research article

Diagnostic accuracy of (1→3)-ß-D-glucan to predict Pneumocystis jirovecii pneumonia in non-HIV-infected patients

Petra Rogina[1], Miha Skvarc[2]

[1] Infectious Disease Department, General Hospital Novo mesto, Novo mesto, Slovenia
[2] Infection Control and Microbiology Unit, General Hospital Jesenice, Jesenice, Slovenia

Radiol Oncol 2020; 54(2): 221-226.

Received 31 December 2019
Accepted 14 April 2020

Correspondence to: Assist. Prof. Miha Skvarč, M.D., Ph.D., General Hospital Jesenice, Cesta maršala Tita 112, Jesenice, Slovenia.
E-mail: mihaskvarc@hotmail.com

Disclosure: No potential conflicts of interest were disclosed.

Background. *Pneumocystis jirovecii* pneumonia (PCP) is a common and potentially fatal opportunistic infection in immunocompromised non-HIV individuals. There are problems with clinical and diagnostic protocols for PCP that lack sensitivity and specificity. We designed a retrospective study to compared several methods that were used in diagnostics of PCP.

Patients and methods. One hundred and eight immunocompromised individuals with typical clinical picture for PCP and suspicious radiological findings were included in the study. Serum samples were taken to measure the values of (1→3)-β-D-glucan (Fungitell, Associates of Cape Cod, USA). Lower respiratory tract samples were obtained to perform direct immunofluorescence (DIF, MERIFLUOR® Pneumocystis, Meridian, USA) stain and real-time PCR (qPCR).

Results. Fifty-four (50%) of the 108 patients in our study had (1→3)-β-D-glucan > 500 pg/ml. Patients that had (1→3)-β-D-glucan concentrations < 400 pg/ml in serum, had mean threshold cycles (Ct) 35.43 ± 3.32 *versus* those that had (1→3)-β-D-glucan concentrations >400 pg/mL and mean Ct of 28.97 ± 5.27 (P < 0.001). If we detected *P. jirovecii* with DIF and qPCR than PCP was proven. If the concentration of (1→3)-β-D-glucan was higher than 400 pg/ml and Ct of qPCR was below 28.97 ± 5.27 than we have been able be certain that *P. jirovecii* caused pneumonia (odds ratio [OR] 2.31, 95% confidence interval [CI] 1.62–3.27, P < 0.001).

Conclusions. Measurement of (1→3)-β-D-glucan or qPCR alone could not be used to diagnose PCP. Diagnostic cut-off value for (1→3)-β-D-glucan > 400pg/ml and qPCR below 30 Ct, allow us to conclude that patient has PCP. If the values of (1→3)-β-D-glucan are < 400 pg/ml and qPCR is above 35 Ct than colonization with *P. jirovecii* is more possible than PCP.

Key words: *Pneumocystis jirovecii* pneumonia; (1→3)-β-D-glucan; DIF; real-time PCR; colonization with *P. jirovecii*; non-HIV-infected patients

Introduction

Pneumocystis jirovecii is a cause of *Pneumocystis* pneumonia (PCP) in immunocompromised patients. PCP most often occurs in human immunodeficiency virus (HIV) infected patients associated with high pathogen burdens but also in non-HIV immunocompromised patients.[1-3] The diagnosis is mainly based on clinical and radiographic examinations, which are in majority of cases inconclusive, so microscopic or molecular detection of *P. jirovecii* in lower respiratory tract samples are necessary.[2,3] The mechanism by which *Pneumocystis* organisms induce lung inflammation remains incompletely understood, but studies imply that *Pneumocystis* spp. cell wall constituent (1→3)-β-D-glucans are released and contribute to the development of extensive lung inflammatory reaction.[4-10] Various studies have reported high serum (1→3)-β-D-glucan levels in patients with PCP.[7-15] The (1→3)-β-D-glucan has

high sensitivity for PCP but, it is a not species-specific marker for *P. jirovecii*.[2,11,13,15]

Detection of *P. jiroveci* DNA in respiratory tract specimens without signs and symptoms of PCP is defined as colonization. Molecular diagnostic techniques like quantitative PCR (qPCR) are more sensitive than staining methods like direct immunofluorescence method (DIF). However, qPCR can give sometimes false signal that patient has PCP, although patient is just colonized with fungi.[16-21] Studies have reported prevalence of colonization in susceptible adults from 10.0% to 43.8%.[17-23] Real-time qPCR assays have been shown as most accurate for discrimination of true PCP from colonization with *P. jirovecii*. Flori *et al*. compared the performance of standard staining, standard PCR, and qPCR using 173 BAL specimens from 150 patients. Real-time qPCR gave the best results.[24]

A systematic review, that evaluated PCR kits to confirm PCP, revealed that positive PCR results do not always confirm PCP. Low amounts of DNA can be expected in patients with *P. jirovecii* colonization of and no clinical signs of PCP.[25]

(1→3)-β-D-glucan can be helpful to distinguish PCP from *P. jirovecii* colonization. However, not as standalone test. (1→3)-β-D-glucan is positive in colonization of gastrointestinal tract with candida. It is positive when patients are colonized with *P. jirovecii*.[13,16] Combining best tests is a advisable in immunocompromised cancer patients to be able to treat patients with the right therapy.

We designed a retrospective multicentric study that included patients from 3 tertiary care hospitals and 2 secondary care hospitals. The objective of our study was to distinguish true PCP from colonization with *P. jirovecii* with diagnostic algorithm that includes measurement of (1→3)-β-D-glucan and real-time PCR (qPCR).

Patients and methods

Patients

We included 108 consecutive non-HIV patients on immunosuppression therapy from 2006 to end of 2014 with clinical diagnosis of possible PCP. Possible PCP diagnosis was given to patients at admission to hospital or during diagnostic process that had typical suggestive symptoms and signs and radiological findings for PCP (fever, dry cough, dyspnoea on exertion and bilateral interstitial pneumonia on chest X-ray, and/or ground glass look on thorax CT scan). Patients receiving antifungal therapy or therapy that works against *P. jirovecii* were excluded from the study. Patients with excessive colonization with *Candida spp.* on mucosal barriers were also excluded from the study.

Methods

Microbiological diagnosis was based on the detection of *P. jirovecii* with DIF (MERIFLUOR® Pneumocystis, Meridian, USA) and on in-house developed qPCR from lower respiratory tract sample (induced sputum, aspiration of trachea or bronchoalveolar lavage – BAL). DNA was extracted from lower respiratory tract samples using QIAamp DNA mini kit (Qiagen, Germany). In-house qPCR developed after article of Alanio *et al*.[16] was performed using a reaction mix Platinum® Quantitative PCR SuperMix-UDG with ROX (Invitrogen, USA) and PCR primers to detect *Pneumocystis* PjFI and PjRI with hybridization probe PjSL (TIB MolBiol, Germany). The qPCR assay developed for this study amplifies a 121-bp fragment of the *P. jirovecii* mitochondrial large-subunit rRNA gene. The primers PjF1 (5′-CTGTTTCCCTTTCGAC TATCTACCTT-3′) and PjR1 (5′-CACTGAATATCTCGAGG GAGTATGAA-3′) and the TaqMan-MGB probe PjSL (5′-TCGCACATAGTCTGATTAT-3′) were designed by using Primer Express software (Applied Biosystems, Foster City, CA, USA). DNA sample volume was 5 μL. PCR reaction with a final volume of 25 μL was carried out with following conditions: 50ºC for 2 min, 95ºC for 2 min, followed by 45 cycles consisting of 15 sec at 95ºC, 60 sec at 60ºC. The results are presented as threshold cycles (Ct). We used Quality Control for Molecular Diagnostics (QCMD, UK) samples from *Pneumocystis jirovecii* pneumonia (PCP) DNA scheme to evaluate our qPCR (sensitivity 97%, specificity 100%). Serum samples were collected to measure the values of (1→3)-β-D-glucan (Fungitell, Associates of Cape Cod, USA).

In all patients 2 sets of blood cultures were collected. Lower respiratory tract samples were collected to detect potential bacterial cause of deteriorating health.

Institutional review board approved of General Hospital Jesenice approved the study under condition that patients' data were anonymized. The results of the study did not influence the course of the treatment.

Statistical analysis

Microbiologically proven PCP was defined as positive DIF and positive qPCR. Colonization was suspected when DIF was negative and if any amount

of DNA with qPCR was found. (1→3)-β-D-glucan in patients with possible colonization with *P. jirovecii* had to be positive (above 80 pg/ml).

Mean values for (1→3)-β-D-glucan and Ct values between different patients' groups and (1→3)-β-D-glucan ranges were compared using an independent T-test. Receiver operating curve (ROC) analysis with area under the curve (AUC) and confidence interval (CI) were performed to calculate diagnostic accuracy. Univariate logistic regression was performed to explore the association of laboratory parameters with the disease. The statistical significance level was set at 0.05 (two-tailed). All analyses were conducted with SPSS version 23.0 statistical software.

Results

One hundred and eight immunosuppressed patients (mean age 53 ± 15 years) with possible PCP were included into final analysis. Majority of patients were in some way immunocompromised. They received immunosuppressive therapy because of transplanted solid organ (53 patients, 49%) with mean (1→3)-β-D-glucan value of 330.01 ± 191 pg/mL. The rest of patients had autoimmune diseases or malignant disease (Table 1).

Patient's data were divided into 6 groups according to (1→3)-β-D-glucan values. In the first group were patients with (1→3)-β-D-glucan value < 100 pg/mL, followed by group of patients with (1→3)-β-D-glucan value from 100 pg/mL till 200 pg/mL and so on till the last group with (1→3)-β-D-glucan values > 500 pg/mL. The largest group of patients (50% of all patients) were in the range of (1→3)-β-D-glucan > 500 pg/mL. Twenty-four patients with possible PCP had (1→3)-β-D-glucan < 100 pg/mL, with mean value of 81.14 ± 3.41 pg/mL (Table 2).

Results for patients in different (1→3)-β-D-glucan ranges and mean qPCR Ct values are presented in Table 3. Patients in the last 2 ranges ((1→3)-β-D-glucan 400-500pg/m and > 500pg/ml) had significantly more *P. jirovecii* DNA detected in lower respiratory tract ((1→3)-β-D-glucan 400-500 pg/mL, mean Ct 29.41 ± 6.25; (1→3)-β-D-glucan > 500 pg/ml, mean Ct 28.90 ± 5.18, P < 0.001) in comparison to other groups of patients. Statistically significant difference in Ct values was noticed when (1→3)-β-D-glucan concentrations exceeded 400pg/ml ((1→3)-β-D-glucan < 400 pg/mL, mean Ct 35.43 ± 3.32 *versus* (1→3)-β-D-glucan > 400 pg/mL, mean Ct 28.97 ± 5.27, P < 0.001). Moreover, analysis showed that majority of patients (57%) had (1→3)-β-D-glucan > 400 pg/ml and had the greatest chance to have PCP ((1→3)-β-D-glucan > 400 pg/mL: OR 2.31 (95% CI 1.62–3.27), p < 0.001).

We compared (1→3)-β-D-glucan values and qPCR Ct of 42 patients with microbiologically confirmed PCP (DIF positive, qPCR positive) to those 66 patients that had possible PCP at initial presentation to predict true PCP (Table 4). Forty-two patients had statistically significant higher concentrations of (1→3)-β-D-glucan and *P. jirovecii* DNA (P < 0.001). Subgroup of 26 patients, that had DIF positive and qPCR Ct < 30, had slightly higher

TABLE 1. Average (1→3)-β-D-glucan values for each group of immunocompromised patients

Diagnosis	N = 108 patients	(1→3)-β-D-glucan (pg/mL) ± SD
Malignant disease	20 (18%)	240.34 ± 162.45
Transplantation	53 (49%)	330.01 ± 191.26
Autoimmune disease	35 (33%)	427.73 ± 152.23

TABLE 2. Average (1→3)-β-D-glucan values for each (1→3)-β-D-glucan range

(1→3)-β-D-glucan group	N = 108 patients	(1→3)-β-D-glucan (pg/mL) ± SD
< 100 pg/mL	24	81.14 ± 3.41
100–200 pg/mL	11	124.4 ± 22.37
200–300 pg/mL	4	238,75 ± 16,55
300–400 pg/mL	7	336.75 ± 20.18
400–500 pg/mL	8	454.13 ± 31.3
> 500 pg/mL*	54	> 500 pg/mL

* Mean could not be calculated since samples dilutions to measure real (1→3)-β-D-glucan concentrations were not performed.

TABLE 3. Average Ct values for each (1→3)-β-D-glucan range

(1→3)-β-D-glucan group	N = 108 patients	Mean Ct ± SD
Group 1 < 100 pg/mL	24	35.43 ± 3.51
Group 2 100–200 pg/mL	11	34.82 ± 3.65
Group 3 200–300 pg/mL	4	37.33 ± 2.36
Group 4 300–400 pg/mL	7	35.27 ± 2.75
Group 5 400–500 pg/mL	8	29.41 ± 6.25*
Group 6 > 500 pg/mL	54	28.90 ± 5.18*

Ct = threshold cycle; SD = standard deviation

* Statistically significant difference seen when Ct from group 1 to 4 are compared to group 5 and 6 combined, P < 0.001. Odds ratio (OR) is the greatest to predict PCP if (1→3)-β-D-glucan > 400 pg/mL, OR 2.31 (95%CI 1.62-3.27), p < 0.001).

TABLE 4. (1→3)-β-D-glucan values for patients with microbiologically confirmed PCP and possible PCP

PCP	108 patients	(1→3)-β-D-glucan (pg/mL) ± SD	qPCR Ct ± SD
Possible PCP	66 (61%)	**256.06 ± 183.48**	**35.01 ± 3.8**
Microbiologically confirmed PCP (DIF and qPCR positive)	42 (39%)	**467.5 ± 95.5**	**27.2 ± 4.6**
Subgroup definite PCP (DIF positive and qPCR Ct < 30 and response to treatment)	26 (24%)	494.81 ± 17.73*	
P value		**< 0.001**	**< 0.001**

Ct = threshold cycle; DIF = direct immunofluorescence; PCP = pneumocystis pneumonia; qPCR = real time PCR; SD = standard deviation

* Area under the curve (AUC) to predict PCP (positive DIF and qPCR) with measurement of (1→3)-β-D-glucan: AUC = 0.817, 95% CI: 0.736–0.898, sensitivity = 83.3%, specificity = 64.6%, cut off 496.45 pg/mL, p < 0.001

(1→3)-β-D-glucan mean values than the group of microbiologically confirmed PCP. The subgroup cut-off for (1→3)-β-D-glucan was set to 496.45 pg/mL, and determined PCP with greatest AUC and statistically significance (AUC = 0.817, 95% CI: 0.736–0.898, sensitivity = 83.3%, specificity = 64.6%, p < 0.001).

Discussion

Our experience with (1→3)-β-D-glucan and qPCR indicated that cut-off of > 400 pg/mL for (1→3)-β-D-glucan could be used to predict microbiologically confirmed PCP with greatest statistical significance. Results of the analysis indicated that colonization is possible when levels of (1→3)-β-D-glucan are moderate, in our case < 400 pg/mL, and low concentrations of *P. jirovecii* DNA with qPCR are detected, in our case > 35 cycles.

Fifty percent of the patients in our study had (1→3)-β-D-glucan > 500 pg/mL and mean qPCR Ct 28.9 ± 5.18. The second largest group of patients (24 patients) with possible PCP were those with (1→3)-β-D-glucan < 100 pg/mL, and mean qPCR Ct 35.43 ± 3.51. Patients with microbiologically confirmed PCP had mean (1→3)-β-D-glucan of 467.5 ± 95.5 and mean qPCr Ct 27.2 ± 4.6. Even more, true PCP where patients with it, had response to therapy, can be predicted when cut-off for (1→3)-β-D-glucan is set to 496.45 pg/ml. At this cut-off, AUC to predict PCP was 0.817.

Similar results were produced in recent studies.[26,27] In an older study cut-off value of 340 copies/mL was used to discriminate probable PCP from colonization. At this qPCR cut-off, (1→3)-β-D-glucan levels were significantly higher in patients with both definite PCP and probable PCP than in colonized patients.[26] It was recently shown that serum (1→3)-β-D-glucan levels of 143 pg/mL can be used to distinguish PCP from colonisation with *P. jirovecii*.[28] To diagnose and treat PCP you have to have a lot of experience.[29] Our results were not concordant with latter studies because we measured much higher (1→3)-β-D-glucan levels in the patients with true PCP. The clinicians in majority of included institutions seldomly get the chance to treat PCP and are not experienced enough.

When PCR was introduced, it remarkably increased sensitivity to diagnose PCP, resulting in an increase in the number of documented PCP cases.[30-34] On the other hand multiple evidences indicate that *P. jirovecii* can colonize the mucosal epithelium of both healthy individuals and those with compromised immunity.[34-36] Results of qPCR in the area where differentiation between PCP and colonization is demanding, if we look at them separately from (1→3)-β-D-glucan. It was suggested that every (1→3)-β-D-glucan values should be interpreted with qPCR and vice versa in patients with possible PCP.[16,26,29]

Montesinos *et al*. also showed with their in-house PCR test cut-off value Ct = 34 to discriminate definite PCP from unlikely PCP with 65% sensitivity and 85% specificity.[37] Other studies have suggested applying two qPCR cut-off values to increase sensitivity and specificity of qPCR. There is a grey zone of unclear clinical significance between these two cut-off values where the differential diagnosis of PCP *versus* colonization cannot be determined.[16,24,38-40] In recent literature two cut-offs were proposed to predict definite PCP. The two cut-offs for qPCR provide 100% sensitivity and 100% specificity to diagnosis definite PCP. The higher cut-off value represents the value below which the diagnosis of PCP is unlikely. The authors suggested that the range between these two cut-off values represents an indeterminate zone.[16,39,41-42]

According to our study, *P. jirovecii* DNA copy number above the cut-off value Ct ≥35 would support a diagnosis of colonization, and a copy number below the cut-off value would support a diagnosis of PCP. However, good sensitivity of qPCR can be misleading, since qPCR detects very low fungal load that can lead to over-diagnosis of PCP due to misinterpretation of findings.[33,42] Our cut-off was determined with the help of (1→3)-β-D-glucan, which is a good indicator for PCP if the values are high and we detect a lot of *P. jirovecii* DNA in lower respiratory tract.

Our study had some limitations. We could not gather the information that treatment with antimicrobials helped patients which would confirm that patients really had PCP for all included individuals. The influence of different samples from lower respiratory tract on qPCR was not assessed. Although our qPCR displayed high accuracy for discriminating colonization from PCP, the cut-off values used in our study should be standardized to copies/ml and compared to other qPCRs. The cut-off values for (1→3)-β-D-glucan was not always measured the same day as qPCR was done which could influence on the concentration of (1→3)-β-D-glucan. For future studies, patients that have their clinical picture and response to therapy consistent with possible PCP, and are randomized according to the analysed samples, should be included to resolve the discrepancy seen in our study.

Conclusions

The protocol we presented here could better support clinicians in their decisions whether patient in front of them has PCP. Our diagnostic cut-off values allow identification of true PCP *versus* colonization with *P. jirovecii* with better diagnostic accuracy. However, according to our results qPCR cannot be used alone to confirm PCP. PCP has to be confirmed with qPCR and with (1→3)-β-D-glucan.

Acknowledgment

We thank Institute of Microbiology and Immunology, Faculty of Medicine, University of Ljubljana for their routine diagnostic support when needed.

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