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

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3 **1 The Fungal PCR Initiative's evaluation of in-house and commercial *Pneumocystis jirovecii***
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5 **2 qPCR assays: towards a standard for a diagnostics assay**
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49 58 **Keywords**

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51 59 *Pneumocystis jirovecii*, Pneumocystosis, PCP, Diagnosis, qPCR, quantification, DNA, Whole
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54 60 nucleic acids, Panel specimens, standardization, efficiency, quantification cycles, Cq,
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56 61 threshold.
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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.

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84 INTRODUCTION

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

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

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3 107 Various molecular assays allowing detection of *P. jirovecii* DNA have been developed in
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6 108 the past 30 years. Real-time quantitative PCR assays (qPCR) are the most suitable options for
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8 109 diagnostic laboratories, providing rapid and quantitative results indispensable for diagnosing
9
10 110 PCP, and a significant reduction in the risk of false positive results by preventing exposure to
11
12
13 111 prior amplicon contamination and excluding post-amplification processing.^{5,11-13} Several
14
15 112 meta-analyses reported the excellent performance of qPCR to aid diagnosis of PCP. However,
16
17 113 detection of *P. jirovecii* DNA in respiratory samples has been observed in asymptomatic
18
19 114 immunocompromised patients leading to the concept of carriage/colonization and the
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21 115 requirement for strategies discriminating between this and active infection.³ Only qPCR has
22
23 116 the potential to be used to differentiate active infection from colonisation, provided fungal
24
25 117 burden thresholds for infection in each patient group can be determined. Currently, definite,
26
27 118 consensual thresholds for PCP PCR in non-HIV patients have not been established and
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29 119 universally used in different diagnostic laboratories,⁵ partially due to the wide diversity of the
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31 120 qPCR assays described and used in diagnostic centres. Some studies have compared the
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33 121 performance of different *P. jirovecii* qPCR assays, but these studies mainly reported intra-
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35 122 laboratory comparison of two to four qPCR assays, including commercial kits and various in
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37 123 house assays.¹⁴⁻²²

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40 124 The *Pneumocystis* working party of the Fungal PCR Initiative, a working group of the
41
42 125 International Society for Human and Animal Mycology, initiated two studies. The first one was
43
44 126 aimed at evaluating the variability of the performance of 20 in-house and commercial assays
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46 127 currently used in reference laboratories with the final goal of providing a consensus method
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48 128 for *P. jirovecii* qPCR, comparable to the process used for *Aspergillus* qPCR.²³ For this purpose,
49
50 129 a panel of five samples with various *P. jirovecii* whole nucleic acid concentrations were tested
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52 130 by 16 reference laboratories. The second study was performed in parallel in one centre testing
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3 131 10 different qPCR assays available at that time in centre E using the same clinical samples and
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6 132 PCR amplification instrument.

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10 134 **MATERIALS AND METHODS**

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13 135 **Multicentre inter-laboratory evaluation of *P. jirovecii* qPCR assays**

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15 136 *Panel specimen production*

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18 137 The broncho-alveolar lavage fluids (BALFs) were obtained through routine diagnostic practice
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20 138 for the patients in Centre E.²⁰ After performing routine investigations, surplus clinical material
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22
23 139 was retained for quality and service evaluation purposes, as permitted by the French Health
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25 140 Public Law (CSP Art L1121-1.1). All further preparation and subsequent dispatching of aliquots
26
27
28 141 were performed in a biosafety cabinet.

29
30 142 Whole nucleic acids (WNA) were extracted from 900 μ L of concentrated BALF pellet (5
31
32 143 min at 10000 g) with addition of 10 μ L/sample of 1:5 diluted internal control (DNA Virus
33
34
35 144 Culture, DICD-CY-L100, Diagenode, Seraing, Belgium) using a Qiasymphony (Qiagen, Hilden,
36
37 145 Germany) with the Virus-Pathogen Kit (Qiagen) following the manufacturer's instructions.
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39
40 146 WNA was eluted in 85 μ L, of which at least 30 μ L was used for the routine diagnosis. Eluates
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42 147 were tested for the presence of *P. jirovecii* WNA using mitochondrial large sub-unit (mtLSU)
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44 148 qPCR assay to detect positive specimens. Mitochondrial small sub-unit (mtSSU) reverse
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47 149 transcriptase (RT)-qPCR (as the most known sensitive method in our centre) was used to select
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50 150 negative specimens. For the qPCR assays, the protocols published by Valero *et al.* were
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52 151 followed.²⁰ All the experiments were performed on a Light Cycler 480 thermocycler (LC480-II;
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54 152 Roche Diagnostics, Mannheim, Germany) with Cq determined with the second derivative
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57 153 method. For the RT-qPCR assay targeting also mtLSU and mtSSU RT-qPCR, the protocol was
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59 154 different to include the reverse transcription step: 1 \times Invitrogen RT-PCR buffer mix
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3 155 (Superscript III One step RT-PCR), 0.3 μ M of each primer, 0.1 μ M of probe, 1 μ M of $MgSO_4$ (to
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6 156 reach a Mg concentration of 4 mM), and 1 μ M of Superscript III Platinum enzyme, in a total of
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8 157 25 μ L with 5 μ L of WNA extract. The amplification consisted of one step of RT-PCR at 50°C for
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10
11 158 15 minutes, followed by qPCR with one activation step at 95°C for 2 minutes and 50 cycles of
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13 159 denaturation at 95°C for 15 seconds and annealing at 60° for 30 seconds. As guided by the
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15 160 internal control values, only the BALFs with no evidence of PCR inhibition were used for
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18 161 further analyses. The amplification results were expressed as quantification cycle (Cq) values.

19
20 162 To provide negative material, WNA extracts from 50 *P. jirovecii* qPCR negative BALFs
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23 163 were pooled. Fifteen microliters of this negative pooled extract were used for the two
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25 164 negative panel specimens. This negative pooled extract was also used to dilute the positive
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28 165 samples, instead of using water, to better mimic the human BALF and maintaining a typical
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30 166 human WNA concentration. As an approximation of the human DNA content, the human
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32 167 albumin gene was quantified by qPCR, as previously reported.²⁴ The result corresponds to 1.6
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35 168 to 1.7×10^7 human cells. We determined that the human to *P. jirovecii* ratio in the 1:1 specimen
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37 169 was 1000:1, (1000-fold more human DNA than *P. jirovecii* DNA).

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40 170 For the positive panel specimens, WNA extracts from residual clinical material of three
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42 171 *P. jirovecii* PCR-positive patients were pooled. This positive pool was diluted in the negative
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44
45 172 pooled extract to obtain different fungal dilutions (pure 1:1; and 1:100; and 1:1000 dilutions),
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47 173 thereafter deemed high, medium, and low fungal loads. Thus, 40 15- μ L positive aliquots were
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50 174 obtained for the multicentre study. The aliquots were stored at -20°C before distribution. To
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52 175 mimic unexpected thawing during transportation, aliquots were thawed and tested
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54 176 immediately after thawing and after 15 days at room temperature.

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57 177 According to the French Health Public Law (CSP Art L1121-1.1), such protocol does not
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59 178 require approval of an ethics committee and is exempt from specific informed consent
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3 179 application. However, the patients were informed and gave consent for the possibility of using
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5 180 leftover material for additional laboratory studies before the broncho-alveolar lavage
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8 181 procedure.

10 182 *Panel distribution to participating laboratories*

13 183 The participating laboratories were 16 reference laboratories anonymised for blind
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15 184 comparison (Centre A to P). The panel consisted of five 15- μ l WNA aliquots comprising three
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17 185 *P. jirovecii* positives and two negatives sent on dry ice. The laboratories were asked to use
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19 186 their own qPCR assay running currently in their lab (listed in Table 1). The primer and probes
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21 187 used in the in-house assays are listed in Supplemental Table 1. In short, 5 μ L of each extract
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23 188 were asked to be tested in duplicate using the qPCR process routinely available in their
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25 189 laboratory. The results were collected using a dedicated form that included requests for the
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27 190 qualitative result (positive/negative), the Cq value for each replicate related to each aliquot
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29 191 and specific information regarding the methods and workflows applied (Supplemental Table
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31 192 2).

37 193 *Quantification cycles (Cq) normalization*

40 194 For comparability between assays, Cq values were normalized as per the volume of nucleic
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42 195 acid tested for each qPCR assay, adjusted to a standard volume of 5 μ L (1 Cq subtracted if 10
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44 196 μ L of extract were recommended). In addition, one commercial assay (Sacace Biotechnologies,
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46 197 Como, Italy) recommended recording fluorescence intensity during qPCR only after 5 first
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48 198 cycles, which artificially decreased the Cq value obtained at the end of the run. So, for
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50 199 comparability, 5 cycles were added to the value obtained with this kit.
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57 201 **Monocentre intra-laboratory evaluation of different qPCR assays using clinical BALFs**

59 202 *Sample preparation*

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3 203 For this monocentre comparison, we selected 10 *P. jirovecii*-negative and 9 *P. jirovecii* –
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5 204 positive BALFs. The 50 µl of the BALF extracts left after routine procedure were combined with
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8 205 70 µl of negative extract (see above), to provide sufficient material for the comparison of the
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10 206 kits. Verification was performed using the mtSSU in house RT-qPCR assay which generated
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13 207 initial Cq values ranging from 17 to 30 cycles for the 9 qPCR-positive BALF extracts . All aliquots
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15 208 were stored at -20°C until use.

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18 209 To calculate the qPCR efficiency of each assay, calibration curves were generated using
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20 210 the highest positive BALF extract (BALF9: initial Cq=17 with the mtSSU in-house RT-qPCR
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22 211 assay). After thawing of an aliquot, a serial dilution of the extract (6 times at 1:5 ratio in
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24 212 negative pooled extract) was tested for each PCR assay.

25 213 *qPCR assays tested*

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28 214 Centre E compared 10 different assays based on the in-house assays available locally
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30 215 and the commercial assays available in his country with the same person and the same qPCR
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32 216 instrument as above. For Centre E in-house assays, the same protocols as above were used.
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34 217 Commercial qPCR assays were performed following the manufacturer's instructions. The
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36 218 major surface glycoprotein (MSG) in-house DNA assay was performed following instruction of
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38 219 the detailed process described in Larsen *et al.*²⁵

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40 220 Technical information on the 10 assays, consisting of 5 in-house assays and 5
41
42 221 commercial kits, are listed in Table 1 and the primers and probes used are available in
43
44 222 Supplemental Table 1. They consisted in 1 commercial and 2 in-house RT-qPCR assays, and 4
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46 223 commercial and 3 in-house qPCR assays. The targets were mtLSU (7 assays); mtSSU (2 assays);
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48 224 and one nucleic gene: Major Surface Glycoprotein (MSG, 1 assays) (Table 1).
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59 226 **Statistical analysis**

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3 227 For the multicentre study, 20 datasets of the five samples tested by each assay in duplicate
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6 228 were produced. Therefore, the total number of data values included was 200. Each replicate
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8 229 was analysed as an independent result. Negative results were allocated a value of 45 cycles.
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10 230 Descriptive statistics were performed calculating summary indexes of Cq at all target loads,
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13 231 pure (1:1) and 1:100, 1:1000 dilutions.

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15 232 An ANOVA model was used to evaluate the effect of a two main covariables: DNA vs.
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17 233 WNA, and target gene. The effects of these categorical covariates were quantified by adding
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20 234 each of them consecutively to a basic ANOVA model. An ANOVA model was applied as (1) the
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23 235 real/absolute amount of the fungal burdens were unknown and the ANOVA approach
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25 236 overcame this by simply assuming that the panel samples 2, 3, and 5 are different; (2) the
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28 237 linearity of the relationship Cq/logRLM was not guaranteed; (3) the difference between the
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30 238 effects exerted by two levels of an added covariate would unlikely be constant across all Cq
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33 239 values. The ANOVA analysis was implemented as a mixed effects regression model, where Cq
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35 240 was the dependent variable, the molecular target level was used as categorical regressor, and
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37 241 the covariate under evaluation was added as an additional factor. The interaction between
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39
40 242 the covariate and the relative genomic load was also included in the model. Anonymization
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42 243 was performed prior to all analysis and identifiers were used to build the categorical grouping.
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44
45 244 The identifier indicated the mixed model categorical grouping variable. As a post-estimation
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47 245 step, the contrasts (predicted mean effects) were calculated for each pair of factor levels,
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50 246 adjusting for each relative genomic load level. They measured the difference between the
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52 247 effects exerted by each member of the factor pair on the signal intensity.
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54 248 For the monocentre study, no specific statistical analysis was applied.
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249 RESULTS**250 Multicentre inter-laboratory evaluation of *P. jirovecii* qPCR assays**

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

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

266 No false-positive amplification was observed (80/80 negative tests) for the two
267 negative aliquots, which set the specificity at 100% (95% CI: 95.4-100). For each positive
268 aliquot tested 40 times (20 assays in duplicates), 40/40 tests were positive at 1:1 (both
269 replicates of the 20 assays) (sensitivity =100%, 95% CI: 91.2-100); 38/40 tests were positive at
270 1:100 (sensitivity=95%, 95% CI: 83.5-98.6), with one replicate negative for 2 assays (#4 and
271 #6). At 1:1000, 33/40 tests were positive (sensitivity=82.5%, 95% CI: 68.1-91.3), with both
272 replicates negative for one assay (#6) and only one replicate negative for three assays across

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3 273 five centres (#2 centre B, C, O, #3 #16) (Table 2, Supplemental Figure 2 and Supplemental
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5
6 274 Table 3 and 4)

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8 275 The maximum variation in the Cq value (excluding negative values) between assays
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10 276 was 12.4 cycles (approximately 10,000-fold variation), 9.2 and 9.4 cycles (approximately 1000-
11
12
13 277 fold variation) for the 1:1, 1:100 and 1:1000 panel specimens, respectively (Supplemental
14
15 278 Figure 2). Of note, mtLSU assays using the same primers and probes,²⁶ but with different
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18 279 reagents and/or protocol, gave a median difference of 3 cycles (i.e. 10-fold variation)
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20 280 (Supplemental Table 5).

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23 281 Two major variables impacted significantly on the results, in addition to minor
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25 282 influences associated with variation in reagents and protocols: (i) the type of qPCR, RT-qPCR
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28 283 vs qPCR, and (ii) the gene targeted for amplification (Figure 1). The RT-qPCR assays based on
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30 284 the detection of WNA (detecting both RNA and DNA) were significantly superior to qPCR
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33 285 assays detecting DNA only at all loads ($p < 0.001$, Supplemental Table 6 and Supplemental
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35 286 Figure 3). The Cq values were also significantly associated to the target gene at all loads with
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38 287 the lowest Cq values associated with mtSSU, successively followed by mtLSU, MSG, and then
39
40 288 TUB (Figure 1, Supplemental Table 7). There was a consistent and significant correlation
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43 289 associated with Cq values generated by mtSSU assays amplifying WNA through to TUB assays
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45 290 amplifying DNA only (ANOVA Kruskal-Wallis $p < 0.05$) across all nucleic acid loads. The Cq values
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48 291 obtained with the 3 panel specimens and the 20 PCR assays are represented Figure 2A. Indeed,
49
50 292 a mean 7 Cqs difference (200-fold variation) was observed between the TUB assay and the
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52 293 assays targeting multicopy genes.

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57 295 **Monocentre intra-laboratory evaluation of 10 *P. jirovecii* qPCR assays**
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3 296 The specificity of the 10 assays was high; nine gave a specificity of 100% (upon duplicate
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6 297 testing of the 10 negative BALFs) and only one assay (assay#12) provided one false-positive
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8 298 result (one of the duplicates) giving a specificity of 95%.

9
10 299 The sensitivity of the assays for the detection of *P. jirovecii* in 9 positive BALFs varied
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12
13 300 between 88.8% (n=5 assays) and 100% (n=2 assays) with 4 assays having a sensitivity of 94.4%
14
15 301 (Supplemental Table 8 and Figure 2B). The lowest Cq values were observed with the mtSSU
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17
18 302 assays amplifying WNA and the highest with the MSG assays amplifying DNA across all positive
19
20 303 BALFs (Figure 2B). Thus, when excluding the negative values (Cq=45), the median of the
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22
23 304 maximum variation of the Cq value between the 10 assays was 8.23 (approximately 500-fold
24
25 305 variation) for a given BALF. Even when considering the 5 commercial assays targeting mtLSU
26
27
28 306 as the target, a median of variation as high as 3.1 (about 10-fold variation) was observed
29
30 307 (Supplemental Table 8 and Figure 2B).

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32 308 By serially diluting BALF#9 WNA extract five times at 1:5 dilutions, the qPCR efficiency
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35 309 for each assay was calculated. The amplification efficiency calculated from the Cq values were
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38 310 all between 94 and 113% (Supplemental Table 8, Supplemental Figure 4). This showed that
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40 311 this factor can be discarded as a possible explanation for the differences observed above.
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3 312 **DISCUSSION**
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6 313 The present study underlines the necessity of comparing different assays for the
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8 314 quantification of the *P. jirovecii* loads. Indeed, large variation was evidenced among all the
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10 315 assays compared. Based on data of 9 commercial kits and 10 in house assays tested in the
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12 316 multicentre and monocentre studies (Table 1), this variation ranged from 500-fold (5 qPCR
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14 317 cycles) to 10,000-fold (12 qPCR cycles) between the assays.

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17 318 This resulted mainly from the target gene (mitochondrial multicopy genes vs. single copy
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19 319 nuclear gene) and the qPCR method/template (qPCR/DNA versus RT-qPCR/WNA). Thus, our
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21 320 studies showed that using mtSSU as the target gene and WNA as the starting material yielded
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23 321 the best sensitivity. Predictably, assays targeting multi copy genes provided the best analytical
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25 322 sensitivity, as already shown.^{16,19,20} This was observed in our study for the single copy gene
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27 323 (beta-tubulin) compared to the mtSSU gene (about 200-fold variation). However, among the
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29 324 repeated genes, MSG assay gave higher/late Cq values than mtLSU or mtSSU, suggesting a
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31 325 lower number of repeats for the MSG gene. The MSG gene sequence is reported to be highly
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33 326 variable,²⁷ which can impacts both the analytical sensitivity/limit of detection (risk of false
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35 327 negative) and qPCR efficiency (risk of variable amplification yield).

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37 328 Among the mitochondrial multicopy assays, the mtSSU assay was two times more
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39 329 sensitive on average than the mtLSU assay, possibly as a result of the higher number of repeats
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41 330 of mtSSU compared to mtLSU in the mitochondrial genome.²⁰ Interestingly, the number of
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43 331 copies of some mitochondrial genes (mtLSU, NAD1 and CYTB) have been shown to vary
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45 332 according to the fungal load (high vs. medium and low fungal loads).²⁰ In contrast, mtSSU
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47 333 seems to remain stable between these conditions suggesting that a more reliable
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49 334 quantification could be achieved with mtSSU rather than with other mitochondrial targets.
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51 335 Indeed, the quantification of unique/single copy gene (nuclear) will better reflect the quantity
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3 336 of microorganism, but the lack of sensitivity of such a target gene hampers its use for clinical
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8 338 Interestingly, even when a given gene is targeted or the same primer/probes utilized,
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10 339 differences can occur. Using the same mtLSU primers and probes and qPCR,²⁶ the
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13 340 quantification obtained from five centres showed here a median 3-fold variation in the Cq (10-
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15 341 fold difference in concentration). In addition, we showed that five commercial mtLSU kits also
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17 342 gave a 3-fold variation in the Cq. Overall, these differences can come from the different primer
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19 343 concentration/amplification kits/enzyme or thermocyclers used. Indeed, technical
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21 344 parameters can affect qPCR efficiency, such as the type of polymerase used, the concentration
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23 345 of oligonucleotides or the method of Cq determination, suggesting that harmonization of the
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25 346 methods requires more than standardization of the target gene or the primer sequences.

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29 347 Although differences were already known among different DNA extraction methods or
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31 348 qPCR assays,¹⁴⁻²² the superiority of WNA over DNA extraction has never been reported for *P.*
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33 349 *jirovecii*, due to lack of prior of investigation. WNA RT-qPCR amplification kits were initially
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35 350 developed for virology to allow cDNA and genomic DNA amplification in the same run to
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37 351 detect both RNA or DNA viruses.^{28,29} Currently, most of the automated extractors allow WNA
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39 352 extraction to test for the presence of both RNA or DNA viruses, and DNA from other
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41 353 pathogenic organisms in the same sample extract as part of a syndromic investigation.³⁰ Such
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43 354 strategy has already been tested with success for detecting *P. jirovecii* DNA in samples
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45 355 dedicated for influenza diagnosis.²⁴ Thus, since WNA extraction does not interfere with the
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47 356 detection of *P. jirovecii* and even provides the best sensitivity in the present study, this
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49 357 extraction method could be recommended.

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52 358 The rationale for supporting the use of the quantitative assays with the highest
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54 359 sensitivity might be contentious as low fungal loads are often considered to represent
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3 360 “carriage/colonization” and therefore of limited clinical significance. However, if the negative
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6 361 predictive value of the assay to exclude PCP is taken into consideration, it seems preferable
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8 362 to rely on optimal sensitivity to exclude PCP, rather than managing false negative results.
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10 363 Indeed, low *P. jirovecii* burden can be associated with severe disease in HIV-negative
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13 364 patients.³¹ Second, prophylaxis could be considered for immunocompromised patients with a
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15 365 low fungal load to avoid subsequent development of full-blown PCP.³² At least, knowing that
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18 366 *P. jirovecii* is detectable can tilt the balance between continuing co-trimoxazole or stopping
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20 367 the drug because of the fear of side effects. Third, patients with low fungal loads in an hospital
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23 368 environment can be part of the onward transmission chain in clinical outbreaks.^{3,31}

24
25 369 There are limitations to this study. To set up a quality control with *P. jirovecii*, one must
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28 370 consider the difficulty of obtaining cultured material. Realistically, only surplus human
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30 371 samples can be used and these are limited in both number and sample volume. Hence the
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33 372 choice of pooling human samples and the limited number panel size used for comparison. In
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35 373 pooling samples, it is acknowledged that potential biases are introduced. Ideally, it would have
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38 374 been preferable to select a specific patient, to have an idea of the sequence of the *P. jirovecii*
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40 375 main target genes and to propose individual panel specimens based on well-defined
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42 376 organisms. Unfortunately, this is impossible due to limited sample volume. Since the BALF
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45 377 material is usually limited after performing thorough routine diagnostics testing, such previous
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47 378 analysis, providing 20 aliquots of several individual BALF extracts is practically impossible. The
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50 379 pooling of BALF extracts also assesses the amplification process in the presence of the genetic
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52 380 variability. In addition, it has been shown that PCP episodes are due to multiple genotypes in
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54 381 more than 70% of the cases,³³⁻³⁶ with known sequence variation in target genes.³³
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57 382 Indeed, false negative results can result from the choice of primers in variable regions of the
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59 383 fungus.³⁷ This is therefore the responsibility of each centre to investigate this when facing a

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3 384 high clinical suspicion of PCP and our quality control did not address this possibility. For
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6 385 example, MGB probes who are very sensitive to polymorphism/mismatch should not be used
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8 386 to avoid the risk of false negative result.³⁷
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10 387 The absolute quantity of *Pneumocystis* DNA added in the initial pooled of samples was not
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13 388 known. Nevertheless, the main purpose of our study was to compare different assays and not
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15 389 to determine the absolute analytic capacity of each assay. Another possible bias in pooling
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18 390 human samples is to dilute PCR inhibitors present in one of the samples for elaborating the
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20 391 panel. Using an internal control is of outmost importance to analyse qPCR results in a
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23 392 diagnostic lab and is strongly advocated^{38,39} and improves the quality of the results, especially
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25 393 to have a strong validation for negative results.⁴⁰ Thus, some PCR assays not controlling for
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28 394 the presence of PCR inhibitors can be falsely over-evaluated in the present study. With limited
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30 395 availability of *Pneumocystis* culture the current methodology based on surplus human
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33 396 samples is the only one possible to perform such evaluations, despite all the inherent potential
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35 397 bias. However, we do not think the possible limitations listed above had a major impact on
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38 398 our comparative studies.

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40 399 The limited quantity of BALF extract available restricted the number of evaluations
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42 400 possible. Thus, some assays were tested only once in a single centre (DNA MSG, DNA TUB,
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44 401 WNA mtSSU, DNA mtSSU) making any evaluation on the reproducibility for these specific
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47 402 assays impossible. Additionally, only the analytical sensitivity of the amplification was tested,
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50 403 not the nucleic acids extraction step, which has a major impact for the global performance of
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52 404 all the amplification procedure.⁴¹ We also did not have the opportunity to evaluate the impact
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55 405 of qPCR platform (thermocycler and Cq determination), because too many parameters varied
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57 406 among the different centres. There are future plans to implement specific studies on these
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3 407 topics to show the impact of extraction, qPCR platform and the use of an internal control on
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5 408 the performance of *P. jirovecii* qPCR.
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10 410 **CONCLUSION**

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13 411 Our present studies confirm the large variation between of qPCR assays to quantify *P. jirovecii*
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15 412 nucleic acids. This large variation, up to 12 cycles (10,000-fold variation) for a given sample,
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17 413 makes reaching a consensus threshold value to distinguish between low and high fungal loads
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19 414 in respiratory samples difficult, but highlights optimal assays in regards to sensitivity. The most
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21 415 sensitive association was shown to be RT-qPCR (amplifying combined RNA and DNA) targeting
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23 416 the mtSSU gene and the less sensitive the qPCR assays (DNA only) targeting Major Surface
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25 417 Glycoprotein and the unique beta-tubulin gene targets using. Interpretative thresholds might
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27 418 be defined in the future, provided centres agree to use the most sensitive method. Other
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29 419 factors not directly related to the PCR process that influence the result, such as the protocol
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31 420 for processing the fluid must, however, be performed. Future harmonization of the results
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33 421 should also take into account the procedure for obtaining broncho-alveolar lavage or other
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35 422 respiratory specimens.
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424 **Acknowledgments**

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427 thank Pr Anne Bergeron who is in charge of the broncho-alveolar lavage procedure at Saint-
428 Louis hospital.

430 **Disclosure of Conflict of Interest**

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431 The authors declare no conflict of interest for this specific study, except GJ who is an employee
432 of OLM Diagnostics and GD who is an employee of PathoNostics. AA and SB are inventors in a
433 patent dealing with Pneumocystis RT-qPCR.
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435 **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
 436 previously²⁶ 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

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

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440 **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

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

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3 443 **Legends to Figures**
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5 444 **Figure 1: Cq variation for each assay regarding the target gene and the type of material**
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8 445 **amplified (DNA or WNA) in all the three positive panel samples at dilutions 1:1 (A), 1:100**
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10 446 **(B), and 1:1000 (C).** It shows the gain in the ability to detect the target (lower Cq) in WNA
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13 447 assays compared to DNA and with a gradient from mtSSU to TUB genes
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15 448 (mtSSU>mtLSU>MSG>TUB)
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20 450 **Figure 2: Cq variation in all assays tested in the multicentre (A) and in the monocentre study**
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22 451 **(B).** For the multicentre study, the panel specimen 1:1 (open circle) 1:100 (open square),
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24 452 1:1000 (black circle) were tested in duplicates among 20 PCR assays tested (assay#2 was
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26 453 tested by 5 centres and assay#12 by 2 centres, see Table 1). For the monocentre study, 9
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28 454 human broncho-alveolar lavage fluids (BALF1 to BALF9 highlighted by different symbols) were
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30 455 tested in duplicates with 10 in house and commercial assays. It shows that assay#10 (mtSSU
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32 456 and RT-qPCR) gives the best Cq values.
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3 459 **Supplemental material**
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8 461 **Supplemental Figure 1: Stability of nucleic acids (mtLSU DNA and mtSSU WNA) obtained in**
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10 462 **all 3 samples (1:1, 1:100, 1:1000) immediately thawing (D0) and after 3 (D3), 8 (D8) or 15**
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12 **(D15) days at room temperature after one thawing/freezing cycle (A) or pooled (B).**
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18 465 **Supplemental Figure 2: Cq variation of the three positive panel specimens at dilutions 1:1,**
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20 466 **1:100, and 1:1000 upon evaluation by the 20 qPCR assays in the multicentre study.** Each dot
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22 represents each Cq value of the duplicate performed in each centre (40 Cq values by
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24 specimen). Negative amplifications have been plotted with a Cq at 45.
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30 470 **Supplemental Figure 3: Cq variation in assays amplifying mtLSU DNA or mtLSU Whole**
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32 **Nucleic Acids (WNA) for each panel sample.** It shows the gain in the ability to detect the target
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34 (lower Cq) in WNA assays compared to DNA.
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40 474 **Supplemental Figure 4: Calibration curves for each of the 11 assays tested using 1:5 dilutions**
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42 475 **of BAL1 harbouring the highest fungal load in the monocentre study.**
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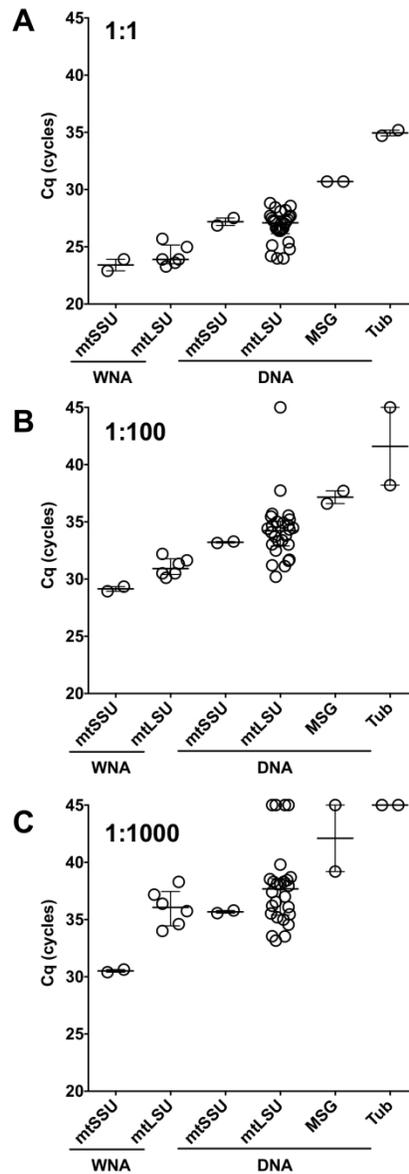
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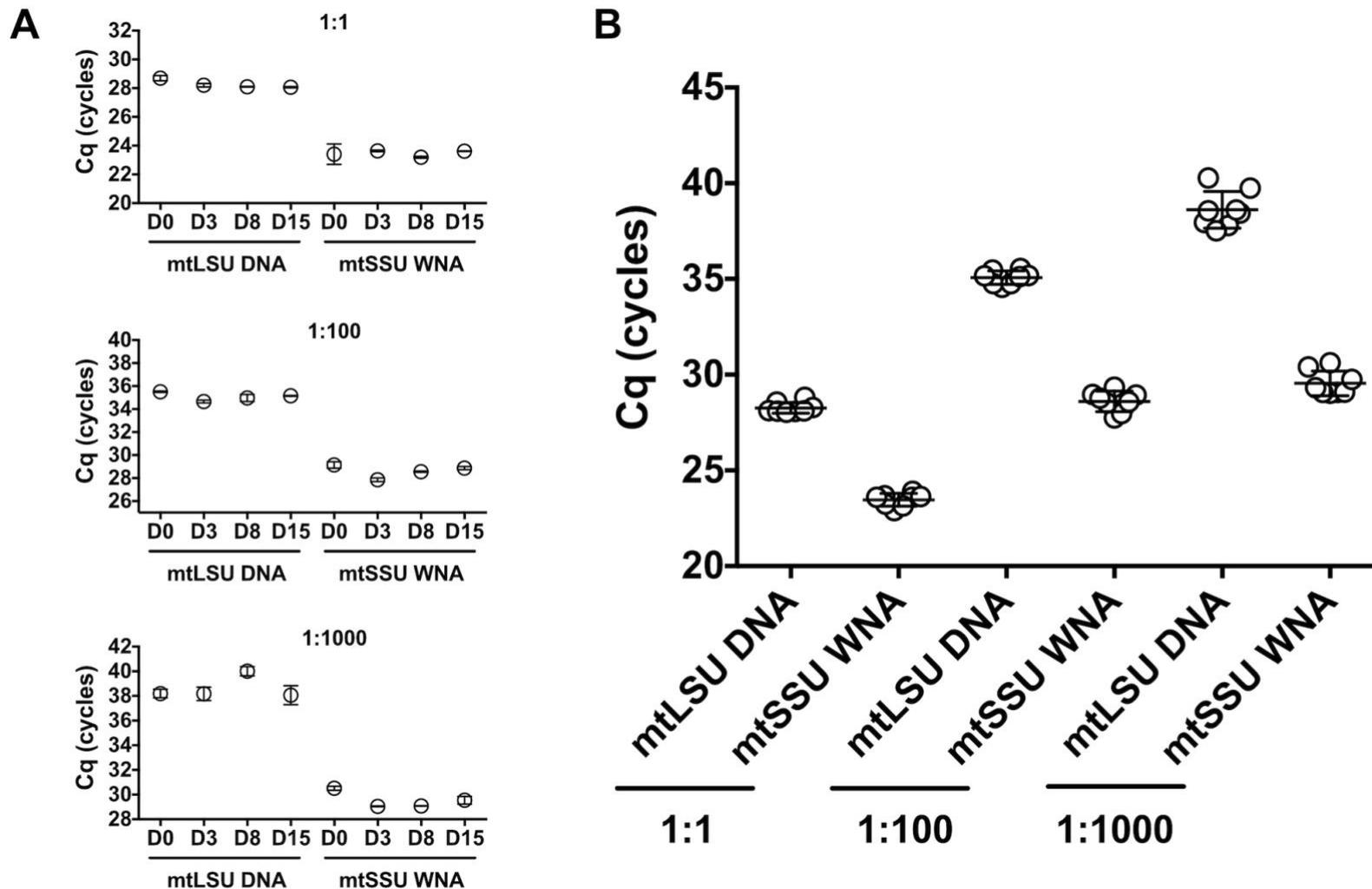
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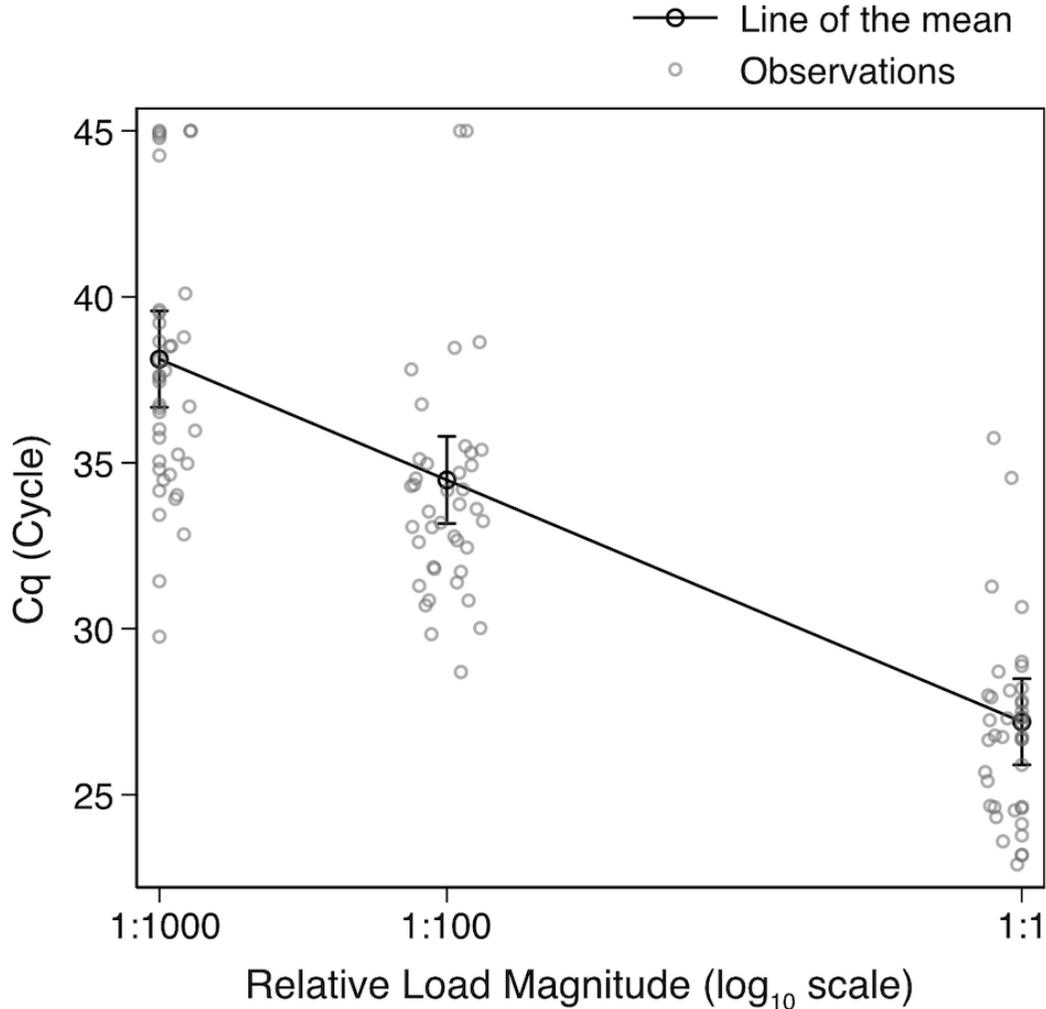
Cq variation for each assay regarding the target gene and the type of material amplified (DNA or WNA) in all the three positive panel samples at dilutions 1:1 (A), 1:100 (B), and 1:1000 (C). It shows the gain in the ability to detect the target (lower Cq) in WNA assays compared to DNA and with a gradient 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).

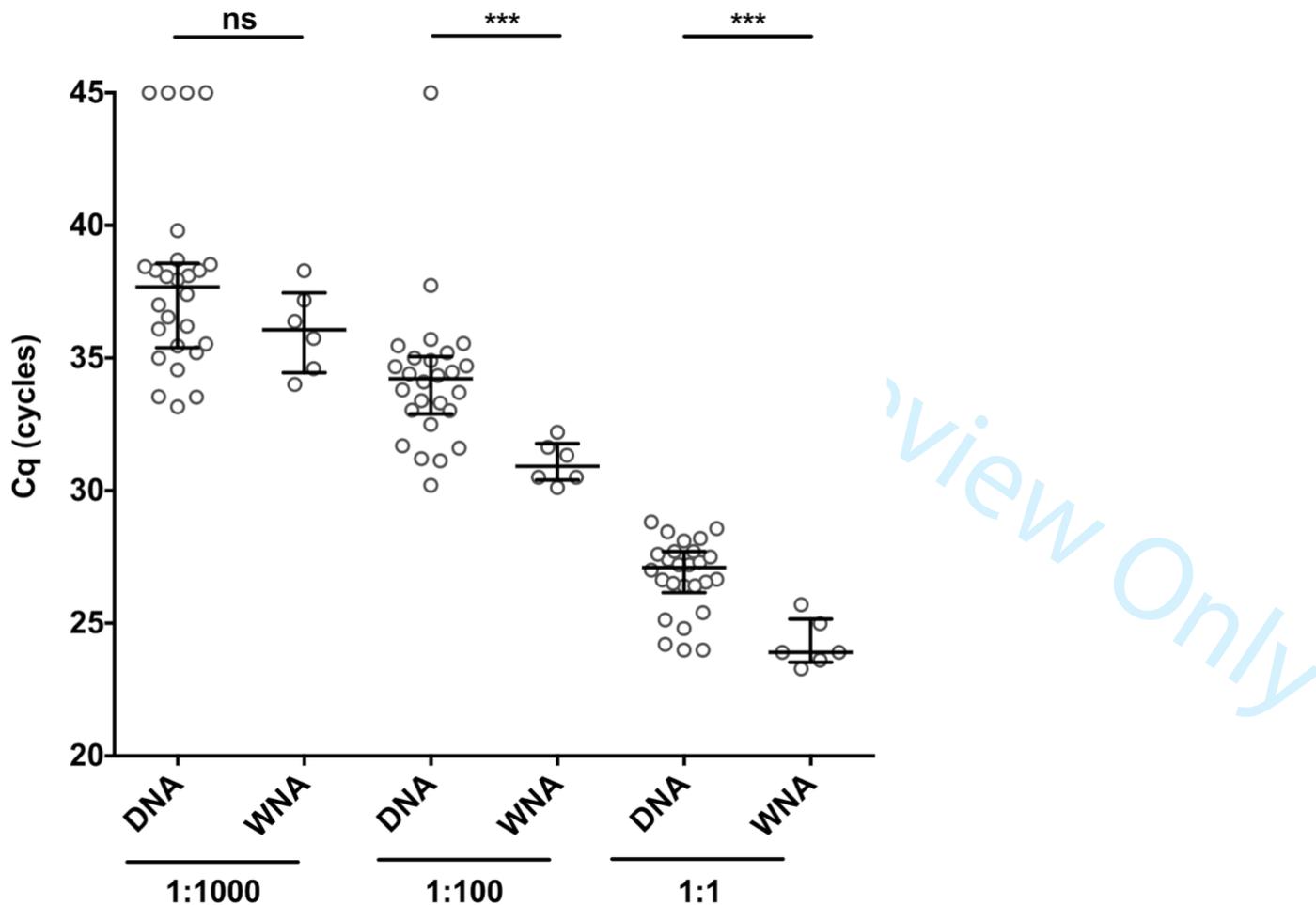


Supplemental Figure 2: Cq variation of the three positive panel specimens at dilutions 1:1, 1:100, and 1:1000 upon evaluation by the 20 qPCR assays in the multicentric study. Each dot represents each Cq value of the duplicate performed in each centre (40 Cq values by specimen). Negative amplifications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

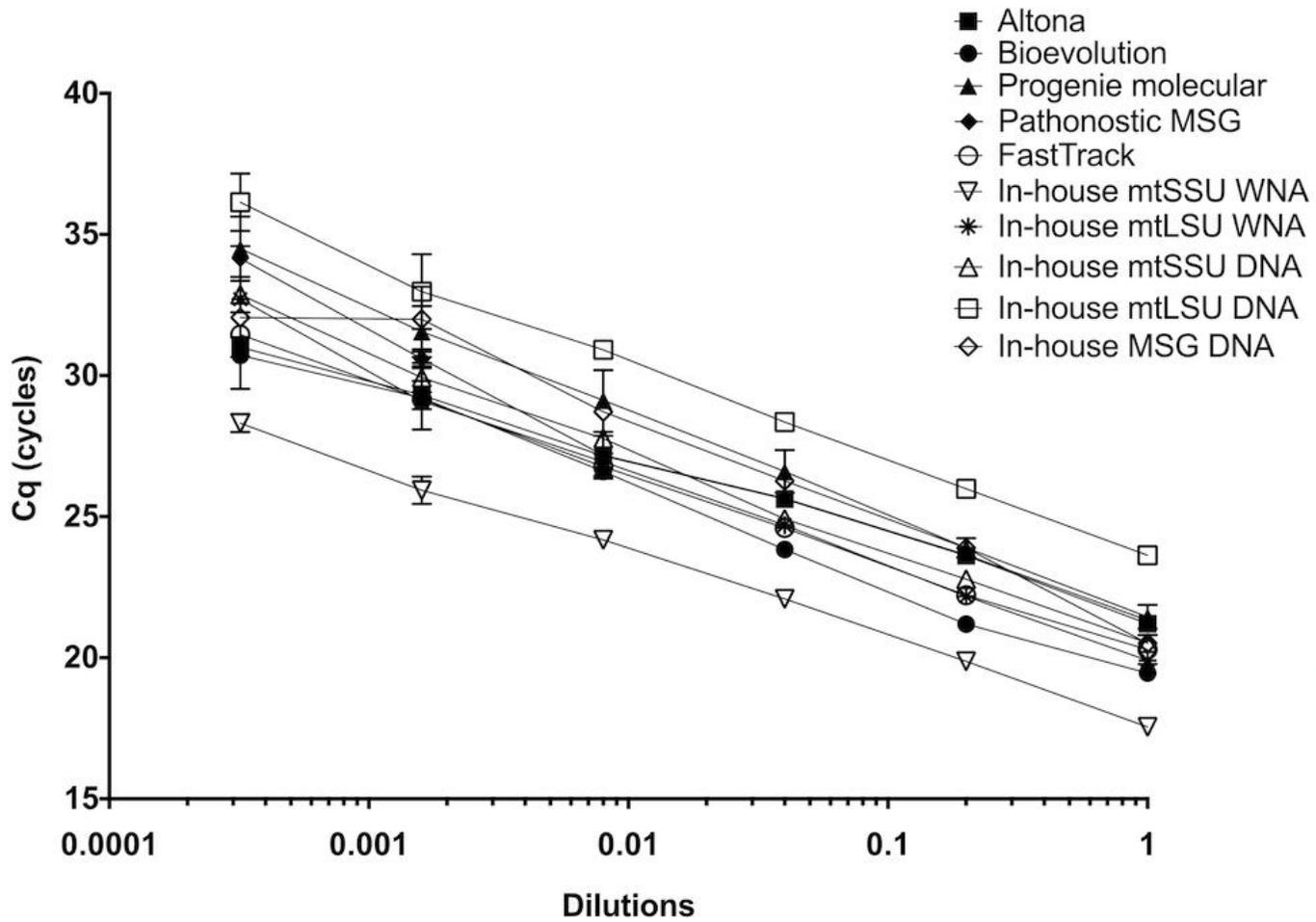


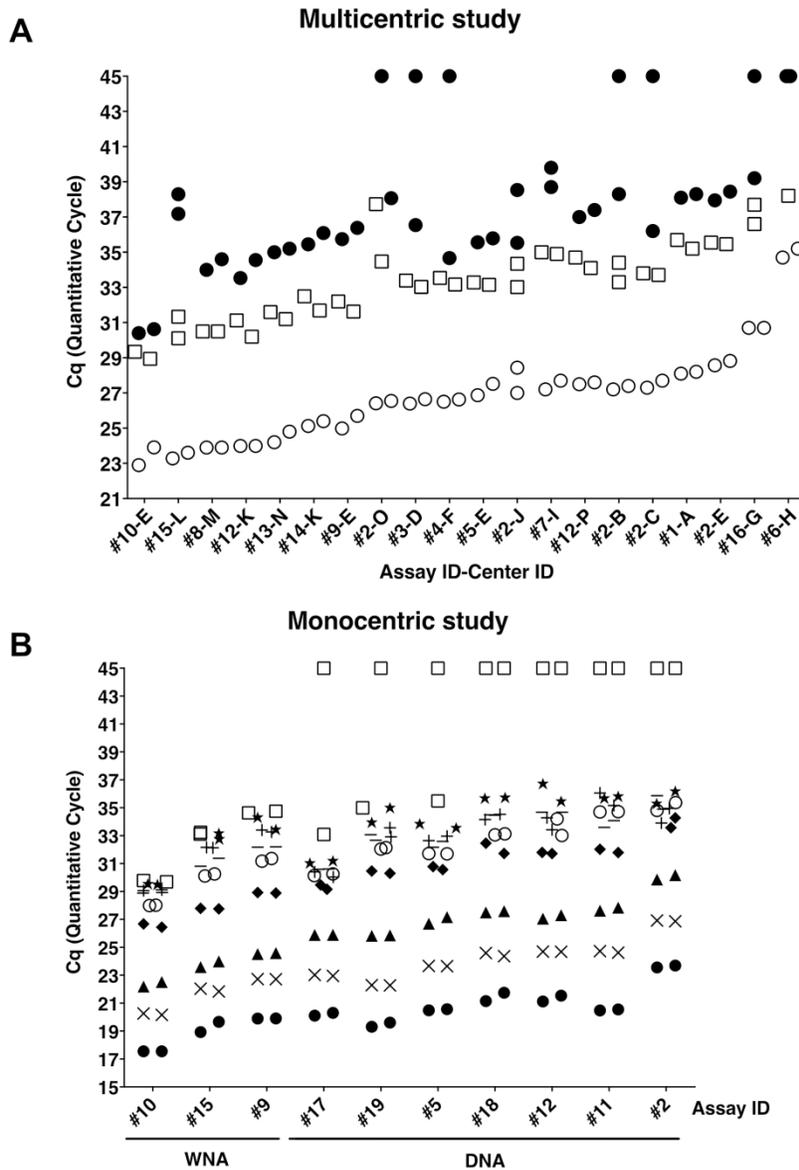
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Supplemental Figure 3: Cq variation in assays amplifying mtLSU DNA or mtLSU Whole Nucleic Acids (WNA) for each panel sample. It shows the gain in the ability to detect the target (lower Cq) in WNA assays compared to DNA.



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 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'-CTGTTTCCCTTTTCGACTATCTACCTT-3'	5'-CACTGAATATCTCGAGGGAGTATGAA-3'	5'-FAM-TCGCACATAGTCTGATTAT-MGB-3'
3	5'-TGCGATAAAGTAGATAGTCGAAAGG-3'	5'-CCCACCTTCTTAAGTGTAGATATCCA-3'	5'-FAM-ACAGCCCAGAACAGTAATTAAGCTCCCA-BHQ1-3'
5	5'-TCATGACCCTTATGAAGTGGGC-3'	5'-GCTCCGACTTCCATCATTGC-3'	5'-FAM-ACGTGCTGCAAAATTTTCTACAATGGG-BHQ1-3'
6	5'-GATCCGAGACATGGTCGCTATT-3'	5'-TTCAACCTCCTTCATGAAACAG-3'	5'-TGTTCAGCGATTTCCGCGTA-3'
7	5'-TGCAAAGTACTCAGAAGAATTGTGGTA-3'	5'-TTCGCAGAAAACCAGCTATATCCT-3'	5'-FAM-CCGATTTGTATTTCACTACT-BHQ1-3'
8	5'-GCACCTGAATATCTCGAGGGAGTATG-3'	5'-TTGGGAGCTTAACTACTGTTCTGG-3'	5'-FAM-TGTTCCCTTCGACTATC-3'-MGB
9	5'-CTGTTTCCCTTTTCGACTATCTACCTT-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'-AAATCATGAACGAAATAACCATTCG-3'	5'-CAAAAATAACAYTSACATCAACRAGGCG-fluorescein-3' 5'-Red 640-TGCAAACCAACCAAGTGTACGACAGG-3'

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3 **Supplemental Table 2: Questions send to each participant**
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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 (µM) 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:

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

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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.

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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 ($^{\circ}\text{C}$)	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

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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) 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.

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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).

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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

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