Evaluation of MucorGenius® mucorales PCR assay for the diagnosis of pulmonary mucormycosis

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Running title: MucorGenius® PCR of pulmonary samples

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Abstract

Objectives: We aimed to assess the clinical relevance of the marketed pan-mucorales real-time PCR assay MucorGenius® (Pathonostics) on pulmonary specimens relative to that of in-house PCR assays and conventional mycology for the diagnosis of mucormycosis.

Methods: In total, 319 pulmonary samples from severely immunosuppressed patients at risk for invasive mold disease (IMD) were retrospectively included. Direct examination, mycological culture, and PCR testing were performed using three genus-specific in-house mucorales real-time PCR assays and MucorGenius®PCR. Results from *Aspergillus* testing, including galactomannan and PCR, were also collected.

Results: The 319 patients were graded according to modified EORTC-MSG criteria as proven/probable mucormycosis (n=6), proven/probable invasive aspergillosis (IA) (n=63), *Aspergillus*–mucorales co-infections (n=4), possible IMD (n=152), and excluded IMD (n=94). The in-house and MucorGenius®PCR assays were positive for 33 (10.3%) and 27 (8.5%) samples, respectively, whereas culture was positive for only 10 (3.1%). The in-house and MucorGenius®PCR assays showed a sensitivity of 100% (10/10) and 90% (9/10) and a specificity of 95.7% and 97.9%, respectively. Both PCR assays allowed the detection of mucorales DNA in samples from 10 possible cases and six IA, all missed by culture.

Conclusions: MucorGenius® showed good performance, despite missing some low fungal burden. Combining mucorales PCR with EORTC-MSG criteria greatly improved the diagnosis of mucormycosis.

Keywords

Real-time PCR, mucorales, MucorGenius®, pulmonary mucormycosis, molecular diagnosis

Introduction

Mucorales infections are life-threatening invasive fungal diseases (IFD) that occur as severe complications in critically ill patients, mainly those who are severely immunosuppressed. Severe traumatic injuries, burns, and diabetes mellitus are other major predisposing risk factors for mucormycosis, despite normal host immunity (1,2). Although considered to be an uncommon IFD (3), the incidence of mucormycosis among patients with hematological malignancies, e.g. in allogeneic hematopoietic stem cell transplantation (HSCT), is high (4), reaching 11% (7/66) for HSCT recipients admitted to an intensive care unit (ICU) (5). The incidence of mucormycosis has significantly increased during the last two decades in Europe (6–8), as illustrated by a French national retrospective study, reporting an increase from 0.7 to 1.2 per million over a ten-year period (1997-2006) (7), with high mortality, ranging from 50 to 100% (4). There are several possible explanations for this trend, such as the longer survival of hematological patients, as well as better knowledge of the disease and improvements in screening for mucorales infections, which are frequently underdiagnosed.

The diagnosis of mucormycosis still relies on clinical and radiological features, which are nonspecific and can be mistaken for invasive aspergillosis (IA), the most common invasive mold disease (IMD) affecting hematological patients in Europe. However, the differentiation between mucormycosis and other IMDs is of critical importance, as it has major therapeutic implications (9). Clinical presentations are multiple, but lung and disseminated forms are a hallmark of mucormycosis in patients with hematological malignancies (10). Although certain radiological findings, such as the "reverse halo sign", have been thought to be suggestive of mucorales infection and useful for the preemptive initiation of antifungal therapy (11), they are not consistently detected (15/27, 55% (12)), and can be associated with other mold infections (11,12). Other factors, such as delays in undergoing computed tomography (CT) after the onset of symptoms or the neutropenic status in hematological patients (12) also affect the contribution of imaging in IMD diagnosis.

Biological screening is therefore of critical importance in demonstrating fungal invasion of the tissue and making the definitive diagnosis. Conventional culture and histopathology-based screening are still the mainstay of the EORTC-MSG biological criteria for IFD (13), but these approaches may be insensitive and may delay the diagnosis. Besides, a promising microculture method has been recently reported to raise drastically the yield of mycological culture in a *Rhizopus*-infected murine model (14). While the critical interest of molecular techniques, combined with the detection of galactomannan or beta-glucan, has been extensively reported for the diagnosis of IA (15,16) and *Pneumocystis* pneumonia (17), respectively, these biomarkers are not contributory to the diagnosis of mucormycosis. Thus, several studies have been conducted to evaluate mucorales PCR on blood samples, showing it to be an early and reliable approach for the noninvasive testing and monitoring of treatment efficacy (18,19). Although microbiological documentation from imaging-guided samples is still of interest, molecular diagnosis from fresh/frozen respiratory specimens or deep tissues has been little investigated (20–22).

Most European evaluations have been based on panfungal ITS-based PCR (23) or multiple genus-specific real-time in-house PCR assays to detect the most relevant mucorales involved in human infection, including *Mucor* spp., *Rhizopus* spp., *Lichtheimia* spp., and *Rhizomucor* spp. (18). These PCR assays still require standardization and clinical validation. A newly marketed mucorales multiplex real-time PCR assay, called MucorGenius® (Pathonostics, Maastricht, the Netherlands), allows the detection of the main mucorales, including *Cunninghamella* spp., but it is yet to be clinically validated.

Here, we aimed to i) evaluate the concordance of the results obtained with the new commercial multiplex PCR MucorGenius® on pulmonary samples with those of in-house PCR assays and conventional mycology procedures and ii) determine the frequency of *Aspergillus* and mucorales co-infection in our cohort of severely immunosuppressed patients.

Materials and methods

Patients and study design

We retrospectively included 319 pulmonary samples collected from 319 patients at high risk for IMD with respiratory symptoms at the Rennes, Toulouse, and Paris-Necker teaching Hospitals (France), between January 2012 and May 2019. Redundant samples for a unique clinical episode were excluded. Results from the biological work-up (based on physician prescriptions) and clinical data, including demographics, hospitalization ward, risk factors for IMD, relevant imaging findings, prophylactic empiric or curative antifungal therapy at the time of sampling, and outcome at day 30 and day 90, were recorded from all centers. According to the French Public Health Laws (24),

protocols of this type do not require approval from an ethics committee and are exempt from the requirement for formal informed consent.

Patients were classified as proven/probable, possible, or excluded IMD, according to the European Organization for the Research and Treatment of Cancer/Mycoses Study Group (EORTC-MSG) criteria (13), modified by the inclusion of additional predisposing host factors, such as severe cirrhosis, concomitant influenza infection, HIV status, and uncontrolled diabetes. The new EORTC-MSG biological criteria include the results of *Aspergillus* but not mucorales PCR. Briefly, IMD was proven when microscopic examination showed hyphae and/or the mycological culture was positive for mold in a normally sterile sample for patients with host factors and imaging consistent with IMD. The IMD was probable when direct examination and/or mycological culture was positive from a non-sterile sample. For IA diagnosis, the detection of galactomannan in serum and/or bronchoalveolar lavage (BAL) and the *Aspergillus* PCR results were also criteria for probable IA. Patients with host and imaging criteria and without any EORTC-MSG biological criteria were graded as possible IMD cases. The other patients were considered as not infected (excluded IMD group). The proportion of breakthrough IMD i.e. infection occurring during antifungal exposure whatever the antifungal spectrum was also estimated according to the recent definitions established by the MSG-ECMM (25).

When available, data of serum mucorales PCR were collected to help interpret positive mucorales PCR results on pulmonary samples.

Processing of pulmonary samples

The study was conducted on a panel of 319 pulmonary samples, including BAL (n = 293), tracheal aspirations (TA, n = 13), sputum samples (n = 5), pleural fluids (n = 3), and lung biopsies (n = 5).

All specimens were divided into two aliquots to perform microscopic examination, mycological culture, and PCR assays. Microscopic examination was performed from a pellet mounted between a slide and coverslip. Cultures consisted of the incubation of a 100-200-µL pellet from liquid samples or a piece of ground biopsy on two Sabouraud plates supplemented with 5% chloramphenicol (Becton Dickinson, Le Pont de Claix, France or Biorad, Marnes-la-Coquette, France) and incubated at 30°C and 37°C for 8 to 14 days according to the center. Fungal isolates were identified at the genus-level using macroscopic and microscopic morphological criteria. When performed, the presence of fungal elements observed after periodic acid-schiff staining or Grocott staining of paraffin-embedded biopsies was also taken in account.

For PCR assays, DNA was extracted from 200 µL of BAL pellet or that of another respiratory fluid or ground biopsy material. According to the center, the extraction method used was the QIAamp® DNA

minikit (Qiagen, les Ulis, France), the High Pure PCR template kit (Roche Diagnostics, Meylan, France) or the EMAG automated device (Biomérieux, Paris, France), after bead-beating, following the manufacturer's recommendations. DNA eluates were kept frozen at -20°C before processing.

Comparison of in-house mucorales PCR and MucorGenius® PCR was performed on the same DNA extract.

Processing of serum samples for mucorales in-house PCR

DNA from 1 mL serum was extracted using MagnaPure Compact (Roche Diagnostics, Meylan, France) or EMAG (Biomérieux, Paris, France) automated devices and eluted in 50 µL. DNA eluates were kept frozen at -20°C before processing.

Mucorales real-time PCR assays

DNA was assessed for mucorales detection in pulmonary samples using a combination of four in-house qPCR assays targeting the 18S rDNA, allowing the detection of the main clinically relevant genera, i.e. *Mucor-Rhizopus* spp., *Lichtheimia* spp., *Rhizomucor* spp., and *Cunninghamella* spp., as previously described (18,19,26). Briefly, each amplification was performed in a 25-µL final volume containing 2X TaqMan® Universal PCR MasterMix, 0.4 µM of each primer, 0.2 µM probe, and 5 µL DNA sample. Amplification was performed using the following thermal conditions: 10 min at 95°C, followed by 50 cycles of 15 s at 95°C and 1 min at 60°C on a StepOne Plus® instrument (Applied Biosystems, Villebon-sur-Yvette, France).

All DNA samples were tested in parallel with the commercial multiplex real-time PCR assay MucorGenius® PN-700 version (PathoNostics, Maastricht, The Netherlands), which simultaneously targets the 18S rDNA region of *Mucor* spp., *Rhizopus* spp., *Rhizomucor* spp.,and *Cunninghamella* spp., without discriminating between genera. Amplification was performed according to the manufacturer's instructions using a LightCycler 480 (Roche) device. Curve analysis was performed using the Derivative program from LC480 software, as recommended.

Discordant results between the in-house and MucorGenius® PCR assays were retested undiluted and diluted 1:10.

***Aspergillus* real-time PCR assays**

Pulmonary samples were tested for *Aspergillus* DNA using an in-house PCR assay targeting the mitochondrial DNA, as previously described (27). Each amplification was performed in a 25-µL final volume containing 2X TaqMan® Universal PCR MasterMix, 0.5 M of each primer, 0.2 µM probe, and 5 µL DNA sample. Amplification was performed using the following thermal conditions: 2 min at 50°C,

10 min at 95°C and 45 cycles of 15 s at 95°C and 1 min at 60°C on a StepOne Plus® instrument (Applied Biosystems).

Galactomann (GM) Platelia *Aspergillus* assay in BAL and serum

The sandwich ELISA for the detection of GM (Platelia Aspergillus®, Bio-Rad, Marnes-la-Coquette, France) was performed on a 390-µL aliquot of BAL or serum sample according to the manufacturer's recommendations. For the classification of patients, BAL was considered to be positive when the optical density index (ODI) was ≥ 0.8, if the concomitant serum GM was positive (ODI ≥ 0.7), and ≥ 1.0 if the serum GM was negative or unavailable (13).

Statistical methods

Data were analyzed using GraphPad Prism software version 5.02. Data are expressed as medians with interquartile ranges [25th; 75th percentile]. The nonparametric Mann-Whitney test was used to compare quantitative data. Fisher's exact test was used to compare proportions. The correlation between Cq values was assessed using the Spearman rank correlation test. The kappa coefficient was used to measure the agreement between PCR tests. A p-value of 0.05 was considered to be statistically significant.

Results

Patient characteristics

According to the new EORTC-MSG criteria, the 319 patients were diagnosed with proven/probable IMD (n = 73), possible IMD (n = 152), or excluded IMD (n = 94), as depicted in Figure 1. Among the 73 patients with proven or probable IMD, there was one proven case of mucormycosis, five probable cases of mucormycosis, four probable coinfections with *Aspergillus* and mucorales and 63 proven/probable cases of IA. Of note, 22 out of the 225 IMD were breakthrough infections occurring while patients received prophylactic (n=15), empiric (n=5) or curative (n=2) antifungal treatment with fluconazole, posaconazole, caspofungin or voriconazole. The remaining IMD were primary (n=186), refractory/persistent IMD (n=10) or not classified (n=7). An antifungal therapy for at least 2 days before sampling was given to 45 out of the 222 proven, probable or possible IMD cases with available data (20.3%); 18 of 45 received mucorales-active drugs (Table1). Complete demographic characteristics for the patients with the 225 proven, probable, or possible IMD cases are presented in Table 1.

Concordance between in-house mucorales PCRs and MucorGenius® PCR

Among the 319 analyzed samples, the in-house PCR assays yielded 33 positive results (10.3%), whereas MucorGenius® PCR was positive for 27 (8.5%) samples (Table 2). The distribution of targets

amplified by the in-house PCR assays was *Mucor-Rhizopus* (n = 18; 54.5%), *Lichtheimia* (n = 7; 21.2%), *Rhizomucor* (n = 5; 15.1%), both *Mucor-Rhizopus* and *Rhizomucor* (n = 2; 6.1%), and *Cunninghamella* (n = 1, 3.0%). The sensitivity of MucorGenius® PCR relative to that of the in-house PCR assay was 81.8% (27/33) and the percentage of concordance between the two PCR assays was 98.1 % (kappa coefficient of 0.890). Among the 27 samples positive by both PCR assays, the median Cq values were not significantly different (30.4 [26.9; 34.9] versus 31.6 [27.9; 34.4], p = 0.574) for the MucorGenius® and in-house PCR assays, respectively. In addition, there was a strong correlation between the Cq values of the in-house and Mucorgenius® PCR assays (r = 0.8816, p < 0.0001). The six samples that were positive by the in-house PCR assay but negative by MucorGenius® PCR contained a low fungal burden, as shown by the high median Cq values of the in-house PCR (37.2, range [36.1; 37.9]). Only one of the six samples grew in culture, with *Mucor* isolate (probable mucormycosis). The five other discordant PCR results were observed for possible IMD cases (n=3) and excluded IMD cases (n=2).

Sensitivity of PCRs compared to direct examination and culture

Both the in-house and MucorGenius® PCR assays dramatically improved the detection of mucorales over that of conventional mycology based on microscopic examination and culture. Both PCR assays increased the number of positive samples by nearly three-fold (Table 3). The sensitivity of both PCR assays in proven and probable cases was excellent, reaching 100% (10/10) and 90% (9/10) for the in-house and MucorGenius® PCR assays, respectively. Identification of the seven mucorales isolates grown in culture was 100% concordant with in-house PCR identification. Thirteen (8.6%) and 10 (6.6%) samples of the 152 possible cases were also positive with the in-house and MucorGenius® PCR assays, respectively (Table 3).

The four remaining samples that were positive by the in-house PCR assay were obtained from patients graded as excluded IMD cases because they were not considered to be immunosuppressed. However, two of these four patients had a positive mucorales culture, of which one was obtained from a sample with mucorales hyphae at microscopic examination.

Although not statistically significant, Cq PCR values tended to correlate with the patient classification, as shown by the lower median Cq value, i.e. higher fungal burden, in proven/probable cases, which was higher for the possible IMD and excluded groups, with Cq values of 31.4 [23.6; 35.6], 33.1 [29.2; 36.5], and 34.0 [30.1; 34.5], 37.5 [32.1; 38.0], respectively (Figure 2).

The simultaneous detection of *Aspergillus* and mucorales was not uncommon, as four patients had criteria for probable IA and probable mucormycosis (Table 4). In addition to a culture grown with mucorales, three patients had a culture positive for *Aspergillus* spp. (*Fumigati* (n = 2) and *Nigri* (n= 1)), including one with a serum sample positive for galactomannan, and the remaining patient had a

positive *Aspergillus* PCR for two consecutive respiratory samples. In addition, six of 63 patients (9.5%) diagnosed with probable IA (alone), according to EORTC-MSG criteria, had at least one positive mucorales PCR (Table 3). Positive targets were *Mucor-Rhizopus (n = 2)*, *Mucor-Rhizopus* and *Rhizomucor (n = 2)*, *Rhizomucor* (n= 1), and *Cunninghamella* (n = 1). None of the six samples grew with mucorales.

Clinical relevance of and outcome of patients with positive mucorales PCRs in pulmonary samples

Interpretation of the isolated positive mucorales PCRs, i.e. to distinguish invasive infection from colonization, was aided by retrospectively collected concomitant results from serum mucorales in-house PCRs, when available. Within the possible IMD group, serum PCR was performed on samples from 11 of 13 patients with isolated positive pulmonary PCR and yielded 10 positive results. Five of the six IA patients with positive pulmonary mucorales PCR results were screened by serum PCR and all were positive. Of note, there was a discordant mucorales identification in one BAL positive for *Mucor-Rhizopus*, whereas the serum collected 10 days before was positive for *Lichtheimia*.

By contrast, the serum PCR was negative for the two "excluded" cases, of which the sputum and tracheal aspirate were both positive by culture and PCR, respectively, suggesting that these positive culture/PCR results probably resulted from colonization rather than an invasive infection. No IMD-favoring factor was found for the first patient, alive at day 90 without any antifungal therapy, consistent with lung colonization. However, the second patient also had a positive microscopic examination; he was admitted to the ICU for severe traumatic injuries following defenestration and heart failure and died the day after sampling, despite the introduction of liposomal amphotericin B. Post-traumatic mucorales inoculation as a source of invasive mucormycosis could not be ruled out. Within the group of excluded IMD, the two other patients with a positive in-house PCR had chronic pulmonary dysfunction, including diffuse bronchiectasis or a lung carcinoma lesion, which could have enhanced fungal colonization (serum PCR not performed). Thus, considering these four patients, the specificity of PCR to detect patients with mucormycosis reached 95.7 % (90/94) and 97.9% (92/94) for the in-house and MucorGenius® PCR assays, respectively.

A positive mucorales PCR result correlated with a poorer prognosis, as shown by the very high mortality of > 50% at day 30 ($p < 0.001$) (Table 1), most of the deaths occurring within the first five days following sample collection (data not shown). There were no statistically significant differences in the mean fungal load according to survival.

Discussion

The poor performance of conventional mycology in diagnosing mucormycosis may result in undiagnosed and fatal infections. Clinical studies of mucorales PCR in deep samples are still scarce, but the relevance of mucorales PCR within the diagnosis algorithm has already been suggested (28). Most studies have focused on in-house PCR assays consisting of several simplex or multiplex 18S-based PCRs, covering the four main genera responsible for human infections. Other targets have been investigated, such as the mitochondrial gene *rnl* (29), the *cytochrome b* gene (30), *28S rDNA* (21), or the mucorales-specific c*oth* gene (31), but their validation requires further clinical studies. These multiple testing approaches still lack standardization and may restrain the implementation of molecular mucorales screening in routine laboratories, stressing the need for developing easy-to-use PCR assays.

Here, we report the first clinical assessment of MucorGenius®, a newly marketed pan-mucorales PCR assay in a series of 319 pulmonary samples, mainly BAL, from immunocompromised patients with pneumonia, in comparison with in-house PCR assays (18,19,26) and conventional mycology. Compared to culture, both the MucorGenius® and in-house mucorales PCR assays allowed the detection of mucorales DNA in 17 and 23 additional samples, respectively, irrespective of the clinical grading. The sensitivity was excellent, especially for probable mucormycosis (90-100%), which was quite predictable, and vastly superior to culture, widely recognized to be a largely insensitive and late method to isolate molds, particularly mucorales (32). Indeed, the sensitivity of BAL culture for IMD diagnosis has been reported to be as low as 30 to 50% (22,33,34). By contrast, a microculture capillary-based assay from both deep tissue and blood samples has been proposed as a promising tool in a murine model of mucormycosis (14). This new process was shown to increase from 31.1% (28/90) to 98.8 % (89/90) the sensitivity of conventional culture from mouse kidney samples after *Rhizopus* intravenous inoculation. Further studies are needed to confirm the relevance of such culture method. In addition, both PCR assays were positive for 10 pulmonary samples from patients without fungal documentation (possible IMD). As serum mucorales PCR has been previously shown to be a highly reliable tool for the diagnosis of invasive mucormycosis in hematological (18,19), burn (35) or other immunosuppressed patients (36), we analyzed the value of isolated positive PCR results for pulmonary specimens, in light of the PCR results for blood, when available. All but one patient with available data had one or several positive mucorales PCR results in serum collected within a three-week period around the lung sampling. All these patients had strong risk factors for mucormycosis, thus we can consider that these positive mucorales PCR results were indicative of invasive mucormycosis, missed by conventional culture-based diagnosis. Thus, combining mucorales in-house PCR with EORTC-MSG criteria would have increased the number of mucormycosis cases from 10 to 29. Similar conclusions were drawn by a recent French multicentric study that emphasized the outstanding performance of such 18S rDNA-targeted in-house PCR assays on BAL from 337

patients, reporting a sensitivity of 100% (15 positive mucorales PCR results against only five positive results based on conventional mycology) and a specificity of 97% (22).

In our multicentric cohort, both the in-house and MucorGenius® PCR assays showed high specificity (> 95%), although probably underestimated due to the patient classification used. Indeed, two of the four supposedly "false-positive" PCR results of the in-house PCRs were obtained from patients graded as excluded cases because they did not fulfill any host risk factors, as defined by EORTC-MSG criteria, although both had biological evidence of mucormycosis, i.e. cultures grown with mucorales (and even a positive microscopic examination).

The marketed MucorGenius® assay proved to be efficient for the diagnosis of pulmonary mucormycosis. This easy-to-use PCR assay allows the possibility to detect all mucorales species involved in most human mucormycosis in Europe in only one well. This technical advantage could widely facilitate the implementation of the molecular diagnosis of mucormycosis in routine laboratories. Here, it allowed the detection of 9/10 cases of proven or probable mucormycosis and 16 additional positive samples missed by culture. Interestingly, the six samples missed by MucorGenius® PCR (all with in-house PCR Cq values > 35.8) were obtained from patients diagnosed with probable mucormycosis (n = 1), possible mucormycosis (n = 3), or excluded mucormycosis (n = 2), who appeared to have a better outcome than the global cohort of patients with positive PCR results. Indeed, only three received adequate antifungal therapy and four of five patients with available follow-up were alive at day 30, which was much higher than the global survival rate for patients with a positive in-house PCR result (< 50%). These data suggest that MucorGenius® PCR has higher specificity than the in-house PCR assays to detect patient with pulmonary mucormycosis, which could avoid the unnecessary use of antifungals. However, it must be noted that a low-burden infection could be missed. Finally, this evaluation focused on pulmonary specimens. The performance of MucorGenius® PCR is yet to be assessed for non-invasive screening, i.e. serum samples.

The main drawback of this assay is that it detects any clinically relevant mucorales, with no information on the genus. As precise identification of mucorales genera is important to guide the choice of antifungal drug, this could prompt the use of the in-house PCR assays, despite heavier technical constraints. Indeed, certain mucorales genera have been associated with a poorer prognosis, as shown by Millon et al., who showed a trend towards higher mortality for *Rhizomucor* spp. infections (18). In addition, certain mucorales genera may have decreased susceptibility to lipid-based amphotericin B or isavuconazole (37). For example, the minimal inhibitory concentration for amphotericin B has been shown to be high for up to 40% of *Cunninghamella* strains (38), which was associated with higher mortality than disease caused by other genera (71% versus 44%) in a recent

meta-analysis of Jeong et al. (39). Susceptibility of *Mucor circinelloides* to triazole antifungals, especially isavuconazole, was also shown to be much lower (40).

Predisposing factors for both pulmonary mucormycosis and IA are common and coinfections have been reported to occur in 20-25% of mucormycosis cases in studies based on PCR testing (18,22). Here, we were unable to draw any reliable conclusions concerning the incidence of *Aspergillus* and mucorales coinfections due to the limitations inherent to the retrospective design of the study, including the inclusion of non-consecutive patients from three centers. Nevertheless, mucorales PCR was positive for six of the 63 patients (9.5%) in our cohort diagnosed with probable IA according to culture-based criteria, including one patient who did not benefit from mucorales PCR at the time of diagnosis and rapidly died under *Aspergillus*-targeted antifungal therapy. This case illustrates the challenge of distinguishing IMD, especially mucorales, from an *Aspergillus* infection, in immunosuppressed patients presenting with nonspecific fever and nodular pulmonary imaging.

These data highlight the need to target the population that would benefit the most from systematic mucorales PCR screening, in addition to *Aspergillus* screening. Here, we included a large series of immunosuppressed patients under a heavy immunosuppressive regimen (80%) with various underlying diseases, mainly hematological malignancy (42%), solid organ transplant (19%), and solid cancer (11%). Commonly admitted as risk factors (10,39), patients with acute leukemia, deep neutropenia, primary immune deficiency, including chronic granulomatous disease or severe combined immune deficiency, or solid organ transplant (SOT) were prone to develop mucormycosis. Remarkably, three of five SOT patients with positive mucorales PCR were lung transplant recipients, underscoring the high vulnerability of these patients, whose previous lung dysfunction allowed mucorales colonization, and the rapid onset of infection after transplantation (41).

This study has a limitation regarding the number of mucormycosis cases in our cohort, but a low incidence of mucormycosis is reported elsewhere in the literature (7). Further evaluation on a larger series would be of interest.

In conclusion, our study supports the relevance of mucorales PCR on lung samples in addition to standard techniques (37) and shows the MucorGenius® PCR assay to be suitable for mucorales screening in pulmonary samples, although it may miss some cases with a low fungal burden. Mucorales PCR should be implemented in combination with *Aspergillus* screening within the global management of targeted patients with a high risk of invasive mold infections, e.g. hematological patients.

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Tables

Table 1. Demographic characteristics of patients with proven, probable, or possible IMD, according to the in-house mucorales PCR assay (N = 225).

		n (%)		p[a]
Clinical features	All N = 225	Positive mucorales PCR N = 29	Negative mucorales PCR N = 196	
Age (years)	58.2 [40.9; 63.6]	58.5 [40.9; 63.6]	58.1 [48.7; 65.7]	0.485
Gender (Sex ratio)	147/78 (1.88)	15/14 (1.07)	132/64 (2.06)	0.142
Hospitalization ward				
Intensive care unit	95 (42.2)	15 (51.7)	80 (40.8)	0.315
Hematology unit	45 (20.0)	5 (17.2)	40 (20.4)	0.807
Pulmonology	32 (14.2)	2 (6.9)	30 (15.3)	0.390
Infectious disease unit	15 (6.7)	1 (3.4)	14 (27.5)	0.700
Other	39 (17.3)	7 (24.1)	32 (16.3)	
Underlying disease				
Hematological malignancy	95 (42.2)	15 (51.7)	80 (40.8)	0.315
Acute leukemia	39 (17.3)	11 (37.9)	28 (14.3)	0.0064**
Lymphoma	23 (10.2)	2 (6.9)	21 (10.7)	0.747
Chronic lymphoid leukemia	9 (4)	1 (3.4)	8 (4.1)	1
Myeloma	11 (4.9)	0	11 (5.6)	0.367
HSCT	25 (11.1)	4 (13.8)	21 (10.7)	0.540
Primary immune deficiency[b]	5 (2.2)	3 (10.3)	2 (1.0)	0.016*
Solid cancer	26 (11.5)	2 (6.9)	24 (12.2)	0.543
SOT	42 (18.7)	5 (17.2)	37 (18.9)	1
Lung	3 (1.3)	3 (10.3)	0	0.002**
Kidney	2 (0.9)	2 (6.9)	12 (6.1)	0.698
Liver	17 (7.5)	0	17 (8.7)	0.138
Heart	8 (3.5)	0	8 (4.1)	0.600
Neutropenia < 0.5G/L	49/216 (22.7)	8/21 (38.1)	41/195 (21.0)	0.098
Immunosuppressive treatment	182 (80.9)	25 (86.2)	157 (80.1)	0.613
Diabetes	18 (8.0)	3 (10.3)	15 (7.6)	0.711
Antifungal therapy ≥2 days before sample				
Any drug	45/222 (20.3)	15/26 (57.7)	30/196 (15.3)	<0.001***
L-AmbI or isavuconazole	4 (1.8)	4 (15.4)	0	
Posaconazole	14 (6.3)	3 (11.5)	11 (5.6)	
Voriconazole or itraconazole	14 (6.3)	3 (11.5)	11 (5.6)	
Overall mortality				
at day 30	44/215 (20.5)	14/26 (53.8)	30/189 (15.9)	<0.0001***
at day 90	57/213 (26.8)	15/26 (57.7)	42/187 (22.4)	0.0005***

HSCT: hematopoietic stem cell transplant, SOT: solid organ transplant, L-Amb: liposomal amphothericin B

[a]p: Comparison between positive and negative in-house PCR groups

[b]chronic granulomatous disease (n = 4), severe combined immunodeficiency (SCID) (n = 1)

Table 2. Concordance between the in-house and MucorGenius® PCR assays (N = 319).

		In-house PCRs n (%)		
		Positive	Negative	Total
MucorGenius® n (%)	Positive	27	0	27
	Negative	6[a]	286	292
	Total	33	286	319

[a]*Mucor-Rhizopus* (n = 4), *Rhizomucor* (n = 2)

Table 3. Diagnostic performance of the in-house mucorales and MucorGenius® PCR assays compared to conventional mycology (N = 319).

	Rate of positive tests according to IMD group n (%), N = 319			
	Proven/probable mucormycosis N = 10[a]	Proven/probable IA N = 63	Possible IMD N = 152	Excluded IMD N = 94
Detection of mucorales by microscopic examination and/or culture N=10	10 (100%)	0	0	2[b] (2.1)
Direct Examination[c]	7 (70.0%)	0	0	1 (1.1)
Positive culture	7[d] (70.0%)	0	0	2 (2.1)
In-house PCR N= 33	10 (100%)	6[e] (9.5)	13[e] (8.6)	4[e] (4.2)
Mucor-Rhizopus, n = 20	4/10	4[f]/6	9/13	3/4
Rhizomucor, n =8	1/10	3[f]/6	3/13	1/4
Lichtheimia, n = 6	5/10	0	1/13	0
Cunninghamella, n = 1	0	1/6	0	0
MucorGenius® PCR N=27	9 (90.0%)	6 (9.5)	10 (6.6%)	2 (2.1)

[a]The four patients diagnosed with coinfection with *Aspergillus* and mucorales are included in the proven/probable mucormycosis group.

[b]These two samples were also positive with both the in-house and MucorGenius® PCR assays.

[c]The microscopic examination (direct smear or histological staining) was positive when broad and non-septate hyphae were observed, excluding "*Aspergillus*"-like hyphae.

[d]*Mucor* (n= 1), *Rhizopus* (n= 3), *Lichtheimia* (n = 3). The three samples with positive microscopy but sterile cultures were positive with *Lichtheimia* PCR (n = 2) and *Rhizomucor* PCR (n = 1).

[e]Serum in-house PCR was performed for patients with a positive PCR on pulmonary samples: 5/6 patients with IA, 11/13 with possible IMD, and 2/4 with excluded IMD. Positive signals were obtained in 5/5 IA, 10/11 possible IMD, and 0/2 patients with excluded IMD.

[f]Two samples were simultaneously positive for both *Mucor-Rhizopus* and *Rhizomucor* by PCR.

Table 4. Rate of positive *Aspergillus* tests in proven, probable, and possible IMD groups according to modified EORTC-MSG criteria (N = 225)

Tests	Rate of positive tests according to IMD group n/N (%)			
	Proven/probable IA N = 63	Co-infection: probable IA and mucormycosis N = 4	Proven/probable mucormycosis N = 6	Possible IMD N = 152
Positive direct examination or culture grown with *Aspergillus*	41[a]/63 (65.1)	3[b]/4	0/6 (0)	1/152 (0.7)
Positive *Aspergillus* BAL PCR	39/57 (68.4)	3/3	0/3 (0)	2/151 (1.3)
Positive BAL galactomannan	22/54 (40.7)	ND	ND	4/142 (2.8)
Positive serum GM	25/58 (43.1)	1/3	0/5 (0)	0/25 (0)

ND: not determined

[a]16/63 (25.1) positive by direct examination and 36/63 (57.1) cultures grown with *Aspergillus* section *Fumigati.*

[b]*Aspergillus* section *Fumigati (n = 2),* section *Nigri (n = 1).*

Figures

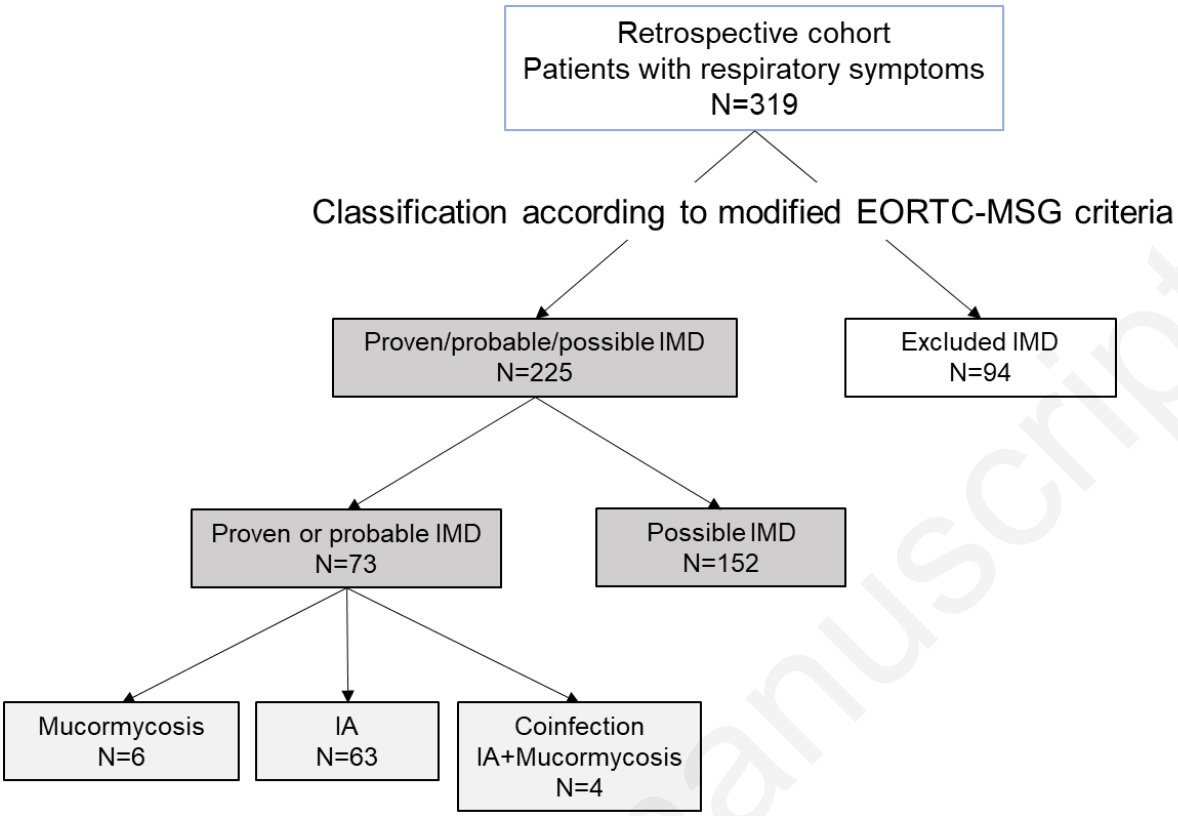

Figure 1. Flow chart of the study population with patient classification according to modified EORTC-MSG criteria (excluding mucorales PCR results) (13).

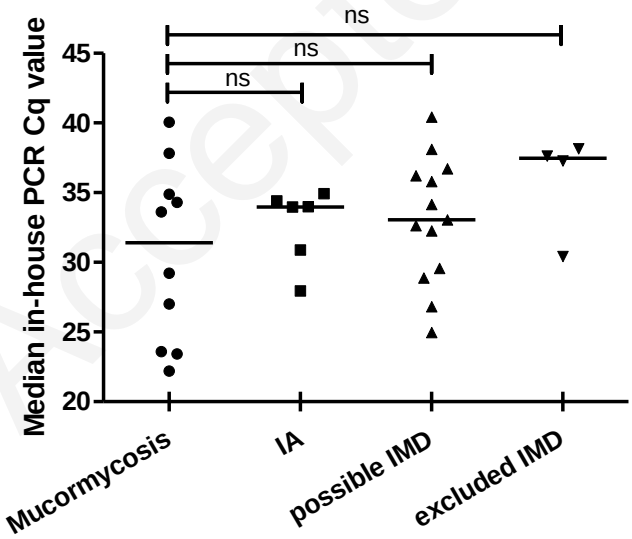

Figure 2. Median in-house mucorales PCR Cq value on pulmonary samples according to IMD group defined by MSG criteria

Legends to figures

Figure 1. Flow chart of the study population with patient classification according to modified EORTC-MSG criteria (excluding mucorales PCR results) (13).

IMD: invasive mold disease; IA: invasive aspergillosis

Figure 2. Median in-house Mucorales PCR Cq value on pulmonary samples according to IMD group defined by MSG criteria

Mucormycosis: proven or probable mucormycosis, IA: proven or probable invasive aspergillosis, IMD: invasive mold disease. For clarity, the four patients diagnosed with coinfection with *Aspergillus* and mucorales were included in the mucormycosis group, ns: not statistically significant