

The Fungal PCR Initiative's evaluation of in-house and commercial Pneumocystis jirovecii qPCR assays: towards a standard for a diagnostics assay

Maud Gits-Muselli, P. Lewis White, Carlo Mengoli, Sharon Chen, Brendan Crowley, Gijs Dingemans, Emilie Fréalle, Rebecca Gorton, Malcom Guiver, Ferry Hagen, et al.

▶ **To cite this version:**

Maud Gits-Muselli, P. Lewis White, Carlo Mengoli, Sharon Chen, Brendan Crowley, et al.. The Fungal PCR Initiative's evaluation of in-house and commercial Pneumocystis jirovecii qPCR assays: towards a standard for a diagnostics assay. 2019. pasteur-02337518

HAL Id: pasteur-02337518

https://pasteur.hal.science/pasteur-02337518

Preprint submitted on 29 Oct 2019

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

The Fungal PCR Initiative's evaluation of in-house and commercial *Pneumocystis jirovecii* qPCR assays: towards a standard for a diagnostics assay

Maud Gits-Muselli,[1,2,3] P. Lewis White[4], Carlo Mengoli,[5] Sharon Chen[6], Brendan Crowley[7], Gijs Dingemans[8], Emilie Fréalle[9], Rebecca Gorton[10], Malcom Guiver[11], Ferry Hagen[12], Catriona Halliday[6], Gemma Johnson[13], Katrien Lagrou[14], Martina Lengerova[15], Willem JG Melchers[16], Lily Novak-Frazer[17], Riina Rautemaa-Richardson[18], Emeline Scherer[19], Joerg Steinmann[20,21], Mario Cruciani[22], Rosemary Barnes[23], J. Peter Donnelly[24], Juergen Loeffler[25], Stéphane Bretagne[1,2,3] and Alexandre Alanio[1,2,3,*]

[1] Institut Pasteur, Molecular Mycology Unit, CNRS UMR2000, Paris, France

[2] Laboratoire de Parasitologie-Mycologie, Hôpital Saint-Louis, Groupe Hospitalier Lariboisière, Saint-Louis, Fernand Widal, Assistance Publique-Hôpitaux de Paris (AP-HP), Paris, France

[3] Université de Paris, Paris, France

[4] Public Health Wales, Microbiology Cardiff, UHW, Heath Park, Cardiff, UK

[5] University of Padua, Padua, Italy

[6] Clinical Mycology reference Laboratory, Centre for Infectious Diseases and Microbiology Laboratory Services, Institute of Clinical Pathology and Medical Research, New South Wales Health Pathology, Westmead Hospital, and the University of Sydney, Australia

[7]Department of Virology, St James's Hospital, Dublin, Ireland

[8] PathoNostics B.V., Maastricht, The Netherlands

For Peer Review Only

[9] CHU Lille, Laboratoire de Parasitologie-Mycologie, F-59000 Lille, France & Univ. Lille, CNRS, Inserm, CHU Lille, Institut Pasteur de Lille, U1019–UMR8204-CIIL-Centre for Infection and Immunity of Lille, F-59000 Lille, France

[10] Regional UK Clinical Mycology Network (UK CMN) Laboratory, Dept. Infection Sciences, Health services laboratories (HSL) LLP, London, UK

[11] Public Health Laboratory, National Infection Service Public Health England, Manchester University NHS Foundation Trust, Manchester, UK

[12] Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; and Department of Medical Microbiology, University Medical Centre Utrecht, Utrecht, The Netherlands

[13] OLM Diagnostics, Newcastle-upon-Tyne, UK

[14] Department of Microbiology, Immunology and Transplantation, KU Leuven, and Department of Laboratory Medicine and National Reference Centre for Mycosis, Excellence Centre for Medical Mycology (ECMM), University Hospitals Leuven, Leuven, Belgium

[15] Department of Internal Medicine – Hematology and Oncology, University Hospital Brno, Brno, Czech Republic

[16] Radboud University Medical Centre, Department of Medical Microbiology, Nijmegen, The Netherlands

[17] Mycology Reference Centre Manchester, Manchester University NHS Foundation Trust; and Division of Infection, Immunity and Respiratory Medicine, Faculty of Biology, Medicine and Health, University of Manchester, UK

For Peer Review Only

[18] Department of Infectious Diseases and the Mycology Reference Centre Manchester, Manchester University NHS Foundation Trust; and Division of Infection, Immunity and Respiratory Medicine, Faculty of Biology, Medicine and Health, University of Manchester, UK

[19] Department of Parasitology-Mycology, University Hospital of Besançon, Besançon, France

[20] Institute of Clinical Hygiene, Medical Microbiology and Infectiology, Klinikum Nürnberg, Paracelsus Medical University, Nuremberg, Germany

[21] Institute of Medical Microbiology, University Hospital Essen, University of Duisburg-Essen, Essen, Germany

[22] Infectious Diseases Unit, San Bonifacio Hospital, Verona, Italy

[23] Emeritus Professor of Medical Microbiology and Infectious Diseases, Cardiff University School of Medicine, Cardiff UK

[24] Nijmegen, The Netherlands

[25]University Hospital Wuerzburg, Medical Hospital II, C11, Wuerzburg, Germany

***Corresponding Author:** Dr Alexandre Alanio, Molecular Mycology unit, Institut Pasteur, 25 rue du Dr Roux 75724 Paris Cedex 15; email: alexandre.alanio@pasteur.fr; Tel: +33140613255; Fax: +33145688420

Keywords

Pneumocystis jirovecii, Pneumocystosis, PCP, Diagnosis, qPCR, quantification, DNA, Whole nucleic acids, Panel specimens, standardization, efficiency, quantification cycles, Cq, threshold.

For Peer Review Only

Abstract

Quantitative real-time PCR (qPCR) is increasingly used to detect *Pneumocystis jirovecii* for the diagnosis of *Pneumocystis* pneumonia (PCP), but there are differences in the nucleic acids targeted, DNA only versus whole nucleic acid (WNA), and also the target genes for amplification. Through the Fungal qPCR Initiative, a working group of the International Society for Human and Animal Mycology, a multicentre and monocentre evaluation of PCP qPCR assays was performed. For the multicentre study, 16 reference laboratories from eight different countries, performing 20 assays analysed a panel consisting of two negative and three PCP positive samples. Aliquots were prepared by pooling residual material from 20 negative or positive- *P. jirovecii* bronchoalveolar lavage fluids (BALFs). The positive pool was diluted to obtain three concentrations (pure 1:1; 1:100; and 1:1000 to mimic high, medium, and low fungal loads respectively). The monocentre study compared five in-house and five commercial qPCR assays testing 19 individual BALFs on the same amplification platform. Across both evaluations and for all fungal loads, targeting WNA and the mitochondrial small sub-unit (mtSSU) provided the earliest Cq values, compared to only targeting DNA and the mitochondrial large subunit, the major surface glycoprotein or the beta-tubulin genes. Thus, Reverse Transcriptase-qPCR targeting the mtSSU gene could serve as a basis for standardizing the *P. jirovecii* load, which is essential if qPCR is to be incorporated into clinical care pathways as the reference method, accepting that additional parameters such as amplification platforms still need evaluation.

For Peer Review Only

INTRODUCTION

Pneumocystis jirovecii is an atypical ascomycetous fungus responsible for *Pneumocystis* pneumonia (PCP), a severe respiratory infection in immunocompromised patients such as those with HIV/AIDS, organ transplant recipients, patients affected by haematological malignancies and patients under immunosuppressive therapies.[1] Healthy humans are a reservoir of *P. jirovecii* resulting in likely continuous inter-individual circulation responsible for further transmission to immunocompromised hosts.[2] Once acquired by a susceptible host, the fungal load increases from undetectable to a high fungal burden leading to full blown pneumonia.[2,3] However, PCP is more severe and mortality rates are significantly higher in non-HIV patients than in HIV-infected/AIDS people, despite a lower fungal load during infection.[4] Therefore, diagnosis of PCP remains a challenge because of the importance of quantification and detection of low fungal loads.

Conventional diagnosis of PCP relies on visualization of asci and trophic forms of *P. jirovecii* by conventional staining (Giemsa, methenamine silver, Toluidine Blue O) or by immunofluorescence using anti-trophic form and/or anti-asci antibodies.[5] These methods depend on staff experience and results can be falsely negative in the case of low fungal load, particularly in non-HIV patients.[4] Recently, the detection in serum of β-1,3-D-glucan (BDG), an antigen present in most fungi including *P. jirovecii*, has been shown to be a valuable biomarker for PCP.[6] However, high BDG titres could be due to the presence of another invasive fungal infection, and the test is prone to numerous causes of false positivity.[7-9] Moreover, serum BDG levels have been shown to be low in non-HIV patients with PCP.[10] Therefore, nucleic acid detection in respiratory specimens appears more reliable for confirming or excluding the diagnosis of PCP.

For Peer Review Only

Various molecular assays allowing detection of *P. jirovecii* DNA have been developed in the past 30 years. Real-time quantitative PCR assays (qPCR) are the most suitable options for diagnostic laboratories, providing rapid and quantitative results indispensable for diagnosing PCP, and a significant reduction in the risk of false positive results by preventing exposure to prior amplicon contamination and excluding post-amplification processing.[5,11-13] Several meta-analyses reported the excellent performance of qPCR to aid diagnosis of PCP. However, detection of *P. jirovecii* DNA in respiratory samples has been observed in asymptomatic immunocompromised patients leading to the concept of carriage/colonization and the requirement for strategies discriminating between this and active infection.[3] Only qPCR has the potential to be used to differentiate active infection from colonisation, provided fungal burden thresholds for infection in each patient group can be determined. Currently, definite, consensual thresholds for PCP PCR in non-HIV patients have not been established and universally used in different diagnostic laboratories,[5] partially due to the wide diversity of the qPCR assays described and used in diagnostic centres. Some studies have compared the performance of different *P. jirovecii* qPCR assays, but these studies mainly reported intra-laboratory comparison of two to four qPCR assays, including commercial kits and various in house assays.[14-22]

The *Pneumocystis* working party of the Fungal PCR Initiative, a working group of the International Society for Human and Animal Mycology, initiated two studies. The first one was aimed at evaluating the variability of the performance of 20 in-house and commercial assays currently used in reference laboratories with the final goal of providing a consensus method for *P. jirovecii* qPCR, comparable to the process used for *Aspergillus* qPCR.[23] For this purpose, a panel of five samples with various *P. jirovecii* whole nucleic acid concentrations were tested by 16 reference laboratories. The second study was performed in parallel in one centre testing

For Peer Review Only

10 different qPCR assays available at that time in centre E using the same clinical samples and PCR amplification instrument.

MATERIALS AND METHODS

Multicentre inter-laboratory evaluation of *P. jirovecii* qPCR assays

Panel specimen production

The broncho-alveolar lavage fluids (BALFs) were obtained through routine diagnostic practice for the patients in Centre E.[20] After performing routine investigations, surplus clinical material was retained for quality and service evaluation purposes, as permitted by the French Health Public Law (CSP Art L1121-1.1). All further preparation and subsequent dispatching of aliquots were performed in a biosafety cabinet.

Whole nucleic acids (WNA) were extracted from 900 µL of concentrated BALF pellet (5 min at 10000 g) with addition of 10 µL/sample of 1:5 diluted internal control (DNA Virus Culture, DICD-CY-L100, Diagenode, Seraing, Belgium) using a Qiasymphony (Qiagen, Hilden, Germany) with the Virus-Pathogen Kit (Qiagen) following the manufacturer's instructions. WNA was eluted in 85 µL, of which at least 30 µL was used for the routine diagnosis. Eluates were tested for the presence of *P. jirovecii* WNA using mitochondrial large sub-unit (mtLSU) qPCR assay to detect positive specimens. Mitochondrial small sub-unit (mtSSU) reverse transcriptase (RT)-qPCR (as the most known sensitive method in our centre) was used to select negative specimens. For the qPCR assays, the protocols published by Valero *et al.* were followed.[20] All the experiments were performed on a Light Cycler 480 thermocycler (LC480-II; Roche Diagnostics, Mannheim, Germany) with Cq determined with the second derivative method. For the RT-qPCR assay targeting also mtLSU and mtSSU RT-qPCR, the protocol was different to include the reverse transcription step: 1× Invitrogen RT-PCR buffer mix

For Peer Review Only

(Superscript III One step RT-PCR), 0.3 µM of each primer, 0.1 µM of probe, 1 µM of $MgSO_4$ (to reach a Mg concentration of 4 mM), and 1 µM of Superscript III Platinum enzyme, in a total of 25 µL with 5 µL of WNA extract. The amplification consisted of one step of RT-PCR at 50°c for 15 minutes, followed by qPCR with one activation step at 95°C for 2 minutes and 50 cycles of denaturation at 95°C for 15 seconds and annealing at 60° for 30 seconds. As guided by the internal control values, only the BALFs with no evidence of PCR inhibition were used for further analyses. The amplification results were expressed as quantification cycle (Cq) values.

To provide negative material, WNA extracts from 50 *P. jirovecii* qPCR negative BALFs were pooled. Fifteen microliters of this negative pooled extract were used for the two negative panel specimens. This negative pooled extract was also used to dilute the positive samples, instead of using water, to better mimic the human BALF and maintaining a typical human WNA concentration. As an approximation of the human DNA content, the human albumin gene was quantified by qPCR, as previously reported.[24] The result corresponds to 1.6 to $1.7x10^7$ human cells. We determined that the human to *P. jirovecii* ratio in the 1:1 specimen was 1000:1, (1000-fold more human DNA than *P. jirovecii* DNA).

For the positive panel specimens, WNA extracts from residual clinical material of three *P. jirovecii* PCR-positive patients were pooled. This positive pool was diluted in the negative pooled extract to obtain different fungal dilutions (pure 1:1; and 1:100; and 1:1000 dilutions), thereafter deemed high, medium, and low fungal loads. Thus, 40 15-µl positive aliquots were obtained for the multicentre study. The aliquots were stored at -20°C before distribution. To mimic unexpected thawing during transportation, aliquots were thawed and tested immediately after thawing and after 15 days at room temperature.

According to the French Health Public Law (CSP Art L1121-1.1), such protocol does not require approval of an ethics committee and is exempt from specific informed consent

For Peer Review Only

application. However, the patients were informed and gave consent for the possibility of using leftover material for additional laboratory studies before the broncho-alveolar lavage procedure.

Panel distribution to participating laboratories

The participating laboratories were 16 reference laboratories anonymised for blind comparison (Centre A to P). The panel consisted of five 15-µl WNA aliquots comprising three *P. jirovecii* positives and two negatives sent on dry ice. The laboratories were asked to use their own qPCR assay running currently in their lab (listed in Table 1). The primer and probes used in the in-house assays are listed in Supplemental Table 1. In short, 5 µL of each extract were asked to be tested in duplicate using the qPCR process routinely available in their laboratory. The results were collected using a dedicated form that included requests for the qualitative result (positive/negative), the Cq value for each replicate related to each aliquot and specific information regarding the methods and workflows applied (Supplemental Table 2).

Quantification cycles (Cq) normalization

For comparability between assays, Cq values were normalized as per the volume of nucleic acid tested for each qPCR assay, adjusted to a standard volume of 5 µL (1 Cq subtracted if 10 µL of extract were recommended). In addition, one commercial assay (Sacace Biotechnologies, Como, Italy) recommended recording fluorescence intensity during qPCR only after 5 first cycles, which artificially decreased the Cq value obtained at the end of the run. So, for comparability, 5 cycles were added to the value obtained with this kit.

Monocentre intra-laboratory evaluation of different qPCR assays using clinical BALFs

Sample preparation

For Peer Review Only

For this monocentre comparison, we selected 10 *P. jirovecii*-negative and 9 *P. jirovecii* – positive BALFs. The 50 µl of the BALF extracts left after routine procedure were combined with 70 µl of negative extract (see above), to provide sufficient material for the comparison of the kits. Verification was performed using the mtSSU in house RT-qPCR assay which generated initial Cq values ranging from 17 to 30 cycles for the 9 qPCR-positive BALF extracts . All aliquots were stored at -20°C until use.

To calculate the qPCR efficiency of each assay, calibration curves were generated using the highest positive BALF extract (BALF9: initial Cq=17 with the mtSSU in-house RT-qPCR assay). After thawing of an aliquot, a serial dilution of the extract (6 times at 1:5 ratio in negative pooled extract) was tested for each PCR assay.

qPCR assays tested

Centre E compared 10 different assays based on the in-house assays available locally and the commercial assays available in his country with the same person and the same qPCR instrument as above. For Centre E in-house assays, the same protocols as above were used. Commercial qPCR assays were performed following the manufacturer's instructions. The major surface glycoprotein (MSG) in-house DNA assay was performed following instruction of the detailed process described in Larsen *et al*.[25]

Technical information on the 10 assays, consisting of 5 in-house assays and 5 commercial kits, are listed in Table 1 and the primers and probes used are available in Supplemental Table 1. They consisted in 1 commercial and 2 in-house RT-qPCR assays, and 4 commercial and 3 in-house qPCR assays. The targets were mtLSU (7 assays); mtSSU (2 assays); and one nucleic gene: Major Surface Glycoprotein (MSG, 1 assays) (Table 1).

Statistical analysis

For Peer Review Only

For the multicentre study, 20 datasets of the five samples tested by each assay in duplicate were produced. Therefore, the total number of data values included was 200. Each replicate was analysed as an independent result. Negative results were allocated a value of 45 cycles. Descriptive statistics were performed calculating summary indexes of Cq at all target loads, pure (1:1) and 1:100, 1:1000 dilutions.

An ANOVA model was used to evaluate the effect of a two main covariables: DNA vs. WNA, and target gene. The effects of these categorical covariates were quantified by adding each of them consecutively to a basic ANOVA model. An ANOVA model was applied as (1) the real/absolute amount of the fungal burdens were unknown and the ANOVA approach overcame this by simply assuming that the panel samples 2, 3, and 5 are different; (2) the linearity of the relationship Cq/logRLM was not guaranteed; (3) the difference between the effects exerted by two levels of an added covariate would unlikely be constant across all Cq values. The ANOVA analysis was implemented as a mixed effects regression model, where Cq was the dependent variable, the molecular target level was used as categorical regressor, and the covariate under evaluation was added as an additional factor. The interaction between the covariate and the relative genomic load was also included in the model. Anonymization was performed prior to all analysis and identifiers were used to build the categorical grouping. The identifier indicated the mixed model categorical grouping variable. As a post-estimation step, the contrasts (predicted mean effects) were calculated for each pair of factor levels, adjusting for each relative genomic load level. They measured the difference between the effects exerted by each member of the factor pair on the signal intensity.

For the monocentre study, no specific statistical analysis was applied.

For Peer Review Only

RESULTS

Multicentre inter-laboratory evaluation of *P. jirovecii* qPCR assays

The effects of shipping on the stability of DNA and WNA in the panel was investigated in Centre E after panel storage for 15 days at room temperature, after a freezing-thawing cycle. Using mtLSU DNA and mtSSU WNA detection, the coefficient of variation was 2.5%, 1% and 1% for mtLSU DNA and 2.2%, 1.4%, 1.9% for mtSSU WNA in samples, respectively (Supplemental Figure 1). All samples were found to be stable indicating that the distribution process did not introduce bias in the results, even when shipping samples from Europe to Australia.

There were 16 participating laboratories across eight countries including seven European countries and Australia. Two centres tested more than one qPCR assays (two in Centre K, and four in Centre E). Thus, 20 qPCR assays were evaluated based on 15 commercial and in-house assays available in literature. In this study, assay#2 was tested by 5 centres (B, C, E, J, O) and #12 by 2 centres (K, P) (Table1). Sixteen qPCR amplifying DNA only and 4 RT-qPCR assays amplifying WNA were tested. Six of 15 assays were commercial, for which two out of six were tested directly by the manufacturers (Table 1, Supplemental Table 1). The targets were mtLSU (11 assays); mtSSU (2 assays); and two nucleic gene: Major Surface Glycoprotein (MSG, one assay) and beta-tubulin (TUB, one assay).

No false-positive amplification was observed (80/80 negative tests) for the two negative aliquots, which set the specificity at 100% (95% CI: 95.4-100). For each positive aliquot tested 40 times (20 assays in duplicates), 40/40 tests were positive at 1:1 (both replicates of the 20 assays) (sensitivity =100%, 95% CI: 91.2-100); 38/40 tests were positive at 1:100 (sensitivity=95%, 95% CI: 83.5-98.6), with one replicate negative for 2 assays (#4 and #6). At 1:1000, 33/40 tests were positive (sensitivity=82.5%, 95% CI: 68.1-91.3), with both replicates negative for one assay (#6) and only one replicate negative for three assays across

For Peer Review Only

five centres (#2 centre B, C, O, #3 #16) (Table 2, Supplemental Figure 2 and Supplemental Table 3 and 4)

The maximum variation in the Cq value (excluding negative values) between assays was 12.4 cycles (approximately 10,000-fold variation), 9.2 and 9.4 cycles (approximately 1000-fold variation) for the 1:1, 1:100 and 1:1000 panel specimens, respectively (Supplemental Figure 2). Of note, mtLSU assays using the same primers and probes,[26] but with different reagents and/or protocol, gave a median difference of 3 cycles (i.e. 10-fold variation) (Supplemental Table 5).

Two major variables impacted significantly on the results, in addition to minor influences associated with variation in reagents and protocols: (i) the type of qPCR, RT-qPCR vs qPCR, and (ii) the gene targeted for amplification (Figure 1). The RT-qPCR assays based on the detection of WNA (detecting both RNA and DNA) were significantly superior to qPCR assays detecting DNA only at all loads ($p<0.001$, Supplemental Table 6 and Supplemental Figure 3). The Cq values were also significantly associated to the target gene at all loads with the lowest Cq values associated with mtSSU, successively followed by mtLSU, MSG, and then TUB (Figure 1, Supplemental Table 7). There was a consistent and significant correlation associated with Cq values generated by mtSSU assays amplifying WNA through to TUB assays amplifying DNA only (ANOVA Kruskal-Wallis $p<0.05$) across all nucleic acid loads. The Cq values obtained with the 3 panel specimens and the 20 PCR assays are represented Figure 2A. Indeed, a mean 7 Cqs difference (200-fold variation) was observed between the TUB assay and the assays targeting multicopy genes.

Monocentre intra-laboratory evaluation of 10 *P. jirovecii* qPCR assays

For Peer Review Only

The specificity of the 10 assays was high; nine gave a specificity of 100% (upon duplicate testing of the 10 negative BALFs) and only one assay (assay#12) provided one false-positive result (one of the duplicates) giving a specificity of 95%.

The sensitivity of the assays for the detection of *P. jirovecii* in 9 positive BALFs varied between 88.8% (n=5 assays) and 100% (n=2 assays) with 4 assays having a sensitivity of 94.4% (Supplemental Table 8 and Figure 2B). The lowest Cq values were observed with the mtSSU assays amplifying WNA and the highest with the MSG assays amplifying DNA across all positive BALFs (Figure 2B). Thus, when excluding the negative values (Cq=45), the median of the maximum variation of the Cq value between the 10 assays was 8.23 (approximately 500-fold variation) for a given BALF. Even when considering the 5 commercial assays targeting mtLSU as the target, a median of variation as high as 3.1 (about 10-fold variation) was observed (Supplemental Table 8 and Figure 2B).

By serially diluting BALF#9 WNA extract five times at 1:5 dilutions, the qPCR efficiency for each assay was calculated. The amplification efficiency calculated from the Cq values were all between 94 and 113% (Supplemental Table 8, Supplemental Figure 4). This showed that this factor can be discarded as a possible explanation for the differences observed above.

For Peer Review Only

DISCUSSION

The present study underlines the necessity of comparing different assays for the quantification of the *P. jirovecii* loads. Indeed, large variation was evidenced among all the assays compared. Based on data of 9 commercial kits and 10 in house assays tested in the multicentre and monocentre studies (Table 1), this variation ranged from 500-fold (5 qPCR cycles) to 10,000-fold (12 qPCR cycles) between the assays.

This resulted mainly from the target gene (mitochondrial multicopy genes vs. single copy nuclear gene) and the qPCR method/template (qPCR/DNA versus RT-qPCR/WNA). Thus, our studies showed that using mtSSU as the target gene and WNA as the starting material yielded the best sensitivity. Predictably, assays targeting multi copy genes provided the best analytical sensitivity, as already shown.[16,19,20] This was observed in our study for the single copy gene (beta-tubulin) compared to the mtSSU gene (about 200-fold variation). However, among the repeated genes, MSG assay gave higher/later Cq values than mtLSU or mtSSU, suggesting a lower number of repeats for the MSG gene. The MSG gene sequence is reported to be highly variable,[27] which can impacts both the analytical sensitivity/limit of detection (risk of false negative) and qPCR efficiency (risk of variable amplification yield).

Among the mitochondrial multicopy assays, the mtSSU assay was two times more sensitive on average than the mtLSU assay, possibly as a result of the higher number of repeats of mtSSU compared to mtLSU in the mitochondrial genome.[20] Interestingly, the number of copies of some mitochondrial genes (mtLSU, NAD1 and CYTB) have been shown to vary according to the fungal load (high vs. medium and low fungal loads).[20] In contrast, mtSSU seems to remain stable between these conditions suggesting that a more reliable quantification could be achieved with mtSSU rather than with other mitochondrial targets. Indeed, the quantification of unique/single copy gene (nuclear) will better reflect the quantity

For Peer Review Only

of microorganism, but the lack of sensitivity of such a target gene hampers its use for clinical diagnosis.

Interestingly, even when a given gene is targeted or the same primer/probes utilized, differences can occur. Using the same mtLSU primers and probes and qPCR,[26] the quantification obtained from five centres showed here a median 3-fold variation in the Cq (10-fold difference in concentration). In addition, we showed that five commercial mtLSU kits also gave a 3-fold variation in the Cq. Overall, these differences can come from the different primer concentration/amplification kits/enzyme or thermocyclers used. Indeed, technical parameters can affect qPCR efficiency, such as the type of polymerase used, the concentration of oligonucleotides or the method of Cq determination, suggesting that harmonization of the methods requires more than standardization of the target gene or the primer sequences.

Although differences were already known among different DNA extraction methods or qPCR assays,[14-22] the superiority of WNA over DNA extraction has never been reported for *P. jirovecii*, due to lack of prior of investigation. WNA RT-qPCR amplification kits were initially developed for virology to allow cDNA and genomic DNA amplification in the same run to detect both RNA or DNA viruses.[28,29] Currently, most of the automated extractors allow WNA extraction to test for the presence of both RNA or DNA viruses, and DNA from other pathogenic organisms in the same sample extract as part of a syndromic investigation.[30] Such strategy has already been tested with success for detecting *P. jirovecii* DNA in samples dedicated for influenza diagnosis.[24] Thus, since WNA extraction does not interfere with the detection of *P. jirovecii* and even provides the best sensitivity in the present study, this extraction method could be recommended.

The rationale for supporting the use of the quantitative assays with the highest sensitivity might be contentious as low fungal loads are often considered to represent

For Peer Review Only

"carriage/colonization" and therefore of limited clinical significance. However, if the negative predictive value of the assay to exclude PCP is taken into consideration, it seems preferable to rely on optimal sensitivity to exclude PCP, rather than managing false negative results. Indeed, low *P. jirovecii* burden can be associated with severe disease in HIV-negative patients.[31] Second, prophylaxis could be considered for immunocompromised patients with a low fungal load to avoid subsequent development of full-blown PCP.[32] At least, knowing that *P. jirovecii* is detectable can tilt the balance between continuing co-trimoxazole or stopping the drug because of the fear of side effects. Third, patients with low fungal loads in an hospital environment can be part of the onward transmission chain in clinical outbreaks.[3,31]

There are limitations to this study. To set up a quality control with *P. jirovecii,* one must consider the difficulty of obtaining cultured material. Realistically, only surplus human samples can be used and these are limited in both number and sample volume. Hence the choice of pooling human samples and the limited number panel size used for comparison. In pooling samples, it is acknowledged that potential biases are introduced. Ideally, it would have been preferable to select a specific patient, to have an idea of the sequence of the *P. jirovecii* main target genes and to propose individual panel specimens based on well-defined organisms. Unfortunately, this is impossible due to limited sample volume. Since the BALF material is usually limited after performing thorough routine diagnostics testing, such previous analysis, providing 20 aliquots of several individual BALF extracts is practically impossible. The pooling of BALF extracts also assesses the amplification process in the presence of the genetic variability. In addition, it has been shown that PCP episodes are due to multiple genotypes in more that 70% of the cases,[33-36] with known sequence variation in targets genes.[33] Indeed, false negative results can result from the choice of primers in variable regions of the fungus.[37] This is therefore the responsibility of each centre to investigate this when facing a

For Peer Review Only

high clinical suspicion of PCP and our quality control did not address this possibility. For example, MGB probes who are very sensitive to polymorphism/mismatch should not be used to avoid the risk of false negative result.[37]

The absolute quantity of *Pneumocystis* DNA added in the initial pooled of samples was not known. Nevertheless, the main purpose of our study was to compare different assays and not to determine the absolute analytic capacity of each assay. Another possible bias in pooling human samples is to dilute PCR inhibitors present in one of the samples for elaborating the panel. Using an internal control is of outmost importance to analyse qPCR results in a diagnostic lab and is strongly advocated[38,39] and improves the quality of the results, especially to have a strong validation for negative results.[40] Thus, some PCR assays not controlling for the presence of PCR inhibitors can be falsely over-evaluated in the present study. With limited availability of *Pneumocystis* culture the current methodology based on surplus human samples is the only one possible to perform such evaluations, despite all the inherent potential bias. However, we do not think the possible limitations listed above had a major impact on our comparative studies.

The limited quantity of BALF extract available restricted the number of evaluations possible. Thus, some assays were tested only once in a single centre (DNA MSG, DNA TUB, WNA mtSSU, DNA mtSSU) making any evaluation on the reproducibility for these specific assays impossible. Additionally, only the analytical sensitivity of the amplification was tested, not the nucleic acids extraction step, which has a major impact for the global performance of all the amplification procedure.[41] We also did not have the opportunity to evaluate the impact of qPCR platform (thermocycler and Cq determination), because too many parameters varied among the different centres. There are future plans to implement specific studies on these

For Peer Review Only

topics to show the impact of extraction, qPCR platform and the use of an internal control on the performance of *P. jirovecii* qPCR.

CONCLUSION

Our present studies confirm the large variation between of qPCR assays to quantify *P. jirovecii* nucleic acids. This large variation, up to 12 cycles (10,000-fold variation) for a given sample, makes reaching a consensus threshold value to distinguish between low and high fungal loads in respiratory samples difficult, but highlights optimal assays in regards to sensitivity. The most sensitive association was shown to be RT-qPCR (amplifying combined RNA and DNA) targeting the mtSSU gene and the less sensitive the qPCR assays (DNA only) targeting Major Surface Glycoprotein and the unique beta-tubulin gene targets using. Interpretative thresholds might be defined in the future, provided centres agree to use the most sensitive method. Other factors not directly related to the PCR process that influence the result, such as the protocol for processing the fluid must, however, be performed. Future harmonization of the results should also take into account the procedure for obtaining broncho-alveolar lavage or other respiratory specimens.

Acknowledgments

We would like to thank Aude Sturny-Leclère, Marion Benazra, and Dirk Schmid for technical assistance and Dr Samia Hamane for her aid in managing the routine diagnosis of PCP. We also thank Pr Anne Bergeron who is in charge of the broncho-alveolar lavage procedure at Saint-Louis hospital.

Disclosure of Conflict of Interest

For Peer Review Only

The authors declare no conflict of interest for this specific study, except GJ who is an employee of OLM Diagnostics and GD who is an employee of PathoNostics. AA and SB are inventors in a patent dealing with Pneumocystis RT-qPCR.

For Peer Review Only

Table 1. Characteristics of the assays used in these studies. Five centres (B, C, E, J, O, E*) used the same in-house mtLSU assay as described previously[26] and two centres (L, E*) used the same commercial assay.

Assay ID	Centre ID (Multicentre study)	Target	PCR Type	Multicentre study	Monocentre study	In House assays	Commercial assays	Name of the kit or publication (PMID)	Manufacturer
1	A	mtLSU	qPCR	X		X		26739439	
2	B, C, E, J, O, E*	mtLSU	qPCR	X	X	X		20946413	
3	D	mtLSU	qPCR	X		X		Unpublished1	
5	E, E*	mtSSU	qPCR	X	X	X		27672381	
6	H	TUB	qPCR	X		X		15767014	
7	I	mtLSU	qPCR	X		X		Unpublished2	
8	M	mtLSU	RT-qPCR	X		X		Unpublished3	
9	E, E*	mtLSU	RT-qPCR	X	X	X		20946413	
10	E, E*	mtSSU	RT-qPCR	X	X	X		27672381	
11	E*	MSG	qPCR		X	X		11825961	
4	F	mtLSU	qPCR	X			X	Pneumocystis jirovecii REAL TIME PCR, PG1905	Ridagene
12	K, P, E*	mtLSU	qPCR	X	X		X	PneumoGenius (PN-600)	Pathonostics
13	N	mtLSU	qPCR	X			X	PneumID (Ref OLM2008)	OLM
14	K	mtLSU	qPCR	X			X	Pneumocystis jirovecii (carinii) Real-TM, P2-50FRT	Sacace Biotechnologies
15	L, E*	mtLSU	RT-qPCR	X	X		X	FTD Pneumocystis jirovecii	Fast Track Diagnostics
16	G	MSG	qPCR	X			X	LightMix Modular Pneumocystis jiroveci	Roche
17	E*	mtLSU	qPCR		X		X	RealStar® Pneumocystis jirovecii PCR Kit 1.0	Altona
18	E*	mtLSU	qPCR		X		X	RealCycler PJIR Kit	Progenie molecular
19	E*	mtLSU	qPCR		X		X	Pneumocystis jirovecii qPCR (BE-A994)	Bioevolution

E*: evaluation in Centre E in the monocentre study ; qPCR: quantitative PCR; RT-PCR: reverse-transcribed quantitative PCR. Mitochondrial Large Sub-Unit (mtLSU); mitochondrial Small Sub-Unit (mtSSU); Major Surface Glycoprotein (MSG); beta-tubulin (TUB).

For Peer Review Only

Table 2. Descriptive statistics.

Fungal load	N	mean	SD	min	p25	p50	p75	max
Negative	80	45	0	45	45	45	45	45
Cq at 1:1000	40	37.704	3.985	30.400	35.325	37.090	38.615	45
Cq at 1:100	40	33.956	3.423	28.940	31.615	33.545	35.100	45
Cq at 1:1	40	26.831	2.682	22.900	24.890	26.760	27.700	35.200

PCR summary results, as Cq means, standard deviations, 25^{th} - 50^{th} -75^{th} percentiles. The number of the DNA assays is indicated (N). Relative genomic loads are indicated as 0, 1:1000, 1:100, and 1:1.

For Peer Review Only

Legends to Figures

Figure 1: Cq variation for each assay regarding the target gene and the type of material amplified (DNA or WNA) in all the three positive panel samples at dilutions 1:1 (A), 1:100 (B), and 1:1000 (C). It shows the gain in the ability to detect the target (lower Cq) in WNA assays compared to DNA and with a gradient from mtSSU to TUB genes (mtSSU>mtLSU>MSG>TUB)

Figure 2: Cq variation in all assays tested in the multicentre (A) and in the monocentre study (B). For the multicentre study, the panel specimen 1:1 (open circle) 1:100 (open square), 1:1000 (black circle) were tested in duplicates among 20 PCR assays tested (assay#2 was tested by 5 centres and assay#12 by 2 centres, see Table 1). For the monocentre study, 9 human broncho-alveolar lavage fluids (BALF1 to BALF9 highlighted by different symbols) were tested in duplicates with 10 in house and commercial assays. It shows that assay#10 (mtSSU and RT-qPCR) gives the best Cq values.

For Peer Review Only

Supplemental material

Supplemental Figure 1: Stability of nucleic acids (mtLSU DNA and mtSSU WNA) obtained in all 3 samples (1:1, 1:100, 1:1000) immediately thawing (D0) and after 3 (D3), 8 (D8) or 15 (D15) days at room temperature after one thawing/freezing cycle (A) or pooled (B).

Supplemental Figure 2: Cq variation of the three positive panel specimens at dilutions 1:1, 1:100, and 1:1000 upon evaluation by the 20 qPCR assays in the multicentre study. Each dot represents each Cq value of the duplicate performed in each centre (40 Cq values by specimen). Negative amplifications have been plotted with a Cq at 45.

Supplemental Figure 3: Cq variation in assays amplifying mtLSU DNA or mtLSU Whole Nucleic Acids (WNA) for each panel sample. It shows the gain in the ability to detect the target (lower Cq) in WNA assays compared to DNA.

Supplemental Figure 4: Calibration curves for each of the 11 assays tested using 1:5 dilutions of BAL1 harbouring the highest fungal load in the monocentre study.

For Peer Review Only

References

1. Thomas CF, Limper AH. *Pneumocystis* pneumonia. *N Engl J Med*. 2004;350(24):2487-2498. doi:10.1056/NEJMra032588.
2. Gigliotti F, Wright TW. *Pneumocystis*: where does it live? *PLoS Pathog*. 2012;8(11):e1003025. doi:10.1371/journal.ppat.1003025.
3. Alanio A, Bretagne S. Pneumocystis jirovecii detection in asymptomatic patients: what does its natural history tell us? *F1000Res*. 2017;6:739. doi:10.12688/f1000research.10619.1.
4. Cordonnier C, Cesaro S, Maschmeyer G, et al. *Pneumocystis jirovecii* pneumonia: still a concern in patients with haematological malignancies and stem cell transplant recipients. *J Antimicrob Chemother*. 2016;71(9):2379-2385. doi:10.1093/jac/dkw155.
5. Alanio A, Hauser PM, Lagrou K, et al. ECIL guidelines for the diagnosis of *Pneumocystis jirovecii* pneumonia in patients with haematological malignancies and stem cell transplant recipients. *J Antimicrob Chemother*. 2016;71(9):2386-2396. doi:10.1093/jac/dkw156.
6. Karageorgopoulos DE, Qu JM, Korbila IP, Zhu YG, Vasileiou VA, Falagas ME. Accuracy of β-D-glucan for the diagnosis of Pneumocystis jirovecii pneumonia: a meta-analysis. *Clin Microbiol Infect*. 2013;19(1):39-49. doi:10.1111/j.1469-0691.2011.03760.x.
7. Marty FM, Lowry CM, Lempitski SJ, Kubiak DW, Finkelman MA, Baden LR. Reactivity of (1-->3)-beta-d-glucan assay with commonly used intravenous antimicrobials. *Antimicrob Agents Chemother*. 2006;50(10):3450-3453. doi:10.1128/AAC.00658-06.
8. Albert O, Toubas D, Strady C, et al. Reactivity of (1→3)-β-d-glucan assay in bacterial bloodstream infections. *Eur J Clin Microbiol Infect Dis*. 2011;30(11):1453-1460. doi:10.1007/s10096-011-1244-8.
9. Azoulay E, Guigue N, Darmon M, et al. (1, 3)-β-D-glucan assay for diagnosing invasive fungal infections in critically ill patients with hematological malignancies. *Oncotarget*. February 2016. doi:10.18632/oncotarget.7471.
10. Nakamura H, Tateyama M, Tasato D, et al. Clinical utility of serum beta-D-glucan and KL-6 levels in Pneumocystis jirovecii pneumonia. *Intern Med*. 2009;48(4):195-202.
11. Bretagne S. Advances and prospects for molecular diagnostics of fungal infections. *Curr Infect Dis Rep*. 2010;12(6):430-436. doi:10.1007/s11908-010-0139-7.
12. Alanio A, Bretagne S. Difficulties with molecular diagnostic tests for mould and yeast infections: where do we stand? *Clin Microbiol Infect*. 2014;20 Suppl 6:36-41. doi:10.1111/1469-0691.12617.
13. Johnson G, Nolan T, Bustin SA. Real-Time Quantitative PCR, Pathogen Detection and MIQE. *Meth Mol Biol*. 2013;943:1-16. doi:10.1007/978-1-60327-353-4_1.
14. Hoarau G, Le Gal S, Zunic P, et al. Evaluation of quantitative FTD-Pneumocystis jirovecii kit for Pneumocystis infection diagnosis. *Diagn Microbiol Infect Dis*. August 2017. doi:10.1016/j.diagmicrobio.2017.08.001.
15. Guillaud-Saumur T, Nevez G, Bazire A, Virmaux M, Papon N, Le Gal S. Comparison of a commercial real-time PCR assay, RealCycler® PJIR kit, progenie molecular, to an in-house

For Peer Review Only

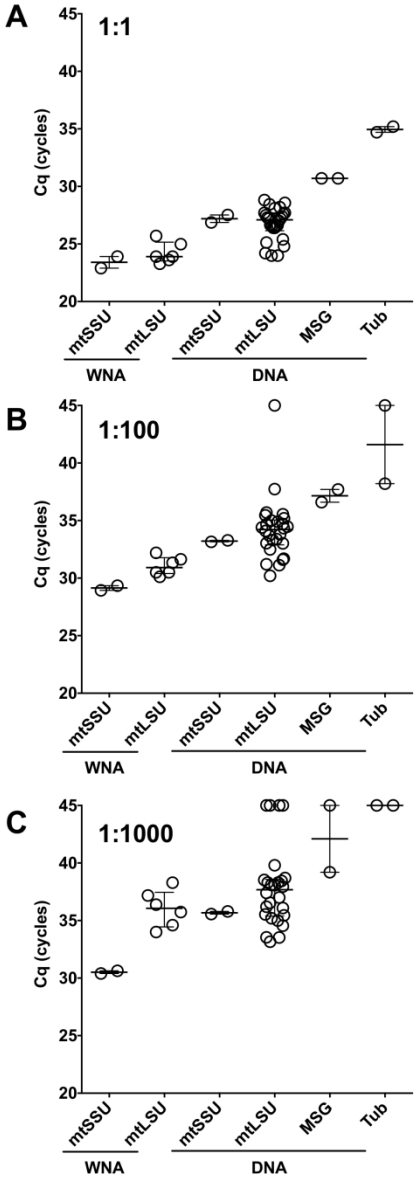
real-time PCR assay for the diagnosis of Pneumocystis jirovecii infections. *Diagn Microbiol Infect Dis*. 2017;87(4):335-337. doi:10.1016/j.diagmicrobio.2017.01.011.

16. Montesinos I, Brancart F, Schepers K, Jacobs F, Denis O, Delforge M-L. Comparison of 2 real-time PCR assays for diagnosis of Pneumocystis jirovecii pneumonia in human immunodeficiency virus (HIV) and non-HIV immunocompromised patients. *Diagn Microbiol Infect Dis*. 2015;82(2):143-147. doi:10.1016/j.diagmicrobio.2015.03.006.
17. Dalpke AH, Hofko M, Zimmermann S. Development and Evaluation of a Real-Time PCR Assay for Detection of Pneumocystis jirovecii on the Fully Automated BD MAX Platform. *J Clin Microbiol*. 2013;51(7):2337-2343. doi:10.1128/JCM.00616-13.
18. Orsi CF, Gennari W, Venturelli C, et al. Performance of 2 commercial real-time polymerase chain reaction assays for the detection of Aspergillus and Pneumocystis DNA in bronchoalveolar lavage fluid samples from critical care patients. *Diagn Microbiol Infect Dis*. 2012;73(2):138-143. doi:10.1016/j.diagmicrobio.2012.03.001.
19. Linssen CFM, Jacobs JA, Beckers P, et al. Inter-laboratory comparison of three different real-time PCR assays for the detection of Pneumocystis jiroveci in bronchoalveolar lavage fluid samples. *J Med Microbiol*. 2006;55(Pt 9):1229-1235. doi:10.1099/jmm.0.46552-0.
20. Valero C, Buitrago MJ, Gits-Muselli M, et al. Copy number variation of mitochondrial DNA genes in *Pneumocystis jirovecii* according to the fungal load in bal specimens. *Front Microbiol*. 2016;7:1413. doi:10.3389/fmicb.2016.01413.
21. Sasso M, Chastang-Dumas E, Bastide S, et al. Performances of four real-time PCR assays for the diagnosis of Pneumocystis jirovecii pneumonia. *J Clin Microbiol*. December 2015:JCM.02876–15. doi:10.1128/JCM.02876-15.
22. Huh HJ, Lim KR, Ki C-S, et al. Comparative Evaluation Between the RealStar Pneumocystis jirovecii PCR Kit and the AmpliSens Pneumocystis jirovecii (carinii)-FRT PCR Kit for Detecting P. jirovecii in Non-HIV Immunocompromised Patients. *Ann Lab Med*. 2019;39(2):176-182. doi:10.3343/alm.2019.39.2.176.
23. White PL, Barnes RA, Springer J, et al. Clinical Performance of Aspergillus PCR for Testing Serum and Plasma: a Study by the European Aspergillus PCR Initiative. Warnock DW, ed. *J Clin Microbiol*. 2015;53(9):2832-2837. doi:10.1128/JCM.00905-15.
24. Guigue N, Alanio A, Menotti J, et al. Utility of adding *Pneumocystis jirovecii* DNA detection in nasopharyngeal aspirates in immunocompromised adult patients with febrile pneumonia. *Med Mycol*. 2015;53(3):241-247. doi:10.1093/mmy/myu087.
25. Larsen HH, Masur H, Kovacs JA, et al. Development and evaluation of a quantitative, touch-down, real-time PCR assay for diagnosing *Pneumocystis carinii* pneumonia. *J Clin Microbiol*. 2002;40(2):490-494.
26. Alanio A, Desoubeaux G, Sarfati C, et al. Real-time PCR assay-based strategy for differentiation between active *Pneumocystis jirovecii* pneumonia and colonization in immunocompromised patients. *Clin Microbiol Infect*. 2011;17(10):1531-1537. doi:10.1111/j.1469-0691.2010.03400.x.
27. Kutty G, Maldarelli F, Achaz G, Kovacs JA. Variation in the major surface glycoprotein genes in *Pneumocystis jirovecii*. *J Infect Dis*. 2008;198(5):741-749. doi:10.1086/590433.

For Peer Review Only

28. Alanio A, Bergeron A, Sturny-Leclère A, et al. Diagnosis of *Pneumocystis* pneumonia : switching from DNA quantification to RNA expression analysis
. In: Lisboa; 2015.
29. Alanio A, Lanternier F, Gits-Muselli M, Bretagne S. Diagnosis of histoplasmosis : switching from microscopy to RNA detection. In: Belgrade; 2017.
30. Alanio A, Bretagne S. Performance evaluation of multiplex PCR including Aspergillus-not so simple! *Med Mycol*. 2017;55(1):56-62. doi:10.1093/mmy/myw080.
31. Robin C, Alanio A, Gits-Muselli M, et al. Molecular demonstration of a *Pneumocystis* outbreak in stem cell transplant patients: evidence for transmission in the daycare centre. *Front Microbiol*. 2017;8:700. doi:10.3389/fmicb.2017.00700.
32. Mori S, Cho I, Sugimoto M. A followup study of asymptomatic carriers of *Pneumocystis jirovecii* during immunosuppressive therapy for rheumatoid arthritis. *The Journal of Rheumatology*. 2009;36(8):1600-1605. doi:10.3899/jrheum.081270.
33. Alanio A, Gits-Muselli M, Mercier-Delarue S, Dromer F, Bretagne S. Diversity of *Pneumocystis jirovecii* during infection revealed by Ultra-Deep Pyrosequencing. *Front Microbiol*. 2016;7:733. doi:10.3389/fmicb.2016.00733.
34. Gits-Muselli M, Peraldi M-N, de Castro N, et al. New short tandem repeat-based molecular typing method for *Pneumocystis jirovecii* reveals intrahospital transmission between patients from different wards. *PLoS One*. 2015;10(5):e0125763. doi:10.1371/journal.pone.0125763.
35. Hauser PM, Blanc DS, Sudre P, et al. Genetic diversity of *Pneumocystis carinii* in HIV-positive and -negative patients as revealed by PCR-SSCP typing. *AIDS*. 2001;15(4):461-466.
36. Parobek CM, Jiang LY, Patel JC, et al. Multilocus microsatellite genotyping array for investigation of genetic epidemiology of *Pneumocystis jirovecii. J Clin Microbiol*. 2014;52(5):1391-1399. doi:10.1128/JCM.02531-13.
37. Le Gal S, Robert Gangneux F, Pépino Y, et al. A misleading false-negative result of Pneumocystis real-time PCR assay due to a rare punctual mutation: A French multicentre study. *Med Mycol*. 2017;55(2):180-184. doi:10.1093/mmy/myw051.
38. Bustin SA, Benes V, Garson JA, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem*. 2009;55(4):611-622. doi:10.1373/clinchem.2008.112797.
39. Hoorfar J, Cook N, Malorny B, et al. Making Internal Amplification Control Mandatory for Diagnostic PCR. *J Clin Microbiol*. 2003;41(12):5835-5835. doi:10.1128/JCM.41.12.5835.2003.
40. White PL, Mengoli C, Bretagne S, et al. Evaluation of Aspergillus PCR protocols for testing serum specimens. *J Clin Microbiol*. 2011;49(11):3842-3848. doi:10.1128/JCM.05316-11.
41. White PL, Perry MD, Loeffler J, et al. Critical stages of extracting DNA from Aspergillus fumigatus in whole-blood specimens. *J Clin Microbiol*. 2010;48(10):3753-3755. doi:10.1128/JCM.01466-10.

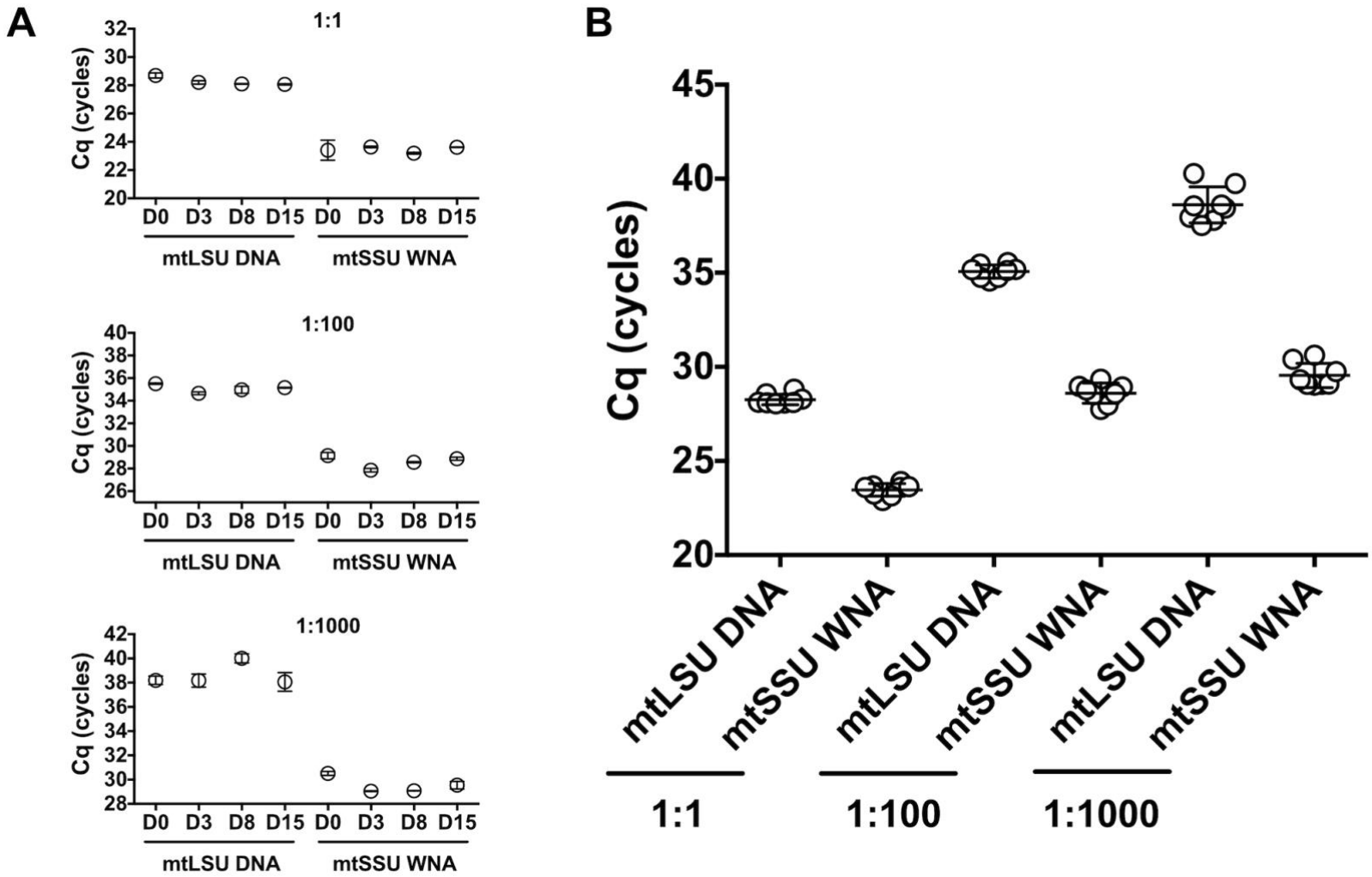
For Peer Review Only



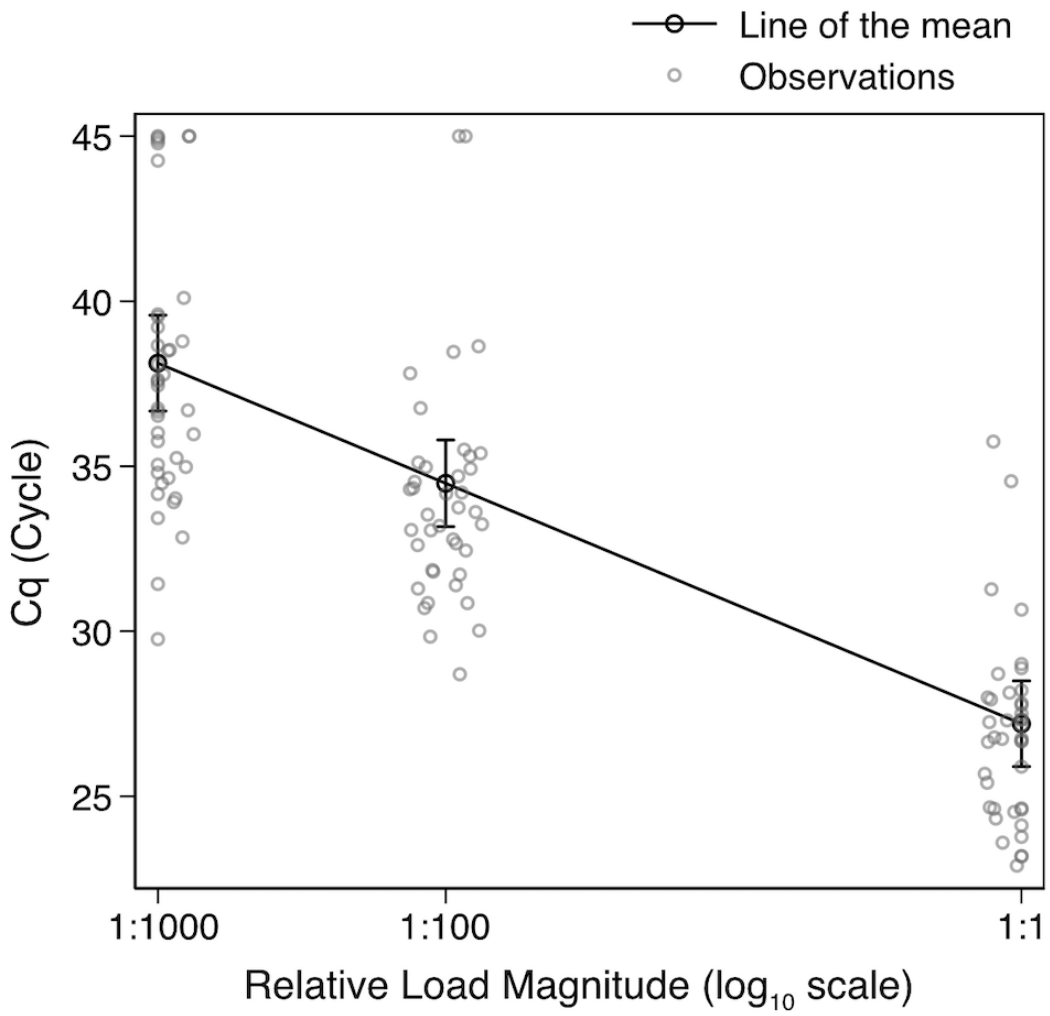
Cq variation for each assay regarding the target gene and the type of material amplified (DNA or WNA) in all the three positive panel samples at dilutions 1:1 (A), 1:100 (B), and 1:1000 (C). It shows the gain in the ability to detect the target (lower Cq) in WNA assays compared to DNA and with a gradient from mtSSU to TUB genes (mtSSU>mtLSU>MSG>TUB)

95x272mm (300 x 300 DPI)

Supplementary Figure 1: Stability of nucleic acids (mtLSU DNA and mtSSU WNA) obtained in all 3 samples (1:1, 1:100, 1:1000) immediately thawing (D0) and after 3 (D3), 8 (D8) or 15 (D15) days at room temperature after one thawing/freezing cycle (A) or pooled (B).

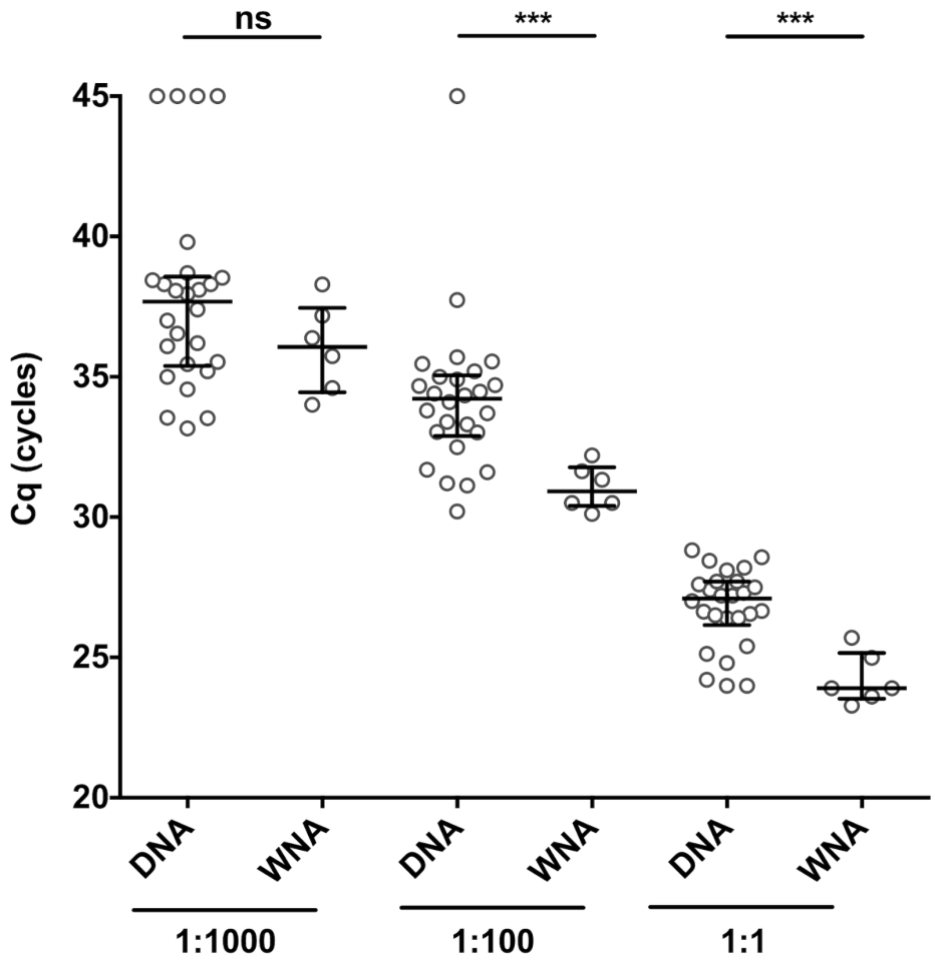


Supplemental Figure 2: Cq variation of the three positive panel specimens at dilutions 1:1, 1:100, and 1:1000 upon evaluation by the 20 qPCR assays in the multicentric study. Each dot represents each Cq value of the duplicate performed in each centre (40 Cq values by specimen). Negative amplifications have been plotted with a Cq at 45.



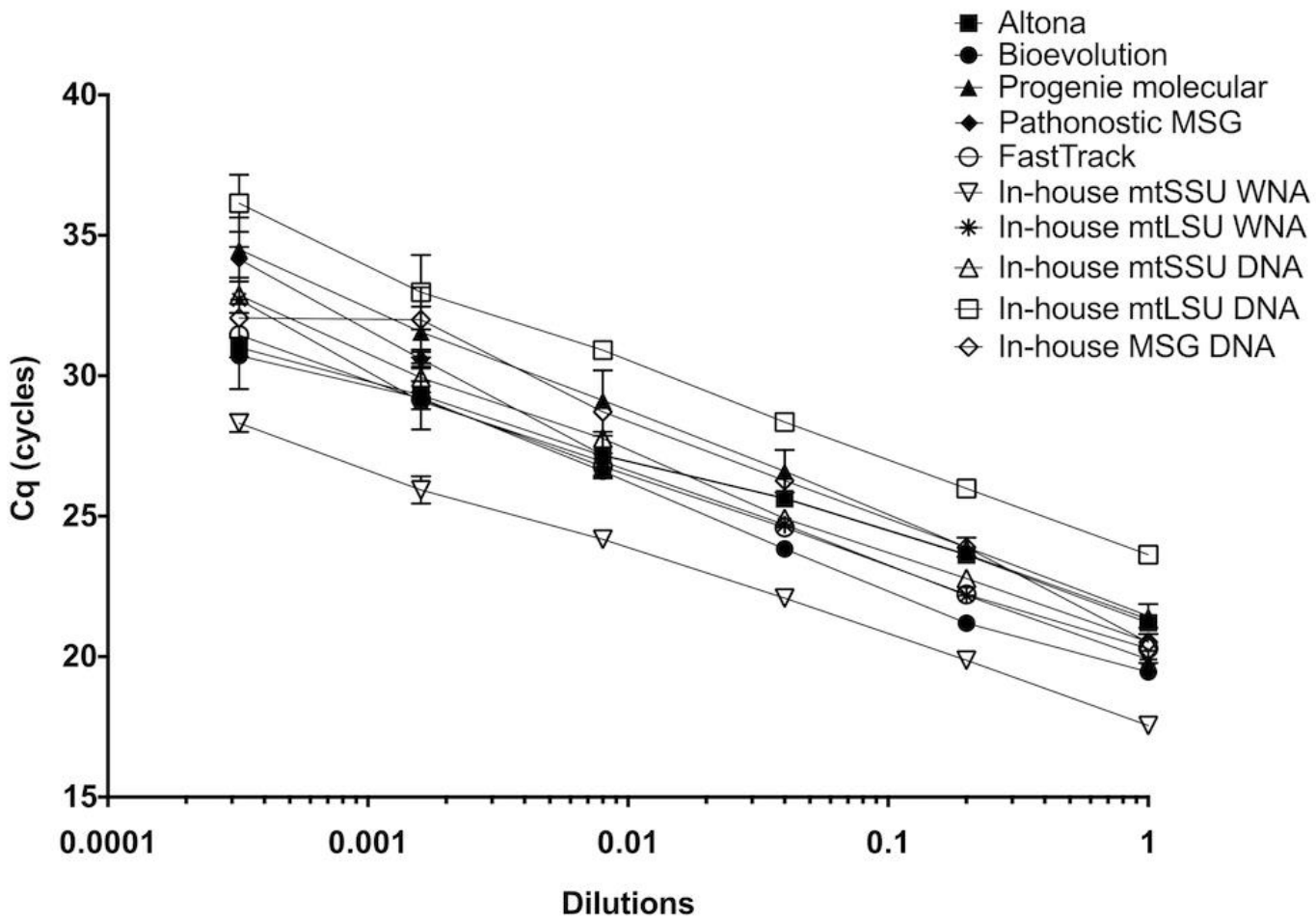
ew Only

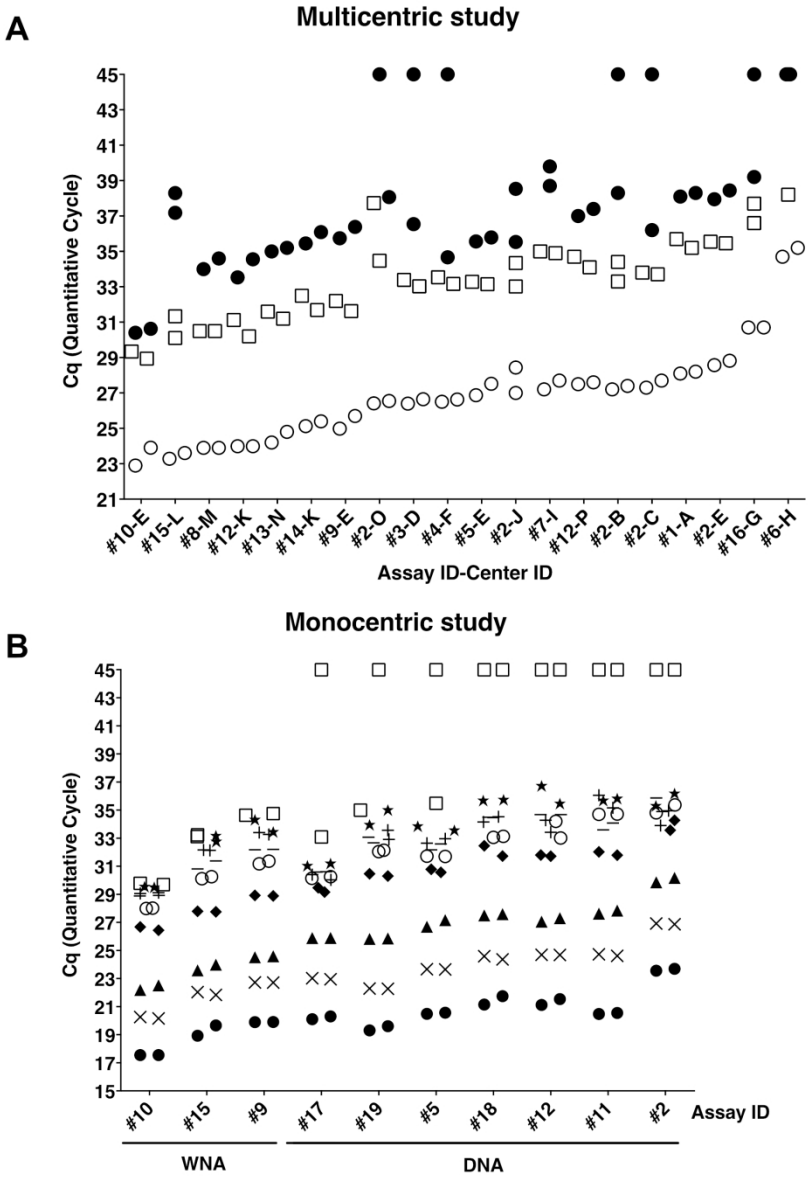
Supplemental Figure 3: Cq variation in assays amplifying mtLSU DNA or mtLSU Whole Nucleic Acids (WNA) for each panel sample. It shows the gain in the ability to detect the target (lower Cq) in WNA assays compared to DNA.



For Review Only

Supplemental Figure 4: Calibration curves for each of the 11 assays tested using 1:5 dilutions of BAL1 harbouring the highest fungal load in the monocentric study.





Cq variation in all assays tested in the multicentre (A) and in the monocentre study (B). For the multicentre study, the panel specimen 1:1 (open circle) 1:100 (open square), 1:1000 (black circle) were tested in duplicates among 20 PCR assays tested (assay#2 was tested by 5 centres and assay#12 by 2 centres, see Table 1). For the monocentre study, 9 human broncho-alveolar lavage fluids (BALF1 to BALF9 highlighted by different symbols) were tested in duplicates with 10 in house and commercial assays. It shows that assay #10 (mtSSU and RT-qPCR) gives the best Cq values.

Supplemental Tables

Supplemental Table 1: Primers of the in-house qPCR assays

Assay ID	Forward primers 5'-3'	Reverse primers 5'-3'	probes 5'-3'
1	5'-TGCAAAGTACTCAGAAGAATTGTGGTA-3'	5'-TTCGCAGAAAACCAGCTATATCCT-3'	5'-FAM–CCGATTTGTATTTCACTACT- NFQ-MGB-3'
2	5'-CTGTTTCCCTTTCGACTATCTACCTT-3'	5'-CACTGAATATCTCGAGGGAGTATGAA-3'	5'-FAM-TCGCACATAGTCTGATTAT- MGB-3'
3	5'-TGCGATAAGGTAGATAGTCGAAAGG-3'	5'-CCCACTTCTTAACTGTTTTAGATATCCA-3'	5'-FAM-ACAGCCCAGAACAGTAATTAAAGCTCCCCA-BHQ1-3'
5	5'-TCATGACCCTTATGAAGTGGGC-3'	5'-GCTCCGACTTCCATCATTGC-3'	5'-FAM-ACGTGCTGCAAAATTTTCTACAATGGG-BHQ1-3'
6	5'-GATCCGAGACATGGTCGCTATT-3'	5'-TTCAACCTCCTTCATGGAAACAG-3'	5'-TGTTGCAGCGATTTTCCGCGGTA-3'
7	5'-TGCAAAGTACTCAGAAGAATTGTGGTA-3'	5'-TTCGCAGAAAACCAGCTATATCCT-3'	5'-FAM-CCGATTTGTATTTCACTACT-BHQ1-3'
8	5'-GCACTGAATATCTCGAGGGAGTATG-3'	5'-TTGGGAGCTTTAATTACTGTTCTGG-3'	5'-FAM-TGTTTCCCTTTCGACTATC-3'-MGB
9	5'-CTGTTTCCCTTTCGACTATCTACCTT-3'	5'-CACTGAATATCTCGAGGGAGTATGAA-3'	5'-FAM-TCGCACATAGTCTGATTAT- MGB-3'
10	5'-TCATGACCCTTATGAAGTGGGC-3'	5'-GCTCCGACTTCCATCATTGC-3'	5'-FAM-ACGTGCTGCAAAATTTTCTACAATGGG-BHQ1-3'
11	5'-GAATGCAAATCYTTACAGACAACAG-3'	5'-AAATCATGAACGAAATAACCATTGC-3'	5'-CAAAAATAACAYTSACATCAACRAGGCG-fluorescein-3' 5'-Red 640-TGCAAACCAACCAAGTGTACGACAGG-3'

For Peer Review Only

Supplemental Table 2: Questions send to each participant

Username
Last name and first name
Name and address of your lab ?
Country of your lab
Country code
Did the samples arrive frozen?
If the samples arrived thawed, indicate the duration of the conservation at 4°C (days)
Sample volume received (µL)
Sample volume tested (µL)
Which PCR cycling platform do you use ?
Which qPCR detection method do you use?
How many cycles did you performed ?
What is the target gene of you PCR ?
What is the hybridization temperature (°C) used?
What is the concentration (ÎµM) of primers and probe (primers/probe) do you use?
Final volume of the PCR reaction
Did you use a rt-PCR step before amplification ?
Which Cq determination method do you use?
Did you tested an Internal control (IC)
If you performed an IC, explain which kit and which protocol
What is the reference paper of your PCR (PMID)?
If in house PCR, what buffer/enzyme kit to you use (add reference) ?
If commercial assay, indicate the exact kit name with reference and batch:
If in house assay unpublished, indicate the primers and probes used:

For Peer Review Only

Supplemental Table 3: Detailed Cq value for each assay tested in the multicentric study

Assay ID	Center ID	1:1 (Cq1/Cq2)	1:100 (Cq1/Cq2)	1:1000 (Cq1/Cq2)
1	A	28.1/28.2	35.2/35.7	38.1/38.3
2	B	27.2/27.4	34.4/33.3	38.3/45
2	C	27.3/27.7	33.7/33.8	36.2/45
2	E	28.57/28.82	35.46/35.55	38.44/37.95
2	J	28.44/27	34.34/33.02	38.53/35.53
2	O	26.55/26.41	34.47/37.73	38.07/45
3	D	26.4/26.65	33.39/33.03	36.54/45
4	F	26.63/26.5	45/34.67	33.16/33.54
5	E	27.52/26.87	33.15/33.28	35.79/35.56
6	H	34.7/35.2	38.2/45	45/45
7	I	27.2/27.7	35/34.9	38.7/39.8
8	M	23.9/23.9	30.5/30.5	34.6/34
9	E	24.98/25.7	32.2/31.63	36.38/35.74
10	E	22.9/23.91	28.94/29.34	30.4/30.63
12	K	23.99/23.99	30.2/31.12	34.55/33.53
12	P	27.5/27.6	34.1/34.7	37/37.4
13	N	24.2/24.8	31.6/31.2	35/35.2
14	K	25.4/25.13	31.69/32.49	35.45/36.09
15	L	23.28/23.61	30.11/31.33	38.29/37.18
16	G	30.7/30.7	36.6/37.7	39.2/45

Cq=45 corresponds to a negative qPCR result

For Peer Review Only

Supplemental Table 4: Prediction model

Cq	Contrast	Std. Err.	z	P	95% Conf. Interval	
1:100 vs 1:1000	-3.814	0.536	-7.11	0.0001	-4.865	-2.763
1:1 vs 1:1000	-11.005	0.564	-19.50	0.0001	-12.111	-9.899
1:1 vs 1:100	-7.191	0.536	-13.41	0.0001	-8.242	-6.140

The mean effect on Cq was evaluated after ANOVA as mean difference between two levels of relative genomic load.

For Peer Review Only

Supplemental Table 5: Detailed protocol for the 5 mtLSU qPCR assays using the same primer/probe target (Alanio et al. CMI 2011)

Center ID	B	C	E	J	O
Sample volume tested (µL)	5	5	5	5	5
Cycler type	ABI 7500 (Applied Biosystem)	Roche LC480	Roche LC480	Roche LC480	ABI 7500 (Applied Biosystem)
qPCR method	Hydrolysis probe (Taqman)	Hydrolysis probe (Taqman)	Hydrolysis probe (Taqman)	Hydrolysis probe (Taqman)	Hydrolysis probe (Taqman)
nb of cycles	45	45	50	50	45
Hybridization temperature (°C)	60	60	60	60	60
Primer/probe concentration (µM)	0.4/0.2	0.4/0.2	0.3/0.1	0.4/0.2	0.4/0.2
Final volume of the PCR reaction (µL)	25	20	25	20	25
Cq determination method	Fit point calculation	2nd derivative calculation	2nd derivative calculation	2nd derivative calculation	Fit point calculation
Internal control tested	No	Yes	Yes	No	Yes
Internal control kit	/	Diagenode - DICU-YD-L100	Diagenode - DICU-YD-L100	/	TaqMan Exogenous Internal Positive Control Reagents
Buffer/enzyme kit	Applied Biosystems TaqMan Fast Universal PCR Master Mix	Takyon No ROX Probe 2X MasterMix UNG Eurogentec	Roche probes master mix	LC480 probe master	TaqMan Universal PCR Master Mix
1:1 (Cq1/Cq2)	27.2/27.4	27.3/27.7	28.57/28.82	28.44/27	26.55/26.41
1:100 (Cq1/Cq2)	34.4/33.3	33.7/33.8	35.46/35.55	34.34/33.02	34.47/37.73
1:1000 (Cq1/Cq2)	38.3/45	36.2/45	38.44/37.95	38.53/35.53	38.07/45

Cq=45 corresponds to a negative qPCR result

For Peer Review Only

Supplemental Table 6: Effects of the WNA/DNA procedures on the signal intensity on the Cq values of *P. jirovecii* detection

Specimen	Procedure 1	Procedure 2	Effects	Std.Error	P	Better
1:1000	RT-qPCR	qPCR	-3.567	1.105	0.001	first
1:100	RT-qPCR	qPCR	-4.125	1.040	0.0001	first
1:1	RT-qPCR	qPCR	-3.539	1.042	0.001	first

The effects of the WNA/DNA procedures on the signal intensity (Cq) ar reported as pairwise contrasts (mean differences), at each target load. Significant ($P < 0.05$) results are included. WNA procedure was superior at all target loads, with higher qPCR signal levels.

For Peer Review Only

Supplemental Table 7: Effect of the target gene on the Cq values of *P. jirovecii* detection

Target load	Gene 1	Gene 2	Contrast	Std. Err.	P	Better
1:1000	mtLSU	MSG	-4.439	2.083	0.033	first
	mtSSU	MSG	-9.023	2.397	0.0001	first
	mtLSU	TUB	-7.339	2.083	0.0001	first
	mtSSU	TUB	-11.923	2.397	0.0001	first
	mtSSU	mtLSU	-4.585	1.298	0.0001	first
1:100	mtSSU	MSG	-5.991	2.397	0.012	first
	mtLSU	TUB	-7.862	2.083	0.0001	first
	mtSSU	TUB	-10.441	2.397	0.0001	first
	mtSSU	mtLSU	-2.579	1.298	0.047	first
1:1	mtLSU	MSG	-4.314	2.083	0.038	first
	mtSSU	MSG	-5.418	2.397	0.024	first
	mtLSU	TUB	-8.564	2.083	0.0001	first
	mtSSU	TUB	-9.668	2.397	0.0001	first

The effects of the target genes on the signal intensity expressed as Cq are reported as pairwise contrasts (mean differences), at each target load. Only significant ($P < 0.05$) results are included. Mitochondrial Large Sub-Unit (mtLSU); mitochondrial Small Sub-Unit (mtSSU); Major Surface Glycoprotein (MSG); beta-tubulin (TUB).

For Peer Review Only

Supplemental Table 8: Detailed Cq values and efficiency for each assay tested in the monocentric intra-laboratory study

Assay ID		Target	BALF1 (Cq1/Cq2)	BALF2 (Cq1/Cq2)	BALF3 (Cq1/Cq2)	BALF4 (Cq1/Cq2)	BALF5 (Cq1/Cq2)	BALF6 (Cq1/Cq2)	BALF7 (Cq1/Cq2)	BALF8 (Cq1/Cq2)	BALF9 (Cq1/Cq2)	PCR Efficiency (%)
2	qPCR	mtLSU	26.92/26.87	33.57/34.27	34.81/35.37	30.18/29.86	33.9/34.95	35.87/34.9	36.18/35.3	45/45	23.7/23.56	97
5	qPCR	mtSSU	23.65/23.67	30.57/30.78	31.72/31.7	26.7/27.17	32.96/32.64	32.17/32.59	33.56/33.85	45/35.49	20.49/20.57	102
9	RT-qPCR	mtLSU	22.73/22.72	28.93/28.89	31.17/31.36	24.52/24.59	33.27/45	32.21/32.18	34.31/33.44	34.64/34.75	19.9/19.91	96
10	RT-qPCR	mtSSU	20.26/20.16	26.44/26.68	27.99/28.02	22.19/22.52	29.06/29.12	28.89/28.93	29.5/29.54	29.79/29.7	17.55/17.55	101,5
11	qPCR	MSG	24.73/24.62	32.03/31.79	34.72/34.7	27.85/27.63	36.06/35.16	33.59/34.08	35.68/35.81	45/45	20.48/20.55	98
12	qPCR	mtLSU	24.69/24.68	31.8/31.72	34.2/33.01	27.06/27.3	34.28/33.42	34.68/34.68	35.46/36.72	45/45	21.54/21.12	113
15	RT-qPCR	mtLSU	23.04/22.84	28.79/28.76	31.26/31.11	24.59/25	33.13/33.16	32.38/31.81	34.17/33.75	34.24/34.11	20.66/19.93	105
17	qPCR	mtLSU	23.95/24.03	30.16/30.47	31.26/31.14	26.89/26.9	31.03/31.4	31.59/31.61	32.03/32.19	45/34.09	21.31/21.11	111,5
18	qPCR	mtLSU	24.59/24.37	31.73/32.46	33.06/33.13	27.5/27.59	34.53/34.16	34.49/34.46	35.68/35.73	45/45	21.75/21.15	94
19	qPCR	mtLSU	22.27/22.29	30.3/30.47	32.04/32.14	25.86/25.83	33.57/32.91	33.07/32.67	33.95/35	35/45	19.31/19.61	96,5

Cq=45 corresponds to a negative qPCR result

For Peer Review Only