 additional patients had other invasive fungal infections (4 probable aspergillosis, 1 proven fusariosis, and 8 possible invasive fungal infections). Only 2/24 patients with a positive PCR result on a bronchoalveolar lavage fluid sample had a positive Mucorales culture. PCR was also positive on serum in 17/24 patients. In most cases, a positive PCR result was first detected using sera (15/17). However, a positive PCR on bronchoalveolar lavage fluid was the earliest and/or the only biological test revealing mucormycosis in 4 patients with a final diagnosis of probable or proven mucormycosis, 3 patients with probable aspergillosis, and one patient with a possible invasive fungal infection. Mucorales PCR performed on bronchoalveolar lavage fluid could provide additional support for earlier administration of Mucorales-directed antifungal therapy, thus improving the outcome of lung mucormycosis cases.

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Mucormycosis is a severe fungal infection caused by molds belonging to the order Mucorales. The disease mainly affects immunocompromised patients and patients with diabetes mellitus. Mucormycosis progresses rapidly and is difficult to diagnose. Lung localization is common in immunosuppressed patients, especially in patients with hematological malignancies (1). Although recent studies have reported that the "reverse halo sign" is strongly suggestive of mucormycosis in patients with acute leukemia (1–3), clinical symptoms and radiological imaging cannot differentiate between the different causes of invasive pulmonary fungal infection in most patients. Cultures of respiratory samples (sputum and bronchoalveolar lavage fluid) can help identify the etiological agent; however, their yield is low, and species identification, which is needed to initiate targeted antifungal therapy, is often missing or delayed (4). Recent reports have indicated that the mortality rates of mucormycosis in hematological patients still range from 25 to 50% (5, 6). Early diagnosis and prompt initiation of directed antifungal therapy are crucial for better patient outcome (7). Early distinction from invasive aspergillosis is essential because the antifungal treatment for each is different (8). Therefore, rapid microbiological documentation is of the utmost importance (9).

Detecting specific fungal DNA using targeted PCR in clinical samples can provide early and accurate information about the etiological agent. Several studies have reported that quantitative PCR (qPCR) detection of Mucorales DNA in serum could anticipate the diagnosis of mucormycosis by an average of 8 days in hematological patients and in critically ill burn patients (3, 9–12). Lengerova et al. showed that the detection of Mucorales DNA in BAL fluid using seminested PCRs followed by high-resolution melt (HRM) analysis presented good sensitivity and specificity (100% and 93%, respectively) for the diagnosis of pulmonary mucormycosis (13). Nested PCR-based techniques require opening PCR tubes between the two runs of amplifications, with an increased risk of false-positive results, and they do not allow access to the yield of amplification (14).

The Mucorales PCR assay (MucPCR) described by our team consists of a combination of 3 qPCR assays with specific hydrolysis probes targeting the main genera involved in mucormycosis in France (*Rhizomucor*, *Mucor/Rhizopus*, and *Lichtheimia*). Amplification protocols were harmonized so that samples could be analyzed with the different targets in a single run of 2 h. MucPCR was evaluated in serum samples and was reported to be positive in 80 to 90% of patients with mucormycosis (9). Although not yet evaluated, this tool is being used more and more to detect Mucorales DNA in BAL fluid, along with the *Aspergillus* PCR, to extend etiologic diagnosis in patients suspected of having a pulmonary invasive fungal infection.

The aim of this study was to assess the contribution of the MucPCR assay on BAL fluid for the diagnosis of pulmonary mucormycosis. For this, we carried out a two-center study based on the retrospective analysis of BAL fluid MucPCR performed in two French University hospitals (Besançon, Toulouse, France) which have major clinical activities in hematology and both of which have already implemented this tool to diagnose invasive mold infections.

MATERIALS AND METHODS

During the study period from January 2013 to May 2017, 405 BAL fluid samples from 337 patients with pneumonia and immunosuppressive conditions were analyzed using the MucPCR assay; 360 BAL fluid samples (from 304 patients) were analyzed at the mycology lab of Besançon University Hospital from January 2013 to May 2017, and 45 BAL fluid samples (from 33 patients) were analyzed at the mycology lab of Toulouse University Hospital. For patients hospitalized in Besançon and Toulouse, clinical and biological data were recorded from clinical files, and patients were classified as having possible, probable, or proven invasive fungal disease (IFD) or without IFD, according to the definitions for invasive fungal diseases of the European Organization for Research and Treatment of

Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) (15), which still does not consider PCR results for IFD classification.

Other additional BAL fluid samples ($n = 45$, from 37 patients) sent from 14 different hospitals in France and Switzerland were also analyzed using MucPCR at the mycology lab of Besançon University Hospital. For these patients, clinical and biological data could be collected only for BAL fluid MucPCR-positive patients.

For mycological culture, 5 ml of BAL fluid was centrifuged, and then 100 μl of the pellet was inoculated on Sabouraud medium. DNA extraction was performed from a BAL fluid pellet as follows: 1.5 ml of BAL fluid was centrifuged, and then the pellet (200 μl) was placed in 2.0-ml tubes (MagNA Lyser green beads; Roche Applied Science, Mannheim, Germany) and shaken in a MagNA Lyser instrument (Roche Applied Science). DNA extraction was then performed using the High Pure PCR template kit (Roche Diagnostics, Meylan, France). Mucorales DNA was detected in BAL fluid using a combination of 3 qPCR assays targeting the main genera involved in mucormycosis (*Rhizomucor* [assay name Rmuc], *Lichtheimia* [assay name Acory], and *Mucor/Rhizopus* [assay name Muc]) (https://irp-cdn.multiscreensite.com/c4e267ab/files/uploaded/gCQnkBNWQuSD96fPIikY_EPA_Technology%20for%20Mold%20Identification%20and%20Enumeration.pdf), as previously described (9).

The PCR mix was prepared in a 20-μl final volume using the LightCycler 480 probes master (Roche Diagnostics, Meylan, France), with 11 μl of master mix containing 0.4 μl of probe (P1, 4 μM), 0.5 μl of primers (F1/R1, 40 μM), and 9 μl of DNA. PCR inhibitors were detected in BAL fluid samples using DiaControlDNA (Diagenode Diagnostics, Liege, Belgium). Quantitative results were expressed by determining the detection threshold, or quantification cycle (C_q), that marked the cycle at which fluorescence of the sample became significantly different from the baseline signal. PCR was positive when C_q was <45 cycles.

MucPCR was also performed on available serum sampled around the date of the first positive BAL fluid MucPCR result (between 10 days before BAL fluid sampling [D−10] and day 10 after sampling [D+10]; $n = 49$). DNA was extracted from serum samples using 1 ml of serum with the MagNA Pure Compact nucleic acid isolation kit I, large volume (Roche Diagnostics, Mannheim, Germany), as previously described (9).

Demographics, underlying conditions, diagnostic tools, EORTC classification, first-line antifungal therapy used, and outcome at day 90 were recorded for all the patients with positive BAL fluid MucPCR results (Tables 1 and 2). Biological material was obtained only for standard diagnosis on the basis of the physicians' prescriptions. Clinical data were made anonymous for analysis. According to the French Public Health Law (24), protocols of this type do not require approval from an ethics committee and are exempt from the requirement for formal informed consent.

A nonparametric test (Wilcoxon rank sum test for paired samples) was used to compare C_q values for BAL fluid and serum using the RStudio software (version 3.2.2; Boston, MA). Statistical analyses were done for 15 patients who had both PCRs (BAL fluid and serum), with a maximum delay of 7 days.

RESULTS

Positive BAL fluid MucPCR results and EORTC classification. Complete data were available only for the 337 patients hospitalized in Besançon and Toulouse. There were 31/337 patients diagnosed with probable or proven IFD. Among them, 5 patients had proven or probable mucormycosis, and 26 patients had probable or proven invasive aspergillosis.

Among these 337 patients, 15 were BAL fluid MucPCR positive, and 322 were negative. The 15 positive patients were the 5 patients with proven or probable mucormycosis, 3 patients with probable invasive aspergillosis (IA), 6 patients with possible IFD, and 1 patient with no IFD. In this group, the sensitivity and specificity of BAL fluid MucPCR for diagnosing probable or proven pulmonary mucormycosis were 100% and 97%, respectively.

Among the 37 patients from the 14 other hospitals, BAL fluid MucPCR was positive for 9 patients (6 patients with probable or proven mucormycosis, 1 patient with fusariosis, and 2 patients with possible IFD). The performance of the test could not be calculated using these data because clinical data from the 28 patients with negative BAL fluid MucPCR had not been collected.

Complete clinical and biological data for the 24 patients with positive BAL fluid MucPCR are shown in Tables 1 and 2. The median C_q was 33 cycles, (range, 24 to 41 cycles). The DNA load in BAL fluid was high for the 2 patients with positive BAL fluid culture for Mucorales (C_q values, 27 and 28 for patients 9 and 10, respectively). The DNA load was not different for patients with histological findings (median C_q, 33 cycles in the proven mucormycosis group).

TABLE 1 Clinical and biological data from patients with proven or probable mucormycosis and positive BAL fluid MucPCR result result[a]

Patient no.	Age (yr)	Sex[b]	Underlying disease[c]	EORTC classification	Radiological sign(s)	Histology site, day, direct exam result, culture result (organism), MucPCR result (C_q)[d]	BAL fluid mycology day, direct exam result, culture result (organism)	Galactomannan serum/BAL fluid result (D−7 to D+7)	BAL fluid MucPCR result, day (C_q)	Serum MucPCR result, day (C_q)	Liposomal amphotericin B day of initiation	Outcome at D90
1	58	F	AML	Proven mucormycosis	Nodules, ground-glass opacity	Lung, D12, Pos*, Neg, *Rhizomucor* (34)	D0, Neg, Neg	Neg	Neg, D–12 (>45), *Rhizomucor*, D0 (38)	Not available	D7	Alive
2	57	M	MDS, HSCT	Proven mucormycosis	Nodules	Lung, D3, Pos*, Neg, not done	D0, Neg, Neg	Neg	*Mucor/Rhizopus*, D0 (41)	Neg, D0 (>45)	D3	Death at D8
3	45	M	ALL	Proven mucormycosis	Nodules	Lung, D90, Pos*, Neg, *Mucor/Rhizopus* (46)	D0, Neg, Neg	Neg	*Mucor/Rhizopus*, D0 (29)	*Mucor/Rhizopus*, D–2 (33)	D0	Alive
4	45	M	AML	Proven mucormycosis	Reverse halo signs, ground-glass opacity	Lung, D13, Pos*, Pos (*Rhizomucor* sp.), not done	D0, Neg, Neg	Neg	Neg, D–5 (>45), *Rhizomucor*, D0 (32)	*Rhizomucor*, D0 (31)	D0	Alive
5	52	M	Liver transplant	Proven mucormycosis	Condensation	Lung, D−25, Pos*, Neg, not done	D0, Pos, Neg	Neg	*Mucor/Rhizopus*, D0 (33)	Neg, D7 (>45)	D0	Death at D34
6	15	F	Diabetes	Proven mucormycosis	Condensation	Lung, D0, Pos*, Neg, *Mucor/Rhizopus* (31)	D−12, Pos, Neg	Neg	*Mucor/Rhizopus* D0 (24)	*Mucor/Rhizopus*, D−12 (34), D−6 (31), D−3 (36), D−1 (35)	D−12	Alive
7	47	F	Burkitt lymphoma	Proven mucormycosis	Nodules	Lung, D4, Pos*, Neg, not done	D0, Neg, Neg	Neg	*Rhizomucor*, D0 (33)	*Rhizomucor*, D−7 (33), D−1 (27), D0 (31), Neg, D7 (>45)	D0	Death at D13
8	30	F	HL allograft	Probable mucormycosis	Nodules	Not done	D0, Pos, Neg	Neg	*Lichtheimia*, D0 (39)	*Lichtheimia*, D−7 (38), D−4 (33), D0 (29), D2 (33), D7 (39)	D2	Death at D28
9	55	M	NHL, HSCT	Probable mucormycosis and aspergillosis	Condensation	Not done	D14, Pos, Pos (*Rhizomucor pusillus*)	Pos (serum 0.64)	*Rhizomucor*, D0 (39), D14 (27)	*Rhizomucor*, D4 (33), D7 (34), D14 (34)	D16	Death at D35
10	23	M	AML, HSCT	Probable mucormycosis and aspergillosis	Nodules, condensation	Not done	D0, Pos, Pos (*Lichtheimia* sp. and *Aspergillus fumigatus*)	Pos (serum 3.5)	*Lichtheimia*, D0 (28)	*Lichtheimia*, D−7 (38), D−1 (38), D4 (40)	D6	Death at D15

[a]Pos, positive; Neg, negative.
[b]F, female; M, male.
[c]AML, acute myeloblastic leukemia; MDS, myelodysplastic syndrome; HSCT, allogeneic hematopoietic stem cell transplant; ALL, acute lymphoblastic leukemia; HL, Hodgkin's lymphoma; NHL, non-Hodgkin's lymphoma.
[d]Asterisk indicates broad nonseptate hyphae.

TABLE 2 Clinical and biological data from patients with other IFD, possible IFD, or no IFD and positive BAL fluid MucPCR result

Patient no.	Age (yr)	Sex[a]	Underlying disease[b]	EORTC classification[c]	Radiological sign(s)	Histology day, direct exam result, culture result (organism)	BAL fluid mycology day, direct exam result, culture (organism)	Galactomannan serum/BAL fluid result (D−7 to D+7)	BAL fluid MucPCR result, day (C_q)	Serum MucPCR result, day (C_q)	Liposomal amphotericin B day of initiation[d]	Outcome at D90
11	60	F	NHL, HSCT	Probable aspergillosis	Nodules	Not done	D0, Neg, Neg	Pos (BAL fluid 0.64)	Neg, D–5 (>45), *Rhizomucor*, D0 (34)	Neg, D0 (>45)	D4 (voriconazole before)	Death at D10
12	73	M	AML	Probable aspergillosis	Cerebral abscess	Not done	D0, Neg, Neg	Pos (serum 0.95, BAL fluid 4.95)	*Rhizomucor*, D0 (35)	*Rhizomucor*, D0 (33)	D3	Death at D26
13	43	M	Aplasia	Probable aspergillosis	Nodules, ground-glass opacity	Not done	D−35, Neg, Pos (*Aspergillus fumigatus*)	Neg	*Mucor/Rhizopus*, D0 (39)	Neg, D6 (>45)	D0 (voriconazole before/ improvement with L-AMB + posaconazole at D16)	Alive
14	63	F	AML	Probable aspergillosis	Condensation	Not done	D0, Pos, Pos (*Aspergillus fumigatus*)	Neg	*Mucor/Rhizopus*, D0 (28); *Rhizomucor*, D0 (34)	Neg, D–2 (>45), *Rhizomucor*, D5 (39); *Mucor/Rhizopus* D10 (37), D12 (38), D16 (37)	D5 (voriconazole before/ improvement with L-AMB + isavuconazole at D9)	Alive
15	28	M	ALL, HSCT	Proven fusariosis	Halo sign, condensation, ground-glass opacity	Skin D8, Pos, *Fusarium* spp.	D0, Neg, Neg	Neg	*Rhizomucor*, D0 (33)	*Rhizomucor*, D0 (39)	D8	Death at D51
16	64	M	IM, HSCT	Possible IFD	Condensation	Not done	D0, Neg, Neg	Neg	*Rhizomucor*, D0 (40)	*Rhizomucor*, D0 (31)	D0	Death at D12
17	58	F	Liver transplant, diabetes	Possible IFD	Condensation	Not done	D0, Neg, Neg	Neg	*Mucor/Rhizopus*, D0 (33)	Neg, D5 (>45)	D3	Alive
18	61	M	AML	Possible IFD	Nodules	Bronchial, D9, Neg, Neg	D0, Neg, Neg	Neg	*Mucor/Rhizopus*, D0 (30)	*Mucor/Rhizopus*, D−6 (38), D−2 (38), D2 (35)	D2	Alive
19	25	M	MDS, HSCT	Possible IFD	Nodules, halo sign	Not done	D−15, Neg, Neg	Neg	*Mucor/Rhizopus*, D0 (36)	*Mucor/Rhizopus*, D0 (41)	D−20	Death at D80
20	60	M	AML	Possible IFD	Nodules	Not done	D0, Neg, Pos (*Candida albicans*)	Neg	*Mucor/Rhizopus*, D0 (27)	Neg, D–5 (>45), *Mucor/ Rhizopus*, D−1 (36), D0 (35), D1 (37), D7 (34)	D7 + posaconazole	Death at D10
21	46	F	CML	Possible IFD	Pleural effusion	Not done	D0, Neg, Neg	Neg	*Rhizomucor*, D0 (39)	*Rhizomucor*, D−1 (35), Neg, D6, D13 (>45)	D0 + isavuconazole (D8)	Death at D15
22	80	M	AML	Possible IFD	Condensation, ground-glass opacity	Not done	D0, Neg, Neg	Neg	*Mucor/Rhizopus*, D0 (29)	*Mucor/Rhizopus*, D−5 (34), D−1 (32)	D6	Death at D7
23	56	M	AML, HSCT	Possible IFD	Reverse halo sign	Not done	D0, Neg, Neg	Neg	*Rhizomucor*, D0 (32)	*Rhizomucor* D0 (31)	D0	Death at D2
24	66	M	Sarcoidosis	No IFD	Not done	Not done	D0, Neg, Neg	Pos (BAL fluid 0.93)	*Lichtheimia*, D0 (41)	Neg, D0 (>45)	None	Death at D10

[a]F, female; M, male.

[b]NHL, non-Hodgkin's lymphoma; HSCT, allogeneic hematopoietic stem cell transplant; AML, acute myeloblastic leukemia; ALL, acute lymphoblastic leukemia; IM, idiopathic myelofibrosis; MDS, myelodysplastic syndrome; CML, chronic myeloblastic leukemia.

[c]IFD, invasive fungal disease. The "proven fusariosis" case had positive hemoculture with *Fusarium* spp. at D45.

[d]L-AMB, liposomal amphotericin B.

Underlying diseases were essentially hematological malignancies (21/24). Other underlying conditions were liver transplant (1/24), diabetes (1/24), and sarcoidosis (1/24). Among these 24 patients, 23 had radiological signs for IFD according to EORTC criteria (presence of nodules, condensation, halo signs, or reverse halo signs on chest computed tomography) (Table 1).

Out of the 23 patients who had radiological criteria for IFD and a positive BAL fluid MucPCR result, there were 10 patients with mucormycosis according to EORTC/MSG criteria (7 proven mucormycosis and 3 probable mucormycosis, two of which were associated with probable aspergillosis) (Table 1). There were 13 additional patients who had a positive BAL fluid MucPCR result but could not be classified as having mucormycosis according to EORTC/criteria; of these, 4 patients had probable aspergillosis, 1 patient had proven fusariosis, and 8 patients had possible IFD (Table 2). The remaining patient classified with no IFD had sarcoidosis; he had been receiving a long-term corticoid treatment and had been diagnosed with chronic aspergillosis (by positive *Aspergillus* serology) (Table 2).

The time between D0 (day of BAL fluid sampling) and the time of treatment initiation depended on how long it took to make results available to clinicians. Our study includes patients hospitalized in 2013 and 2014, when Mucorales PCR was performed once a week in our center; therefore, for some patients, it took up to 7 days to obtain the PCR results, and mycological or histological data were available first. Then progressively, series of Mucorales PCRs were done twice a week (in Besançon in 2015 and in Toulouse in 2017), and PCR results could be obtained sooner (4 to 5 days). In addition, clinicians' confidence in PCR results increased (due to publications from 2016 and 2017 showing the interest of the techniques), and liposomal amphotericin B was initiated earlier (1 to 3 days).

Out of the 24 patients with positive BAL fluid MucPCR results, the mortality rate at D90 was 67% (16/24). The mortality rate was 60% (6/10) among patients with a final diagnosis of probable or proven mucormycosis and was 71% (10/14) among patients classified as other IFD, possible IFD, and no IFD. Death occurred within 15 days in 62% of the patients (10/16).

BAL fluid MucPCR and serum MucPCR. Mucorales genera detected using MucPCR in the 24 positive BAL fluid samples were distributed as follows: *Lichtheimia*, $n = 3$; *Rhizomucor*, $n = 10$; and *Mucor/Rhizopus*, $n = 11$. MucPCR was also positive on sera sampled between D−10 and D+10 in 17/24 patients. Genera detected in BAL fluid and serum were the same in all cases when both BAL fluid and serum MucPCR were positive (17 pairs). A high DNA load was observed in serum samples, as was already observed in previous studies (9, 11, 12). C_q values were not different in BAL fluid and in serum (median C_q, 32 cycles [range, 24 to 40 cycles] in BAL fluid versus 34 cycles [range, 31 to 41 cycles] in serum; P value = 0.14, Wilcoxon rank sum test for paired samples).

Patients with proven or probable mucormycosis according to EORTC/MSG criteria. Serum samples were available at the time of positive BAL fluid MucPCR (D−10 to D+10) for 9/10 patients with a final diagnosis of mucormycosis. For 6/9 patients, an earlier serum sample was available and tested positive before the bronchoscopy examination was performed (median, 7 days [range, 0 to 12 days]).

BAL fluid MucPCR was positive 4 days before serum-positive MucPCR in one patient with probable mucormycosis (patient 9). Serum MucPCR was not done or had a negative result at the time of positive MucPCR BAL fluid testing in 3 patients with proven mucormycosis (patients 1, 2, and 5). For these 4 patients, BAL fluid MucPCR was the earliest available biological test revealing mucormycosis (Table 1).

Patients classified as other IFD according to EORTC/MSG criteria. Serum samples were available at the time of positive BAL fluid MucPCR result (D−10 to D+10) for the 13 other patients with a final diagnosis of IFD. MucPCR serum was detected positive before MucPCR BAL fluid in 9 out of the 13 patients (median, 1 day [range, 0 to 7 days]).

BAL fluid MucPCR was positive 5 days before serum MucPCR in patient 14 with probable aspergillosis and was the earliest biological test associated with mucormy-

cosis; the serum MucPCR had a negative result at the time of positive MucPCR BAL fluid in 2 patients with probable aspergillosis (patients 11 and 13) and one patient with possible IFD (patient 17), and no other subsequent serum samples could be tested. For these 3 patients, MucPCR BAL fluid results were the only biological data associated with mucormycosis (Table 2).

Liposomal amphotericin B was initiated because of a positive BAL fluid MucPCR result in patient 17 with possible IFD, and he was alive at D90. The 3 patients with probable aspergillosis received voriconazole before their positive BAL fluid MucPCR result was known. Radiological findings, with expansion of pulmonary lesions under voriconazole therapy, improved after initiation of liposomal amphotericin B in 2/3 patients (13 and 14). These 2 patients were alive at D90. The third patient received liposomal amphotericin at D4 after positive BAL fluid was sampled and then died at D10.

Mycological direct microscopic examination and BAL fluid culture. Mycological direct examination of BAL fluid with large hyphae characteristic of Mucorales was positive in 5 patients with a final diagnosis of proven or probable mucormycosis (Table 1).

BAL fluid Mucorales culture was positive in 2 patients (patient 9 with *Rhizomucor pusillus* and patient 10 with a *Lichtheimia* sp). Both identifications were in accordance with DNA detected using MucPCR. These 2 patients had a final diagnosis of fungal coinfection (probable mucormycosis and aspergillosis). BAL fluid culture was also positive for *Aspergillus* in patient 10 and in 2 other patients (13 and 14), and it was negative for the other 20 patients with positive BAL fluid MucPCR results (see Tables 1 and 2).

DISCUSSION

Mucorales PCR on BAL fluid can help identify the etiological fungal agent of pneumonia when radiological criteria of IFD are observed, and it can detect mixed Mucorales-*Aspergillus* infection. In our study, 17 patients with a final diagnosis of IFD had positive PCR results both on BAL fluid and serum samples, and serum MucPCR was the earliest argument supporting a diagnosis for mucormycosis in most cases (15/17). Positive BAL fluid MucPCR was the earliest biological test revealing mucormycosis in 4 patients with probable or proven mucormycosis. It was also the earliest and/or the only biological test associated with mucormycosis in 3 patients already diagnosed with probable aspergillosis and in 1 patient with possible IFD. Therefore, this new tool could actually provide essential microbiological arguments for prompt initiation of effective drugs against mucormycosis (liposomal amphotericin B, posaconazole, or isavuconazole).

Mucorales culture was negative for 22/24 BAL fluid samples with MucPCR-positive results. This discrepancy is due to the greater sensitivity of qPCR techniques, which is the main advantage over the culture-based method. The yield of BAL fluid cultures is notoriously low. Among hematopoietic stem cell transplant recipients with a positive molecular test for invasive aspergillosis, only 25 to 50% had a positive culture result (16, 17). Recent multicenter trials also reported a BAL fluid culture sensitivity of only 20 to 50% (18, 19). In our study, essentially including hematological patients, BAL fluid culture was positive in only 2/10 patients with probable or proven mucormycosis. Despite the ability of Mucorales to invade tissue, they are rarely isolated from cultures of blood, urine, sputum, or BAL fluid. Nonseptate hyphae of the fungus can be killed by some lab procedures (refrigeration and sample grinding), and biological samples may often contain few viable organisms (20).

Six out of the 9 patients with probable and proven mucormycosis who had available serum at the time of a positive BAL fluid result also had a positive PCR result on serum, with concordance in the species identified. The BAL fluid and serum results were also concordant for 10 patients with radiological signs of IFD. This finding further enhances the value of the PCR results, and the combination of positive results on serum and BAL fluid should prompt clinicians to complete the diagnosis procedure and initiate specific treatment promptly.

BAL fluid MucPCR could also help detect fungal coinfections, which might be more frequent than previously described. These mixed infections were described in 1 to 4% of patients with invasive mold infections in studies based on conventional microbio-

logical procedures, such as culture and microscopy (21, 22). In the present study, the *Aspergillus*-Mucorales mixed infection was detected using qPCR in 6/24 patients (25%). Only one of the 6 infections had been detected using the culture method. The higher detection rate of mixed infections (about 20%) when PCR assays are used was previously highlighted in other studies that used Mucorales PCR (9, 23).

The sensitivity and specificity of BAL fluid MucPCR were very good. However, performance was evaluated using only partial data from patients in Besançon and Toulouse, and this good performance should be checked in a study including a larger number of mucormycosis cases. The weak performances of culture and histopathological examinations, which are currently used to classify patients as having proven or probable mucormycosis, also hamper any calculation of specificity. However, we did not observe any cross-reactivity of MucPCR with DNA extract from other fungal species, nor from serum samples from patients with aspergillosis and pneumocystosis in a previous study (10). In the present study, 23 patients with probable or proven aspergillosis had negative BAL fluid MucPCR results. Consequently, we believe that the positive result of BAL fluid MucPCR in the 4 patients diagnosed with aspergillosis according to EORTC/MSG criteria was unlikely due to a lack of specificity of the PCR assay, and that these 4 patients actually had a mixed *Aspergillus*-Mucorales infection.

In our opinion, a positive BAL fluid MucPCR result provides a strong argument for the diagnosis of probable mucormycosis, and BAL fluid MucPCR should be included in the diagnostic approach to pulmonary IFD. Detecting mixed infections earlier and more frequently is also crucial to initiating specific antifungal therapy promptly. For two cases of probable aspergillosis, in which voriconazole was replaced by Mucorales-active antifungal treatment when positive BAL fluid MucPCR was known, the radiological findings improved, and so did the outcome. These data also reinforce our position to promote BAL fluid MucPCR.

This retrospective study, performed using BAL fluid MucPCR results obtained from standard diagnoses, has shown that this tool could provide additional arguments favoring earlier initiation of specific antifungal therapy, thus improving the outcome of lung mucormycosis patients. The specific performance of this tool will be assessed more precisely with the prospective multicenter protocol ModiMucor (Prospective evaluation of a new molecular tool for early diagnosis of mucormycosis–PHRC [Projet Hospitalier de Recherche Clinique] national-ModiMucor -French Ministry of Health 2014-A00580-47 [https://www.clinicaltrials.gov/ct2/show/NCT02845934]).

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