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## Combination of $\beta$ -(1, 3)-D-glucan testing in serum and qPCR in nasopharyngeal aspirate for facilitated diagnosis of *Pneumocystis jirovecii* pneumonia

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

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35

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37

38 **ABSTRACT**

39 **Background:** Currently, the biological diagnosis of *Pneumocystis jirovecii* pneumonia (PjP  
40 infection) usually relies on microbiological investigations in bronchial-alveolar lavage fluid  
41 (BALF) by conventional staining methods and/or molecular biology. However, bronchial-  
42 alveolar lavage is sometimes complicated to manage, especially in weakened patients.  
43 Therefore, alternative clinical samples – easier to collect – are warranted in such specific  
44 contexts. **Objective:** Over a four year-period, diagnostic performance of an original method  
45 based on combination of quantitative real-time polymerase chain reaction (qPCR) in  
46 nasopharyngeal aspirate (NPA) with measurement of  $\beta$ -(1, 3)-D-glucan antigen (BDG) in  
47 serum was prospectively assessed in a single center. **Patients/methods:** Results were compared  
48 with those obtained in BALF through direct staining methods and qPCR. True-positives were  
49 defined by an independent committee based on clinical, radiological, and biological data.  
50 Overall, 48 individuals with a definitive diagnosis of PjP infection were included, and 48  
51 controls were selected upon matching for age, sex and underlying disease(s). **Results:** qPCR  
52 results were strongly correlated between BALF and NPA ( $P < 0.0001$ ). Altogether, greater  
53 diagnostic performance was achieved when establishing the positive cutoff of BDG antigen at  
54 143 pg/mL. In such conditions, sensitivity of the testing based on either positive BDG  
55 measurement or positive qPCR in NPA was then calculated at 93.75%,  $_{95\%CI}$  [82.37 –  
56 98.40%], and specificity at 97.87%,  $_{95\%CI}$  [87.66 – 100.00%]. **Conclusions:** Further validation  
57 through multicenter studies is now required, especially for establishing clear cutoffs. However,  
58 one could already state that combination of qPCR in the NPA with BDG measurement in  
59 serum may be a valuable substitute for BALF examination.

60

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

62

## 63 INTRODUCTION

64 *Pneumocystis jirovecii* is an airborne fungus which is responsible for interstitial pneumonia in  
65 humans <sup>1,2</sup>, referred to as PjP (*Pneumocystis jirovecii* pneumonia) and formerly as PCP  
66 (*Pneumocystis carinii* – ancient taxonomic name of the pathogen – pneumonia). PjP infection  
67 occurs mostly in immunocompromised patients, such as subjects infected with human  
68 immunodeficiency virus (HIV) or those suffering from hematological malignancies <sup>3,4</sup>. It has  
69 been also largely described in solid-organ transplant recipients or in individuals receiving  
70 immunosuppressive therapy for autoimmune or inflammatory diseases <sup>5</sup>. Clinical signs of PjP  
71 infection are not specific; they include fever, thoracic pain and cough, and they can  
72 misleadingly mimic other lung infections, mostly those caused by viruses <sup>2,4</sup>.

73 Because *P. jirovecii* is not easy to grow *in vitro* <sup>6</sup>, the biological diagnosis of PjP remains  
74 complex <sup>7</sup>, and is usually based on direct observation of the fungus and/or detection of its  
75 DNA in respiratory fluids <sup>2,7,8</sup>. Since *P. jirovecii* thrives at the surface of type I-pneumocyte  
76 cells in the lung alveoli <sup>9</sup>, bronchial-alveolar lavage fluid (BALF) is primarily acknowledged  
77 as the gold-standard clinical sample <sup>7</sup>. However, one can argue that bronchial-alveolar lavage  
78 (BAL) is a somewhat invasive procedure, and can be complex to manage, especially in  
79 weakened patients. Moreover, there is currently a relevant trend promoting easy collection of  
80 clinical specimens from the upper respiratory tract <sup>10-12</sup>. Unfortunately, so far, superficial  
81 samples have been shown to be less sensitive for the diagnosis of PjP infection, especially  
82 because the fungal burden seems too low inside. Thus, May-Grünwald Giemsa (MGG) or  
83 Gomori-Grocott's methenamine silver (GMS) staining, as well as commercial kits using  
84 monoclonal and fluorescent-conjugated antibodies (IFA, for immunofluorescent assay  
85 allowing detection of *P. jirovecii* cysts (asci)), have never provided reliable results <sup>13,14</sup>: for  
86 example in HIV-positive patients, only 55.3% sputa were found positive by direct methods  
87 during true PjP infection <sup>15</sup>. Eventually, only quantitative real-time polymerase chain reaction

88 (qPCR) appears to be powerful enough to detect efficiently *P. jirovecii* DNA in upper  
89 respiratory samples, but, for technical reasons, is not easily feasible in viscous respiratory  
90 specimens like sputa. Likewise, its diagnostic performance remains somewhat questionable  
91 when considered alone <sup>10</sup>, as it displays real limitations for distinguishing between casual  
92 carriage and true infection <sup>8</sup>. Therefore in upper respiratory samples, qPCR was demonstrated  
93 efficient for the microbiological diagnosis in only 48.0% children <sup>10</sup>, and in 66.2% HIV-  
94 positive adult patients <sup>15</sup>. It should also be noted that some surrogate biomarkers have been  
95 recently developed, such as the measurement of  $\beta$ -(1,3)-D-glucan (BDG) antigen in serum.  
96 Nonetheless, the latter lacks specificity when used alone <sup>16,17</sup>, because BDG is a pan-fungal  
97 cell wall compound which is actually commonly found in most of fungi. Furthermore, the  
98 positive cutoff recommended by the manufacturer at 80 picogram *per* milliliter (pg/mL)  
99 appears actually quite low, and thus is thought to expose to a risk of false-positive results.

100 Altogether in such a context, alternative diagnostic approaches for PjP infection are still  
101 required <sup>11</sup>, especially in patients who cannot undergo BAL. Detection of viral DNA in  
102 nasopharyngeal aspirate (NPA) samples is now a standardized routine practice in cases of  
103 febrile pneumonia <sup>18</sup>. NPA specimens are technically-easy to handle in a laboratory, because  
104 they are less viscous than sputa and because several commercial devices and all-inclusive kits  
105 can automatically detect most moieties of the respiratory pathogens in them. However, *P.*  
106 *jirovecii* has not been included so far in the panel of the distributed kits <sup>19</sup>, except for the Fast  
107 Track Diagnostics Respiratory Pathogen 33® kit (Fast Track Diagnostics, Sliema, Malta) <sup>20</sup>  
108 and one of the High-Plex 24® product for respiratory pathogens involved in pneumonia  
109 (AusDiagnostics, Mascot, Australia).

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

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

115

## 116 **MATERIAL AND METHODS**

### 117 **Study population**

#### 118 *Geographical location*

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

122

#### 123 *Description of cases*

124 All patients with definitive PjP infection were selected as true cases, as long as they were  $\geq 15$   
125 years old and if BALF volume was sufficient for conventional investigations. The definitive  
126 diagnosis of PjP infection had been systematically adjudicated by an independent committee  
127 of physicians (radiologists, intensivists, microbiologists, infectious disease specialists, and  
128 pulmonologists) in light of the medical records. The classification was based on agreement of  
129 the microbiological results obtained in BALF (at least one *P. jirovecii* form by microscopic  
130 method(s) like MGG or calcofluor-blue brightener<sup>21</sup>, and/or a positive Cq value  $\leq 35$  cycles  
131 by qPCR), in association with consistent clinical examination and suggestive medical imaging  
132<sup>1,7</sup>. For instance in subjects with solid tumours, solid-organ transplantation or hematologic  
133 malignancies receiving long-term high-dose steroids ( $\geq 0.3\text{mg/kg/day}$  of prednisone equivalent  
134 for  $\geq 3$  weeks) and/or anti-cancer chemotherapy, the following criteria were systematically  
135 searched: subacute fever associated with hypoxemic and diffuse pulmonary involvement (*e.g.*  
136 bilateral infiltrates and/or ground-glass opacities and/or nodules and/or alveolar condensation

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

148

#### 149 *Selection of controls*

150 Control individuals were selected from patients in the same hospital. Non-inclusion criteria  
151 were similar to those of the cases suffering from PjP infection. Controls were assumed not  
152 infected with *P. jirovecii* on the basis of the clinical examination, the medical imaging and the  
153 biological tests in BALF<sup>7</sup>. They were matched with PjP cases for age, sex and underlying  
154 disease(s).

155

#### 156 **Measurement of $\beta$ -(1, 3)-D-glucan antigen in serum**

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

161 U.S.A.), and 20 $\mu$ L alkaline pre-treatment solution were then added. After a 30 min-  
162 incubation period at 37°C, 100 $\mu$ L reconstituted Fungitell lysate reagent® were introduced  
163 into each well. Kinetic data were then read with the MultiSkan FC® (Thermofischer  
164 Scientifics, Dardilly, France) over 40 min at 37°C. Measurement of BDG antigen  
165 concentration was calculated for each sample according to the values of calibrated standards.  
166 Positive threshold was established by the manufacturer at 80 pg/mL<sup>16</sup>. Samples were re-  
167 tested in case of discrepancy between the two wells.

168

### 169 **Detection of *P. jirovecii* DNA in nasopharyngeal aspirates**

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

179 Detection of a 121 bp fragment of the *P. jirovecii* repeated mitochondrial large subunit  
180 (mtLSU) rRNA gene was performed by qPCR<sup>8,21</sup>. Briefly, DNA was extracted from 200  $\mu$ L  
181 of NPA liquid pellet (after centrifugation, 1,730 x g, 10min). The pellet was first incubated in  
182 10 $\mu$ L lyticase 1 IU/ $\mu$ L for 10 min at 37 °C. The DNA was then extracted using  
183 superparamagnetic beads from the MycoGENIE® DNA extraction kit (Ademtech, Pessac,  
184 France), according to the manufacturer's recommendations. Real-time qPCR was performed

185 in a final volume of 25  $\mu$ L using the GoTaq Probe qPCR Master mix® (Promega,  
186 Charbonnières-les-Bains, France) and 5 $\mu$ L of each DNA extract. The following  
187 oligonucleotides were used: PJF1: 5'-CTGTTTCCCTTTTCGACTATCTACCTT-3' and PJR1:  
188 5'-CACTGAATATCTCGAGGGAGTATGAA-3' for the primers, and 5'-FAM-  
189 TCGCACATAGTCTGATTAT-MGB-3' for the specific TaqMan® probe <sup>8</sup>. The final  
190 concentrations of primers and probe were 0.4  $\mu$ M. The concentration of MgCl<sub>2</sub> was adjusted  
191 to 0.5 mM. Amplifications were performed in duplicate on a LightCycler 480 II® Real-Time  
192 PCR (Roche Applied Science, La Rochelle, France), under the following conditions: initial  
193 decontamination for 2 min at 50 °C, followed by denaturation for 5 min at 95 °C, and then 45  
194 cycles of hybridization/elongation, including 15 s at 95 °C, 1 min at 60°C, and a final cooling  
195 step at 40 °C for 30 s. Inhibition was assessed by an internal control (Universal Inhibition  
196 Control Cy5®, Diagenode Diagnostics, Liège, Belgium) and an endogenous in-house positive  
197 internal control (*P. jirovecii* DNA extract from the local collection). The results were  
198 expressed in quantitative cycle (Cq) values <sup>8</sup>.

199

## 200 **Assay performance and analysis**

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

210 concentration and qPCR C<sub>q</sub> in NPA. The comparison of diagnostic performance was made  
211 using the paired two-sample Student's *t*-test. The Spearman's rank correlation coefficient was  
212 used to assess the correlation of results obtained with the quantitative methods, while the  
213 observer agreement for categorical data was measured through the Cohen's kappa coefficient  
214 ( $\kappa$ ), in light of the interpretation instructions by Landis et Koch<sup>23</sup>. The  $\alpha$ -risk was adjusted at  
215 0.05.

216

## 217 **Ethics**

218 This study was non-interventional, involving no change in standard clinical practices. Only a  
219 form of non-objection was signed by the included patients. Clinical data were anonymized  
220 before analysis. The biological samples were stored in the hospital collection N°DC-20100-  
221 1216, approved by the French Ministry of Research. The study registration number  
222 N°2015\_030 was issued by the French Data Protection Authority (CNIL, Commission  
223 Nationale de l'Informatique et des Libertés). Final approval N°2015 23 was given by the local  
224 Ethics Committee of Tours University Hospital (Espace de Réflexion Ethique, Région Centre-  
225 Val de Loire, France) before submission. The authors complied with the BRISQ guidelines<sup>24</sup>.

226

## 227 **RESULTS**

### 228 **Description of the study population**

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

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

239 For the cases retained with definitive diagnosis of PjP infection, all the characteristics at  
240 baseline are summarized in Table I and Suppl. Material 1. Most involved  
241 immunocompromised males above 60 years of age (median = 64 years old), with a mean  
242 count of 0.12 G/L CD<sub>4</sub><sup>+</sup> lymphocytes at the time of diagnosis. Respiratory symptoms and  
243 fever were the major clinical signs. In BALF, microscopic findings (Suppl. Materials 1 et 2)  
244 and qPCR results were concordant, *i.e.* both positive, in 35 out of the 48 cases (72.92%).  
245 Quantitative PCR appeared insignificantly more performant in BALF than microscopic  
246 observation for the definitive microbiological diagnosis of PjP infection ( $P=0.26$ ). Calcofluor-  
247 blue brightener allowed visual counting  $430 \pm 280$  *P. jirovecii* asci *per* glass slide in average  
248 (Suppl. Material 2). Microscopic examination and qPCR in BALF were systematically  
249 negative (with Cq > 35) in all the control subjects (but two qPCR were detected at 39 cycles,  
250 three at 38, three at 37, and two at 36). In the 48 patients with true PjP infection, mean BDG  
251 was measured at  $527.63 \pm 545.06$  pg/mL vs.  $70.81 \pm 33.98$  pg/mL in the controls ( $P<0.0001$ )  
252 (Figure 2A). The ROC curve allowed calculation of the BDG cutoff value at 143 pg/mL  
253 (Figure 2B).

254

### 255 **Diagnostic performance of the microbiological testing**

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

259 in NPA (Cq = 36 cycles or above). In contrast in the 48 cases asserted with PjP infection, 40  
260 were positive (83.33%; mean Cq calculated at  $32.21 \pm 3.51$  cycles; AUC ROC curve =  $0.96 \pm$   
261  $0.02$ ; Suppl. Materials 1 et 3), whereas four of them had been negative in BALF. Noteworthy  
262 for two patients, the discrepancies were more questionable (Cq=30.12 cycles in NPA vs.  
263 39.50 in BALF, and 31.60 vs. 40.00, respectively): for the first subject, the BALF was of poor  
264 quality (very viscous) which could maybe hamper the reliability of the diagnosis in it, while  
265 the second patient was already given anti-*P.jirovecii* therapy at time of sampling that could  
266 perhaps substantially interfere with the fungus detection. However, no significant differences  
267 were observed between the two types of samples (concordance rate = 86.73%; mean Cq =  
268 32.21 vs. 30.43 cycles, respectively;  $P=0.12$ ): qPCR findings in BALF and in NPA were  
269 statistically correlated (Spearman's rank correlation coefficient = 0.79,  $P<0.0001$  (Figure 3);  
270 Cohen's kappa coefficient ( $\kappa$ ) = 0.77,  $P<0.0001$ ). In all, sensitivity and specificity of the  
271 qPCR in NPA alone were estimated at 81.25%, 95%CI [67.74 – 89.95%] and 100.00%, 95%CI  
272 [90.75 – 100.00%], respectively, while they were just slightly higher in BALF, at 89.58%,  
273 95%CI [77.26 – 95.81] and 100.00%, 95%CI [90.75 – 100.00].

274 When associating the qPCR results in NPA with the BDG measurement in serum, diagnostic  
275 performance of the combined test could vary greatly (Table II). They primarily depended on  
276 the selection of the threshold value for the measurement of BDG antigen: indeed, sensitivity  
277 of BDG alone was 91.67%, 95%CI [79.78 – 97.15%] vs. 85.42%, 95%CI [72.41 – 92.97%] for  
278 the recommended cutoff at 80 pg/mL vs. the calculated cutoff at 143 pg/mL, respectively.  
279 Likewise, specificity shifted from 65.96%, 95%CI [51.59 – 77.82%] to 97.87%, 95%CI [87.66 –  
280 100.00%]. Thus, for combination of qPCR positive in NPA  $\pm$  BDG measurement  $> 80$  pg/mL,  
281 sensitivity was assessed at 95.83%, 95%CI [85.10 – 99.55%], while specificity plunged to  
282 65.96%, 95%CI [51.59 – 77.82%]. There were two false-negative result (4.17%) amongst the  
283 diseased cases with PjP infection, and 16 false-positive (33.33%) amongst the controls. Based

284 on the threshold calculated using the ROC curve, a better diagnostic performance was then  
285 demonstrated: 93.75%,  $_{95\%CI}$  [82.37 – 98.40%] for sensitivity, and 97.87%,  $_{95\%CI}$  [87.66 –  
286 100.00%] for specificity of the testing associating qPCR positive in NPA  $\pm$  BDG  
287 measurement  $>$  143 pg/mL. Three false-negative results were reported among the cases with  
288 true PjP infection (6.25%): one in a male patient suffering from chronic lymphocytic  
289 leukemia (who was undergoing anti-*P. jirovecii* treatment at time of sampling), one in an  
290 individual with solid cancer and for whom the microscopy examination was negative but  
291 qPCR positive in BALF (no mention of any anti-*P. jirovecii* treatment), and one in a 39-year-  
292 old woman with sarcoidosis with direct examination positive but qPCR negative in BALF  
293 (who was undergoing anti-*P. jirovecii* treatment at time of sampling). Only one false-positive  
294 was recorded among the controls (2.08%), with a BDG antigen measured at 484.0 pg/mL  
295 (whereas the *Aspergillus* galactomannan antigen was also very elevated in this patient's  
296 BALF).

297

## 298 **DISCUSSION**

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

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

313 In this study and in contrast to previous ones <sup>11</sup>, the initial inclusion criterion was specifically  
314 based on the clinical suspicion of PjP infection in populations at risk. This means that a  
315 prospective analysis of the results in NPAs and BDG was systematically performed in light of  
316 those obtained with the conventional diagnostic methods carried out in BALFs. Nonetheless,  
317 we would like to emphasize herein the pertinence of brainstorming by experts that should be  
318 initiated regarding the value of the positive cutoff for the BDG concentration. Results of BDG  
319 assay are acknowledged to vary substantially; it's the reason why a "grey zone" should be  
320 recommendable instead of a strict cutoff, like it is currently the case according to the  
321 manufacturer's instructions. In the present study, we demonstrated that the lower threshold  
322 (established by the kit's manufacturer) was largely less specific than the higher one  
323 (calculated at 143 pg/mL), while the sensitivities were actually quite close (just slightly better  
324 for the lower cutoff). In the same way, other authors previously suggested to increase the  
325 cutoff value that is usually recommended by the supplier <sup>27-29</sup>. Regarding the results of *P.*  
326 *jirovecii* qPCR in NPAs, no one can argue that the reliability of positive specimens is  
327 debatable, since all enrolled patients were symptomatic with febrile pneumonia at hospital  
328 admission and/or showed suggestive imaging in a suggestive epidemiological context. In  
329 other words, *P. jirovecii* colonization or casual carriage could be confidently precluded in  
330 them. Furthermore, frequent association of positive qPCR in NPA with BDG concentration  $\geq$   
331 143 pg/mL undoubtedly reinforced the likelihood of definitive diagnosis of PjP infection.  
332 Likewise, we can assert that association of the two tests provides a comforting backup in case  
333 one of them is found to be negative, especially when clinical signs are obvious and/or medical

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

341 One limitation of testing in NPA is that *P. jirovecii* DNA is probably present in lower  
342 amounts than in BALF (even if correlation of the Cq values between the two types of samples  
343 appeared statistically quite strong herein). Consequently to date, strict quantitative  
344 comparison of Cq and fungal burden remains questionable between the two clinical  
345 specimens, given the heterogeneity of their volumes and their consistency; some were more  
346 liquid, while other were rather mucous. As a result, the Cq thresholds that are usually  
347 expressed in BALF should be applied to NPAs with great caution <sup>8</sup>, although the cutoff for the  
348 latter was calculated in this study very close to the one recommended so far in BALF.

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

358

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368

369 **DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST**

370 The authors declare no conflict of interest. The data was obtained as a part of routine work at  
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372

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462 **FIGURE LEGENDS**

463 **Figure 1: Flowchart of the study.**

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

467

468 **Figure 2: Measurement of  $\beta$ -(1, 3)-D-glucan (BDG) antigen concentration in serum. A –**

469 Box plot showing the difference of BDG concentrations between the true cases of  
470 *Pneumocystis jirovecii* pneumonia and the controls. In each group, the mean was calculated at  
471  $527.6 \pm 272.5$  pg/mL [30.0 – 3830.0] and  $70.9 \pm 33.9$  pg/mL [15.6 – 484.0], respectively. The  
472 horizontal dotted line N°1 indicates the threshold suggested by the manufacturer at 80 pg/mL.  
473 The dotted line N°2 shows the upper cutoff determined at 143 pg/mL by the receiver  
474 operating characteristic (ROC) curve of the present study. The grey zone is indicated through  
475 hatched space; B – ROC curve of the measurement of BDG antigen concentration in serum.  
476 The area under the curve (AUC) is estimated at  $0.92 \pm 0.03$ ,  $_{95\%CI}$  [0.87 – 0.98]. The grey thin  
477 arrow indicates the cutoff calculated at 143 pg/mL, thus enabling sensitivity at 85.42%,  $_{95\%CI}$   
478 [72.41 – 92.97%], and specificity at 97.87%,  $_{95\%CI}$  [87.66 - 100.00%].

479

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

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

485 ● 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) <sup>21</sup>.**

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  $0.96 \pm 0.02$ , <sub>95%</sub>CI [0.92 – 1.00]. The grey thin arrow indicates the**  
501 **cutoff calculated at 35.4 cycles.**