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Increased sensitivity of a new commercial reverse transcriptase-quantitative PCR for the detection of *Pneumocystis jirovecii* in respiratory specimens

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19 **Abstract**

20 Optimal sensitivity to detect low *Pneumocystis* loads is of importance to take individual and collective
21 measures to avoid evolution towards *Pneumocystis* pneumonia and outbreaks in immunocompromised
22 patients. This study compares two qPCR procedures, a new automated RTqPCR using the GeneLEAD
23 VIII extractor/thermocycler (GLVIII; ~2.2 hrs workflow) and a previously validated in-house qPCR
24 assays (IH; ~5 hrs workflow) both targeting mtSSU and mtLSU for detecting *P. jirovecii* in 213
25 respiratory samples. GLVIII was found to be more sensitive than IH, detecting 8 more specimens.
26 Bland-Altman analysis between the two procedures showed a Cq bias of 1.17 ± 0.07 in favor of GLVIII.

27 **100**/100 words

28

29 **Lay summary**

30 The fungus *Pneumocystis* needs to be detected early in respiratory samples to prevent pneumonia in
31 immunocompromised hosts. We evaluated a new commercial RTqPCR on 213 respiratory samples to
32 detect *Pneumocystis* and found it more sensitive and faster than our routine sensitive in-house qPCR
33 assay.

34 **298**/300 characters

35 *Pneumocystis jirovecii* is an opportunistic fungal pathogen responsible for *Pneumocystis*
36 pneumonia (PCP).¹ PCP diagnosis relies on X-ray showing bilateral or diffuse ground-glass opacities
37 with interstitial infiltrates as a predominant feature, microscopy of respiratory samples and more and
38 more often on detection of nucleic acids in respiratory samples.² Furthermore, the good negative
39 predictive value of serum β -D-glucan is used as an additional tool to rule out PCP or increase the
40 confidence in the diagnosis when positive.³ Optimal sensitivity to detect low fungal load is of major
41 importance to avoid progression towards PCP and preventing cross-contamination between
42 immunocompromised patients in taking appropriate measures such as cotrimoxazole prophylaxis.^{2,4} An
43 international initiative has recently demonstrated a better sensitivity of reverse transcriptase quantitative
44 PCR (RTqPCR) methods, amplifying whole nucleic acid (WNA, including DNA and RNA) and
45 targeting the mitochondrial small subunit of rRNA gene (mtSSU) over other qPCR methods and targets.⁵
46 We compare here two qPCR procedures both incorporating validation internal controls (DNA or RNA
47 alien target, DIA-EIC/DNA-050/ DIA-EIC/RNA-050 for qPCR and RTqPCR assays),⁶ a new fully
48 automated RTqPCR (~2.2 hrs workflow) and our in-house qPCR assays (~5 hrs workflow: sample
49 preparation and pre-extraction step ~30 min, extraction ~1 hrs 30 min, qPCR mix and plate preparation
50 ~45 min, qPCR ~2 hrs) both targeting mtSSU and mtLSU for detecting *P. jirovecii* in respiratory
51 samples including bronchoalveolar lavage fluids (BAL) and non-BAL specimens. Of note both assays
52 were performed using 45 cycles.

53 Upon reception, samples were centrifuged and pellet was resuspended in 400 μ L and divided in two
54 equal parts. Mucous specimens (mucous BAL, bronchial aspirates, or sputa) were treated with 1X
55 dithiothreitol (dithiothreitol, dTT, digest-EUR, EuroBio) for 15 min at 37°C, centrifuged with the
56 supernatant discarded. To our knowledge, dTT does not impact negatively the results of qPCR, as it is
57 recommended in lysis buffer and used in extraction buffers. The tested procedure included WNA
58 extraction using the GeneLEAD VIII extractor-thermocycler (Precision System Science, Japan) and the
59 new R-DiaPnJ kit (Diagenode, Seraing, Belgium) amplifying mtLSU and mtSSU WNA (designated as
60 GLVIII). The reference procedure included WNA extraction using the DSP virus/Pathogen kit on a
61 QIASymphony apparatus (Qiagen, Hilden, Germany) and amplification using our in-house mtSSU and
62 mtLSU qPCR assays (designated as IH).⁷ This non-interventional study on leftover specimens did not

63 require approval of an ethics committee according to the French Health Public Law (CSP Art L1121-
64 1.1).

65 Full validation of the GLVIII was performed and characteristics are available in [Table S1](#). A
66 total of 213 consecutive respiratory samples were tested from 167 patients described in [Table 1](#).
67 *Pneumocystis jirovecii* nucleic acids were detected in 45 specimens regardless of the PCR procedure. A
68 total of 44 samples were positive with GLVIII and 37 with IH, with 36 samples positive with both
69 methods. Four specimens (2 BAL and 2 non-BAL samples) were deemed invalid based on internal
70 control in the GLVIII procedure with all four being negative using the IH procedure. Agreement
71 between both techniques was >0.81 with a Cohen's kappa = 0.863) ([Table S2](#)). For GLVIII procedure,
72 40/44 (90.91%) were positive with both mtLSU and mtSSU targets, 2/44 with mtLSU only, and 2/44
73 with mtSSU only.

74 Eight samples from eight patients were GLVIII-positive only with mean quantification cycle
75 (Cq) of $35.1 (\pm 0.5)$ for mtLSU and of $34.6 (\pm 1.3)$ for mtSSU targets. The only IH-positive and GVIII-
76 negative sample was not validated by the GLVIII controls, although no PCR inhibitors were evidenced
77 with the IH internal control. All patients' characteristics with discrepancies between GVIII and IH are
78 shown in [Table S3](#). None received PCP prophylaxis. Overall, the Cq values obtained in the 36 samples
79 positive in both methods were significantly lower with the GLVIII than with the IH (mean Cq difference
80 was -3.9 ± 1.7 for mtLSU and -2.5 ± 2.2 for mtSSU) ($p < 0.001$). Bland-Altman analysis between the two
81 procedures showed a Cq bias of 1.17 ± 0.07 in favor of GLVIII. For specific analysis, redundant
82 sampling per patients were excluded and analysis was replicated in BAL (not treated with dTT, $n=23$;
83 Figure 1A and 1C) and non-BAL specimens (treated with dTT, $n=16$; Figure 1B and 1D), separately.
84 Quantification cycle distributions were significantly different in all four assays (non-parametric paired
85 Anova test, $p < 0.0001$). Mean Cq difference between IH and GLVIII was -4.2 ± 1.8 for mtLSU and -3.7
86 ± 1.8 for mtSSU, for BAL specimens, and -3.9 ± 1.5 for mtLSU and -1.6 ± 2.4 for mtSSU, for non-
87 BAL specimens.

88 Despite that the R-DiaPnJ kit was validated only on bronchoalveolar lavage fluids, we performed our
89 study regardless of the specimen type. We finally analyzed 90 non-BAL specimens (37 bronchial

90 aspirate and 53 sputa). A total of 14/16 qPCR-positive specimens (87.5%) were positive with both
91 procedures and two were positive with only one of both assays (1 with GLVIII only and 1 with IH only).
92 GLVIII mtLSU target was the most frequently positive target gene (15/16) as compared to GLVIII
93 mtSSU target (14/16 positive.)

94 Since the PCR targets were identical, differences result potentially from several factors
95 including extraction method, amplification of WNA versus DNA only, platform and mastermix as
96 already described.⁶ Indeed WNA versus DNA extraction and amplification may result in a ΔCq of 5.8
97 for low fungal loads as previously shown.⁶ Depending on master mix selected, Cq may also vary with a
98 ΔCq up to 2.9 between two master mixes with otherwise identical extraction and amplification protocol.⁶

99 The use of two targets in this commercial assay is a significant asset because it strengthens data
100 analysis with a more confident result in case both targets are positive. It also prevents complete non-
101 detection due to mutations in one of the target sequences preventing proper primers/probe annealing.⁸
102 As an example, detection of SARS-CoV-2 variant with mutations on the spike protein could only be
103 performed by multiplex PCR assays targeting other genes.⁹ Furthermore, the manufacturer has disclosed
104 that the mtSSU probe had a Minor Groove Binder (MGB) quencher. MGB probes were designed to
105 increased specificity of detection especially for single base mismatches at elevated hybridization
106 temperatures.¹⁰ A previous *Pneumocystis* qPCR assay using a MBG probe was confronted with false
107 negative results due to an undescribed mutation of the probe hybridization region in the mtLSU gene
108 with an estimated frequency of 0.28%.⁸

109 As part of a previous international initiative, our center compared on the same qPCR
110 thermocycler 10 assays including in-house (n=5) and commercial (n=5) assays.⁵ When our in-house
111 mtLSU qPCR is used as reference to compare all tested assays, GLVIII (mtSSU target) comes in the
112 second (ΔCq of -4.7 ± 1.8) and third position (-3.9 ± 1.7) just after an in-house RT-qPCR targeting
113 mtSSU with a ΔCq of $-6.6 (\pm 0.8)$ ⁷ and before the other commercial assays evaluated (Table S4). GLVIII
114 procedure, giving better detection rate and Cq results, seems to be a good assay to quantify more
115 accurately the fungal load in respiratory samples. Indeed, semi-quantification allows clinical
116 interpretation of fungal loads with high fungal loads associated with PCP and low fungal load to carriage
117 which may require prophylaxis for both individual and collective prevention in immunocompromised

118 populations.^{2,11,12} A prospective clinical study is required to properly determine clinical cut-offs with
119 this assay.

120 In conclusion, we report here an increased sensitivity using a new molecular diagnostic
121 procedure compared to our in-house procedure.¹¹ In addition to a better sensitivity, the diagnosis is
122 achieved within ~2 hours upon reception of the sample using this new procedure. The GeneLEAD
123 VIII/R-DiaPnJ kit is a single sample assay which is clinically relevant when accurate sensitive detection
124 and prompt therapeutic decision are at stake.

125

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1159/1200 words

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128 **Authors contribution**

129 *Writing – Original draft:* SD and AA; *Writing – Review and editing:* All; *Conceptualization:* AA;

130 *Formal Analysis:* SD and AA; *Supervision:* AA

131

132 **Conflict of Interest**

133 AA and SB are owner of a patent on *Pneumocystis jirovecii* diagnosis using RTqPCR

134

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12/20 references

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177 **Table 1.** Patient and sample characteristics.

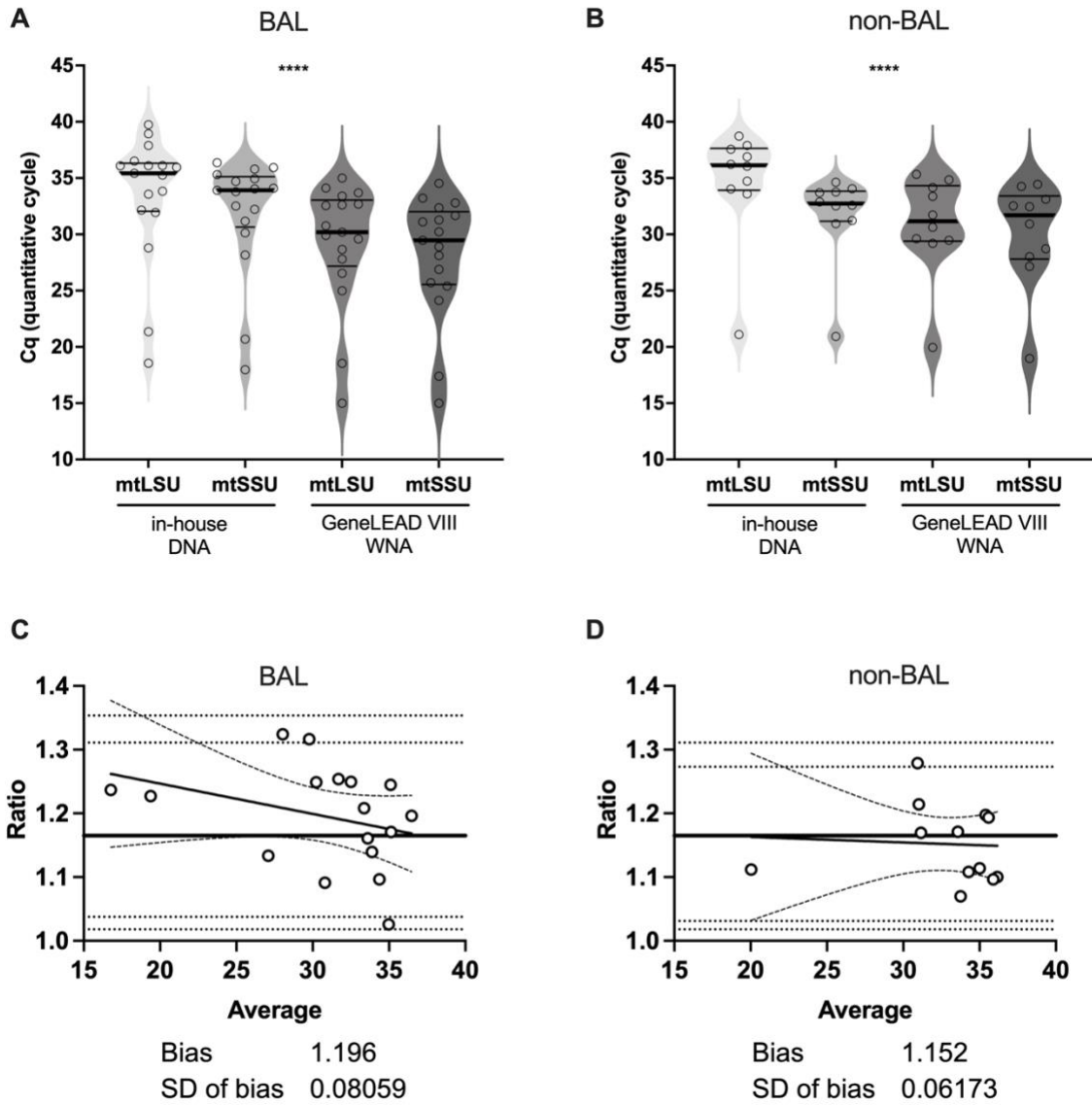
| | Patients (n=167) | Samples (n=213) |
|---|------------------|-----------------|
| Male <i>n</i> (%) | 112 (67.1) | |
| Age median [IQR: Q1-Q3] | 59 [44-67] | |
| Type of respiratory sample | | |
| BAL <i>n</i> (%) | | 123 (57.8) |
| Sputum <i>n</i> (%) | | 52 (24.4) |
| Bronchial aspirate <i>n</i> (%) | | 38 (17.8) |
| <i>P. jirovecii</i> detection rate | | |
| in-house mtLSU | 29 (17.4) | 35 (16.4) |
| in-house mtSSU | 30 (18.0) | 35 (16.4) |
| in-house any target | 31 (18.6) | 37 (17.4) |
| GeneLEAD VIII mtLSU | 36 (21.6) | 42 (19.7) |
| GeneLEAD VIII mtSSU | 36 (21.6) | 42 (19.7) |
| GeneLEAD VIII any target | 38 (22.8) | 44 (20.7) |
| GeneLEAD VIII invalid | 4 (2.4) | 4 (1.9) |

178 BAL: bronchoalveolar lavage; mtLSU, mitochondrial large subunit; mtSSU, mitochondrial small
 179 subunit

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Figure 1. A. *Pneumocystis jirovecii* fungal load (Cq values) according to PCR target and protocol tested for BAL (A) and non-BAL (B) samples. Bland-Altman test between in-house mtLSU DNA and GeneLEAD VIII mtSSU WNA Cq values for BAL (C) and non-BAL (D) samples. MtLSU, mitochondrial large subunit; MtSSU, mitochondrial small subunit; WNA, whole nucleic acids. **** $p < 0.0001$ (Wilcoxon non-parametric test).



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