**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 White4, Carlo Mengoli,5 Sharon Chen6, Brendan Crowley7, Gijs Dingemans8, Emilie Fréalle9, Rebecca Gorton10, Malcom Guiver11, Ferry Hagen12, Catriona Halliday6, Gemma Johnson13, Katrien Lagrou14, Martina Lengerova15, Willem JG Melchers16, Lily Novak-Frazer17, Riina Rautemaa-Richardson18, Emeline Scherer19, Joerg Steinmann20,21, Mario Cruciani22, Rosemary Barnes23, J. Peter Donnelly24, Juergen Loeffler25, Stéphane Bretagne1,2,3 and Alexandre Alanio1,2,3,\*

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

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

8 PathoNostics B.V., Maastricht, The Netherlands

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 Center 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 Center, 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

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

25University 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, Diagnosis, qPCR, Evaluation, DNA, Whole nucleic acids, Panel specimens, standardization, efficiency, quantification cycles, thresholds.

**Abstract**

Quantitative real-time PCR (qPCR) is increasingly used to detect *Pneumocystis jirovecii* for the diagnosis of pneumocystis pneumonia. An evaluation of the performance of different qPCR assays is needed to assist laboratory standardization of quantification. Through the Fungal qPCR Initiative, a working group of the International Society for Human and Animal Mycology, one inter- and one intra-laboratory comparisons of qPCR assays were performed. For the interlaboratory study, sixteen reference laboratories in eight countries running 20 assays analysed a panel consisting of two negative and three positive specimens from a pool of three different *P. jirovecii* positive bronchoalveolar lavage (BAL) fluids at three different dilutions (pure 1:1, 1:100 and 1:1000). The intra-laboratory study compared five in-house and five commercial qPCR assays testing 19 individual BALs on the same amplification platform. The inter-laboratory analytical sensitivity was 100% at 1:1 genomic load, 95% at 1:100, and 82.5% at 1:1000. Analytical specificity was 100% for all assays but yielded a false-positive test (95% specificity). For both evaluations and for all genomic loads, testing whole nucleic acid (RNA plus DNA) using reverse-transcriptase (RT)-qPCR was superior to qPCR (p≤0.001) testing DNA only. The target gene mitochondrial small sub-unit (mtSSU) was significantly more sensitive than the mitochondrial large subunit, the major surface glycoprotein or the beta-tubulin genes. Thus, RT-qPCR targeting the mtSSU gene had the best sensitivity and could serve as a basis for standardizing the *P. jirovecii* load, which is essential if qPCR is to be incorporated into clinical care pathways.

246

**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 of the 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. Various molecular assays allowing detection of *P. jirovecii* DNA have been developed in the past 30 years. Only real-time quantitative qPCR assays (qPCR) are suitable for diagnostic laboratories because of the rapid and quantitative results indispensable for diagnosing PCP, and the dramatic reduction of the risk of false positive results due to amplicon contamination.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 if fungal burden thresholds for infection in each patient group can be determined. Currently, definite thresholds for PCP in non-HIV patients have not been established to be consensual and universally used in different diagnostic laboratories,5 partially due to the wide diversity of the qPCR assays described used in diagnostic centres. Some studies have compared the performance of different *P. jirovecii* qPCR assays but these studies mainly reported monocentric comparison of 2 to 4 qPCR assays including commercial kits and various in house assays.14-22

The *Pneumocystis* working party of the Fungal qPCR 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 the different 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 contents were tested by 16 reference laboratories. The second study was performed in parallel in one centre testing 10 different qPCR assays by one single person using the same clinical samples and thermocycler.

**Materials and Methods**

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

*Participants.*

The participants were 16 reference laboratories anonymised for blind comparison (Centre A to P). The laboratories were asked to use their own qPCR assay running currently in their lab (listed in Table 1 with primer used in Supplemental Table 1). This inter-laboratory evaluation was designed for evaluating the whole qPCR process but the nucleic acid extraction.

*Panel specimen production*

The broncho-alveolar lavage (BAL) fluids were obtained through diagnostic procedure indicated for patient care. According to the French Health Public Law (CSP Art L1121-1.1), such protocol does not require approval of an ethics committee and is exempted from specific informed consent application. However, the patients were informed and gave consent for the possibility of using left-over material for additional laboratory studies. A panel of five specimens of WNA comprising three *P. jirovecii* positives and two negatives were generated. Firstly, WNA extracted from 50 *P. jirovecii* qPCR negative BALFs were pooled. These BALs were consisted of negative specimens within the panel consisted of 15 µL of this pool, dispensed prior to the preparation of any positive samples. In preparing the positive samples, specimens were pooled from three patients to avoid potential biases due to the presence of specific *P. jirovecii* genotypes known to harbour different single nucleotide polymorphisms or different copy numbers of specific genes.20 This pooled positive specimen was diluted in the pooled negative specimens to obtain different fungal loads (pure 1:1; and 1:100; and 1:1000 dilutions), corresponding to high medium and low fungal loads. Prior to distribution to participants, all five specimens in the panel were tested by Centre E responsible for production using mitochondrial large sub-unit (mtLSU) and mitochondrial small sub-unit (mtSSU) qPCR assays and reverse transcriptase (RT)-qPCR.20 On the same day, 15 µL of each sample were dispensed in 40 tubes in a security cabinet and stored at -20°C before distribution. All specimens were deemed free of qPCR inhibitors by performing an internal control qPCR using DNA Virus Culture DNA (DICD-CY-L100, Diagenode, Seraing, Belgium).

*WNA stability experiments*

The stability of the WNA of the specimens was evaluated by performing *P. jirovecii* qPCR after thawing replicate aliquots in different conditions: (i) directly after thawing (d0), and (ii) after thawing on the bench at room temperature for 3 (d3), 8 (d8) and 15 days (d15).

*Panel distribution and workflow*

The panel was sent to each participating laboratory on dry ice. Recipients were asked to test the panel specimens as part of their routine molecular biology workflow. In short, 5 μL of each WNA panel specimen were to be tested in duplicate using the qPCR process routinely available in their laboratory, including —if possible— the use of an internal control qPCR to monitor for qPCR inhibition. The results were collected using a dedicated Google form that included requests for the qualitative result (positive/negative), the quantification cycle (Cq) values for each replicate related to each specimen 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 matrix were recommended). In addition, one commercial assay (Sacace Biotechnologies, Como, Italy) recommended recording fluorescence intensity during qPCR only after 5 Cq which artificially decreased the Cq value obtained at the end of the run. So, for comparability, 5 Cqs were added to the value obtained with this kit.

*Statistical analysis*

For qPCR analysis, 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 duplicate 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 and 0.

*Linear regression curve.* A linear regression model was used to provide a calibration curve of the qPCR, plotting the Cq value versus the log10 relative load of the fungal burden. This was used to generate the efficiency of the qPCR, utilizing the equation e = −1 + 10(−1/slope).

In the absence of a defined fungal burden in each positive sample, some estimations were required in order to set up a linear model between Cq (dependent) and log10 quantity. Interpolating the mean Cq values of the *P. jirovecii* loaded samples (2, 3, and 5) in the calibration curve of *Aspergillus* (EAPCRI BAL panel 2016, ongoing project), generated an estimated fungal burden of approximately 1.6×101, 1.6×104, and 1.6×102 units, respectively. The use of a calibration curve obtained from a different qPCR assay was arbitrary, at best a rough approximation, but confirmed the accuracy of the dilution factors. The dilutions were: 1:100 (sample 3 to sample 5) and 1:10 (sample 5 to sample 2). So, sample 3 was neat and had a relative genomic concentration (RGC) of 1:1 (high), sample 5 had a RGC of 1:100 (intermediate), and sample 2 had a RGC of 1:1000 (low). Simplifying, the order of magnitude was 101 for sample 2, 102 for sample 5, and 104 for sample 3. The respective log10 transforms are 1, 2, and 4. This quantity was called log10 relative load magnitude (logRLM).

A linear regression of a continuous dependent variable (Cq) versus a continuous explanatory variable was performed. A mixed-model was set up, using the designated annotation for each centre (A-P) (“name”) as the categorical grouping variable. In this manner, a tentative calibration curve for the qPCR reaction was obtained. The coefficients of the regression are reported in the table, and the prediction, along with the empirical data, are depicted in the figure.

*ANOVA.* 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; the ANOVA approach avoided the need to know it, simply assuming that the samples 2, 3, and 5 are different; (2) the linearity of the relationship Cq/logRLM was not granted; (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 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.

**Intra-laboratory evaluation of different qPCR assays using clinical BAL specimens**

*Specimens*

In addition to the multicentre study, Centre E compared 10 different assays with the same person and qPCR thermocycler, by testing 10 previously confirmed *P. jirovecii* qPCR-negative and 9 previously confirmed *P. jirovecii* qPCR-positive BALs. Those 10 assays consisted of 5 in-house assays and 5 commercial kits and included 3 RT-qPCR (1 commercial and 2 in-house) and 7 qPCR assays (4 commercial and 3 in-house) (Table 1, Supplemental Table 1).

WNA was extracted from 900 µL of spun BAL pellet (5 min at 10000g) 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. Eluates were tested for the presence of *P. jirovecii* WNA and classified as negative or positive using our in-house diagnostic assay.20 An internal control qPCR (DNA Virus Culture, DICD-CY-L100, Diagenode, Seraing, Belgium) was performed once to determine the presence of compounds inhibitory to the qPCR process and confirm the extraction efficiency of each sample. To obtain a range of *P. jirovecii* concentrations in the panel, selected samples were diluted in a pool of negatively tested, inhibitor-free BALFs eluates, and aliquoted. All extracts and aliquots were stored at -20°C until use.

To calculate the qPCR efficiencies of each assay calibration curves were generated using a single serially diluted positive BALF (diluted 6 times at 1:5 ratio in pooled negatively tested inhibitor-free BALF). 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.

*qPCR assays tested*

The main features of the five commercial qPCR assays and five in-house assays tested including assays targeting DNA only (qPCR) or WNA (RT-qPCR) are listed in Table 1. Commercial qPCR assays were performed following the manufacturer’s instructions. For the mtLSU and mtSSU in-house qPCR assays, the protocols published by Valero *et al.* was followed.20 For mtLSU and mtSSU RT-qPCR the protocol was: 1× Invitrogen RT-PCR buffer mix (Superscript III One step RT-PCR), 0.3 µM of each primer, 0.1 µM of probe, 1 µM of MgSO4 (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 on LC 480-II consisted of one step of RT-PCR at 50°c for 15 minutes, following 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. The major surface glycoprotein (MSG) DNA amplification protocol was performed following instruction of the detailed process described in Larsen *et al*..24

**Results**

**Inter-laboratory evaluation of *P. jirovecii* qPCR assays**

To assess the robustness of the nucleic acids panel specimens, stability of DNA and WNA was first investigated in Center E after 15 days at room temperature after a freezing-thawing cycle. Using mtLSU DNA and mtSSU WNA detection, the coefficient of variation was 2.48%, 1% and 1% for mtLSU DNA and 2.19%, 1.42%, 1.86% 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.

Results from 20 qPCR assays (16 qPCR amplifying DNA only and 4 RT-qPCR assays amplifying WNA) were obtained from 16 laboratories across eight countries (including seven European countries and one Australian). Six of 20 assays were commercial, for which 2/6 were tested directly by the manufacturers (Table 1). One of the four RT-qPCR assays was commercial and three were in-house. The different targets evaluated were mtSSU (n=2), mtLSU (n=16), MSG (n=1) and beta-tubulin (TUB, n=1) (Table 1). Of note, five centres used the same in-house mtLSU assay as described previously25 and two centres used the same commercial assay (Table 1).

Table 2 shows the Cq summary statistics for each *P. jirovecii* relative genomic load. No false-positive amplification was observed (80/80 negative tests, Table 2) for the two negative samples which set the specificity at 100% (95% CI: 95.4-100).

For each positive specimen tested 40 times (20 assays in duplicates), 40/40 tests were positive at the genomic load 1:1 (both replicates of the 20 assays) (sensitivity =100%, 95% CI: 91.2-100); 38/40 tests were positive at genomic load 1:100 (sensitivity=95%, 95% CI: 83.5-98.6), with one duplicate negative for 2 assays. At the genomic load 1:1000, 33/40 tests were positive (sensitivity=82.5%, 95% CI: 68.1-91.3), with both replicates negative for one assay and one replicate negative for five assays (Figure 1).

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 genomic load 1:1, 1:100 and 1:1000, respectively. Of note, mtLSU assays using the same primers25 gave a median difference of 3 cycles (i.e. 10-fold variation).

The ANOVA basic prediction model (Supplemental Table 3), shows, a significant linear relationship between genomic load and Cq value (Table 3, Figure 1), with a gradient of -3.64 ± 0.18 (Supplemental Table 3), equating to a qPCR efficiency of 88.25% across all assays.

The effect of covariates was analysed, with only four covariates impacting significantly the results including (i) the type of qPCR (RT-qPCR vs qPCR), (ii) the gene targeted for amplification, (iii) the thermocycler platform, and (iv) the internal control testing.

The RT-qPCR assays based on the detection of whole nucleic acid (WNA, detecting both RNA and DNA) was shown to be significantly superior to qPCR assays (detecting DNA only) at all genomic loads across all targets (p<0.001, Table 4) and for mtLSU assays at 1:1 and 1:100 genomic loads (Figure 2). The Cq values were significantly associated to the target gene at all genomic loads with the lowest Cq values associated with mtSSU, successively followed by mtLSU, MSG, and then TUB (Table 5). 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 (Figure 3). Indeed, a 7 Cq difference (200-fold variation) was observed between the TUB assay and the assays targeting multicopy genes.

**Intra-laboratory evaluation of 10 *P. jirovecii* qPCR assays**

Among the 9 positive BALs, the median of the maximum variation of the Cq value was 8.23 (approximately 500-fold variation) for all assays (n=10) (Figure 4A, Supplemental Figure 2A-2B) and was 3.09 (about 10-fold variation) for commercial assays using mtLSU (n=5) (Figure 4B). The sensitivity of the assays for the detection of *P. jirovecii* in 9 BAL extracts varied between 88.8% (n=5 assays) and 100% (n=2 assays) with 4 assays having a sensitivity of 94.4% (Supplemental Figure 2A). Specificity among the 10 assays was high; nine gave a specificity of 100% (upon duplicate testing of the 10 negative BAL) and only one assay (PneumoGenius, PathoNostics, Maastricht, the Netherlands) provided one false-positive result (one of the duplicates) giving a specificity of 95%.

The type of assay and the target used gave significantly different results with a decreasing sensitivity from mtSSU assays amplifying WNA and to MSG assays only amplifying DNA (Figure 5A), with mtSSU WNA giving the earliest Cq values and MSG DNA giving the latest across all positive BAL samples (Figure 5B). By serially diluting a *P. jirovecii* positive qPCR BAL extract five times at 1:5 dilutions, the qPCR efficiency for each assay was calculated and varied between 96 and 100% (Figure 6, Supplemental Table 1).

**Discussion**

The present inter-laboratory study shows correct concordance between qualitative results (positive/negative tests) but large variation in the ability to detect a given quantity of *P. jirovecii* nucleic acids among various qPCR assays available for the diagnosis of PCP. Based on comparative data of 9 commercial kits and 14 in house assays, this variation ranged from 500 to 10,000-fold 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/whole nucleic acids), and to a lesser extent, the thermocycler used and the use of an internal control.

Predictably, assays targeting multi copy target genes provided the best analytical sensitivity, as has already been shown.16,19,20 This was observed for the single TUB copy gene compared to the mtSSU gene (about 200 fold variation). However, MSG assay which targets a repeated gene gave higher Cq values than mtLSU or mtSSU suggesting the presence of a lower number of copies. In addition, the MSG gene sequence is reported to be highly variable, which can impact both the analytical sensitivity/limit of detection (risk of false negative) and qPCR efficiency (risk of variable amplification yield).26 Among the mitochondrial multicopy assays, the mtSSU assay was shown to be 2 times more sensitive than the mtLSU assay, possibly as a result of the higher number of repeats of mtSSU compared to mtLSU.20 But even with a given target, differences can occur depending on factors not directly related to the target gene itself. Using mtLSU, the sensitivity of DNA quantification obtained from 5 in-house assays in 5 centres using the same primer set but not the same primer concentration/amplification kits/enzyme showed a median 3-fold variation (10-fold difference) between panel specimens.25 Therefore, technical parameters other than the specific oligonucleotides and subsequent matching to amplification region or secondary structures 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 should include these parameters also.

The difference between the use of WNA or DNA was expected though not apparent from previous comparisons because only DNA extraction and qPCR methods were compared.14-22 , Increases in the number of amplifiable targets in the extract and by employing RT-qPCR to allow the detection of DNA and RNA is a simple way to increase sensitivity by using one step reverse transcriptase qPCR amplification kits. These were initially developed for virology to allow cDNA and genomic DNA amplification in the same run using specific primers and a reverse transcriptase step. Different assays based on this method have been developed to increase the quantity of target.

27,28 Most automated extractors allow whole nucleic acid extraction to test for the presence of RNA or DNA viruses and DNA from other pathogenic organisms in the same sample extract as part of a syndromic investigation.29

In our study, using mtSSU as the target gene and WNA as the starting material yielded the best sensitivity. The rationale of supporting the use of a qPCR assay with the best sensitivity might be contentious as low fungal loads are often considered to represent “carriage/colonization” and therefore of limited clinical significance. However, detection of low numbers of *P. jirovecii*  in an hospital environment can have implications for infection control, as even patients with low fungal loads can form part of the onward transmission chain in clinical outbreaks.3 Moreover, clinically speaking, low *P. jirovecii* burden can be associated with severe disease in HIV-negative patients.30 Prophylaxxis should also be considered for immunocompromised patients with a low fungal load 31 and knowing that *P. jirovecii* is present can tilt the balance between continuing co-trimoxazole or stopping the drug because of the fear of side effects. In any event, if a threshold has to be defined for distinguishing between infection and carriage, it is better to compare quantitative test results than to compare positive with negative results. Moreover, if the negative predictive value of the assay to exclude PCP is taken into consideration, it seems preferable to rely on optimal sensitivity rather than managing false negative results.

There are several limitations to this study. First, due to the lack of culture system, the panel has to be constructed from clinical specimens containing variable numbers of *P. jirovecii* and variable content of human DNA without any possibility of reproducing the same panel. Given the possible diversity of the genotypes even in a given patient32, one can hypothesise that the BALs selected can be less well amplified by a given assay. We expected to have overcome this possibility in pooling three different BAL fluids for the inter-laboratory study. Second, 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 this specific assay impossible. Third, in this study, only the analytical sensitivity of the amplification was tested, not the nucleic acids extraction step which can differ between platforms and modifies the global performance of commercial kits. We also did not have the opportunity to evaluate the impact of qPCR platform (thermocycler and Cq determination) or of the internal control on the efficiency of the different qPCR assays because too much parameters varied among the different centres. This study would require qPCR assays to be run on each thermocycler and with each internal control which was not possible to implement. Using an internal control is of outmost importance to analyse qPCR results in a diagnostic lab and is strongly advocated33,34 and improve the quality of the results35. There are future plans to implement specific studies on these topics to show the impact of extraction, qPCR plateform and the use of an internal control on the performance of *P. jirovecii* qPCR.

**Conclusion**

The large variations in the ability of different procedures and qPCR assays to quantify *P. jirovecii* nucleic acids makes reaching a consensus threshold value to distinguish between low and high fungal loads in respiratory samples difficult. RT-qPCR amplifying combined RNA and DNA targeting the mtSSU gene is the most sensitive technique and MSG and unique gene targets should be avoided. Interpretative thresholds might be defined in the future, provided centres use the same target and proper account is taken of the other factors not directly related to the PCR process that influence the result such as the procedure for obtaining BAL and the protocol for processing the fluid.

**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 BAL procedure in Saint Louis hospital.

**Tables**

**Table 1. Characteristics of the assays used in these studies**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Assay ID** | **Center ID** | **Target** | **PCR Type** | **In House assay** | **Commercial kit** | **Multicentric study** | **Monocentric study** |
| 1 | A | mtLSU | qPCR | X |  | X |  |
| 2 | B, C, E, J, O | mtLSU | qPCR | X |  | X | X |
| 3 | D | mtLSU | qPCR | X |  | X |  |
| 5 | E | mtSSU | qPCR | X |  | X | X |
| 6 | H | TUB | qPCR | X |  | X |  |
| 7 | I | mtLSU | qPCR | X |  | X |  |
| 8 | M | mtLSU | RT-qPCR | X |  | X |  |
| 9 | E | mtLSU | RT-qPCR | X |  | X | X |
| 10 | E | mtSSU | RT-qPCR | X |  | X | X |
| 11 | E | MSG | qPCR | X |  |  | X |
| 4 | F | mtLSU | qPCR |  | X | X |  |
| 12 | K, P, E | mtLSU | qPCR |  | X | X | X |
| 13 | N | mtLSU | qPCR |  | X | X |  |
| 14 | K | mtLSU | qPCR |  | X | X |  |
| 15 | L, E | mtLSU | RT-qPCR |  | X | X | X |
| 16 | G | MSG | qPCR |  | X | X |  |
| 17 | E | mtLSU | qPCR |  | X |  | X |
| 18 | E | mtLSU | qPCR |  | X |  | X |
| 19 | E | mtLSU | qPCR |  | X |  | X |

**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, 25th - 50th -75th percentiles. The number of the DNA assays is indicated (N). Relative genomic loads are indicated as 0, 1:1000, 1:100, and 1:1.

**Table 3: 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.

**Table 4: Effect of the qPCR type on the Cq values of *P. jirovecii* detection**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Target load | 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) are 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.

**Table 5: 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 (Cq) are reported as pairwise contrasts (mean differences), at each target load. Only significant (P < 0.05) results are included. mtLSU, mitochondrial Large Sub-Unit; mtSSU, mitochondrial Small Sub-Unit; MSG, Major Surface Glycoprotein; TUB, beta-tubulin

**Legends to Figure**

**Figure 1: Cq variation of the three positive panel samples at dilutions 1:1, 1:100, and 1:1000 upon evaluation by the 20 qPCR assays in the inter-laboratory 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.

**Figure 2: 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.

**Figure 3: 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, 1:100, and 1:1000.** 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 4: Cq variation from 9 human broncho-alveolar lavage fluids (BAL1 to BAL9) among all 11 assays (A) of XX mtLSU (B) assays in the intra-laboratory evaluation.**

**Figure 5: Cq variation of the 9 tested BAL by the 11 qPCR assays in the intra-laboratory study including all duplicates (A) or the mean of each replicate in different qPCR assays categories (different targets, mtSSU, mtLSU, MSG, DHPS, and nucleic acids, DNA vs. WNA).** In A, each dot represents each Cq value of the duplicate performed in each centre (40 Cq values by specimen).In B, the mean values of each qPCR obtained from each BAL (BAL1 to BAL9) are connected in all categories of assays.

**Supplemental material**

**Supplemental Figure 1: Results of the stability of nucleic acids (mtLSU DNA and mtSSU WNA) obtained in all 3 samples (1:1, 1:100, 1:1000) obtained 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 tested from 11 assays among 9 human brocho-alveolar lavage fluids (BAL1 to BAL9) (A) or from 9 BAL among 11 qPCR assays in the intra-laboratory evaluation.** Each of the 9 BALs in A or 11 assays in B are connected.

**Supplemental Figure 3: Calibration curves for each of the 11 assays tested using 1:5 dilutions of BAL1 harbouring the highest fungal load in the intra-laboratory evaluation.**

**Supplemental Table 1: Characteristics of all qPCR assay evaluated in the interlaboratory and the intra-laboratory evaluations.**

**Supplemental Table 2: Items evaluated for the interlaboratory study.**

**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 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. 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.

25. 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.

26. 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.

27. Alanio A, Bergeron A, Sturny-Leclère A, et al. Diagnosis of *Pneumocystis* pneumonia : switching from DNA quantification to RNA expression analysis. In: 7th Trends in Medical Mycology, Lisboa; 2015.

28. Alanio A, Lanternier F, Gits-Muselli M, Bretagne S. Diagnosis of histoplasmosis : switching from microscopy to RNA detection. In: 8th Trends in Medical Mycology, Belgrade; 2017.

29. 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.

30. 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 center. *Front Microbiol*. 2017;8:700. doi:10.3389/fmicb.2017.00700.

31. 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.

32. 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.

33. 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.

34. 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.

35. 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.