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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Key Words

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.

Introduction

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

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

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

DNA per microliter of serum in patients with probable and proven mucormycosis (median Cq was 34 cycles (range 23-41 cycles)), while the *Aspergillus* DNA concentrations found in patients with invasive aspergillosis were <0.1fg/µL of serum (median 40 cycles (range 33-45 cyles)) [7, 9].

This large DNA load makes an accurate quantification for therapeutic monitoring possible[7]. An increasing number of studies has demonstrated that Mucorales qPCR is very helpful in optimizing the management of mucormycosis[13-15]. However, studies evaluating and comparing analytical performance between methods are lacking, limiting the standardized optimal methods, necessary for inclusion as mycological criterion in future EORTC/MSG definitions.

The aim of the ISHAM working group the European *Aspergillus* PCR Initiative (EARPCI) was to standardize *Aspergillus* PCR[16, 17], for inclusion as microbiological criterion for defining probable aspergillosis in the EORTC/MSG definitions[5]. As this has now been achieved, the initiative expanded its remit include the molecular detection of *Candida*, Mucorales, *Pneumocystis*[18] and fungi in tissue and changed its name accordingly to the Fungal PCR Initiative (FPCRI, www.fpcri.eu). The Mucorales Laboratory Working party organised the distribution of two separate series of inter-laboratory simulated serum panels for the molecular detection of Mucorales DNA.

These inter-laboratory studies were performed in 2017 and 2018 with two main objectives: 1) to evaluate qualitative diagnosis (positive/negative) and to assess the reproducibility of methods currently used and 2) to assess qPCR performance according to protocols used. Twenty-three European laboratories participated in these studies. This large collaboration allowed comparison of 4 main qPCR assays, with 26 different technical protocols, with various combinations of DNA extraction methods, qPCR targets, qPCR platforms and qPCR reagents and helped identify procedural factors associated with the best qPCR performance.

Materials and Methods

1) Participants

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

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

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

2) DNA source material

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

Lichtheimia corymbifera (IHEM 3809) strains were grown on Sabouraud dextrose agar medium (37°C, 5 days). Species identification was confirmed by ITS sequencing (V9D and LS266 primers[20]). DNA was extracted from cultures using the DNeasy Plant Mini Kit™ (Qiagen®, Hilden, Germany) and DNA concentration was measured using a Nanodrop® (Thermo Fisher Scientific®, Waltham, MA, USA). These DNA solutions were used to spike sera.

3) Preparation of simulated serum panels

Serum was obtained from healthy donors, volunteering to donate their blood specifically for research purpose, according to procedure and ethical rules of the Bourgogne Franche-Comté Blood Transfusion Center (BTC). Detection of infectious agents was performed according to usual protocols of the BTC. For each panel the serum from up to three donors was pooled and was tested for contamination using specific Mucorales qPCR targeting the most frequent genera[6, 21] before processing. All processing of material took place in a category II laminar flow cabinet to minimize the risk of contamination by environmental fungal spores.

Two panels (#1 and #2) including 1mL-serum samples were sent to each of the 23 laboratories (Table 1). Both panels were stored at -20°C before shipping (-20°C for international shipping, +4°C for shipping in France). Panels were sent in July 2017 (panel #1) and July 2018 (panel #2). All panels were delivered within 48h and stored below +4°C before being analysed.

The first panel (panel #1, four 1mL-serum samples) aimed at assessing qualitative diagnosis (positive/negative). Three sera were spiked with DNA from three different species (*R. pusillus* (27 pg/mL of serum), *L. corymbifera* (30 pg/mL of serum), *R. oryzae* (116 pg/mL of serum)). The second panel (panel #2, seven 1mL-serum samples) was designed to assess qPCR performance. To this end, 3 concentrations of *R. pusillus* and *L. corymbifera* (1, 10 and 100

genomes equivalent/mL) were prepared as previously described by the European *Aspergillus* PCR Initiative group[22]. The concentrations chosen generated Cq values comparable to those observed in patients diagnosed with mucormycosis (range 23-41 cycles)[7]. DNA was extracted from *R. pusillus* and *L. corymbifera* conidia and serial dilutions were performed considering that one conidia had one genome and that the extraction efficiency from the respective culture was 100%. Each panel included a blind negative-control serum.

A form was distributed with each panel to collect results and obtain technical information, including DNA extraction method, sample volume used, DNA elution volume, qPCR method, qPCR template input volume, qPCR total reaction volume, qPCR amplification platform, and qPCR reagents used.

4) DNA extraction and qPCR assays

While recommendations were given to French laboratories participating in the Modimucor study (DNA extraction from 1mL of serum with an elution volume of 50µL; specific qPCR assay[7, 23]), all other participants used their own methodology for both DNA extraction and qPCR amplification. All the participants used the qPCR platform and reagents available in their own laboratories and provided all protocol details on the technical form.

The qPCR assays used in panel #1 were distributed across four categories: qPCR A, genus-specific assay described by Millon et al.[7, 23]; qPCR B, mucorales-specific assay described by Springer at al.[8] ; qPCR C, species-specific assay described by Lengerova et al.[24] and other qPCR assays not published.

In panel #2, the same qPCR assays A, B, C and others (not published) were used. In addition, qPCR D was assigned to participants using the Pathonostics MucorGenius kit (mucorales-specific assay). Description of gene targets, primers and probes, cycling parameters and level

of identification allowed by each of 4 main qPCR assays (A, B, C, D) are provided in Tables 2A and 2B.

The results were expressed in quantification cycles (Cq), with higher values indicating the smaller the amount of DNA in the sample. Any detectable amount of DNA (i.e., Cq < 45) was considered a positive result.

The combination of DNA extraction methods, qPCR targets, qPCR platforms, qPCR mix reagents, elution volumes and qPCR volumes resulted in 18 different protocols used in panel #1 and 26 in panel #2 (Table 2C) .

The large diversity of reagents and platforms used for extraction and amplification meant that some variables had to be grouped together for statistical analysis. For example, DNA extraction methods were grouped in four categories according to manufacturer and type of extraction (i.e. automated or manual). Master-mix reagents were grouped in 3 categories according to manufacturer (Applied biosystems, Roche and others), and qPCR platform grouped in six manufacturer aligned categories (Applied biosystems, Bio-Rad, Cepheid, Rotorgene, Roche (for microplate technology, LightCycler480) and Roche2 (for capillary technology, LightCycler 2.0). Grouped data are provided in supplemental data (S1) for panel #1, and in Table 2C for panel #2; details of methods for panel #2 are provided in supplemental data (S2).

5) Statistical analysis

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

Data from panel #2 monitored qPCR performance across all laboratories using different qPCR assays, before focusing on performance in laboratories using a same qPCR assay (assay A). Only the Cq values corresponding to the detection of the correct target (defined according to the qPCR assay used, as described above) were included in the statistical tests (e.g. if the assay detected *R. pusillus* with a Cq of 35 cycles but the sample contained *L. corymbifera* DNA it was excluded from analysis). A Cq value of 46 was assigned to the negative results. Statistical analyses were performed using the statistical software R-3.4.4 for Microsoft® Windows.

To analyse Cq values in panel #2, statistical models assessed the potential benefit of inclusion of random effects in the models (e.g. influence of the "genomic load" and/or spiked species). A first linear mixed effect model (LMM)[25] was used to model Cq values in function of log transform genomic loads, with a grouping variable "laboratory" and a random effect "genomic load". To assess the impact of different species in the diagnostic sensitivity, a second LMM (species-specific model) was created adding spiked species as a covariate in the fixed effect part of the model. Interaction between genomic load and fungal species was also analyse.

The species-specific model was significantly better when compared to the first model ($p<0.001$). Differences in detection rates between spiked species were observed (Fig. S3 in supplemental data), with *L. corymbifera* being better detected (irrespective of the protocol) than *R. pusillus*. Thus, for the following statistical analysis, the species-specific model was used.

The influence of the qPCR assay was then investigated by adding this variable to the species-specific model and qPCR assays were pairwise compared using differences of least squares means (marginal effects) and confidence intervals with lmerTest library[26].

To determine whether technical parameters influence performance, the 16 protocols using qPCR assay A were arranged according to the Cq value. A full LMM with the serum tested in random part of the model was then undertaken using Cq values and the different available

variables (group of DNA extraction method, mix reagent, platform, elution ratio for DNA extraction (elution volume / volume of sample extracted) and qPCR volume ratio (qPCR input volume / final volume of qPCR reaction). A backward stepwise selection was performed to select variables to include in the final model. Factors selected in the final model were pairwise compared using differences of least squares means as previously mentioned.

Results

All participating centres returned results and the completed form with technical information within 3 months.

1) Evaluation of the qualitative detection of a range of Mucorales species (panel #1)

For panel #1 (three spiked serum and one negative control), results from 18 laboratories (corresponding to 18 protocols) were analysed. Correct detection and identification of the target in serum was 94.4% (17/18, 95% CI: 74.2-99.0) for sera spiked with DNA from *R. pusillus* and *L. corymbifera*, and 100% (18/18, 95% CI: 82.4-100) for sera spiked with DNA from *R. or*yzae (Table 3). The two labs that failed to give a positive signal were laboratory L15 with protocol 15 (supplemental data S1) for serum S1-1 spiked with DNA from *R. pusillus* and laboratory L13 with protocol 13 for serum S4-1 spiked with DNA from *L. corymbifera.* These two laboratories used qPCR C and B respectively. A single laboratory (L7 in supplemental data S1) using qPCR A gave an additional positive signal for *Mucor/Rhizopus* assay for serum S1-1 (spiked with *R. pusillus*), with high Cq values (44.14). No false positive results were observed for the negative-control serum. Qualitative results (positive or negative) were correct in 97% of cases (70/72).

Despite the huge diversity of methods, Mucorales DNA detection in sera was highly reproducible with a very low inter-laboratory variability in Cq values (SD = 1.89 cycles [range 1.3; 2.9]).

2) Determination of Mucorales qPCR performance (panel #2)

For panel #2 (six spiked serum and one negative control), results from 21 laboratories were analysed, corresponding to a total of 26 different protocols (combined DNA extraction and qPCR amplification methods, Table 2C and supplemental data S2). Three centres tested two protocols, one centre tested three protocols and 17 centres tested a single protocol.

2.1) Comparison of performance between different qPCR assays

Across all qPCR assays, the detection rates were high (77-100%) for 5/6 of spiked serum (S2-2, S3-2, S4-2, S5-2, S6-2 (Table 4)). The 6% of false positive rate recorded with negative control when qPCR assay A was used (Table 4) correspond to only one laboratory (L7 with protocol 7 in Table 2C). This laboratory detected *Mucor/Rhizopus* in the control serum (Cq = 38.68) and was the same laboratory that had a cross detection in panel #1 (additional positive signal for *Mucor/Rhizopus* assay for serum S1-1 spiked with *R. pusillus*).

For serum S7-2 spiked with 1 equivalent genome of *R. pusillus*/mL, one laboratory (L6, protocol 6 using qPCR A) gave an additional positive signal (cross detection) for *Mucor/Rhizopus* assay (Cq = 36) and one laboratory (L3, protocol 3 using qPCR A) just gave a positive signal for *Lichtheimia* assay (Cq = 36.7 and 38). The qPCR detection rate for this serum (S7-2) was 50%.

For assay A which was used in 15 laboratories (16/26 protocols), global sensitivity and specificity were 89.6% and 97%, respectively (100% and 94.1% for *Lichtheimia*, and 79.1%

and 100% for *Rhizomucor*). For assay D which was used in 4 laboratories (4/26 protocols), sensitivity and specificity were 84% and 100%, respectively.

Figure 1 illustrates the level of Cq value according to the qPCR assay, accounting for the genomic load and the fungal species. Assays A and D gave fewer negative results and lower (earlier) Cq values (increasing the likelihood of detection). Pairwise comparisons showed that "other" qPCR gave significant higher (later) Cq value compared to assays A, C and D ($p=0.002$, 0.01 and 0.006, respectively). However, these results should be interpreted with caution given the modest number of observations from some qPCR systems (e.g. B and C).

2.2) The influence of differing technical aspects on the performance of qPCR assay A

Fifteen laboratories used qPCR assay A. Results obtained for the six spiked serums constituting panel #2 are presented in Figure 2.

Among technical information (DNA elution ratio, qPCR volume ratio, master-mix reagents and qPCR platform), the stepwise backward selection process identified only the qPCR platform variable as significant for inclusion in the final model. Pairwise comparisons of qPCR platforms are presented in Figure 3. Higher values (associated with worse performance) was observed for the Cepheid platform, compared to all other qPCR platforms ($p<0.001$). This qPCR platform (Cepheid's SmartCycler® instrument) was used by only two laboratories (10 and 12, right-hand side in Fig. 2). Higher values were also observed for Roche compared to Applied biosystems ($p<0.05$) and Rotor-Gene ($p<0.001$); a significant lower value (consequently superior performance) was observed for Rotor-Gene compared to Bio-Rad ($p<0.05$) and Roche2 ($p<0.01$).

Discussion

The improved efficiency of real time qPCR techniques and the removal post-qPCR processing shortens time for analysis and reduces false positive results, leading us to recommend the use of qPCR assays for the detection of circulating Mucorales DNA in serum. Consequently, only results from qPCR–based protocols were analysed in the current study.

Because of the severity of mucormycosis and the impact of any delay in treatment on prognosis, any detectable amount of DNA (i.e., Cq < 45) was considered a positive result. Indeed, in clinical setting, a first positive result should at least lead to increased biological, clinical and radiological surveillance. This strategy improves early diagnosis and help to initiate early appropriate treatment.

In panel #1, correct detection and identification of the target in serum was 94-100% when testing strong positive samples ($30 \leq Cq \leq 34$). It was 77-100% when testing strong positive samples (10 and 100 genome/mL) from panel #2, and 50-85% when testing weakly positives samples (1 genome/mL). Out of the two panels, there were only 4 false positives which are probably due to inter-sample contamination (<2% of all the qPCR results). Inter-laboratory variability was minimal and Cq values were consistent, regardless of fungal load (panels #1 and #2 SD < 3cyles). The main result of our study is the demonstration of very good inter-laboratory concordance despite the considerable diversity of methods used (26 different combinations). This was also the case for *Aspergillus* PCR[22].

However, some differences were observed. Firstly, the performance of qPCR varied according to the Mucorales species spiked in serum. When serum was spiked with DNA from *L. corymbifera*, a larger number of laboratories gave positive results compared with serum spiked with *R. pusillus*, even at low DNA quantities. Variability at the time of preparation of spore suspensions, extraction and serial dilutions cannot be excluded. Alternatively, this could be due

to a larger copy numbers of the 18S rDNA in *L. corymbifera*. Further investigations are required to resolve copy number differences between species, using qPCR for a single-copy control gene *vs*. 18S[27].

Regarding the qPCR performance, assays A and D seem to provide better analytical sensitivity (fewer negative results and lower Cq values). But, but this has not been confirmed by statistical analysis (just some significant pairwise comparisons "other" vs A, C and D). Moreover, the number of the protocols using others qPCR assays than assay A, weakening the evidence concerning the related comparisons.

When focussing on assay A (16/26 protocols in this study) certain protocols generated lower Cq values. Among tested variables (DNA extraction method, qPCR amplification and platforms), we observed higher Cq values for some qPCR platforms (Cepheid and to a lesser degree Roche vs Applied biosystems) and lower for Rotor-Gene compared with Roche and Bio-Rad.

However, it is probably a combination of several parameters (larger volume of serum extracted AND optimal elution ratio AND optimal DNA input and qPCR reaction volume, AND optimal qPCR platform along with high qPCR efficiency), that determines better performance. Laboratory nine generally provided the earliest Cq values (left side in Fig. 2) and was able to detect low genomic loads. Subsequently, the protocol used was scrutinized in detail (Table 2C). Although the variables listed were not all significant in the statistical analyses, it is possible the combination of factors is associated with optimal performance (large sample volume: 1000 µL of serum; small elution volume: 50 µL; >25% ratio of DNA (template 9 µL, to final qPCR volume 25 µL); Rotor-Gene Q® platform). Future inter-laboratory trials focusing on individual steps of the whole molecular process (extraction and amplification) will help highlight each of these elements.

Comparisons in the current study should be undertaken with caution because of the variable number of laboratories that used each of the assays. In addition, certain technical steps, and optimal combination, may have more impact on performance than a particular assay or platform. Another limitation in respect to optimal clinical performance is the structure of the DNA detected. The detection of Mucorales DNA in contrived samples is not the same as detecting circulating Mucorales DNA from serum of infected patients (with potentially fragmented DNA in human serum, as shown for *Aspergillus fumigatus* strains and *Aspergillus* infections[28]). Especially, extraction efficiency is probably different according to the type of targeted DNA (free DNA in serum sample, fungal DNA extracted from grown colonies, then spiked in contrived sampled) and the type of samples (whole blood, serum, plasma) and this may impact qPCR results. Samples from these inter-laboratory panels were exclusively serum samples. Based on the experience gained by the *Aspergillus* PCR working group, the use of serum is less technical than testing of whole blood and it allows the use of a single sample for galactomannan enzyme-linked immunosorbent assay (ELISA), b-D-glucan, and PCR analysis, thereby reducing costs if high-throughput screening of high-risk patients is required[22]. More recently, the sensitivity of *Aspergillus* PCR using plasma was shown to be superior to that using serum, and this should be also tested for Mucorales PCR[17].

Further studies, with an equivalent number of laboratories using specific assays and methodological recommendations, specifically designed to assess the impact of certain steps (input volume, elution volume, DNA/qPCR-volume ratio, and platform) are required to improve the performance of detection of circulating Mucorales DNA using qPCR. However, the robust inter-laboratory reproducibility demonstrated in this study, and very good performance when detecting clinically relevant DNA concentrations in most of the laboratories support the use of Mucorales qPCR as part of the diagnostic strategy for mucormycosis.

Acknowledgements

This work was supported by a grant from the French Ministry of Health PHRC (Projet Hospitalier de Recherche Clinique) national-ModiMucor 2014-A00580-47.

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.

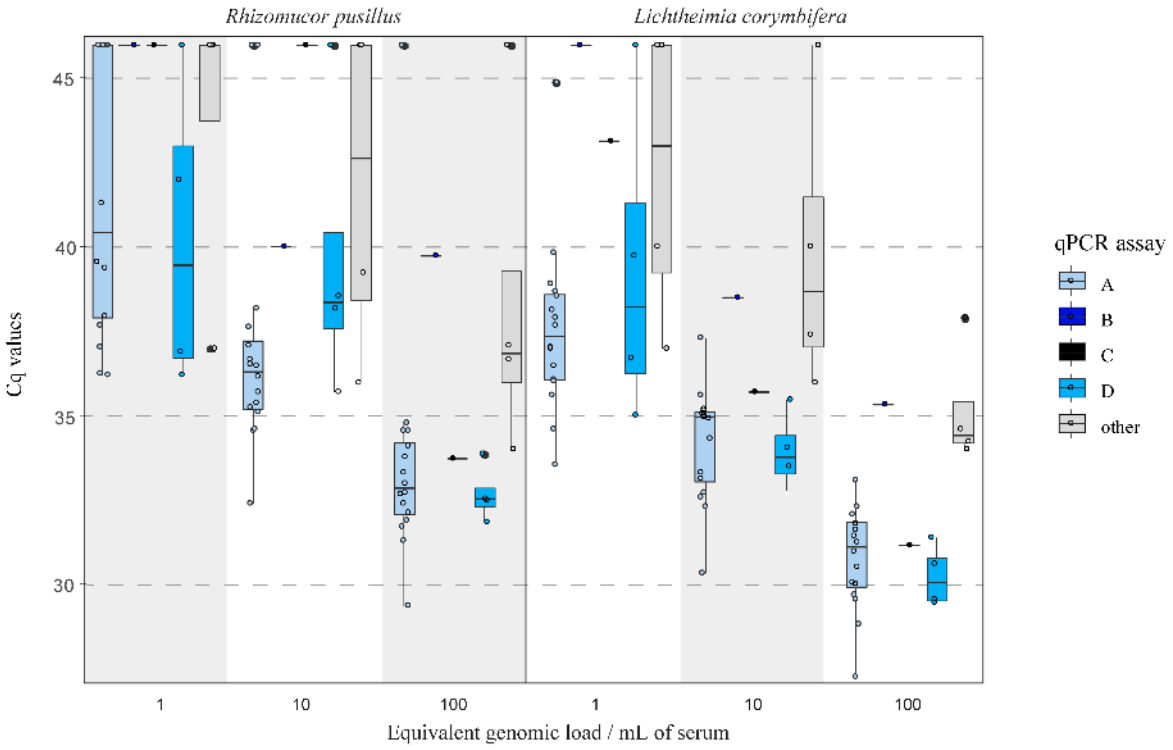

Figure 1: Distribution of Cq values according to the spiked quantity (genome), the species and the qPCR used for detection (panel #2).
qPCR assays: "A" (n=16 protocols): qPCR described by Millon et al.[7, 23]; "B" (n=1 protocol): qPCR described by Springer et al.[8]; "C" (n=1 protocol): qPCR described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

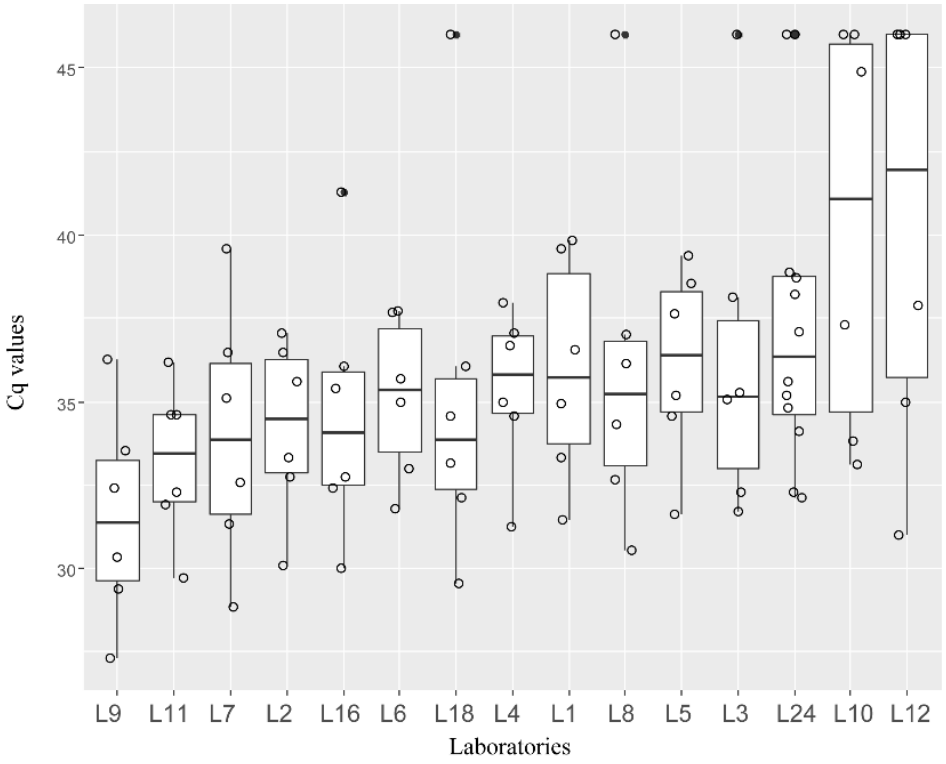

Figure 2: Cq results obtained by the 15 different laboratories using qPCR assay A[7, 23]. A Cq value of 46 was assigned to the negative results.

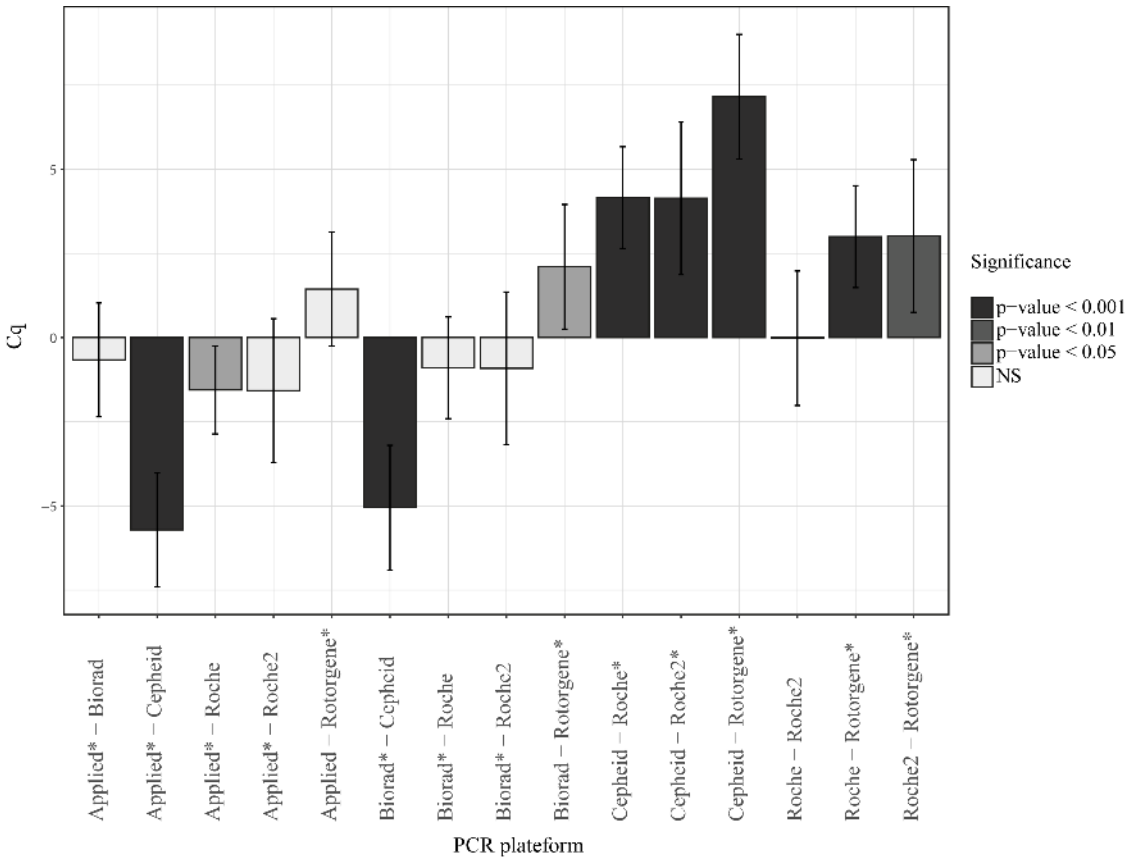

Figure 3: Pairwise comparison of qPCR platforms (least squares means and confidence intervals between the qPCR platforms included in the fixed part of linear mixed effects model). Significance of differences is mentioned with grey intensities. When the difference had a negative value, the first qPCR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

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