



Comparison of 2 real-time PCR assays for diagnosis of *Pneumocystis jirovecii* pneumonia in human immunodeficiency virus (HIV) and non-HIV immunocompromised patients

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ABSTRACT

A total of 120 bronchoalveolar lavage specimens from HIV and non-HIV immunocompromised patients, positive for *Pneumocystis jirovecii* by an “in house” real-time polymerase chain reaction (PCR), were evaluated by the Bio-Evolution *Pneumocystis* real-time PCR, a commercial quantitative assay. Patients were classified in 2 categories based on clinical and radiological findings: definite and unlikely *Pneumocystis* pneumonia (PCP). For the “in house” PCR, cycle threshold 34 was established as cut-off value to discriminate definite PCP from unlikely PCP with 65% and 85% of sensitivity and specificity, respectively. For the Bio-Evolution quantitative PCR, a cut-off value of 2.8×10^5 copies/mL was defined with 72% and 82% of sensitivity and specificity, respectively. Overlapped zones of results for definite and unlikely PCP were observed. Quantitative PCR is probably a useful tool for PCP diagnosis. However, for optimal management of PCP in non-HIV immunocompromised patients, operational thresholds should be assessed according to underlying diseases and other clinical and radiological parameters.

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1. Introduction

Pneumocystis jirovecii remains an important cause of pneumonia in acquired immune deficiency syndrome (AIDS) and other immunocompromised patients. In human immunodeficiency virus (HIV)-infected patients, *P. jirovecii* pneumonia (PCP) has decreased after the widespread use of highly active antiretroviral therapy and the use of routine prophylaxis against *P. jirovecii*; nevertheless, it remains one of the leading causes of morbidity and mortality (Walzer et al., 2008). Non-HIV immunocompromised patients (non-HIV patients) are also at risk for PCP including patients undergoing transplantation, patients with haematological malignancies, solid tumours, and connective tissue diseases (Roblot et al., 2002; Tasaka and Tokuda, 2012). Non-HIV patients with PCP differ in several clinical manifestations and laboratory aspects from those patients with HIV. In non-HIV patients, PCP usually develops more rapidly with severe respiratory failure (Nüesch et al., 1999; Roblot et al., 2002). In addition, non-HIV patients with PCP have a lower burden of *P. jirovecii* than those with AIDS resulting in false negative direct microscopic examination (Tasaka and Tokuda, 2012).

Direct microscopy examination (DME) by staining techniques such Giemsa or immunofluorescence-targeted antibodies have been the reference method for PCP diagnosis. The development of polymerase chain reaction (PCR) assays has markedly improved diagnostic accuracy that can be especially helpful in those cases of false-negative DME (Sing et al., 1999). However, 1 disadvantage of PCR assays is the detection of positive results in patients with asymptomatic carriage (Ribes et al., 1997). PCR has been crucial in the study of *Pneumocystis* colonisation, defined as the detection of *Pneumocystis* organisms or their DNA in pulmonary specimens from individuals without signs or symptoms of pneumonia. One example is the patients with chronic obstructive pulmonary diseases (COPDs) that have an increased prevalence of *Pneumocystis* colonisation (Gutierrez et al., 2011; Morris et al., 2008). Several studies have shown that the copy number of *P. jirovecii*, measured by real-time quantitative PCR, interpreted in parallel with clinical manifestations and radiological and laboratory findings, may be useful in discriminating colonisation and infection (Alanio et al., 2011; Botterel et al., 2012; Flori et al., 2004; Matsumura et al., 2012; Tia et al., 2012).

We investigated the ability of a commercial quantitative real-time PCR assay, the Bio-Evolution *Pneumocystis jirovecii* real-time PCR (Bio-Evolution qPCR) (Bry-sur-Marne, France), to discriminate PCP from *P. jirovecii* colonisation in HIV and non-HIV immunocompromised patients, in comparison with an “in house” TaqMan-based real-time PCR.

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2. Materials and methods

2.1. Patients and clinical samples

This study took place at the Erasme University Hospital in Brussels (Belgium), which counts 858 beds. Between January 2010 and September 2014, bronchoalveolar lavage specimens (BALs) from 1082 patients were collected at our hospital for diagnosis of PCP in the context of undetermined pneumonia. For each BAL, 3 aliquots of 50 mL were sampled and diluted in saline: the second or third aliquot was sent to the microbiology laboratory for PCP diagnosis. During this period, *P. jirovecii* was detected by our “in house” real-time PCR in 120 patients. Patients' characteristics were acquired retrospectively by medical chart review including: age, gender, underlying disease, immunosuppressive therapy and PCP prophylaxis during the month before sample collection, laboratory results, radiological presentation, and final clinical diagnosis.

2.2. Clinical probability of PCP

Patient classification was performed by an infectious disease physician with experience in infectious lung disease in immunocompromised hosts and blinded to quantitative PCR results obtained by commercial kit (Bio-Evolution *Pneumocystis* real-time PCR).

Definite PCP was diagnosed in patients receiving anti-pneumocystis treatment if all following additional characteristics were present: 1) compatibility of clinical signs (at least 2 symptoms: subtle onset of progressive dyspnoea, pyrexia, and nonproductive cough) amongst patients with underlying immunodeficiency; 2) presence of hypoxia (arterial oxygen saturation <93% and/or need for supplementary oxygen of >2 L/min and/or mechanical ventilation); 3) compatible radiological findings (chest radiograph and/or high-resolution computed tomographic scan findings of interstitial infiltrates); and 4) response to anti-pneumocystis treatment (favourable outcome in 7 days) with absence of alternative diagnosis (negative results on BAL for alternative pulmonary pathogens).

Unlikely PCP was diagnosed in 1) patients without all criteria described above, 2) patients with respiratory symptoms not typical for PCP or no typical radiological infiltrations, and 3) patients with treatment response to other antimicrobial agents or other definite diagnosis.

2.3. Microscopy diagnosis

The slides were prepared by cytocentrifugation and stained with rapid Giemsa-like staining (Diff Quick, Dade Behring, Germany). The smears were examined by experienced individuals for the presence of cysts and/or trophic forms.

2.4. DNA extraction

DNA was extracted from 200 µL of BAL samples, with a QIAamp DNA Blood Mini Kit (Qiagen, Westburg, The Netherlands) according to manufacturers' instructions (elution volume: 200 µL).

2.5. Determination of *P. jirovecii* by real-time PCR assays

Two real-time PCR assays were used for detection of *P. jirovecii* in this study.

2.5.1. “In house” real-time PCR

We performed an “in house” TaqMan-based real-time PCR selecting the beta-tubulin gene like target using the LC480 sequence detector as described before by Brancart et al. (2005). DICO Extra r-gene (Argene, Verniolle, France) was used as extraction and inhibition control for check the entire real-time process. Positive and negative controls were included in each PCR. The samples were tested in duplicate. The cycle threshold value (Ct value) was registered for positive samples.

2.5.2. Bio-Evolution *Pneumocystis* real-time PCR

The Bio-Evolution *Pneumocystis* real-time PCR kit is a commercially available qualitative and quantitative real-time PCR method that targets the *P. jirovecii* mitochondrial ribosomal large subunit. Real-time PCR was performed according to manufacturers' instructions using a LC480 sequence detector system. The quantified copy numbers were expressed as copy numbers per mL.

2.6. Statistical analysis

Qualitative variables were analysed using the chi-square test. Statistical assessment of differences of the means of *P. jirovecii* DNA concentration (Ct value or copies/mL) from definite PCP and unlikely PCP patients was performed by Mann–Whitney test. Correlation between 2 real-time PCR results was analysed by Spearman's coefficient of rank correlation test. A receiver operating characteristic (ROC) curve was constructed and used to define optimal cut-off values in order to discriminate the definite PCP from the unlikely PCP groups. The comparison of the area under the curve (AUC) of the 2 ROC curves and Kappa statistics were also analysed. A *P* value <0.05 was considered statistically significant. Statistical analysis was performed with GraphPad Prism 4 software (La Jolla, CA, USA) and MedCalc software (Mariakerke, Belgium).

3. Results and discussion

3.1. Characteristics of patients

P. jirovecii was detected in 120 patients by our “in house” real-time PCR. Definite PCP was diagnosed in 34 patients and unlikely PCP in 86 patients. Clinical and demographic characteristics and results of the different techniques used for the PCP diagnosis are shown in Table 1. Underlying disease and other characteristics of patients were not different from other studies (Li et al., 2014; Maillet et al., 2014; Nüesch et al., 1999; Ribes et al., 1997; Robert-Gangneux et al., 2014). The incidence of PCP in HIV patients has declined after widespread use of highly active antiretroviral therapy and PCP prophylaxis (Kaplan et al., 2000). The occurrence of PCP in patients who are compliant with

Table 1

Clinical and demographic characteristics of patients and results of different techniques used for PCP diagnosis.

Clinical characteristics and laboratory results	Definite (n = 34)	Unlikely (n = 86)	<i>P</i> value
Age (mean)	58	56	0.18
Male sex (%)	27 (79%)	60 (70%)	0.2
Underlying disease			
HIV infection (%)	8 (23%)	0	<0.001
Organ transplantation (%)	6 (17%)	35 (41%)	0.019
Renal transplantation	5	19	
Lung–heart transplantation	1	12	
Liver transplantation	0	1	
Solid malignancy (%)	4 (11%)	9 (10%)	1
Haematological malignancy (%)	4 (11%)	11 (13%)	1
Autoimmune disease (%)	4 (11%)	4 (4%)	0.22
COPD (%)	4 (11%)	9 (10%)	1
Acute alcoholic hepatitis (%)	1 (2%)	9 (10%)	0.27
Others	3 (9%)	9 (10%)	
Corticosteroids	23 (67%)	71 (82%)	0.07
Other immunosuppressive agents	9 (26%)	41 (47%)	0.034
Anti-tumour chemotherapy	9 (26%)	16 (18%)	0.3
Mortality ^a	5 (15%)	3 (3%)	0.02
Positive DME (Giemsa)	14 (41%)	0	<0.001
Mean Ct value “in house” PCR ^b (SEM)	32 (0.8)	37 (0.25)	<0.001
Mean copy number/mL Bio-Evolution PCR ^c (SEM)	2.5 × 10 ⁸ (1.2 × 10 ⁸)	4.7 × 10 ⁵ (1.4 × 10 ⁵)	<0.001

^a Mortality after 1 month of *P. jirovecii* detection.

^b Ct value obtained by “in house” real-time PCR.

^c Copy number per milliliter obtained by commercial *P. jirovecii* real-time PCR.

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