

## *Pneumocystis jirovecii* (*Pj*) quantitative PCR to differentiate *Pj* pneumonia from *Pj* colonization in immunocompromised patients

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**Abstract** Conventional polymerase chain reaction (PCR) in respiratory samples does not differentiate between *Pneumocystis* pneumonia (PCP) and *Pneumocystis jirovecii* (*Pj*) colonization. We used *Pj* real-time quantitative PCR (qPCR) with the objective to discriminate PCP from *Pj* colonization in immunocompromised patients. All positive *Pj* qPCR [targeting the major surface glycoprotein (MSG) gene] obtained in respiratory samples from immunocompromised patients presenting pneumonia at the Grenoble University Hospital, France, were collected between August 2009 and April 2011. Diagnoses were retrospectively determined by a multidisciplinary group of experts blinded to the *Pj* qPCR results. Thirty-one bronchoalveolar lavages and four broncho aspirations positive for the *Pj* qPCR were obtained from 35 immunocompromised patients. Diagnoses of definite, probable, and possible PCP, and pneumonia from another etiology

were retrospectively made for 7, 4, 5, and 19 patients, respectively. Copy numbers were significantly higher in the “definite group” (median 465,000 copies/ml) than in the “probable group” (median 38,600 copies/ml), the “possible group” (median 1,032 copies/ml), and the “other diagnosis group” (median 390 copies/ml). With the value of 3,160 copies/ml, the sensitivity and specificity of qPCR for the diagnosis of PCP were 100 % and 70 %, respectively. With the value of 31,600 copies/ml, the sensitivity and specificity were 80 % and 100 %, respectively. The positive predictive value was 100 % for results with more than 31,600 copies/ml and the negative predictive value was 100 % for results with fewer than 3,160 copies/ml. qPCR targeting the MSG gene can be helpful to discriminate PCP from *Pj* colonization in immunocompromised patients, using two cut-off values, with a gray zone between them.

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### Introduction

The incidence of *Pneumocystis* pneumonia (PCP) in human immunodeficiency virus (HIV)-infected patients has decreased since the introduction of chemoprophylaxis and antiretroviral therapy [1]; meanwhile, the incidence of PCP in non-HIV immunocompromised patients is increasing [2]. The standard laboratory method to diagnose PCP remains the microscopic identification of *Pneumocystis jirovecii* (*Pj*) by staining methods in respiratory samples. However, studies highlighted the low burden of *Pj* in non-HIV immunocompromised patients [3] and the lack of sensitivity of microscopic methods [4]. This justifies the increasing use of polymerase chain reaction (PCR) methods for diagnosis [5, 6]. The detection of *Pj* in individuals presenting without pneumonia or with pneumonia from another etiology has been defined as colonization or “carriage” [7]. Conventional *Pj* PCR is qualitative and very

sensitive, but does not differentiate between active PCP and *Pj* colonization.

The aim of this study was to use *Pj* real-time quantitative PCR (qPCR) in order to differentiate PCP and *Pj* colonization in immunocompromised patients.

## Patients and methods

### Patients and clinical samples

All positive *Pj* qPCR obtained in respiratory samples from immunosuppressed patients presenting with pneumonia at the Grenoble University Hospital, France, were collected between August 2009 and April 2011. We retrospectively studied the clinical histories of the concerned patients.

### Clinical data

The collected clinical data included: age, sex, underlying diseases, immunosuppression therapy during the previous months, PCP prophylaxis, clinical symptoms, routine laboratory data, anti-pneumocystis treatment, stay in a critical care unit, mechanical ventilation, hypoxemia ( $\text{PaO}_2 < 70$  mmHg in room air or requirement for supplemental oxygen), use of other anti-infectious agents, and outcome.

### Staining methods

The bronchoalveolar lavage (BAL) and broncho aspirations samples were stained and examined by a qualified microscopist at the Laboratory of Parasitology-Mycology, with 400  $\mu\text{l}$  of fluid analyzed by Fast Giemsa and 800  $\mu\text{l}$  observed after Gomori–Grocott staining.

### Real-time qPCR

A DNA extract solution from each sample was tested with a *Pj* qPCR targeting the major surface glycoprotein (MSG) gene of *Pj* as previously described [4, 8]. The results were expressed as the number of MSG copies/ml of BAL fluid.

### Diagnosis of *Pj* pneumonia and *Pj* colonization

A group of six experts, including three infectious diseases specialists, one internist, one hematologist, and one mycologist, classified the clinical histories of these patients in one of the following categories: definite, probable, and possible PCP, or other diagnosis (case definitions in Table 1). The experts were blinded to the *Pj* PCR results.

### Statistical analysis

Statistical analyses were performed with the StatView software (for Windows, SAS Institute Inc., Cary, NC, USA). Categorical variables were compared using the Fisher's exact test. Continuous variables were compared using the Kruskal–Wallis test. A receiver operating characteristic (ROC) curve for copy numbers was constructed and used to define cut-off values in order to discriminate the definite and probable PCP groups from the colonized group (possible PCP group and other diagnosis). A  $p$ -value  $< 0.05$  was considered statistically significant. The Youden's index ( $\text{Se} + \text{Sp} - 1$ ) was calculated for each cut-off value, as well as the number of correctly classified patients, in order to determine an optimal cut-off value.

## Results

Thirty-one BAL samples and four broncho aspirations with positive *Pj* qPCR were obtained from 35 immunocompromised patients. Broncho aspirations were used when BAL could not be performed. All of these patients had clinical presentations such as fever, cough, sputum, or dyspnea, and suspected pneumonia.

Seven patients met all the criteria for definite PCP, four patients for probable PCP, and five for possible PCP; another diagnosis was finally made for 19 patients. Prophylaxis therapy was used in one of the 35 patients (2 %). All definite,

**Table 1** Diagnosis definitions used by the experts

Definite PCP	- clinical signs of progressive pneumonia - ground glass opacities in chest computed tomography - microscopical identification of <i>Pj</i> with Musto or RAL staining methods
Probable PCP	- clinical signs of progressive pneumonia - ground glass opacities in chest computed tomography - complete resolution of symptoms after a full course of anti-PCP treatment - absence of microscopical identification of <i>Pj</i> with staining methods
Possible PCP	- clinical signs of progressive pneumonia and: - either compatible radiological signs - or complete resolution of symptoms after anti-PCP treatment - absence of microscopical identification of <i>Pj</i> with staining methods
Other diagnosis	none of the above criteria

The “definite PCP” and “probable PCP” groups were then re-assigned to the “final diagnosis of PCP” group, and the “possible PCP” and “other diagnosis” groups were re-assigned to the “non-PCP” group in order to construct a receiver operating characteristic (ROC) curve

possible, and probable patients had received anti-PCP treatment. Microscopic examination was positive in 8 of 32 samples of BAL fluid samples (one patient's retained diagnosis was allergic bronchopulmonary aspergillosis).

### Clinical features

The clinical features for each group are described in Table 2. There was no statistical difference between the four groups.

### Real-time qPCR

qPCR copy numbers were significantly higher in the definite group (median 465,000 copies/ml, range 99,900–750,290,000) than in the probable group (median 38,600 copies/ml, range 7,750–148,982) and the possible group (median

1,032 copies/ml, range 731–13,000) or the other diagnosis group (median 390 copies/ml, range 145–2,833) (Fig. 1). The difference was significant between the four groups ( $p < 0.0001$ ).

ROC curve analysis gave an area under the curve of 0.97 (Fig. 2), confirming the ability of qPCR to discriminate PCP from colonization. The sensitivity and specificity of qPCR on BAL fluids for discrimination between PCP and colonization were estimated with different qPCR cut-off values. Two cut-off values were determined to give 100 % positive and negative predictive values, with a gray zone between them. The estimated cut-off values were 31,600 and 3,160 copies/ml, respectively. The sensitivity and specificity of qPCR for the diagnosis of PCP are specified in Fig. 2 for each cut-off value. The Youden's index and the rate of correctly classified patients for each cut-off value provided an optimal cut-off value of 36,100 copies/ml for the diagnosis of PCP (Fig. 3).

**Table 2** Clinical features

Characteristics	Definite PCP <sup>a</sup> ( <i>n</i> =7)	Probable PCP ( <i>n</i> =4)	Possible PCP ( <i>n</i> =5)	Other diagnosis <sup>d</sup> ( <i>n</i> =19)	<i>p</i> -Value
Age, years	66 (47–69)	66 (52–75)	65 (60–67)	49 (42–66)	0.34
Male sex	3 (43 %)	1 (25 %)	2 (40 %)	9 (47 %)	0.874
Underlying disease					
Hematological malignancy	4 (57 %)	4 (100 %)	4 (80 %)	13 (68 %)	0.46
Solid malignancy	1 (14 %)	0 (0 %)	0 (0 %)	1 (5 %)	0.68
Organ transplantation	0 (0 %)	0 (0 %)	0 (0 %)	4 (21 %)	0.28
HIV <sup>b</sup> infection	2 (29 %)	0 (0 %)	0 (0 %)	1 (5 %)	0.2
Inflammatory disease	0 (0 %)	0 (0 %)	1 (20 %)	0 (0 %)	0.1
Corticosteroids	2 (29 %)	1 (25 %)	2 (40 %)	4 (21 %)	0.85
Anti-tumor chemotherapy	2 (29 %)	2 (50 %)	2 (40 %)	11 (58 %)	0.59
Immunosuppressive agents other than corticosteroids or chemotherapy	1 (14 %)	1 (25 %)	2 (40 %)	3 (16 %)	0.64
PCP prophylaxis	0 (0 %)	0 (0 %)	0 (0 %)	1 (5 %)	0.83
Hypoxia	7 (100 %)	3 (75 %)	5 (100 %)	11 (58 %)	0.19
Stay in ICU <sup>c</sup>	2 (29 %)	2 (50 %)	0 (0 %)	5 (26 %)	0.39
Anti-PCP treatment	7 (100 %)	4 (100 %)	5 (100 %)	12 (63 %)	0.06
Mechanical ventilation	2 (29 %)	0 (0 %)	0 (0 %)	5 (26 %)	0.38
Laboratory findings					
Leucocytes, /mm <sup>3</sup>	2,100 (1,700–9,825)	7,150 (6,600–8 750)	9,100 (750–10 025)	3,700 (2,325–5,900)	0.4
Neutrophils, /mm <sup>3</sup>	1,400 (1,125–5,650)	5,850 (5,200–7,250)	4,650 (1,800–8,150)	2,600 (2,200–5,525)	0.28
Lymphocytes, /mm <sup>3</sup>	400 (225–600)	950 (450–1,300)	400 (350–2,150)	500 (225–800)	0.77
C-reactive protein, mg/dl	11.8 (2.6–17.9)	10.5 (4.95–14.1)	10.7 (4.5–20)	9.3 (3.9–6.2)	0.99
Creatinine, mg/dl	0.8 (0.61–1)	0.84 (0.74–1.37)	0.71 (0.6–1.3)	0.97 (0.63–1.1)	0.91
Death	0 (0 %)	0 (0 %)	0 (0 %)	3 (16 %)	0.43

Data are presented as *n* (%) or median (interquartile range)

<sup>a</sup> *Pneumocystis pneumonia*

<sup>b</sup> Human immunodeficiency virus

<sup>c</sup> Intensive care unit

<sup>d</sup> The “other diagnoses” group included bacterial pneumonia (*n*=2), atypical pneumonia (*n*=2), influenza virus pneumonia (*n*=1), acute respiratory distress syndrome (ARDS) due to sepsis (*n*=1), candidemia (*n*=1), bronchiolitis obliterans organizing pneumonia (*n*=1), drug-induced pneumonia (*n*=1), bronchial hamartoma (*n*=1), multifactorial pulmonary fibrosis (*n*=1), allergic bronchopulmonary aspergillosis (*n*=1), pulmonary lymphoma (*n*=2), and undetermined (*n*=5)

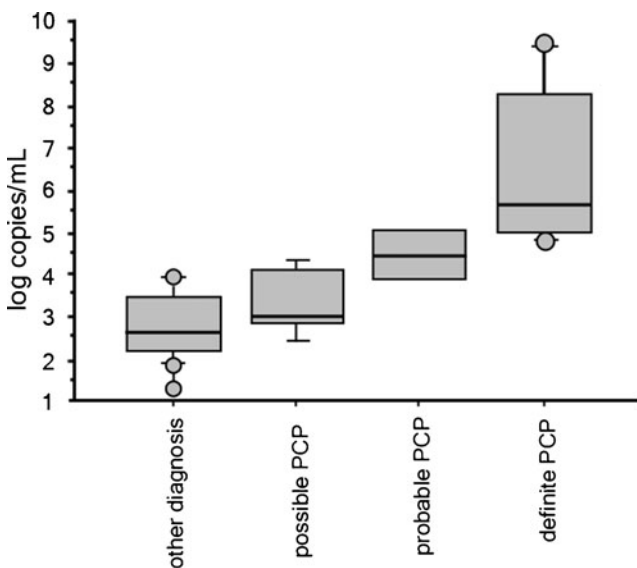
## Discussion

The diagnostic performance of the microscopic visualization of *Pj* is dependent on the quality and type of sample, the number of organisms, and the experience of the microscopist [9]. The higher sensitivity of PCR for *Pj* detection has been demonstrated previously [4]. The main risk of using conventional PCR for the diagnosis of PCP is a positive result for colonized patients having pneumonia from another etiology [7, 10–17].

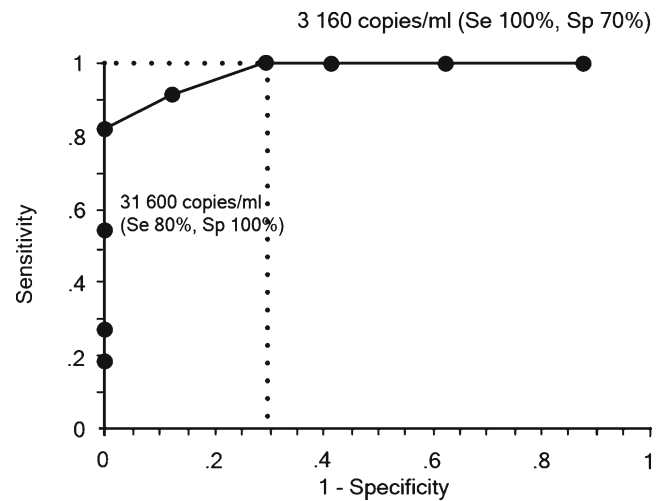
In this study, we observed that the qPCR results in the probable PCP group were significantly higher than those in the possible PCP group and in the group with another etiology, and were significantly lower than those in the definite PCP group. These results suggest that qPCR can help discriminate PCP from colonization and are in concordance with the hypothesis that the burden of *Pj* is lower in colonized people than in patients with PCP [18]. This is particularly interesting for non-HIV-infected immunocompromised patients, who often present with PCP with a low burden of *Pj* organisms and may not be diagnosed using microscopic examination [3].

qPCR values between 3,160 and 31,600 copies/ml fall within a gray zone, in which curative or prophylactic treatment should be discussed individually. It included nine out of the 35 (25 %) patients. Nevertheless, the Youden's index and the rate of correctly classified patients allowed determining the optimal cut-off value of 31,600 copies/ml, maximizing the accuracy for the diagnosis of PCP.

A recent study concluded that early diagnosis and treatment are crucial for the survival of PCP patients without HIV infection [19], suggesting that a rapid and accurate PCP diagnosis method such as qPCR should be used in these patients



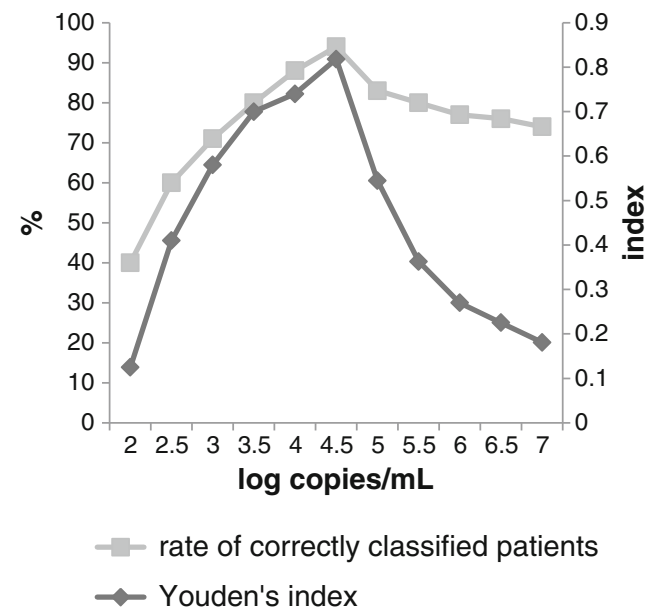
**Fig. 1** Box and whisker plots of *Pneumocystis jirovecii* copy numbers according to patient group. The boxes contain 50 % of the sample data, with the median value indicated by a horizontal bar



**Fig. 2** Receiver operator characteristic (ROC) curve of *P. jirovecii* copy numbers for the diagnosis of *Pneumocystis* pneumonia (PCP). The arrows indicate cut-off values of 3,160 copies/ml, and 31,600 copies/ml

with a low burden of *Pj*. In our study, the cut-off value of 3,160 copies/ml would have avoided 13 of 17 (76 %) treatments prescribed to patients whose final diagnosis was not PCP. This cut-off would allow physicians to stop probabilistic treatment, and, therefore, limit adverse effects, cost of treatment, and development of resistances [7, 20].

A meta-analysis showed that PCR has good diagnosis accuracy and may be a useful tool for the diagnosis of PCP in immunocompromised patients [21]. Previous studies determined various cut-offs of qPCR targeting different genes to discriminate PCP from colonization: for MSG gene, Larsen et al. proved that a cut-off value of 50 copies/tube for qPCR in



**Fig. 3** Rate of correctly classified patients and Youden's index for each cut-off value

oral-wash samples increased the specificity for diagnosis of PCP to 100 % [18]. Flori et al.'s study established that a cut-off of  $10^3$  copies of DNA per capillary of BAL (PCR targeting the MSG gene) increased the specificity from 84.9 to 98.6 % with 100 % sensitivity [4]. Other studies highlighted the usefulness of qPCR in discriminating colonization from PCP: Alanio et al. proposed two cut-off values (120 and 1,900 trophic forms equivalents/ml) with a qPCR targeting the mitochondrial large subunit ribosomal RNA gene [22]; for Matsumura et al., the value of 1,300 copies/ml distinguished PCP from colonization with a sensitivity of 100 % and a specificity of 80 %, using qPCR targeting the DHPS (dihydropteroate synthase) gene [23].

We need to consider limitations when interpreting our results: this is a single-center study, the number of patients is relatively small, and we combined the data from the BAL and the broncho aspiration samples to evaluate the qPCR. Without a test capable of confirming or excluding the diagnosis of PCP, the classification of patients is uncertain. However, one quality of our study is that patients were classified by a multidisciplinary group of experts, ruling by consensus in view of all the clinical and complementary data. The fact that the experts did not know the qPCR results avoids classification bias. In spite of the small number of patients, the observed differences between the groups are statistically significant, which suggests that these differences are important. In our study, we used qPCR targeting the MSG gene, which is appropriate in discuss PCP diagnosis: the sensitivity is excellent, and it has been proved that qPCR analysis and the MSG target have the highest specificity compared with other PCR assays [24].

We can note that the rate of anti-pneumocystis prophylaxis was very low in our population. There is no current recommendation for anti-PCP prophylaxis or empirical therapy for non-HIV immunocompromised patients. It is known that colonization can sometimes lead to the development of PCP [7]. We propose to treat patients with a high probability of PCP and *Pj* burdens in the gray zone, and to monitor patients with low burdens and low probability of PCP with biological markers such as  $\beta$ -D-glucan, while prescribing a prophylactic treatment [23, 25, 26]. The proportion of patients having a hematological malignancy was very high in our population. We hypothesize that cut-off values may differ with the underlying diseases and may vary from one center to another.

In conclusion, our study shows that our qPCR targeting the MSG gene in respiratory samples can help discriminate PCP from pneumonia with *Pj* colonization in immunocompromised patients, using two cut-off values of 3,160 and 31,600 copies/ml, with a gray zone between them. Cut-off values should be determined for each laboratory and each population of patients.

**Conflict of interest** The authors declare they have no conflict of interest.

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