Association Between Circulating DNA, Serum (1→3)-β-D-Glucan, and Pulmonary Fungal Burden in Pneumocystis Pneumonia

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Circulating Pneumocystis jirovecii DNA and (1→3)-β-D-glucan determined in 70 serum samples from immunocompromised patients were compared to fungal load in bronchoalveolar lavage fluids assessed using quantitative polymerase chain reaction. Both serum biomarkers are influenced by pulmonary fungal load, which should be taken into account when diagnosing Pneumocystis infection.

Pneumocystis pneumonia (PCP), caused by the opportunistic fungus Pneumocystis jirovecii, is usually diagnosed by means of microscopic observation of P. jirovecii cysts or trophic forms on slides prepared using bronchoalveolar lavage (BAL) fluid [1]. However, serum tests for PCP have also been developed for noncompliant patients or those too fragile for invasive procedures. The (1→3)-β-D-glucan (hereafter, β-glucan) test was used for several years as a noninvasive alternative to microscopic diagnosis [1]. β-Glucan is a common antigen component of the cell wall in most fungi and a main structural component of the cell wall in P. jirovecii cysts [1].

The performance of β-glucan tests is usually assessed in relation to positive microscopy and compatible clinical presentation as the gold standard [2, 3]. However, quantitative polymerase chain reaction (qPCR) assays are increasingly being used for diagnosis on account of the low probability of false-positive results due to the closed tube format and access to the amplification yield for quantifying results. Thus, qPCR has the potential to replace microscopy for diagnosing PCP if quantitative thresholds can be defined. To assess the association between circulating DNA, serum β-glucan, and pulmonary fungal burden in PCP, we tested serum samples when developing our qPCR assay for P. jirovecii DNA detection in BAL fluids [4].

METHODS

As part of a retrospective single-center, hospital-based cohort study involving all patients undergoing BAL to investigate the cause of pneumonia [4], we examined concomitant serum samples submitted to the laboratory for purposes other than PCP diagnosis (mainly detecting antigens or antibodies). For patients undergoing several BAL procedures, only those performed at least 15 days apart were considered. This study was approved by the Henri Mondor Hospital Ethics Review Committee (IMC-H20-569). The BAL procedure and qPCR from BAL fluids were performed as reported elsewhere [4]. Microscopic diagnosis included the identification of P. jirovecii on slides prepared from BAL fluid and stained using indirect immunofluorescence assay (Monofluo kit P. jirovecii, Bio-Rad, Marnes la Coquette, France).

Serum samples were stored at −40°C as part of routine procedure. After thawing, serum β-glucan levels were measured using the Fungitell assay (Associates of Cape Cod, Inc, Cape Cod, Massachusetts). Manipulation was performed in accordance with the manufacturer’s instructions. The β-glucan results were categorized as either positive (>80 pg/mL) or negative (≤80 pg/mL), with values >500 pg/mL being censored at 500 pg/mL. Additional dilutions were not performed.

In parallel, DNA was extracted from 1 mL of serum using the Total Nucleic Acid LV extraction procedure on the MagNA Pure Compact instrument (Roche Diagnostics, Meylan, France). Finally, DNA was eluted in 50 µL of elution buffer, with 10 µL being used per PCR reaction. The qPCR assay targeted the mtLSU gene, including the enzymatic prevention of contamination with internal control and quantification of copy numbers with customized plasmid [4].

Fisher exact test and χ2 test were used to compare categorical variables, and Student t test was used for continuous variables. Differences were considered significant at P < .05. Statistical analyses were performed using Prism 4.0 (GraphPad Software).
RESULTS

A total of 70 serum samples were collected from 63 patients, with 1 patient having 4 BALs and 4 patients 2 BALs (median intervals, 16 days; range, 15–318). The samples were divided in 3 groups according to the microscopy and qPCR BAL results: group 1 represented microscopy-positive and qPCR-positive, group 2 microscopy-negative and qPCR-positive, and group 3 microscopy-negative and qPCR-negative (Table 1). The time interval between the serum and BAL sampling did not significantly differ between the 3 groups. The samples from the same patient were evenly distributed among the 3 groups (Table 1).

Nine of the 10 group 1 serum samples were qPCR-positive, with a median copy number 103 times lower (range, 119; 70–538) than in BAL fluid. The only qPCR-negative serum of this group corresponded to the BAL with the lowest copy number (3.3 log copies/µL). The β-glucan level was >500 pg/mL for all 10 serum samples and imaging showed diffuse radiological patterns for all of them.

BAL results in group 2 exhibited a lower pulmonary fungal load compared with group 1, all being ≤3 log copies/µL. The corresponding serum samples were all qPCR-negative. Six (23.1%) serum samples had β-glucan levels >500 pg/mL for BAL, with a mean log copy number of 2.183 (standard error of the mean [SEM], 0.399). Nineteen (73.1%) serum samples with β-glucan levels >80 and ≤500 pg/mL corresponded to BALs with a lower mean log copy of 0.8526 (SEM, 0.184) than the 6 BAL samples corresponding to β-glucan >500 pg/mL (P = .003). Only 1 serum sample was β-glucan-negative (52 pg/mL) in this group and corresponded to the BAL with the lowest fungal burden (40 copies/µL).

In group 3, 10 serum samples had β-glucan levels >80 pg/mL. The number of probable or proven invasive aspergillosis classified according to European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group definitions [5] did not differ between the 3 groups. No candidemia was observed during the 10-day interval before or after the BAL procedure.

The sensitivity and specificity of serum β-glucan for the diagnosis of PCP were 1.0 (95% confidence interval [CI], .69–1) and 0.42 (95% CI, .29–.55), respectively, and 0.97 (95% CI, .86–.99) and 0.71 (95% CI, .52–.85), respectively, when positive microscopy or positive qPCR was taken as the standard.

DISCUSSION

In this article, the detection of circulating P. jirovecii DNA using qPCR was influenced by pulmonary fungal load.

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Table 1. Serum Pneumocystis jirovecii DNA and β-β-Glucan (β-Glucan) Titers According to Microscopy and P. jirovecii Quantitative Polymerase Chain Reaction Results From 70 Bronchoalveolar Lavage Samples From 63 Patients

<table>
<thead>
<tr>
<th>Comorbid conditions</th>
<th>Group 1: Microscopy-Positive, qPCR-Positive BAL (n = 10), No. (%)</th>
<th>Group 2: Microscopy-Negative, qPCR-Positive BAL (n = 26), No. (%)</th>
<th>Group 3: Microscopy-Negative, qPCR-Negative BAL (n = 34), No. (%)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIDS (n = 8, 11%)</td>
<td>5 (50)</td>
<td>1 (4)</td>
<td>2 (6)</td>
<td>.28</td>
</tr>
<tr>
<td>Acute leukemia (n = 28, 40%)</td>
<td>2 (20)</td>
<td>12 (46)</td>
<td>14 (41)</td>
<td></td>
</tr>
<tr>
<td>Chronic lymphoproliferative disorders (n = 8, 11%)</td>
<td>0</td>
<td>2 (8)</td>
<td>6 (18)</td>
<td></td>
</tr>
<tr>
<td>Solid organ transplantation (n = 11, 16%)</td>
<td>2 (20)</td>
<td>3 (11)</td>
<td>6 (18)</td>
<td></td>
</tr>
<tr>
<td>Systemic inflammatory diseases (n = 11, 16%)</td>
<td>1 (10)</td>
<td>7 (27)</td>
<td>3 (9)</td>
<td></td>
</tr>
<tr>
<td>Solid cancer (n = 2, 3%)</td>
<td>0</td>
<td>1 (4)</td>
<td>1 (3)</td>
<td></td>
</tr>
<tr>
<td>Other (n = 2, 3%)</td>
<td>0</td>
<td>0</td>
<td>2 (6)</td>
<td></td>
</tr>
<tr>
<td>Time interval between serum and BAL sampling, days (SEM)</td>
<td>1.0 (0.88)</td>
<td>−0.08 (0.32)</td>
<td>−0.21 (0.51)</td>
<td>.28</td>
</tr>
<tr>
<td>No. with qPCR-positive serum sample (%)</td>
<td>9 (90)</td>
<td>0</td>
<td>0</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Mean log10 BAL P. jirovecii DNA copy, No./µL (SEM)</td>
<td>4.6 (0.34)</td>
<td>1.1 (0.20)</td>
<td>...</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>No. with β-glucan &gt;500 pg/mL (%)</td>
<td>10 (100)</td>
<td>6 (23)</td>
<td>2 (6)</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>No. with β-glucan &gt;80 and ≤500 pg/mL (%)</td>
<td>0</td>
<td>19 (73)</td>
<td>8 (24)</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>No. with β-glucan ≤80 pg/mL (%)</td>
<td>0</td>
<td>1 (4)</td>
<td>24 (71)</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>No. with concomitant probable or proven invasive aspergillosis (%)</td>
<td>1 (10)</td>
<td>4 (15)</td>
<td>5 (15)</td>
<td>.91</td>
</tr>
<tr>
<td>Diffuse radiological patterns</td>
<td>10 (100)</td>
<td>7 (27)</td>
<td>7 (21)</td>
<td>.19</td>
</tr>
</tbody>
</table>

Abbreviations: BAL, bronchoalveolar lavage; qPCR, quantitative polymerase chain reaction; SEM, standard error of the mean.

* When BAL was performed several times from a given patient, the sample distribution was as follows: 1 sample in groups 1 and 3 for 1 patient; 2 samples in group 1 for 1 patient; 2 samples in group 2 for 1 patient; 1 sample in group 2, and 3 samples in group 3 for 1 patient; and 2 samples in group 3 for 1 patient.
Provided that pulmonary load was >3.3 log copies number/µL, all 9 of the serum samples were qPCR-positive. The other main finding of our study was that the majority (25 of 26, 96%) of serum samples corresponding to microscopy-negative and qPCR-positive BALs were β-glucan-positive.

Detecting *P. jirovecii* DNA in serum was previously investigated, but with contrasting results. None or very few of the samples were found to be PCR-positive when the diagnosis was microscopically proven using BAL fluids [6–9], or PCR-positive results were obtained from patients who were neither infected nor colonized with *P. jirovecii* [6, 7, 9]. Because these PCR assays did not include enzymatic prevention of contamination and were nested PCR, false-positive results cannot be ruled out for non-PCP patients [7, 9]. Similarly, because no internal control was used, false-negative results cannot be excluded on account of residual PCR inhibitors in order to explain the low rate of PCR-positive serum samples [6, 7, 9]. In contrast, 2 studies reported that almost all PCP patients tested had at least 1 PCR-positive sample [10, 11]. Because the standard for diagnosis was microscopy, these results are consistent with our own. Indeed, we found positive serum qPCR corresponding to pulmonary fungal loads to be always associated with positive microscopy [4]. The circulating DNA detection could be seen as a noninvasive diagnostic method. Not all patients with high pulmonary loads were human immunodeficiency virus positive, suggesting that fungal load, rather than the underlying disease, was the cause of the serum qPCR positivity.

Serum β-glucan appeared to be a good marker for PCP [1]. In this article, we showed a clear association between BAL fungal load and serum β-glucan levels, as was recently reported by de Ber et al [12]. However, false-positive results may occur in up to 35% of cases [3]. Indeed, β-glucan is not specific for PCP, as it is included, for example, in the criteria for deep-seated fungal diseases, such as invasive candidiasis and invasive aspergillosis [5]. The number of invasive fungal infections cannot explain the differences observed between the 3 groups in our study (Table 1). In addition, numerous other causes of false-positive results have been previously cited [1]. Our results, however, suggested another probable cause, which was not investigated in previous studies, namely the presence of *P. jirovecii* in BAL fluids without microscopic evidence. If the diagnosis of PCP is based on positive microscopy corresponding to a threshold of >3 log copies/µL as in the present study, then serum samples positive for β-glucan with BAL below this threshold should be considered false-positive. Thus, β-glucan specificity decreased from 70.6% to 41.7% depending on whether the microscopy-negative and qPCR-positive BAL samples were considered as true-positives or false-positives, respectively. The high rate of false-positives may in fact stem from the clinical decision to exclude the diagnosis of PCP when microscopy is negative and qPCR positive [1, 3].

We are well aware of the limits of this retrospective study on patients with a high pretest probability, which may bias the calculated performance, as may the low number of samples evaluated. Another bias may be the selection of patients for whom only 1 simultaneous serum sample was available, although this probably did not lead to a selection bias in the population. Finally, the kinetics of serum markers were unknown, meaning that they may have changed within a few days. Nonetheless, the interval between serum and BAL fluid collection did not differ between the 3 sample groups.

In addition to improving our knowledge of PCP pathophysiology, the detection of β-glucan and *P. jirovecii* DNA could be usefully combined as serum markers for the diagnosis of PCP in patients with severe diseases or other conditions precluding the BAL procedure. Further studies with serial serum sampling should permit an exploration of β-glucan and DNA kinetics following treatment in order to determine whether they may assist clinicians in therapy assessment.

**Notes**

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**References**


