Real-time PCR assay-based strategy for differentiation between active *Pneumocystis jirovecii* pneumonia and colonization in immunocompromised patients

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Abstract

Diagnosis of pneumocystosis usually relies on microscopic demonstration of *Pneumocystis jirovecii* in respiratory samples. Conventional PCR can detect low levels of *P. jirovecii* DNA but cannot differentiate active pneumonia from colonization. In this study, we used a new real-time quantitative PCR (qPCR) assay to identify and discriminate these entities. One hundred and sixty-three bronchoalveolar lavage fluids and 115 induced sputa were prospectively obtained from 238 consecutive immunocompromised patients presenting signs of pneumonia. Each patient was classified as having a high or a low probability of *P. jirovecii* pneumonia according to clinical and radiological presentation. Samples were processed by microscopy and by a qPCR assay amplifying the *P. jirovecii* mitochondrial large-subunit rRNA gene; qPCR results were expressed as trophic form equivalents (TFEq)/mL by reference to a standard curve obtained from numbered suspensions of trophic forms. From 21 samples obtained from 16 patients with a high probability of *P. jirovecii* pneumonia, 21 were positive by qPCR whereas only 16 were positive by microscopy. Fungal load ranged from 134 to 1.73×10^6 TFEq/mL. Among 257 specimens sampled from 222 patients with a low probability of *P. jirovecii* pneumonia, 222 were negative by both techniques but 35 were positive by qPCR (0.1–1840 TFEq/mL), suggesting *P. jirovecii* colonization. Two cut-off values of 120 and 1900 TFEq/mL were proposed to discriminate active pneumonia from colonization, with a grey zone between them. In conclusion, this qPCR assay discriminates active pneumonia from colonization. This is particularly relevant for patient management, especially in non-human immunodeficiency virus (HIV)-infected immunocompromised patients, who often present low-burden *P. jirovecii* infections that are not diagnosed microscopically.

Keywords: Colonization, immunocompromised host, *Pneumocystis jirovecii*, pneumonia, real-time PCR
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Introduction

Pneumocystis pneumonia (PCP) is an opportunistic infection caused by the ascomycetous fungus *Pneumocystis jirovecii* [1]. Human immunodeficiency virus (HIV)-infected individuals with low CD4 counts are at considerable risk of developing PCP. Despite the extensive use of co-trimoxazole prophylaxis and marked advances in the control of HIV infection, resulting in immune reconstitution, PCP remains one of the most prevalent infections in patients with AIDS [2]. PCP also occurs in non-HIV-infected immunocompromised (IC) patients, including individuals receiving immunosuppressive medication for autoimmune or inflammatory diseases, patients with haematological or solid malignancies, and transplant recipients [2–7]. In non-HIV-infected IC patients, PCP is typically more acute and severe than in patients with AIDS. Overall, PCP carries a mortality rate of 35–55% in patients without AIDS [8], as compared with 10–20% in patients with AIDS [9].

Diagnosis of pneumocystosis usually relies on microscopic demonstration of *P. jirovecii* in bronchoalveolar lavage (BAL) fluid or induced sputum samples by the use of staining methods. However, the lack of sensitivity of microscopic methods, owing to the low burden of *P. jirovecii* in non-HIV-infected IC patients, has justified the increasing use of PCR-based methods for diagnosis [10]. These methods also revealed the possibility of *P. jirovecii* lung colonization in HIV-infected and non-HIV-infected IC patients, defined as the

detection of *Pneumocystis* organisms or their DNA in pulmonary specimens from individuals without clinical signs or symptoms of PCP [11–16].

Conventional PCR methods can detect low levels of *P. jirovecii* DNA, but are not quantitative and cannot differentiate PCP from colonization. Real-time quantitative PCR (qPCR), a more appropriate method for DNA quantification, has been developed for the diagnosis of PCP [17–20]. However, interpretation of fungal burden estimates by qPCR remains puzzling with regard to patient management.

In this study, we aimed to assess the ability of a new qPCR method to discriminate PCP from *P. jirovecii* colonization in non-HIV-infected and HIV-infected IC patients.

Materials and Methods

Patients and clinical samples

Samples were prospectively collected during a 7-month period from 238 consecutive IC patients, for whom a search for *P. jirovecii* was performed in a context of undetermined pneumonia. They consisted of 163 BAL fluids and 115 induced sputa (IS). Patients' clinical characteristics are presented in Table 1. Sixty-nine patients were HIV-infected and 169 were non-HIV-infected IC patients.

Fibreoptic bronchoscopy was performed after patients gave their informed consent. The site of BAL was guided by lung high-resolution computed tomography, and BAL was performed with four 50-mL aliquots of sterile saline solution following a standardized protocol. IS were obtained with the help of experienced respiratory physiotherapists.

TABLE 1. Patient characteristics

Characteristic	All patients (n = 238)	PCP patients (n = 16)
Age (years), median (range)	48 (7–88)	42 (22–82)
Sex, male/female	160/78	12/4
Underlying diseases		
HIV infection	69	9
Haematological malignancies (including 35 allogeneic HSCT)		
Lymphoma	47	2
Chronic lymphocytic leukaemia	12	0
Acute lymphoblastic leukaemia	7	0
Acute myeloid leukaemia	24	0
Myeloproliferative syndrome	8	0
Myelodysplasia	8	0
Myeloma	9	0
Haemolytic anaemia	3	1
Solid organ transplantation		
Kidney transplantation	14	2
Kidney + pancreas transplantation	1	1
Solid malignancies	14	0
Autoimmune diseases with immunosuppressive regimens	22	1

HIV, human immunodeficiency virus; HSCT, haematopoietic stem cell transplantation; PCP, *Pneumocystis* pneumonia.

Clinical probability of PCP

As no international standardized score is available to classify patients for the diagnosis of PCP, we estimated the probability of PCP for each patient and allocated them to high-probability and low-probability groups. According to well-known risk factors for PCP and features suggesting PCP in HIV-infected and non-HIV-infected IC patients [5,21], the following data were recorded: type of immunodeficiency, long-term steroid therapy, immunosuppressive therapy, pneumocystosis prophylaxis, hypoxaemia, chest radiograph findings, high-resolution computed tomography scan findings, microscopic detection of *Pneumocystis*, other microbiological recovery on respiratory samples, aetiological treatment and favourable outcome after 14 days, and final diagnosis of the episode retained by the physician. Patient assignation was performed independently by two physicians, without knowledge of the results of qPCR.

Specimen processing and analysis

Respiratory specimens were centrifuged at 2800 ***g*** for 10 min. Part of the resuspended pellet (about 200 μL) was used for microscopic methods, and 200 μL was used for PCR. DNA extraction was performed by using a QIAamp DNA Mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions.

Microscopy diagnosis

Fifty to 75 μL of the resuspended pellet diluted in phosphate-buffered saline was cytocentrifuged at 1100 ***g*** for 5 min on each of three microscopy slides. The first slide was stained with Giemsa, and the second one was subjected to an indirect immunofluorescence (IIF) assay (Monofluo kit *Pneumocystis*; Bio-Rad, Marnes-la-Coquette, France). Both smears were examined by two experienced microscopists for the demonstration of cysts and/or trophic forms. In a few cases of negative microscopy results in patients presenting several other features in favour of an ongoing PCP, the third slide was subjected to a direct immunofluorescence assay (Monofluo *P. jirovecii* IFA test kit; Bio-Rad) and examined for the presence of trophic forms and cysts.

Real-time PCR assay

The real-time PCR assay developed for this study amplifies a 121-bp fragment of the *P. jirovecii* mitochondrial large-subunit rRNA gene. The primers PjF1 (5′-CTGTTTCCCTTTCGACTATCTACCTT-3′) and PjR1 (5′-CACTGAATATCTCGAGGGAGTATGAA-3′) and the TaqMan-MGB probe PjSL (5′-TCGCACATAGTCTGATTAT-3′) were designed by using Primer Express software (Applied Biosystems, Foster City, CA, USA). The qPCR assay was performed on an Applied