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Guillaume Desoubreaux, Adélaïde Chesnay, Victor Mercier, José Bras-Cachinho, Parastou Moshiri, et al.. Combination of β -(1, 3)-D-glucan testing in serum and qPCR in nasopharyngeal aspirate for facilitated diagnosis of *Pneumocystis jirovecii* pneumonia. *Mycoses*, 2019, 62 (11), pp.1015-1022. 10.1111/myc.12997 . hal-02439410

HAL Id: hal-02439410

<https://hal.science/hal-02439410v1>

Submitted on 21 Jan 2020

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Interests of combination testing based on qPCR in nasopharyngeal aspirate and measurement of β -(1, 3)-D-glucan antigen concentration in serum for a facilitated diagnosis of *Pneumocystis jirovecii* pneumonia.

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Short-running title: Aspirate plus glucan for *P. jirovecii* pneumonia diagnosis

Word count: 2881 (typographical signs)

Figure count: 3

Table count: 2

Supplementary material count: 3

30 **Acknowledgement:** The authors are grateful to the native English speaker reviewed the
31 English syntax and the editing of the manuscript

32

33 **Author contribution:** GD and EB conceived the ideas; AC, VM, JBC, SE, PM, MAK, and
34 AL collected the data; GD, AC, VM, JBC, SE and PM analyzed the data; GD led the writing.

35

36 **Conflicts of interest:** the authors have no conflicts of interest to declare

37

ABSTRACT

Background: Currently, the biological diagnosis of *Pneumocystis jirovecii* pneumonia (PjP infection) usually relies on microbiological investigations in bronchial-alveolar lavage fluid (BALF) by conventional staining methods and/or molecular biology. However, bronchial-alveolar lavage is sometimes complicated to manage, especially in weakened patients. Therefore, alternative clinical samples – easier to collect – are warranted in such specific contexts. **Objective:** Over a four year-period, diagnostic performance of an original method based on combination of quantitative real-time polymerase chain reaction (qPCR) in nasopharyngeal aspirate (NPA) with measurement of β -(1, 3)-D-glucan antigen (BDG) in serum was prospectively assessed in a single center. **Patients/methods:** Results were compared with those obtained in BALF through direct staining methods and qPCR. True-positives were defined by an independent committee based on clinical, radiological, and biological data. Overall, 48 individuals with a definitive diagnosis of PjP infection were included, and 48 controls were selected upon matching for age, sex and underlying disease(s). **Results:** qPCR results were strongly correlated between BALF and NPA ($P < 0.0001$). Altogether, greater diagnostic performance was achieved when establishing the positive cutoff of BDG antigen at 143 pg/mL. In such conditions, sensitivity of the testing based on either positive BDG measurement or positive qPCR in NPA was then calculated at 93.75%, $_{95\%CI}$ [82.37 – 98.40%], and specificity at 97.87%, $_{95\%CI}$ [87.66 – 100.00%]. **Conclusions:** Further validation through multicenter studies is now required, especially for establishing clear cutoffs. However, one could already state that combination of qPCR in the NPA with BDG measurement in serum may be a valuable substitute for BALF examination.

Keywords: Uvitex; calcofluor-blue; fungal infection; virus; respiratory samples.

INTRODUCTION

Pneumocystis jirovecii is an airborne fungus which is responsible for interstitial pneumonia in humans^{1,2}, referred to as PjP (*Pneumocystis jirovecii* pneumonia) and formerly as PCP (*Pneumocystis carinii* – ancient taxonomic name of the pathogen – pneumonia). PjP infection occurs mostly in immunocompromised patients, such as subjects infected with human immunodeficiency virus (HIV) or those suffering from hematological malignancies^{3,4}. It has been also largely described in solid-organ transplant recipients or in individuals receiving immunosuppressive therapy for autoimmune or inflammatory diseases⁵. Clinical signs of PjP infection are not specific; they include fever, thoracic pain and cough, and they can misleadingly mimic other lung infections, mostly those caused by viruses^{2,4}.

Because *P. jirovecii* is not easy to grow *in vitro*⁶, the biological diagnosis of PjP remains complex⁷, and is usually based on direct observation of the fungus and/or detection of its DNA in respiratory fluids^{2,7,8}. Since *P. jirovecii* thrives at the surface of type I-pneumocyte cells in the lung alveoli⁹, bronchial-alveolar lavage fluid (BALF) is primarily acknowledged as the gold-standard clinical sample⁷. However, one can argue that bronchial-alveolar lavage (BAL) is a somewhat invasive procedure, and can be complex to manage, especially in weakened patients. Moreover, there is currently a relevant trend promoting easy collection of clinical specimens from the upper respiratory tract^{10–12}. Unfortunately, so far, superficial samples have been shown to be less sensitive for the diagnosis of PjP infection, especially because the fungal burden seems too low inside. Thus, May-Grünwald Giemsa (MGG) or Gomori-Grocott's methenamine silver (GMS) staining, as well as commercial kits using monoclonal and fluorescent-conjugated antibodies (IFA, for immunofluorescent assay allowing detection of *P. jirovecii* cysts (asci)), have never provided reliable results^{13,14}: for example in HIV-positive patients, only 55.3% sputa were found positive by direct methods during true PjP infection¹⁵. Eventually, only quantitative real-time polymerase chain reaction

(qPCR) appears to be powerful enough to detect efficiently *P. jirovecii* DNA in upper respiratory samples, but, for technical reasons, is not easily feasible in viscous respiratory specimens like sputa. Likewise, its diagnostic performance remains somewhat questionable when considered alone¹⁰, as it displays real limitations for distinguishing between casual carriage and true infection⁸. Therefore in upper respiratory samples, qPCR was demonstrated efficient for the microbiological diagnosis in only 48.0% children¹⁰, and in 66.2% HIV-positive adult patients¹⁵. It should also be noted that some surrogate biomarkers have been recently developed, such as the measurement of β -(1,3)-D-glucan (BDG) antigen in serum. Nonetheless, the latter lacks specificity when used alone^{16,17}, because BDG is a pan-fungal cell wall compound which is actually commonly found in most of fungi. Furthermore, the positive cutoff recommended by the manufacturer at 80 picogram *per* milliliter (pg/mL) appears actually quite low, and thus is thought to expose to a risk of false-positive results.

Altogether in such a context, alternative diagnostic approaches for PjP infection are still required¹¹, especially in patients who cannot undergo BAL. Detection of viral DNA in nasopharyngeal aspirate (NPA) samples is now a standardized routine practice in cases of febrile pneumonia¹⁸. NPA specimens are technically-easy to handle in a laboratory, because they are less viscous than sputa and because several commercial devices and all-inclusive kits can automatically detect most moieties of the respiratory pathogens in them. However, *P. jirovecii* has not been included so far in the panel of the distributed kits¹⁹, except for the Fast Track Diagnostics Respiratory Pathogen 33® kit (Fast Track Diagnostics, Sliema, Malta)²⁰ and one of the High-Plex 24® product for respiratory pathogens involved in pneumonia (AusDiagnostics, Mascot, Australia).

Therefore in this study, we assessed over a four year-period the diagnostic performance of an in-house qPCR carried out in NPAs, in combination with measurement of BDG antigen in serum, for the diagnosis of PjP infection. Results were systematically discussed in light of the

definitive diagnosis obtained according to the conventional methods, primarily based on microbiological investigations in BALFs, as well as on radiology findings and clinical signs.

MATERIAL AND METHODS

Study population

Geographical location

This study was carried out over a four-year period [02-06-2014 – 07-31-2018] in a French university hospital (lat.: 46.449070 – long.: 6.865345) which hosts 2,008 inpatient beds for the whole panel of surgical and medical specialties.

Description of cases

All patients with definitive PjP infection were selected as true cases, as long as they were ≥ 15 years old and if BALF volume was sufficient for conventional investigations. The definitive diagnosis of PjP infection had been systematically adjudicated by an independent committee of physicians (radiologists, intensivists, microbiologists, infectious disease specialists, and pulmonologists) in light of the medical records. The classification was based on agreement of the microbiological results obtained in BALF (at least one *P. jirovecii* form by microscopic method(s) like MGG or calcofluor-blue brightener²¹, and/or a positive Cq value ≤ 35 cycles by qPCR), in association with consistent clinical examination and suggestive medical imaging^{1,7}. For instance in subjects with solid tumours, solid-organ transplantation or hematologic malignancies receiving long-term high-dose steroids ($\geq 0.3\text{mg/kg/day}$ of prednisone equivalent for ≥ 3 weeks) and/or anti-cancer chemotherapy, the following criteria were systematically searched: subacute fever associated with hypoxemic and diffuse pulmonary involvement (*e.g.* bilateral infiltrates and/or ground-glass opacities and/or nodules and/or alveolar condensation

on high-resolution computed tomography scan)²². For HIV-positive patients, the following data were specifically screened for: resting oxygen saturation <90% and diffuse radiology shadowing (*e.g.* bilateral infiltrates and/or ground-glass opacities and/or nodules and/or cystic changes on high-resolution computed tomography scan), as well as a history of anti-*P. jirovecii* primary chemoprophylaxis¹⁴. The independent committee also assessed clinical outcome after 14 days of anti-*P. jirovecii* curative treatment. All the cases of PjP infection were subsequently reported to the French centre of surveillance for fungal diseases (Centre national de reference des mycoses et antifongiques, Institut Paris, France: <https://www.pasteur.fr/en/institut-pasteur>). Finally, only diseased patients who were sampled for NPA and blood, within three days before or after the BAL, were retained for further analysis and comparison.

Selection of controls

Control individuals were selected from patients in the same hospital. Non-inclusion criteria were similar to those of the cases suffering from PjP infection. Controls were assumed not infected with *P. jirovecii* on the basis of the clinical examination, the medical imaging and the biological tests in BALF⁷. They were matched with PjP cases for age, sex and underlying disease(s).

Measurement of β -(1, 3)-D-glucan antigen in serum

Beta-(1, 3)-D-glucan antigen was tested in each patient serum that had been collected \leq three days before or after the BAL, using the Fungitell assay® serum test (Cape Cod, East Falmouth – MA, U.S.A.) and glucan-free material. Briefly, 5 μ L serum samples were dispensed in duplicate into a 96-well Pyroplate® microplate (CapeCod, East Falmouth – MA,

U.S.A.), and 20µL alkaline pre-treatment solution were then added. After a 30 min-incubation period at 37°C, 100µL reconstituted Fungitell lysate reagent® were introduced into each well. Kinetic data were then read with the MultiSkan FC® (Thermofischer Scientifics, Dardilly, France) over 40 min at 37°C. Measurement of BDG antigen concentration was calculated for each sample according to the values of calibrated standards. Positive threshold was established by the manufacturer at 80 pg/mL¹⁶. Samples were re-tested in case of discrepancy between the two wells.

Detection of *P. jirovecii* DNA in nasopharyngeal aspirates

Practically, NPA was collected ≤ three days before or after the BAL using a standardized procedure: for each patient, a catheter was inserted into his nostrils as far as the posterior pharynx, and a mural vacuum pump was used for suction of mucus. Every aspirate specimen was introduced in a sterile tube using a mucus extractor device. All NPA specimens were first addressed to the Microbiology laboratory where virus detection for 26 respiratory pathogens was carried out using routine medical practice with the panel included in the Allplex® Respiratory Panel Assays kit (Seegene, Seoul, Korea; *P. jirovecii* is not included in this panel), and then they were subsequently transferred to the Parasitology-Myecology department specifically for the purpose of the study.

Detection of a 121 bp fragment of the *P. jirovecii* repeated mitochondrial large subunit (mtLSU) rRNA gene was performed by qPCR^{8,21}. Briefly, DNA was extracted from 200 µL of NPA liquid pellet (after centrifugation, 1,730 x g, 10min). The pellet was first incubated in 10µL lyticase 1 IU/µL for 10 min at 37 °C. The DNA was then extracted using superparamagnetic beads from the MycoGENIE® DNA extraction kit (Ademtech, Pessac, France), according to the manufacturer's recommendations. Real-time qPCR was performed

in a final volume of 25 μ L using the GoTaq Probe qPCR Master mix® (Promega, Charbonnières-les-Bains, France) and 5 μ L of each DNA extract. The following oligonucleotides were used: PJF1: 5'-CTGTTTCCCTTTTCGACTATCTACCTT-3' and PJR1: 5'-CACTGAATATCTCGAGGGAGTATGAA-3' for the primers, and 5'-FAM-TCGCACATAGTCTGATTAT-MGB-3' for the specific TaqMan® probe⁸. The final concentrations of primers and probe were 0.4 μ M. The concentration of MgCl₂ was adjusted to 0.5 mM. Amplifications were performed in duplicate on a LightCycler 480 II® Real-Time PCR (Roche Applied Science, La Rochelle, France), under the following conditions: initial decontamination for 2 min at 50 °C, followed by denaturation for 5 min at 95 °C, and then 45 cycles of hybridization/elongation, including 15 s at 95 °C, 1 min at 60°C, and a final cooling step at 40 °C for 30 s. Inhibition was assessed by an internal control (Universal Inhibition Control Cy5®, Diagenode Diagnostics, Liège, Belgium) and an endogenous in-house positive internal control (*P. jirovecii* DNA extract from the local collection). The results were expressed in quantitative cycle (Cq) values⁸.

Assay performance and analysis

Epidemiological data were provided by the Service d'Information Médicale, Épidémiologie et Économie de la Santé (SIMEES) from Tours University Hospital. Statistical analyses were performed using XLStat v.18.06® software (Addinsoft, Paris, France). Missing data were managed by the method of mean imputation. A true-positive was defined as a positive result that was in agreement with the definitive diagnosis of PjP infection that had been adjudicated by the scientific independent committee. Diagnostic measures were calculated to assess performance: sensitivity (Se), specificity (Sp), predictive values (PPV and NPV), and likelihood ratios (+LR and -LR), as well as receiver operating characteristic (ROC) curves and their respective areas under the curve (AUC) for measurement of BDG antigen

concentration and qPCR Cq in NPA. The comparison of diagnostic performance was made using the paired two-sample Student's *t*-test. The Spearman's rank correlation coefficient was used to assess the correlation of results obtained with the quantitative methods, while the observer agreement for categorical data was measured through the Cohen's kappa coefficient (κ), in light of the interpretation instructions by Landis et Koch²³. The α -risk was adjusted at 0.05.

Ethics

This study was non-interventional, involving no change in standard clinical practices. Only a form of non-objection was signed by the included patients. Clinical data were anonymized before analysis. The biological samples were stored in the hospital collection N°DC-20100-1216, approved by the French Ministry of Research. The study registration number N°2015_030 was issued by the French Data Protection Authority (CNIL, Commission Nationale de l'Informatique et des Libertés). Final approval N°2015 23 was given by the local Ethics Committee of Tours University Hospital (Espace de Réflexion Ethique, Région Centre-Val de Loire, France) before submission. The authors complied with the BRISQ guidelines²⁴.

RESULTS

Description of the study population

Overall, 1,118 distinct patients underwent a BAL during the study period for high suspicion of PjP infection (Figure 1). Ten and 32 were not included because they were children or the total volume of the collected BALF was insufficient for investigation. One hundred and nineteen subjects were eventually adjudicated by the scientific committee as consistent with PjP infection. Thus, the overall incidence of PjP infection in our university hospital was

estimated at 2.21 episodes *per* 1,000 patient-days. Finally, only 48 subjects with PjP infection were actually sampled for NPA and blood concomitantly to the BALF \pm three days, and then were selected for further analysis. Meanwhile, the NPAs were all tested negative with the Allplex® Respiratory Panel Assays kit (Seegene, Seoul, Korea). Forty-eight control subjects were selected upon stringent matching with the PjP cases (Figure 1).

For the cases retained with definitive diagnosis of PjP infection, all the characteristics at baseline are summarized in Table I and Suppl. Material 1. Most involved immunocompromised males above 60 years of age (median = 64 years old), with a mean count of 0.12 G/L CD₄⁺ lymphocytes at the time of diagnosis. Respiratory symptoms and fever were the major clinical signs. In BALF, microscopic findings (Suppl. Materials 1 et 2) and qPCR results were concordant, *i.e.* both positive, in 35 out of the 48 cases (72.92%). Quantitative PCR appeared insignificantly more performant in BALF than microscopic observation for the definitive microbiological diagnosis of PjP infection ($P=0.26$). Calcofluor-blue brightener allowed visual counting 430 ± 280 *P. jirovecii* asci *per* glass slide in average (Suppl. Material 2). Microscopic examination and qPCR in BALF were systematically negative (with Cq > 35) in all the control subjects (but two qPCR were detected at 39 cycles, three at 38, three at 37, and two at 36). In the 48 patients with true PjP infection, mean BDG was measured at 527.63 ± 545.06 pg/mL vs. 70.81 ± 33.98 pg/mL in the controls ($P<0.0001$) (Figure 2A). The ROC curve allowed calculation of the BDG cutoff value at 143 pg/mL (Figure 2B).

Diagnostic performance of the microbiological testing

Cutoff for qPCR Cq in NPA was calculated at 35.4 cycles (Suppl. Material 3; for more comfort, value at 35 cycles was retained for further analyses). For every specimen, no amplification inhibition was noticed. In the 48 controls, all qPCR results were found negative

in NPA ($C_q = 36$ cycles or above). In contrast in the 48 cases asserted with PjP infection, 40 were positive (83.33%; mean C_q calculated at 32.21 ± 3.51 cycles; AUC ROC curve = 0.96 ± 0.02 ; Suppl. Materials 1 et 3), whereas four of them had been negative in BALF. Noteworthy for two patients, the discrepancies were more questionable ($C_q=30.12$ cycles in NPA vs. 39.50 in BALF, and 31.60 vs. 40.00, respectively): for the first subject, the BALF was of poor quality (very viscous) which could maybe hamper the reliability of the diagnosis in it, while the second patient was already given anti-*P.jirovecii* therapy at time of sampling that could perhaps substantially interfere with the fungus detection. However, no significant differences were observed between the two types of samples (concordance rate = 86.73%; mean $C_q = 32.21$ vs. 30.43 cycles, respectively; $P=0.12$): qPCR findings in BALF and in NPA were statistically correlated (Spearman's rank correlation coefficient = 0.79, $P<0.0001$ (Figure 3); Cohen's kappa coefficient (κ) = 0.77, $P<0.0001$). In all, sensitivity and specificity of the qPCR in NPA alone were estimated at 81.25%, 95%CI [67.74 – 89.95%] and 100.00%, 95%CI [90.75 – 100.00%], respectively, while they were just slightly higher in BALF, at 89.58%, 95%CI [77.26 – 95.81] and 100.00%, 95%CI [90.75 – 100.00].

When associating the qPCR results in NPA with the BDG measurement in serum, diagnostic performance of the combined test could vary greatly (Table II). They primarily depended on the selection of the threshold value for the measurement of BDG antigen: indeed, sensitivity of BDG alone was 91.67%, 95%CI [79.78 – 97.15%] vs. 85.42%, 95%CI [72.41 – 92.97%] for the recommended cutoff at 80 pg/mL vs. the calculated cutoff at 143 pg/mL, respectively. Likewise, specificity shifted from 65.96%, 95%CI [51.59 – 77.82%] to 97.87%, 95%CI [87.66 – 100.00%]. Thus, for combination of qPCR positive in NPA \pm BDG measurement > 80 pg/mL, sensitivity was assessed at 95.83%, 95%CI [85.10 – 99.55%], while specificity plunged to 65.96%, 95%CI [51.59 – 77.82%]. There were two false-negative result (4.17%) amongst the diseased cases with PjP infection, and 16 false-positive (33.33%) amongst the controls. Based

on the threshold calculated using the ROC curve, a better diagnostic performance was then demonstrated: 93.75%, $_{95\%}\text{CI}$ [82.37 – 98.40%] for sensitivity, and 97.87%, $_{95\%}\text{CI}$ [87.66 – 100.00%] for specificity of the testing associating qPCR positive in NPA \pm BDG measurement > 143 pg/mL. Three false-negative results were reported among the cases with true PjP infection (6.25%): one in a male patient suffering from chronic lymphocytic leukemia (who was undergoing anti-*P. jirovecii* treatment at time of sampling), one in an individual with solid cancer and for whom the microscopy examination was negative but qPCR positive in BALF (no mention of any anti-*P. jirovecii* treatment), and one in a 39-year-old woman with sarcoidosis with direct examination positive but qPCR negative in BALF (who was undergoing anti-*P. jirovecii* treatment at time of sampling). Only one false-positive was recorded among the controls (2.08%), with a BDG antigen measured at 484.0 pg/mL (whereas the *Aspergillus* galactomannan antigen was also very elevated in this patient's BALF).

DISCUSSION

Pneumocystis jirovecii pneumonia (PjP, formerly PCP) is a fungal infection with potentially severe consequences ^{1,2}: mortality rates are still estimated at about 20% ²⁵. More accurate and rapid diagnosis would allow a prompter initiation of effective antifungal treatment, therefore, substantially reducing subsequent complications ²⁶. Alternative samples are expected to circumvent the actual limitations of BAL, which is still nowadays the gold-standard clinical sampling for diagnosis of PjP infection. For example in the past, induced sputa have been quite frequently used for such purposes in patients infected with HIV ¹³, although their induction can provoke nausea and bronchospasm. More recently, NPAs were shown to be relevant for detection of viral and bacterial DNA, because they are less viscous than sputa and largely standardized for urgent practice in cases of febrile pneumonia ¹⁸. Nonetheless, global

sensitivity of direct methods or qPCR alone was shown to be quite low in all samples from the upper respiratory tract ¹⁴, including NPAs ¹⁰. Therefore in situations when BAL is not possible, it seems to make sense to combine *P. jirovecii* DNA detection in NPA ¹¹ with another diagnostic tool, such as the measurement of BDG antigen concentration in serum ¹⁷.

In this study and in contrast to previous ones ¹¹, the initial inclusion criterion was specifically based on the clinical suspicion of PjP infection in populations at risk. This means that a prospective analysis of the results in NPAs and BDG was systematically performed in light of those obtained with the conventional diagnostic methods carried out in BALFs. Nonetheless, we would like to emphasize herein the pertinence of brainstorming by experts that should be initiated regarding the value of the positive cutoff for the BDG concentration. Results of BDG assay are acknowledged to vary substantially; it's the reason why a "grey zone" should be recommendable instead of a strict cutoff, like it is currently the case according to the manufacturer's instructions. In the present study, we demonstrated that the lower threshold (established by the kit's manufacturer) was largely less specific than the higher one (calculated at 143 pg/mL), while the sensitivities were actually quite close (just slightly better for the lower cutoff). In the same way, other authors previously suggested to increase the cutoff value that is usually recommended by the supplier ²⁷⁻²⁹. Regarding the results of *P. jirovecii* qPCR in NPAs, no one can argue that the reliability of positive specimens is debatable, since all enrolled patients were symptomatic with febrile pneumonia at hospital admission and/or showed suggestive imaging in a suggestive epidemiological context. In other words, *P. jirovecii* colonization or casual carriage could be confidently precluded in them. Furthermore, frequent association of positive qPCR in NPA with BDG concentration \geq 143 pg/mL undoubtedly reinforced the likelihood of definitive diagnosis of PjP infection. Likewise, we can assert that association of the two tests provides a comforting backup in case one of them is found to be negative, especially when clinical signs are obvious and/or medical

imaging is suggestive. This situation actually occurred in this work in 27.1% of the patients with definitive diagnosis of PjP infection. In all, thanks to this combined approach, only 4.2% patients of the whole cohort were misdiagnosed. False-negative results were obtained in HIV-negative patients with cancer or auto-immune disease who are acknowledged to host lower fungal burden²¹. Furthermore, two of them were already been given anti-*P. jirovecii* therapy at time of diagnosis: we previously demonstrated that antifungal treatment can substantially reduce the sensitivity of qPCR (while not affecting so much the microscopic approach)²¹.

One limitation of testing in NPA is that *P. jirovecii* DNA is probably present in lower amounts than in BALF (even if correlation of the Cq values between the two types of samples appeared statistically quite strong herein). Consequently to date, strict quantitative comparison of Cq and fungal burden remains questionable between the two clinical specimens, given the heterogeneity of their volumes and their consistency; some were more liquid, while other were rather mucous. As a result, the Cq thresholds that are usually expressed in BALF should be applied to NPAs with great caution⁸, although the cutoff for the latter was calculated in this study very close to the one recommended so far in BALF.

In conclusion, we propose the following strategy for a convenient diagnostic approach of PjP infection: as soon as the initial screening has showed BDG antigen concentration ≥ 143 pg/mL in serum of patients at risk, NPA should be in priority investigated if BAL is not realizable. Positive result of qPCR in NPA is then highly suggestive to support rapid instauration of anti-*P. jirovecii* curative therapy. Of course, further validation through wide multi-center studies is now expected. Besides, our results also suggest that adding *P. jirovecii* to all the panels of respiratory microorganisms tested by automated commercial devices seems to be critical. Globally, this would generate limited additional costs, as all the nucleic acids are already extracted for the other tests in the panel.

ACKNOWLEDGMENTS

The authors thank Prof. Jacques Chandenier for his valuable scientific opinion. They are grateful to the technicians of the Virology department for their help in collecting the nasopharyngeal aspirate specimens.

A native English speaker reviewed the English syntax and the editing of the manuscript.

FUNDING

This work was supported by none. The authors did not receive any specific research funding for this study.

DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The authors declare no conflict of interest. The data was obtained as a part of routine work at the University Hospital in Tours, France.

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FIGURE LEGENDS

Figure 1: Flowchart of the study.

Abbreviations: BALF, bronchial-alveolar lavage fluid; NPA, nasopharyngeal aspirate; PjP, *Pneumocystis jirovecii* pneumonia; * matched with cases for age, sex and underlying disease(s).

Figure 2: Measurement of β -(1, 3)-D-glucan (BDG) antigen concentration in serum. A –

Box plot showing the difference of BDG concentrations between the true cases of *Pneumocystis jirovecii* pneumonia and the controls. In each group, the mean was calculated at 527.6 ± 272.5 pg/mL [30.0 – 3830.0] and 70.9 ± 33.9 pg/mL [15.6 – 484.0], respectively. The horizontal dotted line N°1 indicates the threshold suggested by the manufacturer at 80 pg/mL. The dotted line N°2 shows the upper cutoff determined at 143 pg/mL by the receiver operating characteristic (ROC) curve of the present study. The grey zone is indicated through hatched space; B – ROC curve of the measurement of BDG antigen concentration in serum. The area under the curve (AUC) is estimated at 0.92 ± 0.03 , 95%CI [0.87 – 0.98]. The grey thin arrow indicates the cutoff calculated at 143 pg/mL, thus enabling sensitivity at 85.42%, 95%CI [72.41 – 92.97%], and specificity at 97.87%, 95%CI [87.66 - 100.00%].

Figure 3: Comparison of the Cq values between qPCR carried out in bronchial-alveolar

lavage fluids and qPCR in nasopharyngeal aspirates. Results are presented in box plot showing correlation curve according to Spearman inside a confidence ellipse. All the dots that appear at Cq = 40 cycles actually correspond to qPCR with no detection of *P. jirovecii* DNA (Cq > 40 cycles, *i.e.* no inflexion of the amplification curve).

● data from control-patients with no diagnosis of *P. jirovecii* pneumonia

486 ♦ data from case-patients with diagnosis of *P. jirovecii* pneumonia.

487 Abbreviations: Cq, quantitative cycle; qPCR, quantitative real-time polymerase chain reaction.

488

489 **Supplementary material 1: Individual details of the case-patients with asserted**
490 ***Pneumocystis jirovecii* pneumonia.**

491

492 **Supplementary material 2: Example of positive microscopic observation during direct**
493 **examination showing *Pneumocystis jirovecii* cysts (asci) in bronchial-alveolar lavage**
494 **fluids, using calcofluor-blue brightener (Fluorescent Brightener 28®, Sigma, Saint-Louis,**
495 **MO, U.S.A.), at magnification x1250. Note the specific “coffee-bean” shape of the cyst and**
496 **sporadic presence of a halo (example pointed by the thin arrow) ²¹.**

497

498 **Supplementary material 3: ROC curve of the *P. jirovecii* qPCR in naso-pharyngeal**
499 **aspirates expressed in number of quantitative cycles (Cq). The area under the curve**
500 **(AUC) is estimated at 0.96 ± 0.02 , _{95%}CI [0.92 – 1.00]. The grey thin arrow indicates the**
501 **cutoff calculated at 35.4 cycles.**