Differentiation between *pneumocystis jirovecii* pneumonia and colonization in immunocompromised patients

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Background The diagnosis of *Pneumocystis jirovecii* pneumonia (PJP) is mainly based on microscopic detection of *P. jirovecii* in the respiratory samples. Quantitative PCR (qPCR) can detect low levels of *P. jirovecii* DNA but cannot differentiate between infection and colonization. Therefore a new and more accurate assay have to be used. 1,3 BD-glucan (BD-glucan) with a threshold value of 100 pg/ml can differentiate *P. jirovecii* infection from colonization.

Aim The aim of this study is to evaluate the diagnostic accuracy of qPCR and BD-glucan assays in differentiating pneumocystis infection from colonization in immunocompromised patients with help of radiological pulmonary infiltrates.

Patients and methods This study consisted of 75 immunocompromised patients (37 renal transplanted patients and 38 HIV patients) who were admitted for radiological pulmonary infiltrates and who presented a clinical picture suspecting PJP. They were investigated using microscopic staining of their respiratory samples (induced sputum or bronchoalveolar lavage).By applying both qPCR and serum BD-glucan assays we can differentiate between *P. jirovecii* infection from colonization.

Results In this study, the first group of 25 patients were diagnosed as definite PJP, the second group of 20 patients were diagnosed as having pneumonia with *P. jirovecii* colonization, and a third group of 30 patients were diagnosed as having pneumonia without colonization. The number of copies of fungal DNA detected by qPCR were significantly higher in a definite PJP than in those with pneumonia accompanied with *P. jirovecii* colonization. Also BD-glucan assays were significantly higher in definite PJP by applying a threshold value of 100 pg/ml. The sensitivity and specificity of qPCR for differentiation of PJP infection from colonization were 100 and 64%, respectively, whereas the sensitivity and specificity of BD-glucan were 100 and 96%, respectively.

Conclusion Both BD-glucan and qPCR assays had high diagnostic values in differentiating definite PJP from *P. jirovecii* colonization, and by applying qPCR with two cutoff values combined with serum BD-glucan using a threshold value of 100 pg/ml.

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Keywords: colonization, immunocompromised host, *Pneumocystis jirovecii* pneumonia, quantitative PCR, BD-glucan, respiratory tract infection

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Introduction

Pneumocystis jirovecii pneumonia (PJP) is an opportunistic infection caused by the fungus *P. jirovecii* [1]. The PJP remains one of the most prevalent infections in immunocompromised patients, including individuals receiving immunosuppressive medication for inflammatory or autoimmune diseases, patients with solid or hematological malignancies, and organ transplanted recipients [2].

The standard method for laboratory diagnosis of PJP is the microscopic detection of *P. jirovecii* in respiratory samples, including induced sputum or bronchoalveolar lavage (BAL) by staining method. But sometimes the respiratory samples commonly had a low fungal burden that may lead to false-negative results [3]. To overcome this problem, PCR, which is a highly sensitive technique, has been used for PJP diagnosis. Conventional PCR methods can detect low levels of *P. jirovecii* DNA, but are not quantitative and cannot differentiate PJP infection from colonization. Then the more advanced method, Real-time quantitative PCR (qPCR), was used for DNA quantification [4]. qPCR cutoff values were applied to differentiate between PJP infection from colonization [5].

1,3 BD-glucan (BD-glucan) represents a major structural component of the cell wall of most fungi [6]. Serum measurement of BD-glucan is based on the polysaccharide level within the cell wall of pneumocystis and other fungi. It was reported that serum BD-glucan level is the best test for PJP diagnosis [7]. Some studies had assessed the accuracy of BD-glucan assay for differentiating PJP infection from colonization [8].

Aim

The aim of this study is to evaluate the diagnostic accuracy of BD-glucan and qPCR assays in differentiating pneumocystis infection from colonization in immuno-

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compromised patients presented with a clinical picture of acute respiratory infection and radiological pulmonary infiltrates.

Patients and methods

In this research we are responding autonomy and maximizing patient benefit. Giving the mentioned points this research was approved by the Kuwaiti ethical approval committee. We conducted this study at the Infectious Disease Hospital and Organ Transplant Center in Kuwait, between February 2014 and May 2016. A total of 75 patients were enrolled for this study, presented by clinical manifestations of recent lower respiratory tract infection, such as fever, cough, expectoration, exertional dyspnea, desaturation (PaO_2<70 mmHg) in some patients (others were admitted with normal oxygenation but desaturated latter on after progression of pneumonia), new radiological infiltrates in either chest radiograph, or computed tomography of chest. All the patients were immunocompromised due to immunosuppressive drugs administered for renal transplantation or due to HIV infection. PJP was suspected in those patients and thefollowing process was carried out:

(1) Analysis of past clinical history of repeated chest infection (either upper or lower respiratory tract infection) and the degree of immunosuppression (by reviewing the level of immunosuppressive drugs in case of renal transplantation and CD4 count for HIV patients).
(2) Comprehensive clinical examination.
(3) Complete blood count, liver function tests, kidney function tests, and fasting blood sugar.
(4) Serum level of procalcitonin.
(5) Chest radiograph and high-resolution computed tomography chest.
(6) Septic workup (blood, sputum,urine culture, and sensitivity) [9].
(7) Induction of sputum for PJP staining (using Gomori methenamine silver staining) and Ziehl–Neelsen stain for acid-fast bacilli. Induction of sputum done by nebulization of 6 ml of 3% hypertonic saline three times daily, then percussing the back of the patients to potentiate them to expectorate [10].
(8) Sputum study for:
    (a) Gram stain for bacterial detection.
    (b) Ziehl–Neelsen stain (three successive days) for acid-fast bacilli.
    (c) Culture and sensitivity.
    (d) PCR for a panel of respiratory viruses.
(9) About 5–10 ml of venous blood was collected by sterile venipuncture, allowed to be clotted, and the sera were separated and kept frozen at −20°C until they were used for:
    (a) Galactomanan for aspergillus antigen detection.
    (b) PCR for cytomegalovirus, herpes simplex virus, and Epstein–Barr virus.
    (c) Serology for atypicals (*Mycoplasma* spp., *Legionella* spp., and *Chlamydia* spp.).
(10) Blood examination was done to examine:
    (a) Culture and sensitivity.
    (b) Fungal culture.
(11) Fibro-optic bronchoscopy (for some patients) for BAL and for PJP staining. Bronchoscopy was performed after written consent from the patients or their relatives. An Olympus BF260 videobronchoscope (Olympus Medical Systems Corporation, Tokyo, Japan) was used to perform airway evaluation and BAL was done, according to radiological assessment: if the disease is localized, BAL was done from the affected segment, and if the infiltrate is generalized in the whole lung field BAL was done from the middle lobe or lingual segment. BAL was performed using 120 ml of sterile saline in six equal aliquots, and the samples were sent for microbiological examination including PJP staining [11].
(12) In the respiratory samples (sputum and or BAL), both qualitative and qPCR were done. Qualitative PCR assay targeting the mitochondrial large subunit (mtLSU) rRNA gene of pneumocystis. Then *P. jirovecii* DNA was quantified. Plasmid suspensions were used as standards for quantification after being prepared by cloning the (mtLSU) rRNA insert into the plasmid vector. After propagation of the plasmids, the (mtLSU) rRNA gene copy number (number of copies per microliter) was derived. Results were expressed as numbers of *P. jirovecii* DNA copies per microliter of extracted DNA. The upper cutoff value of qPCR was 2×10^4 copies per microliter and the lower cutoff value was 1.6×10^3 copies per microliter.
(13) About 5 ml of venous blood was collected by sterile venipuncture, allowed to be clotted, and sera were separated and kept frozen at −20°C until they were used for detection of BD-glucan. Estimation of serum BD-glucan concentrations was calculated automatically by using a calibration curve. BD-glucan levels higher than 100 pg/ml were considered to be positive as defined by the manufacturer.

The patients included in this study were classified into three groups:

The first group (definite PJP), they were characterized by positive microscopic stain and positive qPCR for *P. jirovecii* in respiratory samples (BAL and/or induced sputum), positive serum BD-glucan, no evidence of bacterial pneumonia and those patients showed clinical improvement after giving anti-PJP treatment. The second group (pneumonia with *P. jirovecii* colonization), they were characterized by negative microscopic stain for *P. jirovecii* in respiratory samples, positive qPCR assay, negative serum BD-glucan, alternative diagnosis was documented like bacterial, viral, or fungal pneumonia. Those patients showed clinical improvement with other antimicrobial treatment (without anti-PJP treatment). The third group (pneumonia without colonization). They were characterized by negative detection of *P. jirovecii* in respiratory samples by microscopic stain and qPCR, negative serum BD-glucan, alternative diagnosis other than PJP was documented and clinical improvement was shown with other antimicrobial treatment.

Statistical analysis

Data analysis was performed using statistical package for the social sciences, version 8.0 (SPSS; SPSS Inc., Chicago, USA). The *t* test was used to evaluate the significance of differences between mean values of the study variables. The significance of differences between proportions was performed using the χ^2 test. Significant differences were expressed at *P* value less than 0.05.

Results

Seventy-five patients with clinical suspicion of PJP were studied. They were classified into three groups; the first group (definite PJP) included 25 patients. The second group (pneumonia with *P. jirovecii* colonization) included 20 patients. The third group (pneumonia without *P. jirovecii* colonization) consisted of 30 patients.

Significant differences were found between the three groups with respect to the inflammatory markers as shown in Table 1. Median lactate dehydrogenase level was 542 IU/l in the first group with a high significant difference when compared with other groups. Procalcitonin and C-reactive protein were significantly higher in the colonization group than the definite PJP group. There was no significant differences with respect tooxygen saturation between the different groups. Positive microscopic stain for *P. jirovecii* was found only in the definite PJP group (Table 1).

For the second group (pneumonia with *P. jirovecii* colonization), an alternative diagnosis was found as bacterial pneumonia (*n*=9), acute respiratory distress syndrome (*n*=2), atypical pneumonia (*n*=3), and viral

Table 1 Comparison between studied groups at the time of presentation

	n (%)			*P* value		
	Group 1 (definite PJP) (25 patients)	Group 2 (pneumonia with *P. jirovecii* colonization) (20 patients)	Group 3 (pneumonia without *P. jirovecii* colonization) (30 patients)	P_1	P_2	P_3
Age (years)	49 (36–62)	56 (45–71)	51 (42–67)	0.71	0.89	0.83
Sex						
Male	15 (60)	12 (60)	17 (56.6)	1.0	0.64	0.64
Female	10 (40)	8 (40)	13 (43.4)	1.0	0.71	0.71
Underlying diseases						
RTX	10 (40)	11 (55)	16 (53.3)	0.45	0.43	0.93
HIV	15 (60)	9 (45)	14(46.7)	0.46	0.45	0.98
CRP (mg/dl)	28 (13–79)	76 (47–165)	94 (54–197)	0.05	0.01	0.67
PCT (ng/ml)	0.7 (0.3–2.25)	7.9 (3.05–11.7)	10.3 (5.4–16.7)	0.03	0.05	0.11
LDH (IU/l)	542 (262–588)	314 (280–389)	208 (119–322)	0.05	0.01	0.12
O_2 saturation	20 (80)	15 (75)	23 (76.6)	0.23	0.21	0.98
Respiratory samples (BAL/IS) (*N*)	17/8	9/11	12/18	–	–	–
Positive microscopic stain of *P. jirovecii*	25 (100)	0 (0.0)	0 (0.0)	0.001	0.001	1.0

BAL, bronchoalveolar lavage; CRP, C-reactive protein; IS, induction sputum; LDH, lactate dehydrogenase; PCT, procalcitonin; PJP, *Pneumocystis jirovecii* pneumonia; RTX, renal transplant. P_1, group 1 versus group 2. P_2, group 1 versus group 3. P_3, group 2 versus group 3. The values for CRP, PCT, and LDH are presented as the median value then the range between brackets.

pneumonia (*n*=6). For the third group (pneumonia without *P. jirovecii* colonization), the alternative diagnosis was bacterial pneumonia (*n*=12), atypical pneumonia (*n*=7), drug-induced pneumonitis (*n*=2), alveolar hemorrhage (*n*=2), cryptogenic organizing pneumonia (*n*=3), and viral pneumonia (*n*=4).

The qPCR for *P. jirovecii* DNA in respiratory samples are shown in Table 2, the upper cutoff value of qPCR was 2×10^4 copies per microliters and the lower cutoff value of qPCR was 1.6×10^3 copies per microliters.

Twenty-one (84%) patients from the definite PJP group showed high levels of qPCR (as discussed above, the level of upper cutoff value) with highly significant differences when compared with the other two groups. Four (16%) patients in the first group and 20 (100%) patients in the second group showed levels between the upper cutoff value and lower cutoff value of qPCR and there were significant differences when compared with the third group. All patients (100%) of the third group were below the lower cutoff value of qPCR and high significant differences were observed when compared with the other two groups (Table 2). At cutoff value of 1.6×10^3, the sensitivity and specificity of qPCR for differentiation of PJP infection from colonization were 100 and 64%, respectively.

With respect to serum BD-glucan levels, we found significantly higher levels of the BD-glucan in the definite PJP when compared with the other two groups (Table 2).

All patients of the first group presented with serum BD-glucan levels higher than 100 pg/ml. Only four patients in the colonization group showed BD-glucan levels, which ranged between 80 and 100 pg/ml. All patients in the third group had serum BD-glucan levels lower than 80 pg/ml. The positive threshold defined by the laboratory for serum BD-glucan was 100 pg/ml (Fig. 1).

Discussion

P. jirovecii is a transmissible fungus that causes severe pneumonia (PJP), especially in immunecompromised patients, which include organ transplanted patients and HIV-infected patients. The diagnosis was mainly based on microscopic visualization of cysts in the respiratory samples. In common, the fungal load in the respiratory samples can be low, which makes the microscopic stain negative, so detection of the fungus requires highly sensitive techniques, such as DNA amplification (PCR assay) [6].

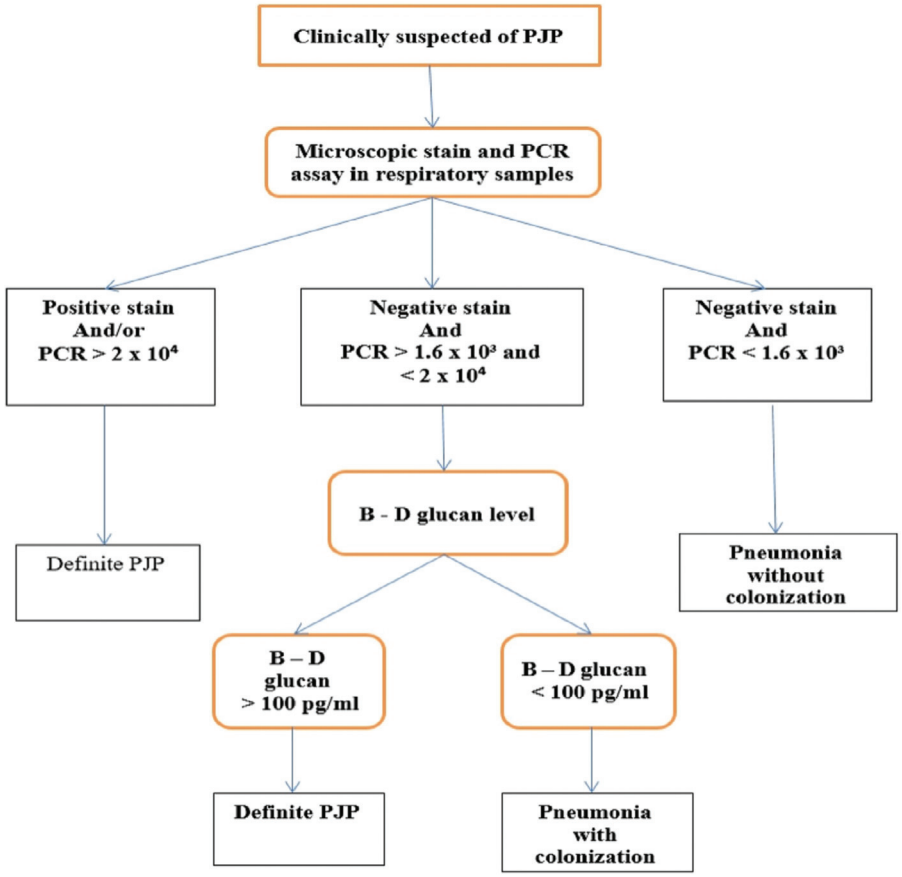
Pneumocystis colonization was defined as detection of the organism or its DNA without signs or symptoms of pneumonia [12]. In the current study, some patients with radiological pulmonary infiltrates, who were PCR positive for *P. jirovecii*, were diagnosed as having pneumonia caused by other etiologies rather than PJP (an alternative diagnosis was found). They were considered as having pneumonia with *P. jirovecii* colonization. Therefore, positive PCR results cannot differentiate between PJP and colonization because it is a qualitative assay. Many studies supported the development of qPCR assays for quantification of *P. jirovecii* in the respiratory samples [13,14]. Many studies reported cutoff values for *P. jirovecii* DNA copy numbers to differentiate PJP from colonization [8,15]. As shown in Table 2, our study showed that

Table 2 Comparison of quantitative PCR and 1,3 BD-glucan in the studied groups

	n (%)			P value		
	Group 1 (definite PJP) (25 patients)	Group 2	Group 3 (pneumonia without *P. jirovecii* colonization) (30 patients)	P_1	P_2	P_3
Quantitative PCR (copies/μl)						
$>2\times10^4$ (upper cutoff value)	21 (84)	0 (0)	0 (0)	0.000	0.000	1.0
1.6×10^3–2×10^4 (gray zone)	4 (16)	20 (100)	0 (0)	0.001	0.05	0.000
$<1.6\times10^3$ (lower cutoff value)	0 (0)	0 (0)	30 (100)	1.0	0.000	0.000
BD-glucan						
>100 pg/ml (upper cutoff value)	25 (100)	0 (0)	0 (0)	0.000	0.000	1
80–100 pg/ml	0 (0)	4 (20)	0 (0)	0.05	1	0.34
<80 pg/ml (lower cutoff value)	0 (0)	16 (80)	30 (100)	0.001	0.000	0.12

PJP, *Pneumocystis jirovecii* pneumonia. P_1, group 1 versus group 2. P_2, group 1 versus group 3. P_3, group 2 versus group 3.

Figure 1



Flow diagram describing the steps of diagnosing *Pneumocystis jirovecii* infection and differentiating definite PJP, pneumonia with colonization and pneumonia without colonization. PJP, *Pneumocystis jirovecii* pneumonia.

qPCR assay was significantly higher in the first group than the other groups. However, the cutoff values differed between the different researches due to differences in the laboratory methods [5,16]. Other studies determined a single cutoff value for *P. jirovecii* DNA copy numbers [8]. Applying different single cutoff values, the sensitivity and specificity were found to be changed. Other studies increased the specificity and sensitivity of qPCR assays for PJP diagnosis by applying two cutoff values [16]. The value by which the diagnosis of PJP can be ruled out is the lower cutoff value for qPCR, therefore the diagnosis of pneumocystis colonization can be established. On the other, the value above which the diagnosis of PJP can be confirmed is the upper cutoff value for qPCR. However, there is a gray zone between these two cutoff values. Values in this gray zone cannot identify infection from colonization. The current study revealed that the sensitivity and specificity of qPCR were 100 and 64%, respectively, and these results were consistent with that found by Matsumura *et al.* [8]. The low specificity of qPCR assay could be explained by its inability to discriminate live from the dead organism.

These findings enabled us to search for a more accurate assay for differentiation between infection and colonization. BD-glucan assay provided a more accurate test. All patients classified as the definite PJP group showed serum BD-glucan levels higher than 100 pg/ml [17]. On the other colonization can be ruled out with serum BD-glucan levels lower than 80 pg/ml. In the current study, we had a third group of patients who were classified to have pneumonia without colonization based on the negative microscopic staining and negative results for both qPCR assay and BD-glucan assay. In our study, positive BD-glucan with positive qPCR assays results and negative results of microscopic stain, could be explained by low load of *P. jirovecii* and absence of cysts in the lungs. Desmet *et al.* [18] found that the specificity of the BD-glucan assay for PJP diagnosis was higher when the serum level of BD-glucan above 100 pg/ml was taken as a cutoff value. Our results are consistent with these findings as all PJP patients had serum levels of BD-glucan more than 100 pg/ml, whereas the colonization group patients had negative BD-glucan results (<100 pg/ml) with negative microscopic stain and qPCR levels in the

gray zone. The sensitivity and specificity of BD-glucan for discriminating the definite PJP from the colonized *P. jirovecii* were 100 and 96%, respectively; these findings were in line with the results found by Damiani *et al.*, [6]. This means that positive BD-glucan with high clinical suspicion of PJP was highly sensitive to confirm pneumocystis infection. The BD-glucan assay showed higher specificity than qPCR. BD-glucan could be positive for other fungal infection, so its interpretation should be in the view of clinical scenario, furthermore, it should be taken into consideration that the value of more than 100 pg/ml is a more accurate cutoff value for better discrimination between infection and colonization [6], other studies showed that a cutoff value of BD-glucan higher than 80 pg/ml was considered positive [19].Moreover, in this study, we found that the alternative diagnosis of the colonized patients included bacterial pneumonia (n=9), acute respiratory distress syndrome due to sepsis (n=2), atypical pneumonia (n=3), and viral pneumonia (n=6). Whereas the final diagnoses of the noncolonized patients, included, bacterial pneumonia (n=12), atypical pneumonia (n=7), drug-induced pneumonitis (n=2), alveolar hemorrhage (n=2), cryptogenic organizing pneumonia (n=3), and viral pneumonia (n=4). For differentiation between PJP and colonization by using two cutoff values for qPCR assay in combination with a threshold value of 100 pg/ml for BD-glucan level, an accurate identification of our patients either to PJP or colonization can be established easily [6].

Conclusion

Both qPCR assay and BD-glucan assay could be used as markers for detection of *P. jirovecii* infection, but BD-glucan assay was more specific than qPCR in discriminating definite PJP from pneumonia with *P. jirovecii* colonization.

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Conflicts of interest

There are no conflicts of interest.

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