



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

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Abstract

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

100/100 words

Lay summary

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

298/300 characters

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

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

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

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

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

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

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

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

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

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

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

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

1159/1200 words

Authors contribution

Writing – Original draft: SD and AA; *Writing – Review and editing:* All; *Conceptualization:* AA; *Formal Analysis:* SD and AA; *Supervision:* AA

Conflict of Interest

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

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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

180

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).

