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Interlaboratory evaluation of Mucorales PCR assays for testing serum specimens: A study by the fungal PCR Initiative and the Modimucor study group

Short Title : Interlaboratory evaluation of serum Mucorales PCR assays

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84
85 **Key Words**
86 Mucorales PCR - circulating DNA – inter-laboratory assay – standardization

Abstract

Inter-laboratory evaluations of Mucorales qPCR assays were developed to assess the reproducibility and performance of methods currently used. The participants comprised 12 laboratories from French university hospitals (nine of them participating in the Modimucor study) and 11 laboratories participating in the Fungal PCR Initiative.

For panel #1, three sera were each spiked with DNA from three different species (*Rhizomucor pusillus*, *Lichtheimia corymbifera, Rhizopus oryzae*). For panel #2, six sera with three concentrations of *R. pusillus* and *L. corymbifera* (1, 10 and 100 genomes/mL) were prepared. Each panel included a blind negative-control serum. A form was distributed with each panel to collect results and required technical information, including DNA extraction method, sample volume used, DNA elution volume, qPCR method, qPCR template input volume, qPCR total reaction volume, qPCR platform, and qPCR reagents used.

For panel #1, assessing 18 different protocols, qualitative results (positive or negative) were correct in 97% of cases (70/72). A very low inter-laboratory variability in Cq values (SD = 1.89 cycles) were observed. For panel #2 assessing 26 different protocols, the detection rates were high (77-100%) for 5/6 of spiked serum. There was a significant association between the qPCR platform and performance. However, certain technical steps and optimal combinations of factors may also impact performance.

The good reproducibility and performance demonstrated in this study support the use of Mucorales qPCR as part of the diagnostic strategy for mucormycosis.

108 **Introduction**

109 Mucormycosis is a severe invasive disease caused by species associated to the order Mucorales
110 (main clinically relevant genera are: *Rhizopus, Mucor, Rhizomucor*, and *Lichtheimia* (formerly
111 *Absidia*[1]). The diagnosis of this life-threatening infection is challenging. Clinical and
112 radiological signs are not specific and can be confused with other, more common invasive
113 mould infections, such as invasive aspergillosis (IA). Early initiation of specific treatment is
114 essential to improve prognosis[2]. However, voriconazole, recommended as a first-line treatment
115 for IA, is not effective for mucormycosis. Therefore, obtaining early, aetiological specific
116 diagnostic evidence is essential.

117 Molecular detection of circulating DNA was not considered as a mycological criterion for
118 defining probable invasive fungal disease in the original and revised EORTC/MSG consensus
119 definitions[3, 4], because of a lack of methodological standardization and limited clinical
120 validation. Advances in both have led recently to the acceptance of *Aspergillus* PCR as
121 mycological evidence for defining probable IA[5]. If molecular methods for the detection of other
122 fungal pathogens are to be included, it is paramount that they attain the same level of
123 standardization.

124 Quantitative PCR (qPCR) detection of Mucorales DNA in serum, plasma and BAL has been
125 shown to be a sensitive and early tool for diagnosing mucormycosis[6-12]. Mucorales DNA can
126 be detected using qPCR an average of 8 days before conventional mycological and histological
127 techniques in patients with haematological malignancies or who are critically ill because of
128 burns[7, 9, 10], and an average of 4 days before radiological signs (reverse halo sign) in patients
129 with acute leukaemia[11]. The good sensitivity of these techniques is probably due to the large
130 load of circulating Mucorales DNA observed in mucormycosis which is estimated to be 10 to
131 100-fold higher than that has been observed for *Aspergillus* in IA[7]. Indeed, previous studies
132 showed that the concentrations calculated after the positive control were 1-10fg of Mucorales

133 DNA per microliter of serum in patients with probable and proven mucormycosis (median Cq
134 was 34 cycles (range 23-41 cycles)), while the *Aspergillus* DNA concentrations found in
135 patients with invasive aspergillosis were <0.1fg/µL of serum (median 40 cycles (range 33-45
136 cyles)) [7, 9].
137 This large DNA load makes an accurate quantification for therapeutic monitoring possible[7]. An
138 increasing number of studies has demonstrated that Mucorales qPCR is very helpful in
139 optimizing the management of mucormycosis[13-15]. However, studies evaluating and comparing
140 analytical performance between methods are lacking, limiting the standardized optimal
141 methods, necessary for inclusion as mycological criterion in future EORTC/MSG definitions.
142
143 The aim of the ISHAM working group the European *Aspergillus* PCR Initiative (EARPCI) was
144 to standardize *Aspergillus* PCR[16, 17], for inclusion as microbiological criterion for defining
145 probable aspergillosis in the EORTC/MSG definitions[5]. As this has now been achieved, the
146 initiative expanded its remit include the molecular detection of *Candida*, Mucorales,
147 *Pneumocystis*[18] and fungi in tissue and changed its name accordingly to the Fungal PCR
148 Initiative (FPCRI, www.fpcri.eu). The Mucorales Laboratory Working party organised the
149 distribution of two separate series of inter-laboratory simulated serum panels for the molecular
150 detection of Mucorales DNA.
151 These inter-laboratory studies were performed in 2017 and 2018 with two main objectives: 1)
152 to evaluate qualitative diagnosis (positive/negative) and to assess the reproducibility of methods
153 currently used and 2) to assess qPCR performance according to protocols used. Twenty-three
154 European laboratories participated in these studies. This large collaboration allowed
155 comparison of 4 main qPCR assays, with 26 different technical protocols, with various
156 combinations of DNA extraction methods, qPCR targets, qPCR platforms and qPCR reagents
157 and helped identify procedural factors associated with the best qPCR performance.

158

159 **Materials and Methods**

160 **1) Participants**

161 Twenty-three different laboratories participated in at least one of the two trials (20 in panel #1
162 and 22 in panel #2, Table 1). The participants comprised: 12 laboratories from French university
163 hospitals, nine of them participating in the French national prospective Modimucor study
164 evaluating the qPCR detection of circulating DNA for the diagnosis of Mucormycosis (Projet
165 Hospitalier de Recherche Clinique national-ModiMucor 2014-A00580-47)[19], who were asked
166 to follow several technical recommendations (see below); and 11 laboratories participating in
167 the FPCRI/Mucorales PCR Laboratory working group, who were free to use their own method
168 without any specific recommendation. Two laboratories participated in both groups
169 (Modimucor study and FPCRI/Mucorales PCR group).

170 For the final analysis, only qPCR results as indicated by quantitative cycle (Cq) value were
171 included. Laboratories that used conventional or nested PCR were excluded from further
172 analysis. Therefore, results were analysed from 18 laboratories for panel #1, and from 21
173 laboratories for panel #2 (Table 1).

174 All laboratories were designated with a numerical code to allow blinded review of individual
175 methodological procedures, determination of performance and statistical analysis. After each
176 trial, all participants were given the identity of each sample and their own individual
177 performance, together with the average results from other participating laboratories for
178 comparison.

179

180 **2) DNA source material**

181 *Rhizomucor pusillus* (Centre de Ressources Biologiques - Filière Microbiologique, Besançon
182 (CRB-FMB), Biobanque BB-0033-00090), *Rhizopus oryzae syn. arrhizus* (CBS 32947) and

183 *Lichtheimia corymbifera* (IHEM 3809) strains were grown on Sabouraud dextrose agar medium
184 (37°C, 5 days). Species identification was confirmed by ITS sequencing (V9D and LS266
185 primers[20]). DNA was extracted from cultures using the DNeasy Plant Mini Kit™ (Qiagen®,
186 Hilden, Germany) and DNA concentration was measured using a Nanodrop® (Thermo Fisher
187 Scientific®, Waltham, MA, USA). These DNA solutions were used to spike sera.
188
189 **3) Preparation of simulated serum panels**
190 Serum was obtained from healthy donors, volunteering to donate their blood specifically for
191 research purpose, according to procedure and ethical rules of the Bourgogne Franche-Comté
192 Blood Transfusion Center (BTC). Detection of infectious agents was performed according to
193 usual protocols of the BTC. For each panel the serum from up to three donors was pooled and
194 was tested for contamination using specific Mucorales qPCR targeting the most frequent
195 genera[6, 21] before processing. All processing of material took place in a category II laminar flow
196 cabinet to minimize the risk of contamination by environmental fungal spores.
197
198 Two panels (#1 and #2) including 1mL-serum samples were sent to each of the 23 laboratories
199 (Table 1). Both panels were stored at -20°C before shipping (-20°C for international shipping,
200 +4°C for shipping in France). Panels were sent in July 2017 (panel #1) and July 2018 (panel
201 #2). All panels were delivered within 48h and stored below +4°C before being analysed.
202
203 The first panel (panel #1, four 1mL-serum samples) aimed at assessing qualitative diagnosis
204 (positive/negative). Three sera were spiked with DNA from three different species (*R. pusillus*
205 (27 pg/mL of serum), *L. corymbifera* (30 pg/mL of serum), *R. oryzae* (116 pg/mL of serum)).
206 The second panel (panel #2, seven 1mL-serum samples) was designed to assess qPCR
207 performance. To this end, 3 concentrations of *R. pusillus* and *L. corymbifera* (1, 10 and 100

208 genomes equivalent/mL) were prepared as previously described by the European *Aspergillus*
209 PCR Initiative group[22]. The concentrations chosen generated Cq values comparable to those
210 observed in patients diagnosed with mucormycosis (range 23-41 cycles)[7]. DNA was extracted
211 from *R. pusillus* and *L. corymbifera* conidia and serial dilutions were performed considering
212 that one conidia had one genome and that the extraction efficiency from the respective culture
213 was 100%. Each panel included a blind negative-control serum.
214 A form was distributed with each panel to collect results and obtain technical information,
215 including DNA extraction method, sample volume used, DNA elution volume, qPCR method,
216 qPCR template input volume, qPCR total reaction volume, qPCR amplification platform, and
217 qPCR reagents used.
218
219 **4) DNA extraction and qPCR assays**
220 While recommendations were given to French laboratories participating in the Modimucor
221 study (DNA extraction from 1mL of serum with an elution volume of 50µL; specific qPCR
222 assay[7, 23]), all other participants used their own methodology for both DNA extraction and
223 qPCR amplification. All the participants used the qPCR platform and reagents available in their
224 own laboratories and provided all protocol details on the technical form.
225 The qPCR assays used in panel #1 were distributed across four categories: qPCR A, genus-
226 specific assay described by Millon et al.[7, 23]; qPCR B, mucorales-specific assay described by
227 Springer at al.[8] ; qPCR C, species-specific assay described by Lengerova et al.[24] and other qPCR
228 assays not published.
229 In panel #2, the same qPCR assays A, B, C and others (not published) were used. In addition,
230 qPCR D was assigned to participants using the Pathonostics MucorGenius kit (mucorales-
231 specific assay). Description of gene targets, primers and probes, cycling parameters and level

232 of identification allowed by each of 4 main qPCR assays (A, B, C, D) are provided in Tables
233 2A and 2B.
234 The results were expressed in quantification cycles (Cq), with higher values indicating the
235 smaller the amount of DNA in the sample. Any detectable amount of DNA (i.e., Cq < 45) was
236 considered a positive result.
237 The combination of DNA extraction methods, qPCR targets, qPCR platforms, qPCR mix
238 reagents, elution volumes and qPCR volumes resulted in 18 different protocols used in panel
239 #1 and 26 in panel #2 (Table 2C) .
240 The large diversity of reagents and platforms used for extraction and amplification meant that
241 some variables had to be grouped together for statistical analysis. For example, DNA extraction
242 methods were grouped in four categories according to manufacturer and type of extraction (i.e.
243 automated or manual). Master-mix reagents were grouped in 3 categories according to
244 manufacturer (Applied biosystems, Roche and others), and qPCR platform grouped in six
245 manufacturer aligned categories (Applied biosystems, Bio-Rad, Cepheid, Rotorgene, Roche
246 (for microplate technology, LightCycler480) and Roche2 (for capillary technology,
247 LightCycler 2.0). Grouped data are provided in supplemental data (S1) for panel #1, and in
248 Table 2C for panel #2; details of methods for panel #2 are provided in supplemental data (S2).
249

250 **5) Statistical analysis**

251 The aim of the first panel was to check that laboratories were able to detect presence/absence
252 of DNA from Mucorales in each serum samples and to evaluate the reproducibility of detection
253 between the different laboratories. According to the qPCR assay used, the positive answer could
254 be "presence of DNA from Mucorales" for qPCR assay D; "presence of DNA from specific
255 genera (*Mucor/Rhizopus* or *Rhizomucor*, or *Lichtheimia*)" for qPCR assay A; or "presence of
256 DNA from a specific species (list in Table 2A)" for qPCR assays B and C)".

257 Data from panel #2 monitored qPCR performance across all laboratories using different qPCR
258 assays, before focusing on performance in laboratories using a same qPCR assay (assay A).
259 Only the Cq values corresponding to the detection of the correct target (defined according to
260 the qPCR assay used, as described above) were included in the statistical tests (e.g. if the assay
261 detected *R. pusillus* with a Cq of 35 cycles but the sample contained *L. corymbifera* DNA it
262 was excluded from analysis). A Cq value of 46 was assigned to the negative results. Statistical
263 analyses were performed using the statistical software R-3.4.4 for Microsoft® Windows.
264
265 To analyse Cq values in panel #2, statistical models assessed the potential benefit of inclusion
266 of random effects in the models (e.g. influence of the "genomic load" and/or spiked species).
267 A first linear mixed effect model (LMM)[25] was used to model Cq values in function of log
268 transform genomic loads, with a grouping variable "laboratory" and a random effect "genomic
269 load". To assess the impact of different species in the diagnostic sensitivity, a second LMM
270 (species-specific model) was created adding spiked species as a covariate in the fixed effect
271 part of the model. Interaction between genomic load and fungal species was also analyse.
272 The species-specific model was significantly better when compared to the first model
273 ($p<0.001$). Differences in detection rates between spiked species were observed (Fig. S3 in
274 supplemental data), with *L. corymbifera* being better detected (irrespective of the protocol) than
275 *R. pusillus*. Thus, for the following statistical analysis, the species-specific model was used.
276 The influence of the qPCR assay was then investigated by adding this variable to the species-
277 specific model and qPCR assays were pairwise compared using differences of least squares
278 means (marginal effects) and confidence intervals with lmerTest library[26].
279 To determine whether technical parameters influence performance, the 16 protocols using
280 qPCR assay A were arranged according to the Cq value. A full LMM with the serum tested in
281 random part of the model was then undertaken using Cq values and the different available

282 variables (group of DNA extraction method, mix reagent, platform, elution ratio for DNA
283 extraction (elution volume / volume of sample extracted) and qPCR volume ratio (qPCR input
284 volume / final volume of qPCR reaction). A backward stepwise selection was performed to
285 select variables to include in the final model. Factors selected in the final model were pairwise
286 compared using differences of least squares means as previously mentioned.
287
288 **Results**
289 All participating centres returned results and the completed form with technical information
290 within 3 months.
291
292 **1) Evaluation of the qualitative detection of a range of Mucorales species (panel #1)**
293 For panel #1 (three spiked serum and one negative control), results from 18 laboratories
294 (corresponding to 18 protocols) were analysed. Correct detection and identification of the target
295 in serum was 94.4% (17/18, 95% CI: 74.2-99.0) for sera spiked with DNA from *R. pusillus* and
296 *L. corymbifera,* and 100% (18/18, 95% CI: 82.4-100) for sera spiked with DNA from *R. or*yzae
297 (Table 3). The two labs that failed to give a positive signal were laboratory L15 with protocol
298 15 (supplemental data S1) for serum S1-1 spiked with DNA from *R. pusillus* and laboratory
299 L13 with protocol 13 for serum S4-1 spiked with DNA from *L. corymbifera.* These two
300 laboratories used qPCR C and B respectively. A single laboratory (L7 in supplemental data S1)
301 using qPCR A gave an additional positive signal for *Mucor/Rhizopus* assay for serum S1-1
302 (spiked with *R. pusillus*), with high Cq values (44.14). No false positive results were observed
303 for the negative-control serum. Qualitative results (positive or negative) were correct in 97% of
304 cases (70/72).

305 Despite the huge diversity of methods, Mucorales DNA detection in sera was highly
306 reproducible with a very low inter-laboratory variability in Cq values (SD = 1.89 cycles [range
307 1.3; 2.9]).
308
309 **2) Determination of Mucorales qPCR performance (panel #2)**
310 For panel #2 (six spiked serum and one negative control), results from 21 laboratories were
311 analysed, corresponding to a total of 26 different protocols (combined DNA extraction and
312 qPCR amplification methods, Table 2C and supplemental data S2). Three centres tested two
313 protocols, one centre tested three protocols and 17 centres tested a single protocol.
314
315 **2.1) Comparison of performance between different qPCR assays**
316 Across all qPCR assays, the detection rates were high (77-100%) for 5/6 of spiked serum (S2-
317 2, S3-2, S4-2, S5-2, S6-2 (Table 4)). The 6% of false positive rate recorded with negative
318 control when qPCR assay A was used (Table 4) correspond to only one laboratory (L7 with
319 protocol 7 in Table 2C). This laboratory detected *Mucor/Rhizopus* in the control serum (Cq =
320 38.68) and was the same laboratory that had a cross detection in panel #1 (additional positive
321 signal for *Mucor/Rhizopus* assay for serum S1-1 spiked with *R. pusillus*).
322 For serum S7-2 spiked with 1 equivalent genome of *R. pusillus*/mL, one laboratory (L6,
323 protocol 6 using qPCR A) gave an additional positive signal (cross detection) for
324 *Mucor/Rhizopus* assay (Cq = 36) and one laboratory (L3, protocol 3 using qPCR A) just gave
325 a positive signal for *Lichtheimia* assay (Cq = 36.7 and 38). The qPCR detection rate for this
326 serum (S7-2) was 50%.
327
328 For assay A which was used in 15 laboratories (16/26 protocols), global sensitivity and
329 specificity were 89.6% and 97%, respectively (100% and 94.1% for *Lichtheimia*, and 79.1%

330 and 100% for *Rhizomucor*). For assay D which was used in 4 laboratories (4/26 protocols),
331 sensitivity and specificity were 84% and 100%, respectively.
332
333 Figure 1 illustrates the level of Cq value according to the qPCR assay, accounting for the
334 genomic load and the fungal species. Assays A and D gave fewer negative results and lower
335 (earlier) Cq values (increasing the likelihood of detection). Pairwise comparisons showed that
336 "other" qPCR gave significant higher (later) Cq value compared to assays A, C and D (p=0.002,
337 0.01 and 0.006, respectively). However, these results should be interpreted with caution given
338 the modest number of observations from some qPCR systems (e.g. B and C).
339
340 **2.2) The influence of differing technical aspects on the performance of qPCR assay A**
341 Fifteen laboratories used qPCR assay A. Results obtained for the six spiked serums constituting
342 panel #2 are presented in Figure 2.
343 Among technical information (DNA elution ratio, qPCR volume ratio, master-mix reagents and
344 qPCR platform), the stepwise backward selection process identified only the qPCR platform
345 variable as significant for inclusion in the final model. Pairwise comparisons of qPCR platforms
346 are presented in Figure 3. Higher values (associated with worse performance) was observed for
347 the Cepheid platform, compared to all other qPCR platforms ($p<0.001$). This qPCR platform
348 (Cepheid's SmartCycler® instrument) was used by only two laboratories (10 and 12, right-hand
349 side in Fig. 2). Higher values were also observed for Roche compared to Applied biosystems
350 ($p<0.05$) and Rotor-Gene ($p<0.001$); a significant lower value (consequently superior
351 performance) was observed for Rotor-Gene compared to Bio-Rad ($p<0.05$) and Roche2
352 ($p<0.01$).
353
354 **Discussion**

355 The improved efficiency of real time qPCR techniques and the removal post-qPCR processing
356 shortens time for analysis and reduces false positive results, leading us to recommend the use
357 of qPCR assays for the detection of circulating Mucorales DNA in serum. Consequently, only
358 results from qPCR–based protocols were analysed in the current study.
359
360 Because of the severity of mucormycosis and the impact of any delay in treatment on prognosis,
361 any detectable amount of DNA (i.e., Cq < 45) was considered a positive result. Indeed, in
362 clinical setting, a first positive result should at least lead to increased biological, clinical and
363 radiological surveillance. This strategy improves early diagnosis and help to initiate early
364 appropriate treatment.
365 In panel #1, correct detection and identification of the target in serum was 94-100% when
366 testing strong positive samples (30 ≤ Cq ≤ 34). It was 77-100% when testing strong positive
367 samples (10 and 100 genome/mL) from panel #2, and 50-85% when testing weakly positives
368 samples (1 genome/mL). Out of the two panels, there were only 4 false positives which are
369 probably due to inter-sample contamination (<2% of all the qPCR results). Inter-laboratory
370 variability was minimal and Cq values were consistent, regardless of fungal load (panels #1 and
371 #2 SD < 3cyles). The main result of our study is the demonstration of very good inter-laboratory
372 concordance despite the considerable diversity of methods used (26 different combinations).
373 This was also the case for *Aspergillus* PCR[22].
374
375 However, some differences were observed. Firstly, the performance of qPCR varied according
376 to the Mucorales species spiked in serum. When serum was spiked with DNA from *L.*
377 *corymbifera*, a larger number of laboratories gave positive results compared with serum spiked
378 with *R. pusillus*, even at low DNA quantities. Variability at the time of preparation of spore
379 suspensions, extraction and serial dilutions cannot be excluded. Alternatively, this could be due

380 to a larger copy numbers of the 18S rDNA in *L. corymbifera*. Further investigations are required
381 to resolve copy number differences between species, using qPCR for a single-copy control gene
382 *vs*. 18S[27].
383 Regarding the qPCR performance, assays A and D seem to provide better analytical sensitivity
384 (fewer negative results and lower Cq values). But, but this has not been confirmed by statistical
385 analysis (just some significant pairwise comparisons "other" vs A, C and D). Moreover, the
386 number of the protocols using others qPCR assays than assay A, weakening the evidence
387 concerning the related comparisons.
388 When focussing on assay A (16/26 protocols in this study) certain protocols generated lower
389 Cq values. Among tested variables (DNA extraction method, qPCR amplification and
390 platforms), we observed higher Cq values for some qPCR platforms (Cepheid and to a lesser
391 degree Roche vs Applied biosystems) and lower for Rotor-Gene compared with Roche and Bio-
392 Rad.
393 However, it is probably a combination of several parameters (larger volume of serum extracted
394 AND optimal elution ratio AND optimal DNA input and qPCR reaction volume, AND optimal
395 qPCR platform along with high qPCR efficiency), that determines better performance.
396 Laboratory nine generally provided the earliest Cq values (left side in Fig. 2) and was able to
397 detect low genomic loads. Subsequently, the protocol used was scrutinized in detail (Table 2C).
398 Although the variables listed were not all significant in the statistical analyses, it is possible the
399 combination of factors is associated with optimal performance (large sample volume: 1000 µL
400 of serum; small elution volume: 50 µL; >25% ratio of DNA (template 9 µL, to final qPCR
401 volume 25 µL); Rotor-Gene Q® platform). Future inter-laboratory trials focusing on individual
402 steps of the whole molecular process (extraction and amplification) will help highlight each of
403 these elements.
404

405 Comparisons in the current study should be undertaken with caution because of the variable
406 number of laboratories that used each of the assays. In addition, certain technical steps, and
407 optimal combination, may have more impact on performance than a particular assay or
408 platform. Another limitation in respect to optimal clinical performance is the structure of the
409 DNA detected. The detection of Mucorales DNA in contrived samples is not the same as
410 detecting circulating Mucorales DNA from serum of infected patients (with potentially
411 fragmented DNA in human serum, as shown for *Aspergillus fumigatus* strains and *Aspergillus*
412 infections[28]). Especially, extraction efficiency is probably different according to the type of
413 targeted DNA (free DNA in serum sample, fungal DNA extracted from grown colonies, then
414 spiked in contrived sampled) and the type of samples (whole blood, serum, plasma) and this
415 may impact qPCR results. Samples from these inter-laboratory panels were exclusively serum
416 samples. Based on the experience gained by the *Aspergillus* PCR working group, the use of
417 serum is less technical than testing of whole blood and it allows the use of a single sample for
418 galactomannan enzyme-linked immunosorbent assay (ELISA), b-D-glucan, and PCR analysis,
419 thereby reducing costs if high-throughput screening of high-risk patients is required[22]. More
420 recently, the sensitivity of *Aspergillus* PCR using plasma was shown to be superior to that using
421 serum, and this should be also tested for Mucorales PCR[17].
422 Further studies, with an equivalent number of laboratories using specific assays and
423 methodological recommendations, specifically designed to assess the impact of certain steps
424 (input volume, elution volume, DNA/qPCR-volume ratio, and platform) are required to
425 improve the performance of detection of circulating Mucorales DNA using qPCR. However,
426 the robust inter-laboratory reproducibility demonstrated in this study, and very good
427 performance when detecting clinically relevant DNA concentrations in most of the laboratories
428 support the use of Mucorales qPCR as part of the diagnostic strategy for mucormycosis.
429

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Conflicts of Interest

A. Alanio reports speakers fees from Gilead and travel grant from Astellas. F Botterel reports speakers fees from Pfizer and travel grants from Gilead. F. Dalle reports meeting sponsorship from Pfizer. F. Morio reports speakers fees and travel grants from Basilea, Gilead, Pfizer and MSD. P.L.White performed diagnostic evaluations and received meeting sponsorship from Bruker, Dynamiker, and Launch Diagnostics; speakers fees, expert advice fees and meeting sponsorship from Gilead; and speaker and expert advice fees from F2G and speaker fees MSD and Pfizer; is a founding member of the European *Aspergillus* PCR Initiative. P. Donnely reports speaker fees from Gilead, Pfizer; consultancy from F2G. L. Millon reports speakers fees and meeting sponsorship from Gilead, Pfizer, Basilea and MSD. All other authors declare no conflict of interest relevant to this manuscript.

Tables and Figures

Table 1: Constitution and participants for each panel

Panels	Date	Participants		Participants with results included in analysis
1 (4-1mL serum)	2017	20 laboratories	12 French laboratories (including 9 participating in Modimucor group), named L1 to L12 *	18 laboratories using qPCR assays (2 laboratories using conventional PCR excluded)
			8 laboratories from Fungal PCR Initiative group named L13 to L20	
2 (7-1mL serum)	2018	22 laboratories	12 French laboratories (including 9 participating in Modimucor group), named L1 to L12 *	21 laboratories using qPCR assays (1 laboratory using conventional PCR excluded)
			10 laboratories from Fungal PCR Initiative group, named L13 - 18, 20, 24 - 26.	

* L1, L2 and L11 also participating in the FPCRI.

Table 2A: Characteristics of the 4 main qPCR assays used by the participants.

Assays	Target	Type of assays	Cycling parameter	Reaction mix	Reference	Genera /species detected	Type of result
A	18S rRNA	Combination of 3 targeted qPCR assay: Muc assay RMuc assay ACor Assay	Light cycler 480 Instrument II Hydrolysis probes Cycling condition 50 cycles of 15 seconds at 95°C, and 1 minute at 60°C	Light cycler 480 probes Master (Roche Diagnostic)	Millon et al.[7, 23]	*Rhizopus* spp., *Mucor* spp., *Rhizomucor* spp., *Lichtheimia* spp.	List of species detected by primers/probes is provided by EPA and available online[29].
B	18S rRNA	Mucorales-specific assay	StepOnePlus thermocycler (applied biosystem) Hydrolysis probes Cycling condition 50 cycles of 15 seconds at 95°C, and 1 minute at 60°C.	Taqman genEx master mix (Applied biosystem)	Springer et al.[8]	*Rhizopus* spp., *Mucor* spp., *Rhizomucor* spp., *Lichtheimia* spp. *Cunninghamella* spp.	Detection of Mucorales DNA Identification of species by an additional step of sequencing
C	ITS2	Combination of 6 targeted assays	Rotor-Gene 6000 Hydrolysis probes Cycling condition 50 cycles of 15 seconds at 95°C, and 1 minute at 60°C.	ABsolute QPCR ROX mix (Thermo Scientific, UK),	Lengerova et al.[24]	*Rhizopus microsporus* *Rhizopus oryzae* *Mucor spp.* *Lichtheimia corymbifera*	Detection of specific species of mucorales *Identification of* *Rhizopus microsporus* *Rhizopus oryzae* *Mucor spp.* *Lichtheimia corymbifera*
D	28S rRNA	Pan-mucorales assay	LightCycler 480 II (Roche) Rotor-Gene Q (Qiagen) CFX96 (Biorad) Mic qPCR (BMS) QuantStudio 5 (Thermo Fisher Scientific) Probes and cycling condition : NA	NA	Mucorgenius commercial kit	*Rhizopus* spp., *Mucor* spp., *Rhizomucor* spp., *Lichtheimia* spp. and C*unninghamella* spp.	Detection of Mucorales DNA *No further identification*

Table 2B: Primers and probes sequences (5'–3') of the 4 main qPCR assays used by the participants

	Forward Primer	Reverse Primer	Probes
Assay A (Millon et al.[7, 23])			
Muc assay	CACCGCCCGTCGCTAC	CCTAGTTTGCCATAGTTCTCTGCAG	FAM-CCGATTGAATGGTTATAGTGAGCATATGGGATC-TAMRA
RMuc assay	CACCGCCCGTCGCTAC	GTAGTTTGCCATAGTTCGGCTA	VIC-TTGAATGGCTATAGTGAGCATATGGGAGGCT-TAMRA
Acory assay	CACCGCCCGTCGCTAC	GCAAAGCGTTCCGAAGGACA	FAM-ATGGCACGAGCAAGCATTAGGGACG-TAMRA
Assay B (Springer at al.[8])			
18S based qPCR assay*	TTACC**R**TGAGCAAATCAGA**R**TG	AATC**Y**AAGAATTTCACCTCTAGCG	T**YRR**(G)G(G)**B**(A)T(T)T(G)T(A)TTT
Assay C (Lengerova at al.[24])			
Rhizopus microsporus	TTCGTGAATCATCGAGTCTTTGA	AGCAAGCGTACTCTATAGAAGATCCA	6-FAM-CGCAGCTTGCACTCT-MGBNFQ*b*
Rhizopus oryzae	AGCAAAGTGCGATAACTAGTGTGAA	TGAAGCAGGCGTACTCTATAGAAAAA	6-FAM-CGCAGCTTGCACTCT-MGBNFQ
Mucor spp.	GCAACTTGCGCTCATTGGTA	GGATAGAGGGTTTGTTTTGATACTGAA	6-FAM-CCAATGAGCACGCCTG-MGBNFQ
Rhizomucor pusillus	CCGTTCAAGCTACCCGAACA	AATGCAAGCCCTCAAGGAAA	6-VIC-TTTGTATGTTGTTGACCCTTG-MGBNFQ
Lichtheimia corymbifera (assay 1)	TTCAGTTGCTGTCATGGCCTTA	CATCCGGCAAATGACTAAAGC	6-FAM-ATACATTTAGTCCTAGGCAATT-MGBNFQ
Lichtheimia corymbifera (assay 2)	GTTGAGTTGGAACTGGGCTTCT	AGGACATTGATTTAAGGCCATGA	6-FAM-TTGATGGCATTTAGTTGCT-MGBNFQ
Assay D (MucorGenius)			
	NA	NA	NA

*Nucleotides in bold case are wobble nucleotides: **R** stands for A or G; **W** for A or T; **Y** for C or T; **B** for G, C or T.
a MGB, minor-groove binder.
b 6-FAM, 6-carboxyfluorescein; MGBNFQ, minor-groove binder nonfluorescent quencher.

Table 2C: An overview of the protocols used when testing panel #2. Some methods were grouped together (by type of extraction, manufacturer, mix reagents and qPCR platform) for statistical analyses. Detailed methods are provided in supplemental data (S2).

Protocols	Laboratories	Sample volume (ml)	DNA extraction method	Elution vol (µl)	qPCR platform	qPCR assay*	Mix reagents	Input qPCR vol (µl)	Final qPCR vol (µl)
1	L1	1.2	Qiagen automated	85	Roche	A	Roche	9	25
2	L2	1	Roche automated (large volume)	50	Roche	A	other	9	20
3	L3	1	Qiagen automated	60	Applied	A	Applied	9	20
4	L4	1	Biomerieux automated	50	Roche	A	Roche	9	20
5	L5	1	Biomerieux automated	50	Roche	A	Roche	9	20
6	L6	1	Roche automated (large volume)	50	Bio-Rad	A	other	9	20
7	L7	1	Biomerieux automated	50	Applied	A	Applied	9	20
8	L8	1	Roche automated (large volume)	50	Roche 2	A	Roche	9	20
9	L9	1	Roche automated (large volume)	50	Rotorgene	A	Applied	9	25
10	L10	1	Roche automated (large volume)	50	Cepheid	A	other	5	25
11	L11	1	other	100	Applied	A	Roche	9	20
12	L12	1	Biomerieux automated	100	Cepheid	A	other	5	25
13	L13	0.2	other	100	Roche	other	other	8	20
14	L13	0.2	other	100	Roche	other	Applied	8	20
15	L14	1	other	70	Applied	B	Applied	5	20
16	L15	0.4	other	50	Rotorgene	C	Applied	5	25
17	L15	0.4	other	100	Rotorgene	D	other	5	25
18	L16	0.5	Biomerieux automated	60	Rotorgene	D	other	5	25
19	L16	1	Biomerieux automated	100	Rotorgene	D	other	5	25
20	L16	1	Biomerieux automated	100	Rotorgene	A	Roche	20	50
21	L17	0.5	other	100	Roche 2	other	Roche	5	20
22	L18	1	Roche automated (large volume)	50	Bio-Rad	A	other	7	20
23	L24	1	Roche automated (large volume)	50	Roche	A	Roche	10	25
24	L24	1	Roche automated (large volume)	50	Roche	A	Roche	5	20
25	L25	0.75	Biomerieux automated	50	Roche	D	other	5	25
26	L26	1	Biomerieux automated	50	Applied	other	Applied	5	30

*qPCR assays: “A”: qPCR described by Millon et al.[7, 23]; “B”: qPCR described by Springer et al.[8]; “C”: qPCR described by Lengerova et al.[24]; “D”: MucorGenius kit and “other”: qPCR assay not published.
For DNA extraction method, “other” is manual extraction methods or Roche automated methods with small volume.

Table 3: Results for panel #1 and Cq values for the most commonly used qPCR and for all qPCRs combined.

Spiked DNA	serum N°	Positivity rate (correct identification to the genus level)	Average Cq (SD) Laboratories using qPCR A (n=14) *	Average Cq (SD) All laboratories -All qPCR assays (n=18) *
R. pusillus	S1-1	94%	34.0 (2.45)	34.7 (3.0)
Negative control	S2-1	100%	/	/
R. oryzae	S3-1	100%	30.1 (1.74)	31.2 (2.8)
L. corymbifera	S4-1	94%	33.3 (1.61)	33.7 (1.9)

* Only Cq values for good identification were used calculation of the mean and standard deviation (SD)
qPCR assays: "A": qPCR described by Millon et al.[7, 23].

Table 4: Composition (DNA quantity in genome equivalent /mL) of serum panel #2 (S1 to S7) and percentage of laboratories that generated positive qPCR result for each individual sample.

		Serum N°	Positive samples qPCR A (n=16)	Positive samples qPCR D (n=4)	Positive samples qPCR B, C and other (n=6)	Positive samples all qPCR (n=26)
Composition of panel	Negative control (**no DNA**)	S1-2	6%	0%	0%	4%
	Rhizomucor pusillus (**100** genomes/mL)	S5-2	94%	100%	83%	92%
	Rhizomucor pusillus (**10** genomes/mL)	S2-2	88%	75%	50%	77%
	Rhizomucor pusillus (**1** genome/mL)	S7-2	56%	75%	17%	50%
	Lichtheimia corymbifera (**100** genomes/mL)	S3-2	100%	100%	100%	100%
	Lichtheimia corymbifera (**10** genomes/mL)	S6-2	100%	100%	83%	96%
	Lichtheimia corymbifera (**1** genome/mL)	S4-2	100%	75%	50%	85%

qPCR assays: "A": qPCR described by Millon et al.[7, 23]; "B": qPCR described by Springer et al.[8]; "C": qPCR described by Lengerova et al.[24]; "D": MucorGenius kit and "other": qPCR assay not published.

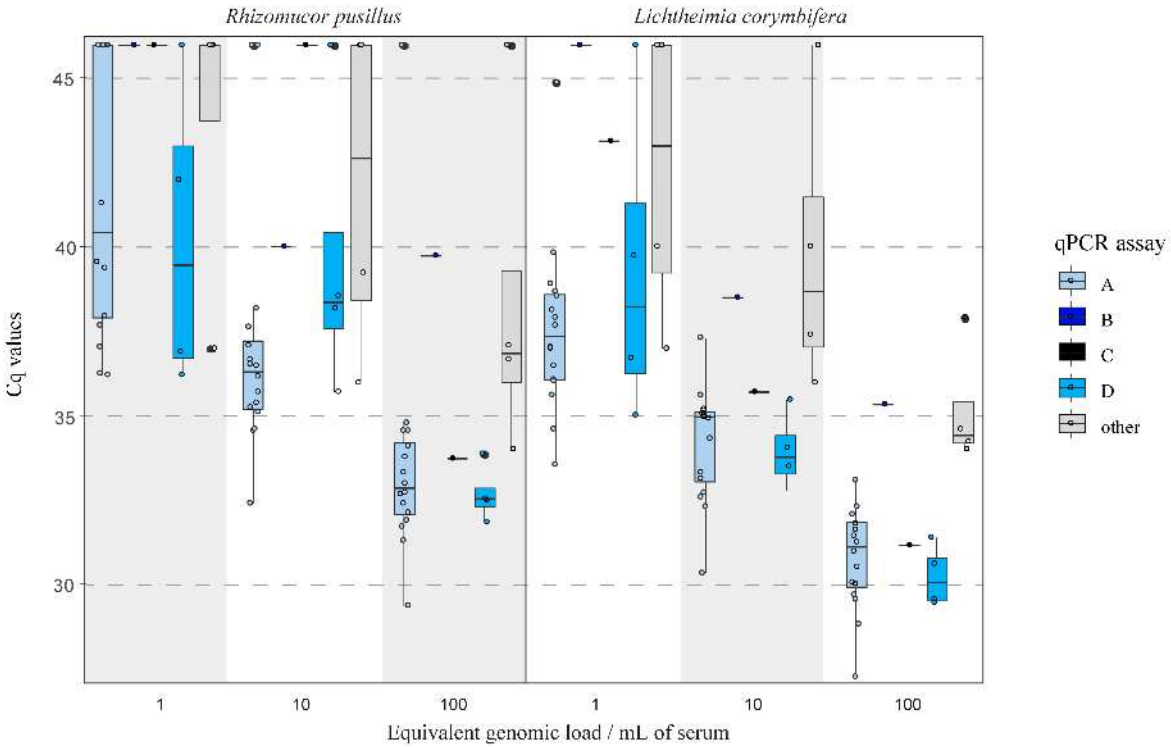

Figure 1: Distribution of Cq values according to the spiked quantity (genome), the species and the qPCR used for detection (panel #2).
qPCR assays: "A" (n=16 protocols): qPCR described by Millon et al.[7, 23]; "B" (n=1 protocol): qPCR described by Springer et al.[8]; "C" (n=1 protocol): qPCR described by Lengerova et al.[24]; "D" (n=4 protocols): MucorGenius kit and "other" (n=4 protocols): qPCR assay not published.

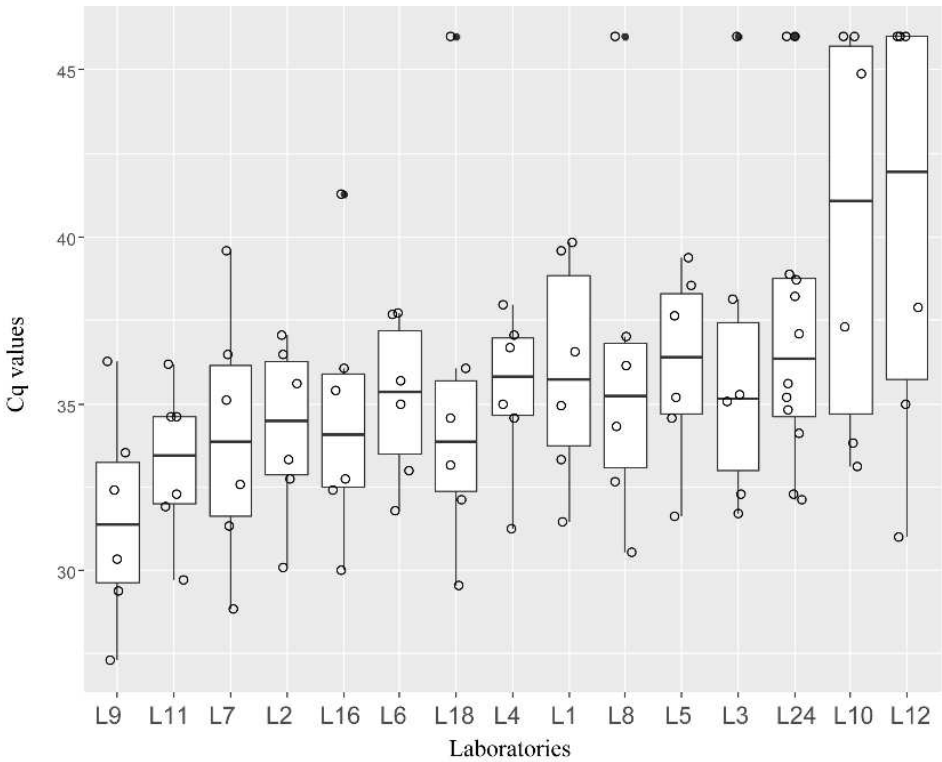

Figure 2: Cq results obtained by the 15 different laboratories using qPCR assay A[7, 23]. A Cq value of 46 was assigned to the negative results.

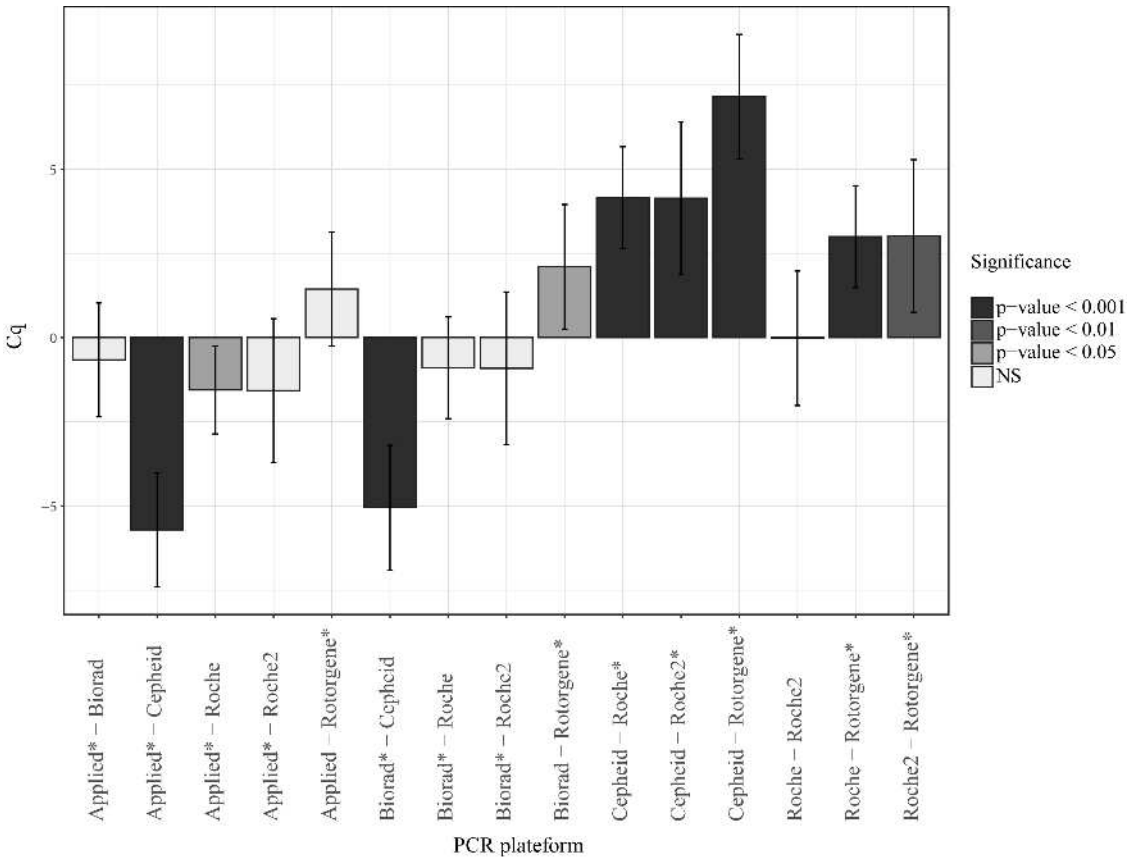

Figure 3: Pairwise comparison of qPCR platforms (least squares means and confidence intervals between the qPCR platforms included in the fixed part of linear mixed effects model). Significance of differences is mentioned with grey intensities. When the difference had a negative value, the first qPCR platform had a best performance.
*: best platform in each pairwise comparison.
NS: No significant difference.

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