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

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.

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

42

43 **Keywords**

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

45

46 **Introduction**

47 Mucorales infections are life-threatening invasive fungal diseases (IFD) that occur as severe
48 complications in critically ill patients, mainly those who are severely immunosuppressed. Severe
49 traumatic injuries, burns, and diabetes mellitus are other major predisposing risk factors for
50 mucormycosis, despite normal host immunity (1,2). Although considered to be an uncommon IFD (3),
51 the incidence of mucormycosis among patients with hematological malignancies, e.g. in allogeneic
52 hematopoietic stem cell transplantation (HSCT), is high (4), reaching 11% (7/66) for HSCT recipients
53 admitted to an intensive care unit (ICU) (5). The incidence of mucormycosis has significantly
54 increased during the last two decades in Europe (6–8), as illustrated by a French national
55 retrospective study, reporting an increase from 0.7 to 1.2 per million over a ten-year period (1997-
56 2006) (7), with high mortality, ranging from 50 to 100% (4). There are several possible explanations
57 for this trend, such as the longer survival of hematological patients, as well as better knowledge of
58 the disease and improvements in screening for mucorales infections, which are frequently
59 underdiagnosed.

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

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

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

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

95 **Materials and methods**

96 **Patients and study design**

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

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

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

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

122

123 **Processing of pulmonary samples**

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

126 All specimens were divided into two aliquots to perform microscopic examination, mycological
127 culture, and PCR assays. Microscopic examination was performed from a pellet mounted between a
128 slide and coverslip. Cultures consisted of the incubation of a 100-200- μ L pellet from liquid samples or
129 a piece of ground biopsy on two Sabouraud plates supplemented with 5% chloramphenicol (Becton
130 Dickinson, Le Pont de Claix, France or Biorad, Marnes-la-Coquette, France) and incubated at 30°C and
131 37°C for 8 to 14 days according to the center. Fungal isolates were identified at the genus-level using
132 macroscopic and microscopic morphological criteria. When performed, the presence of fungal
133 elements observed after periodic acid-schiff staining or Grocott staining of paraffin-embedded
134 biopsies was also taken in account.

135

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

138 minikit (Qiagen, les Ulis, France), the High Pure PCR template kit (Roche Diagnostics, Meylan, France)
139 or the EMAG automated device (Biomérieux, Paris, France), after bead-beating, following the
140 manufacturer's recommendations. DNA eluates were kept frozen at -20°C before processing.
141 Comparison of in-house mucorales PCR and MucorGenius® PCR was performed on the same DNA
142 extract.

143 **Processing of serum samples for mucorales in-house PCR**

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

147

148 **Mucorales real-time PCR assays**

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

157

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

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

166

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

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

172 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
173 (Applied Biosystems).

174

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

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

181

182 **Statistical methods**

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

189 **Results**

190 **Patient characteristics**

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

203

204 **Concordance between in-house mucorales PCRs and MucorGenius® PCR**

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

207 amplified by the in-house PCR assays was *Mucor-Rhizopus* (n = 18; 54.5%), *Lichtheimia* (n = 7; 21.2%),
208 *Rhizomucor* (n = 5; 15.1%), both *Mucor-Rhizopus* and *Rhizomucor* (n = 2; 6.1%), and *Cunninghamella*
209 (n = 1, 3.0%). The sensitivity of MucorGenius® PCR relative to that of the in-house PCR assay was
210 81.8% (27/33) and the percentage of concordance between the two PCR assays was 98.1 % (kappa
211 coefficient of 0.890). Among the 27 samples positive by both PCR assays, the median Cq values were
212 not significantly different (30.4 [26.9; 34.9] versus 31.6 [27.9; 34.4], p = 0.574) for the MucorGenius®
213 and in-house PCR assays, respectively. In addition, there was a strong correlation between the Cq
214 values of the in-house and Mucorgenius® PCR assays (r = 0.8816, p < 0.0001). The six samples that
215 were positive by the in-house PCR assay but negative by MucorGenius® PCR contained a low fungal
216 burden, as shown by the high median Cq values of the in-house PCR (37.2, range [36.1; 37.9]). Only
217 one of the six samples grew in culture, with *Mucor* isolate (probable mucormycosis). The five other
218 discordant PCR results were observed for possible IMD cases (n=3) and excluded IMD cases (n=2).

219 **Sensitivity of PCRs compared to direct examination and culture**

220 Both the in-house and MucorGenius® PCR assays dramatically improved the detection of mucorales
221 over that of conventional mycology based on microscopic examination and culture. Both PCR assays
222 increased the number of positive samples by nearly three-fold (Table 3). The sensitivity of both PCR
223 assays in proven and probable cases was excellent, reaching 100% (10/10) and 90% (9/10) for the in-
224 house and MucorGenius® PCR assays, respectively. Identification of the seven mucorales isolates
225 grown in culture was 100% concordant with in-house PCR identification. Thirteen (8.6%) and 10
226 (6.6%) samples of the 152 possible cases were also positive with the in-house and MucorGenius® PCR
227 assays, respectively (Table 3).

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

232 Although not statistically significant, Cq PCR values tended to correlate with the patient classification,
233 as shown by the lower median Cq value, i.e. higher fungal burden, in proven/probable cases, which
234 was higher for the possible IMD and excluded groups, with Cq values of 31.4 [23.6; 35.6], 33.1 [29.2;
235 36.5], and 34.0 [30.1; 34.5], 37.5 [32.1; 38.0], respectively (Figure 2).

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

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

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

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

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

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

270

271 **Discussion**

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

282 Here, we report the first clinical assessment of MucorGenius®, a newly marketed pan-mucorales PCR
283 assay in a series of 319 pulmonary samples, mainly BAL, from immunocompromised patients with
284 pneumonia, in comparison with in-house PCR assays (18,19,26) and conventional mycology.
285 Compared to culture, both the MucorGenius® and in-house mucorales PCR assays allowed the
286 detection of mucorales DNA in 17 and 23 additional samples, respectively, irrespective of the clinical
287 grading. The sensitivity was excellent, especially for probable mucormycosis (90-100%), which was
288 quite predictable, and vastly superior to culture, widely recognized to be a largely insensitive and late
289 method to isolate molds, particularly mucorales (32). Indeed, the sensitivity of BAL culture for IMD
290 diagnosis has been reported to be as low as 30 to 50% (22,33,34). By contrast, a microculture
291 capillary-based assay from both deep tissue and blood samples has been proposed as a promising
292 tool in a murine model of mucormycosis (14). This new process was shown to increase from 31.1%
293 (28/90) to 98.8 % (89/90) the sensitivity of conventional culture from mouse kidney samples after
294 *Rhizopus* intravenous inoculation. Further studies are needed to confirm the relevance of such
295 culture method. In addition, both PCR assays were positive for 10 pulmonary samples from patients
296 without fungal documentation (possible IMD). As serum mucorales PCR has been previously shown
297 to be a highly reliable tool for the diagnosis of invasive mucormycosis in hematological (18,19), burn
298 (35) or other immunosuppressed patients (36), we analyzed the value of isolated positive PCR results
299 for pulmonary specimens, in light of the PCR results for blood, when available. All but one patient
300 with available data had one or several positive mucorales PCR results in serum collected within a
301 three-week period around the lung sampling. All these patients had strong risk factors for
302 mucormycosis, thus we can consider that these positive mucorales PCR results were indicative of
303 invasive mucormycosis, missed by conventional culture-based diagnosis. Thus, combining mucorales
304 in-house PCR with EORTC-MSG criteria would have increased the number of mucormycosis cases
305 from 10 to 29. Similar conclusions were drawn by a recent French multicentric study that emphasized
306 the outstanding performance of such 18S rDNA-targeted in-house PCR assays on BAL from 337

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

309 In our multicentric cohort, both the in-house and MucorGenius® PCR assays showed high specificity
310 (> 95%), although probably underestimated due to the patient classification used. Indeed, two of the
311 four supposedly “false-positive” PCR results of the in-house PCRs were obtained from patients
312 graded as excluded cases because they did not fulfill any host risk factors, as defined by EORTC-MSG
313 criteria, although both had biological evidence of mucormycosis, i.e. cultures grown with mucorales
314 (and even a positive microscopic examination).

315 The marketed MucorGenius® assay proved to be efficient for the diagnosis of pulmonary
316 mucormycosis. This easy-to-use PCR assay allows the possibility to detect all mucorales species
317 involved in most human mucormycosis in Europe in only one well. This technical advantage could
318 widely facilitate the implementation of the molecular diagnosis of mucormycosis in routine
319 laboratories. Here, it allowed the detection of 9/10 cases of proven or probable mucormycosis and
320 16 additional positive samples missed by culture. Interestingly, the six samples missed by
321 MucorGenius® PCR (all with in-house PCR Cq values > 35.8) were obtained from patients diagnosed
322 with probable mucormycosis (n = 1), possible mucormycosis (n = 3), or excluded mucormycosis (n =
323 2), who appeared to have a better outcome than the global cohort of patients with positive PCR
324 results. Indeed, only three received adequate antifungal therapy and four of five patients with
325 available follow-up were alive at day 30, which was much higher than the global survival rate for
326 patients with a positive in-house PCR result (< 50%). These data suggest that MucorGenius® PCR has
327 higher specificity than the in-house PCR assays to detect patient with pulmonary mucormycosis,
328 which could avoid the unnecessary use of antifungals. However, it must be noted that a low-burden
329 infection could be missed. Finally, this evaluation focused on pulmonary specimens. The
330 performance of MucorGenius® PCR is yet to be assessed for non-invasive screening, i.e. serum
331 samples.

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

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

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

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

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

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

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515 **Tables**516 **Table 1. Demographic characteristics of patients with proven, probable, or possible IMD, according**
517 **to the in-house mucorales PCR assay (N = 225).**

Clinical features	All N = 225	n (%)		p ^a
		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***

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

519 ^ap: Comparison between positive and negative in-house PCR groups520 ^bchronic granulomatous disease (n = 4), severe combined immunodeficiency (SCID) (n = 1)

521

522

523 **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

524 ^a*Mucor-Rhizopus* (n = 4), *Rhizomucor* (n = 2)

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526 **Table 3. Diagnostic performance of the in-house mucorales and MucorGenius® PCR assays**
 527 **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)
<i>Mucor-Rhizopus</i> , n = 20	4/10	4 ^f /6	9/13	3/4
<i>Rhizomucor</i> , n =8	1/10	3 ^f /6	3/13	1/4
<i>Lichtheimia</i> , n = 6	5/10	0	1/13	0
<i>Cunninghamella</i> , n = 1	0	1/6	0	0
MucorGenius® PCR N=27	9 (90.0%)	6 (9.5)	10 (6.6%)	2 (2.1)

528 ^aThe four patients diagnosed with coinfection with *Aspergillus* and mucorales are included in the
 529 proven/probable mucormycosis group.

530 ^bThese two samples were also positive with both the in-house and MucorGenius® PCR assays.

531 ^cThe microscopic examination (direct smear or histological staining) was positive when broad and non-septate
 532 hyphae were observed, excluding "*Aspergillus*"-like hyphae.

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

535 ^eSerum in-house PCR was performed for patients with a positive PCR on pulmonary samples: 5/6 patients with
 536 IA, 11/13 with possible IMD, and 2/4 with excluded IMD. Positive signals were obtained in 5/5 IA, 10/11
 537 possible IMD, and 0/2 patients with excluded IMD.

538 ^fTwo samples were simultaneously positive for both *Mucor-Rhizopus* and *Rhizomucor* by PCR.

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540

541 **Table 4. Rate of positive *Aspergillus* tests in proven, probable, and possible IMD groups according**
 542 **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 <i>Aspergillus</i>	41 ^a /63 (65.1)	3 ^b /4	0/6 (0)	1/152 (0.7)
Positive <i>Aspergillus</i> 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)

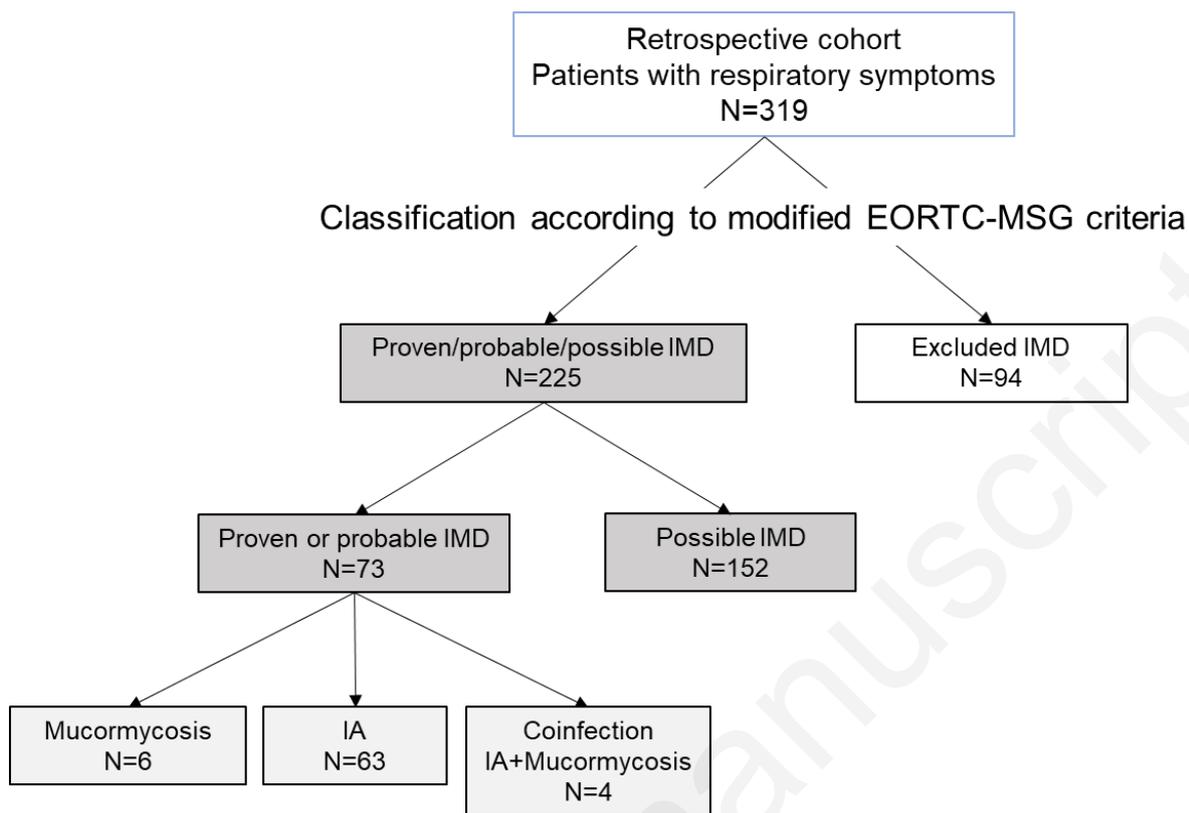
543 ND: not determined

544 ^a16/63 (25.1) positive by direct examination and 36/63 (57.1) cultures grown with *Aspergillus* section *Fumigati*.

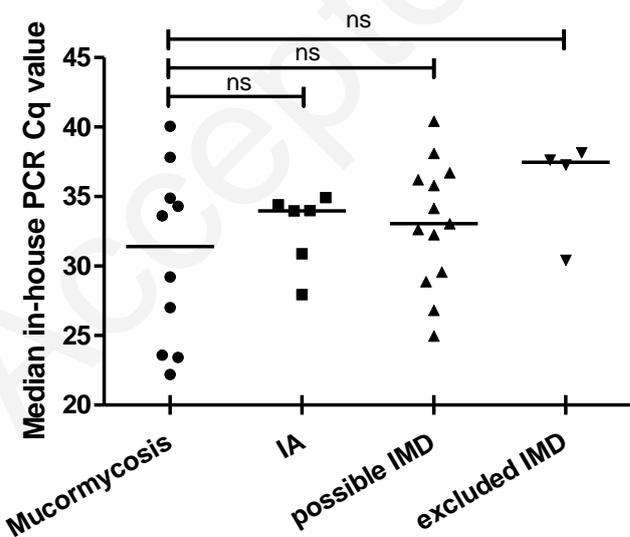
545 ^b*Aspergillus* section *Fumigati* (n = 2), section *Nigri* (n = 1).

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549
550 **Figure 1. Flow chart of the study population with patient classification according to modified**
551 **EORTC-MSG criteria (excluding mucorales PCR results) (13).**



553
554 **Figure 2. Median in-house mucorales PCR Cq value on pulmonary samples according to IMD group**
555 **defined by MSG criteria**

557 **Legends to figures**

558

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

561 IMD: invasive mold disease; IA: invasive aspergillosis

562

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

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

568