

Inter-laboratory comparison of three different real-time PCR assays for the detection of *Pneumocystis jiroveci* in bronchoalveolar lavage fluid samples

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Pneumocystis jiroveci pneumonia (PCP) is an opportunistic infection affecting immunocompromised patients. While conventional diagnosis of PCP by microscopy is cumbersome, the use of PCR to diagnose PCP has great potential. Nevertheless, inter-laboratory validation and standardization of PCR assays is lacking. The aim of this study was to evaluate the inter-laboratory agreement of three independently developed real-time PCR assays for the detection of *P. jiroveci* in bronchoalveolar lavage fluid samples. Therefore, 124 samples were collected in three tertiary care laboratories (Leiden University Medical Center, Maastricht Infection Center and Radboud University Nijmegen Medical Centre) and were tested by both microscopy and real-time PCR. Of 41 samples positive for *P. jiroveci* by microscopy, 40 were positive in all three PCR assays. The remaining sample was positive in a single assay only. Out of 83 microscopy-negative samples, 69 were negative in all three PCR assays. The other 14 samples were found positive, either in all three assays ($n=5$), in two ($n=2$) or in one of the assays ($n=7$). The data demonstrate high inter-laboratory agreement among real-time PCR assays for the detection of *P. jiroveci*.

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INTRODUCTION

Pneumocystis carinii is an opportunistic pathogen that was classified as a fungus in 1988 (Edman *et al.*, 1988). Although human-derived *P. carinii* (*P. carinii* f. sp. *hominis*) has recently been renamed *Pneumocystis jiroveci*, the abbreviation PCP (now referring to *Pneumocystis* pneumonia) remains in use (Stringer *et al.*, 2001). Patients at risk for *P. jiroveci* pneumonia can be divided into two categories: HIV positive and HIV negative. Several risk factors have been identified in the HIV-negative group, such as immunosuppressive medication, or an inherited or acquired immunodeficiency (Kovacs *et al.*, 2001). Since untreated PCP is

associated with a high morbidity and mortality (Sepkowitz, 2002; Yale & Limper, 1996), especially in HIV-negative patients, a rapid and reliable diagnosis is mandatory. Current diagnosis of PCP relies on tinctorial and/or immunofluorescent staining of induced sputum or bronchoalveolar lavage (BAL) fluid samples (Djamin *et al.*, 1998; Thomas & Limper, 2004). Using these methods, sensitivity and specificity rates are reached which exceed 95% (Amin *et al.*, 1992; Chandra *et al.*, 1988; Elvin *et al.*, 1988; Kovacs *et al.*, 2001). Major drawbacks of microscopy, however, are that it is cumbersome and requires trained microscopists. The latter makes it essential that positive samples are encountered regularly in order to maintain an expertise in microscopy. In recent years, the incidence of PCP has declined significantly, especially in HIV-positive patients. Due to the introduction of highly active anti-retroviral therapy, PCP chemoprophylaxis, and an increase of patients receiving chemotherapy, the future trend will be towards

Abbreviations: BAL, bronchoalveolar lavage; DHPS, dihydroperoxide synthase; LUMC, Leiden University Medical Center; MINC, Maastricht Infection Center; MSG, major surface glycoprotein; PCP, *Pneumocystis jiroveci* pneumonia; RUNMC, Radboud University Nijmegen Medical Centre.

samples with relatively low *P. jiroveci* burdens, making the diagnosis of PCP even more difficult (Limper *et al.*, 1989; Miller, 1999). Therefore, a rapid diagnostic technique which can identify the presence of a low number of cysts is needed.

Nucleic acid amplification tests, such as PCR, play an increasing role in the detection of *P. jiroveci* (Larsen *et al.*, 2002b; Wakefield *et al.*, 1990). In particular, real-time PCR is highly suitable for the diagnosis of PCP, since this technique allows the generation of quantitative results. This is crucial, as *P. jiroveci* may be present in low quantities in some asymptomatic individuals (Maskell *et al.*, 2003; Nevez *et al.*, 2001). Consequently, it is of utmost importance to be able to discriminate between asymptomatic carriage and clinically relevant infection. In recent years, molecular tests to detect *P. jiroveci* have shifted from research to diagnostic applications (Flori *et al.*, 2004; Larsen *et al.*, 2004; Olsson *et al.*, 2001). Nevertheless, quality control panels for validation and standardization of such tests are currently lacking. Therefore, a study was initiated to compare the performance of three independently developed real-time PCR assays for the detection of *P. jiroveci* in three different tertiary care centres in The Netherlands. This retrospective study assessed the routine diagnostic performance of these assays on a collection of BAL fluid samples of which the microscopic evaluation for the presence of *P. jiroveci* had already been performed.

METHODS

Design of the study. Three diagnostic medical microbiology laboratories participated in this study, i.e. Maastricht Infection Center (MINC), Radboud University Nijmegen Medical Center (RUNMC) and Leiden University Medical Center (LUMC). All three laboratories are university tertiary care centres located in the Netherlands. Each of these laboratories supplied *P. jiroveci*-positive and -negative samples. Diagnosis was based on microscopy. The materials were collected by one of the authors (C. F. M. Linssen),

encoded, and distributed to the other laboratories. Each laboratory received a set of samples and performed its own real-time PCR in a blinded fashion.

BAL fluid sampling and conventional diagnosis of PCP.

Samples obtained from patients suspected of PCP were collected over the period August 1999 to April 2004. Bronchoscopy with BAL was performed with sterile saline, in four aliquots of 50 ml (MINC), three aliquots of 20 ml (LUMC) and three aliquots of 50 ml (RUNMC). Samples were immediately transported to the laboratory and processed upon arrival. At the LUMC and RUNMC, aliquots of 10 ml were centrifuged at 3000 g and smears were made from the sediment. At the MINC, cytocentrifuged monolayer preparations were made as previously described (Jacobs *et al.*, 2001). Subsequently, slides were subjected to Giemsa and methenamine silver staining (LUMC), Giemsa and direct immunofluorescence staining (PneumoCel, CeLLabs) (RUNMC), or May-Grünwald Giemsa and methenamine silver staining (MINC).

Nucleic acid extraction and real-time PCR analysis.

At the MINC, 200 µl BAL fluid was used for DNA isolation using the Wizard Genomic DNA Purification kit (Promega). Purified DNA was resuspended in a final volume of 120 µl. A real-time PCR was designed that targeted the major surface glycoprotein (MSG) gene (Larsen *et al.*, 2002a). Assays were performed in 96-well Optical Reaction plates [Applied Biosystems (ABI)] in a 50 µl volume containing 0.6 µM each of primers PCPFor and PCPRev (Table 1), 0.15 µM probe PCPProbe, 1× TaqMan Universal Master Mix (ABI) and 20 µl purified DNA. Thermal cycling was carried out on an ABI PRISM 7000 Sequence Detection system (ABI) as follows: 2 min at 50 °C, 10 min at 95 °C, followed by 42 cycles of 15 s at 95 °C and 1 min at 60 °C. Quantification was performed using the ABI PRISM software and was based on extrapolation of data to standard curves, which were generated by amplification of quantified dilutions of the plasmid pPCP, which contains the *P. jiroveci* PCR fragment.

At the RUNMC, the MagnaPure LC Isolation station (Roche Applied Science) was used for nucleic acid isolation. A 100 µl aliquot was isolated using the Total Nucleic Acid isolation kit (Roche Applied Science). Nucleic acids were resuspended in 50 µl H₂O. Real-time PCR for *P. jiroveci* was performed as described by Larsen *et al.* (2002a) using the MSG gene as target. All reactions were performed in a volume of 20 µl, consisting of 1× LightCycler FastStart DNA Master

Table 1. Description of primers and probes used by the three laboratories

The sequences for primers and probes used by MINC and RUNMC were obtained from Larsen *et al.* (2002).

Laboratory	Target	Primer type*	Sequence (5'-3') (name)	GenBank accession no.	Position on gene
MINC	MSG	F	CAAAAATAACAYTSACATCAACRAGG (PCPFor)	AF372980	223-248
		P	FAM-TGCAAACCAACCAAGTGTACGACAGG-TAMRA (PCPProbe)		252-277
		R	AAATCATGAACGAAATAACCATTCG (PCPRev)		378-354
LUMC	DHPS	F	ATGATTCTATATTAATGGATGTGGAG (PJIRs)	AJ586567	148-173
		P	MB-CGCGCTGGGCGACGATAATTGATATTGGTGGAGCGCG-FAM (578PJIR)		190-214
		R	AGATATTTTATAGCAGGAATAACTCG (PJIRas)		290-268
RUNMC	MSG	F	GAATGCAAATCYTTACAGACAACA (JJK114/15)	AF37298	135-158
		P1	CAAAAATAACAYTSACATCAACRAGGC (PCMSGFRET1U)		223-248
		P2	TGCAAACCAACCAAGTGTACGACAGG (PCMSGFRET1D)		252-277
		R	AAATCATGAACGAAATAACCATTCG (JJK17)		378-354

*F, forward primer; P, probe; R, reverse primer.

Table 2. Origin of BAL fluid samples included in this study

Microscopy result for <i>P. jiroveci</i>	Patients at risk*		Patients not at risk		Total
	HIV positive	HIV negative	Ventilator-associated pneumonia	Sarcoidosis†	
Positive	21	20	0	0	41
Negative	10	33	20	20	83

*HIV-infected patients, or patients with a known risk factor for PCP, such as haematological malignancy, bone marrow or organ transplantation, Wegener's granulomatosis, and immunosuppressive or corticosteroid therapy.

†Sarcoidosis patients were not receiving immunosuppressive therapy or corticosteroids.

Hybridization Probes reaction mixture (Roche), 4 mM MgCl₂, 1.0 µM each of primers JKK14/15 and JKK17 (Table 1), 0.2 µM each of probes PCMSGFRET1U and PCMSGFRET1D, and 5 µl template. The PCR thermal profile consisted of an initial incubation at 95 °C for 10 min, followed by a touch-down procedure, consisting of 11 cycles of 5 s at 95 °C and 10 s at temperatures decreasing from 65 to 50 °C. This was followed by 35 cycles of 5 s at 95 °C and 10 s at 50 °C, and a final step of 15 s at 72 °C. Amplification, detection and data analysis were executed using the LightCycler v2.0 system (Roche).

At the LUMC, nucleic acids were extracted from 200 µl samples using the Qiagen whole blood DNA extraction kit (Qiagen). Each sample was eluted using 200 µl elution buffer. Real-time PCR for *P. jiroveci* was targeted at the dihydroperoxide synthase (DHPS) gene, and was performed in 50 µl reaction mixtures, consisting of 1 × HotstarTaq mastermix (Qiagen), 3.5 mM MgCl₂, 0.4 µM each primer, 0.34 µM molecular beacon probe and 10 µl purified DNA. The PCR thermal profile consisted of 15 min at 95 °C, followed by 50 cycles of 30 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C. Amplification, detection and data analysis were performed with an iCycler IQ Real-Time Detection system (Bio-Rad).

Additional analyses. Since two of the three PCR assays were based on a multicopy target (MSG) and one assay on a single-copy target (DHPS), comparison of quantification was performed on the basis threshold cycle (C_t) values rather than absolute quantifications. In 26 samples (positive samples from the MINC), the *P. jiroveci* burden was also quantified microscopically (Kovacs *et al.*, 1988). For these samples, microscopic quantification was compared to PCR quantification using the MINC assay (described above).

Statistical analysis. Qualitative inter-assay agreement was assessed by pairwise comparisons of test results from the three laboratories by calculating the crude percentage agreement and the kappa statistic. For this comparison, the results obtained on the initial run for each sample were taken and any C_t value obtained was considered as a positive result. Further pairwise correlations between the C_t values of the different laboratories for each sample were calculated using the Pearson correlation coefficient. Correlation between microscopy quantification and real-time PCR quantification was also expressed as a correlation coefficient.

RESULTS AND DISCUSSION

Origin of BAL fluid samples

A total of 124 BAL fluid samples collected during the period August 1999 to April 2004 were included. They were recovered either from HIV-positive or HIV-negative patients with a known risk factor for PCP, such as (haematological)

malignancy, bone marrow or organ transplantation, Wegener's granulomatosis, and immunosuppressive or corticosteroid therapy (Miller, 1999; Thomas *et al.*, 2004). In addition, a number of BAL fluid samples obtained from patients with no known risk factor for PCP were included (Table 2). These samples were obtained from patients with newly diagnosed sarcoidosis or ventilator-associated pneumonia. The samples were obtained from the LUMC (*n* = 20), RUNMC (*n* = 18) and MINC (*n* = 86). As shown in Table 2, 41 samples (33.1 %) were found to be positive for *P. jiroveci* by microscopy.

Qualitative agreement between the three *P. jiroveci* real-time PCR assays

All 124 samples were subjected to real-time PCR at each of the three participating laboratories. For 114 (91.9 %) of the 124 samples, the three laboratories obtained identical qualitative results (Table 3). Forty out of 41 (97.6 %) microscopy-positive samples were found positive in all three PCR assays. The remaining microscopy-positive sample was

Table 3. Comparison of the three real-time PCR assays for the detection of *P. jiroveci*

+, Positive; -, negative.

Number of samples	Real-time PCR result for:		
	MINC	LUMC	RUNMC
Microscopy-positive samples (<i>n</i> = 41)			
40	+	+	+
1	+	-	-
Microscopy-negative samples (<i>n</i> = 83)			
69	-	-	-
5	+	+	+
4	-	-	+
2	+	-	-
1	+	+	-
1	+	-	+
1	-	+	-

only positive in a single assay, but showed a relatively high C_t value (36.6). This sample was obtained from a patient with a low parasite burden (one cyst in one out of three investigated microscopic slides). This patient had been diagnosed with PCP a week earlier and had been treated with cotrimoxazole during the week previous to the bronchoscopy.

Of the 83 microscopy-negative samples, 69 (83.1%) were found negative in all three PCR assays (Table 3). The remaining 14 samples were PCR positive in at least one of the three assays. Five of these were positive in all three assays, two samples were positive in two of the assays, and seven samples were positive in a single assay only. The majority (12/14) of the patients with microscopy-negative/PCR-positive results were HIV-negative patients with one or more risk factors for PCP (data not shown). An additional patient was HIV-positive, and the remaining patient did not have any known risk factors. The most likely explanation for the microscopy-negative/PCR-positive results is the higher sensitivity of PCR in comparison with microscopy.

The agreement between the three *P. jiroveci* real-time PCR assays was assessed by pairwise comparisons of the qualitative test results. The highest agreement was found between the MINC and LUMC assays, with a percentage agreement of 96.8% and a kappa value of 0.93. The agreement between the other assays was also excellent, with a percentage agreement of 94.4% and a kappa value of 0.88, both between the MINC and RUNMC assays and between the RUNMC and LUMC assays.

Quantitative comparison between the three real-time PCR assays

To compare the quantitative performance of the three real-time PCR assays, pairwise correlations between C_t values generated on samples that were scored positive in all three assays ($n=45$; Table 3) were calculated. As shown in Fig. 1, there was a good correlation between the C_t values produced by each of the three assays. The correlation coefficients were 0.84, 0.90 and 0.99 between the LUMC and MINC assays, the MINC and RUNMC assays, and the LUMC and RUNMC assays, respectively. While the RUNMC and LUMC assays generated similar C_t values (Fig. 1a), the C_t values produced by the MINC assay were somewhat lower than those produced by the other assays (Fig. 1a, c).

Quantitative comparison between microscopy and real-time PCR

To examine the correlation between microscopy and real-time PCR in quantitative detection, the results from these assays were compared for a selection of positive samples ($n=26$). Since a comparison with C_t values might be difficult to interpret, these values were first converted to copy-number equivalents of a plasmid (pPCP) containing the *P. jiroveci* amplicon. This was achieved in the MINC real-time PCR assay by generating a standard curve using this

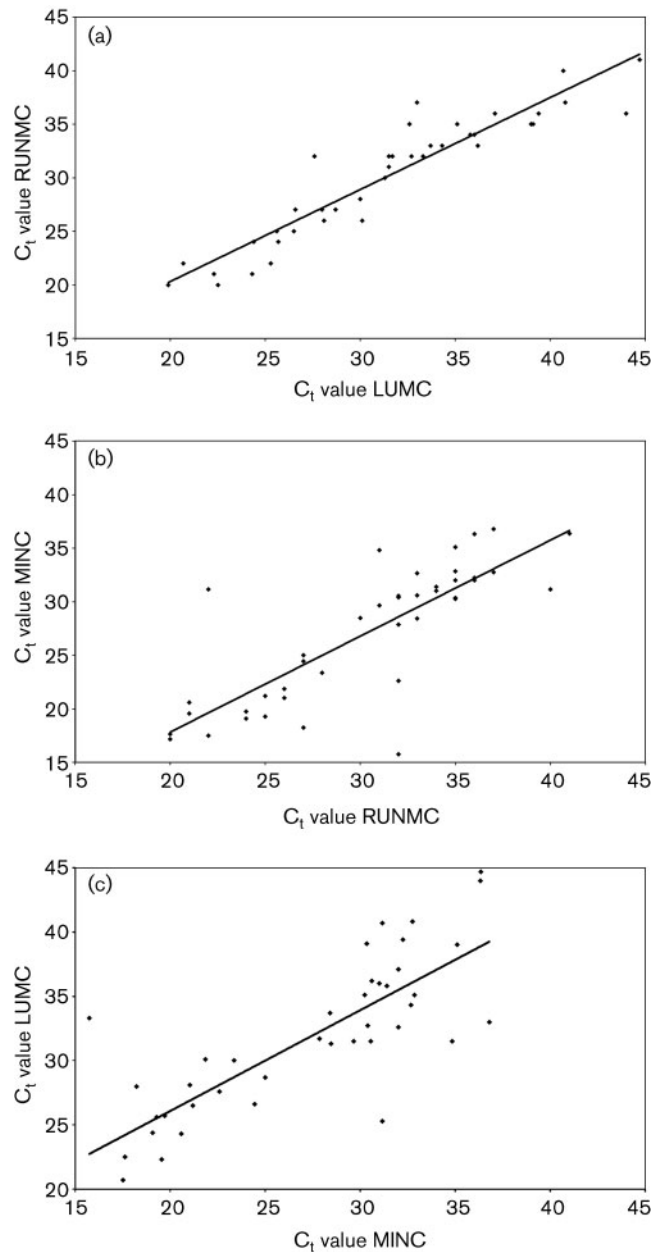


Fig. 1. Pairwise comparison between the different C_t values for all samples ($n=45$) that were found to be positive in all three real-time PCR assays. (a) Comparison of C_t values from the RUNMC assay (y axis) and the LUMC assay (x axis); (b) comparison of the C_t values from the MINC assay (y axis) and the RUNMC assay (x axis); (c) comparison of the C_t values from the LUMC assay (y axis) and the MINC assay (x axis).

plasmid (Fig. 2a, b). Subsequently, the copy-number equivalents of the 26 *P. jiroveci*-positive samples were plotted against the microscopically quantified *P. jiroveci* burden (expressed as clusters per cytospin spot). As shown in Fig. 2(c), there was a good correlation between both methods, which was expressed as a correlation coefficient of 0.83.

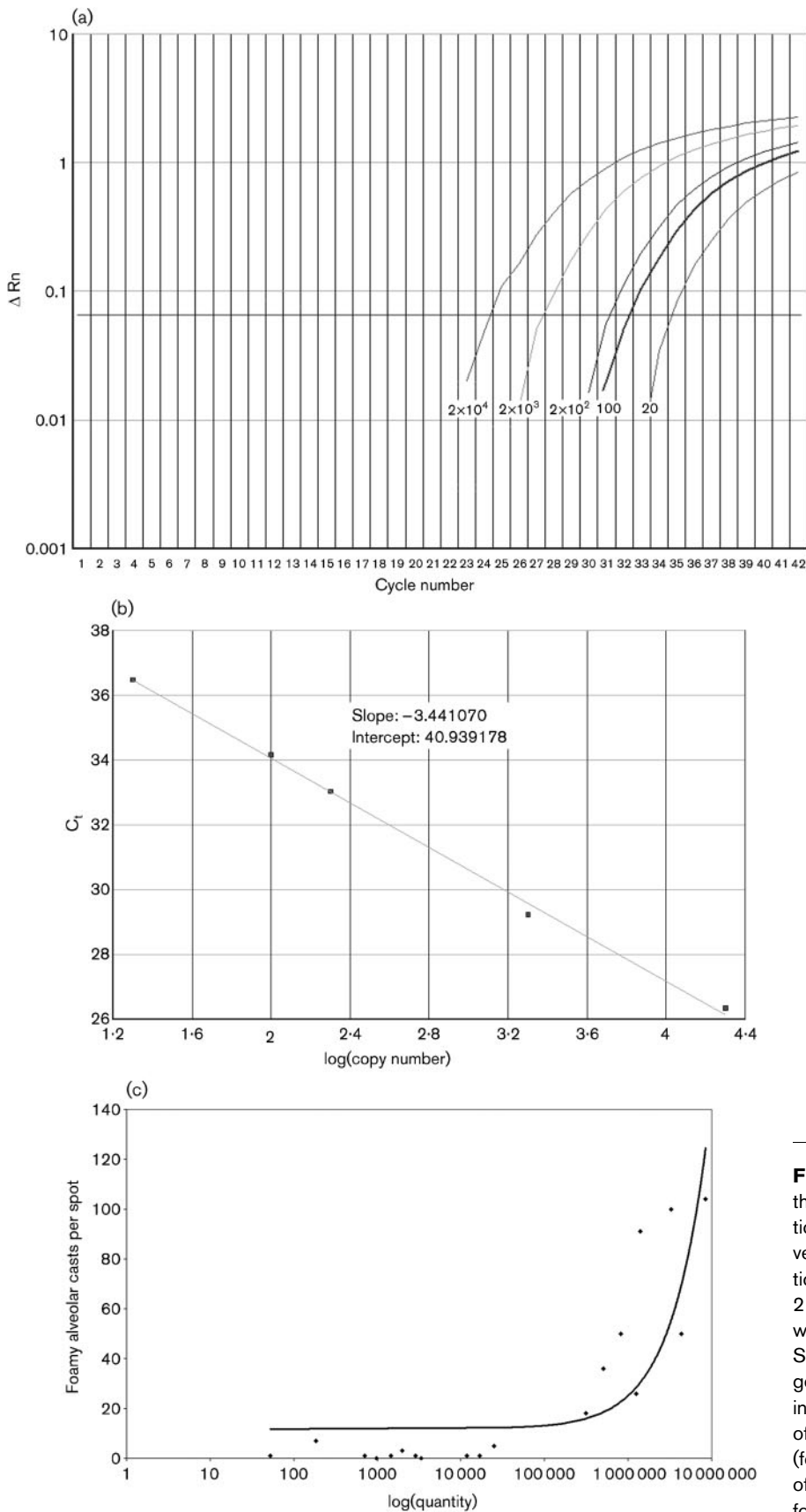


Fig. 2. Correlation between microscopy and the MINC real-time PCR assay in quantification of *P. jiroveci*. (a) Amplification plot (ΔRn versus cycle number) generated with dilutions of plasmid pPCP (2×10^4 , 2×10^3 , 2×10^2 , 100 and 20 copies per reaction), which contains the *P. jiroveci* amplicon. (b) Standard curve [C_t versus $\log(\text{copy number})$] generated from the amplification plot shown in (a). (c) Correlation between the number of *P. jiroveci* clusters per cytospin spot (foamy alveolar casts per spot) and the log of the *P. jiroveci* copy number [$\log(\text{quantity})$] for 26 PCR-positive BAL fluid samples.

***P. jiroveci* carriership versus clinically relevant infection**

An important issue in the diagnosis of PCP is the distinction between apparent asymptomatic *P. jiroveci* carriers and patients with clinically obvious PCP. In most studies, carriers have been defined as patients in whom *P. jiroveci* DNA can be detected in the absence of clinical signs of *P. jiroveci* infection, and without microscopically detectable *P. jiroveci* cysts in BAL fluid samples. In the present study, we found an overlap between C_t values in samples obtained from potential carriers of *P. jiroveci* on the one hand and some of the samples from patients with clinically and microscopically proven PCP on the other. This is in line with earlier reports, in which a reliable cut-off value for the differentiation between disease and carrier states could not be firmly established (Flori *et al.*, 2004; Larsen *et al.*, 2004). Nevertheless, it is possible to divide patients into three categories. The first category includes patients with clinical symptoms indicative of PCP, and with a positive PCR result and/or a positive microscopy result; these patients are diagnosed with PCP. The second category includes patients who have no clinical symptoms and do not have any indication of infection with *P. jiroveci*, as indicated by negative results in both microscopy and PCR. The third group is more complex, and consists of patients who do not have typical clinical symptoms of PCP, but show a positive PCR result (usually with high C_t values). We hypothesize that the patients without risk factors for PCP should be regarded as asymptomatic carriers and do not require treatment for PCP. In the case of patients belonging to the at-risk group with negative microscopy results, the microscopy should be re-evaluated and the patient should be followed clinically and receive therapy at the appearance of any clinical symptoms indicative of PCP.

Previously, only immunocompromised patients, such as HIV-positive patients, were considered to be potential carriers of *P. jiroveci* (Weig *et al.*, 1996, 1997). More recently, however, immunocompetent individuals have also been found to be putative carriers (Sing *et al.*, 1999; Visconti *et al.*, 2000). Miller and coworkers investigated health-care workers who had come into contact with patients with PCP and found among them a carrier rate of 30.5% (Miller, 1999). In particular, health-care workers taking BAL or induced sputum samples were found to be at risk of developing a carrier status, which in one case persisted for 27 months (Miller *et al.*, 2001). All *P. jiroveci*-DNA-positive patients from our study belonged to the group at risk, except for a single patient who was admitted to the intensive care unit and diagnosed with a community-acquired pneumonia caused by *Streptococcus pneumoniae*.

In conclusion, we have compared the performance of three different in-house-developed real-time PCR assays for *P. jiroveci*. Interestingly, while these three assays employ different methods for nucleic acid isolation, amplification and detection, an excellent agreement in performance was found between the assays, both qualitatively (the diagnosis

of PCP) and quantitatively (the *P. jiroveci* burden). Likewise, a good correlation was found between the *P. jiroveci* quantities determined by real-time PCR and microscopic quantification. A cut-off value to discriminate between disease and carrier status for *P. jiroveci* could not be established in the present retrospective study. A future prospective study is needed to investigate whether quantitative PCR results can be employed to differentiate between PCP and carriership of *P. jiroveci*. Finally, in order to monitor the performance of the different in-house PCR assays currently used in microbiology laboratories, the availability of quality control panels is of utmost importance. The assays described here could serve as reference assays in the development and maintenance of such panels.

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