

Comparison between real-time PCR, conventional PCR and different staining techniques for diagnosing *Pneumocystis jiroveci* pneumonia from bronchoalveolar lavage specimens

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Between January 2002 and July 2003, 173 bronchoalveolar lavage (BAL) specimens from 150 patients (19 HIV-infected and 131 non-HIV-infected patients) were evaluated for identification of *Pneumocystis jiroveci* (formerly known as *Pneumocystis carinii* f. sp. *hominis*) using staining techniques, conventional PCR (*mtLSUrRNA* gene) and real-time PCR (*MSG* gene). Test results were compared to *Pneumocystis* pneumonia (PCP) confirmed by typical clinical findings and response to treatment. Sensitivity and specificity of the techniques were 60 and 100% for staining (where either one or both techniques were positive), 100 and 87.0% for conventional PCR and 100 and 84.9% for real-time PCR, respectively. The use of a concentration of 10³ copies of DNA per capillary of BAL as a cut-off (determined by real-time PCR) increased specificity from 84.9 to 98.6% without reducing the sensitivity of the technique. This technique is rapid (<3 h) and therefore of major interest in differentiating between asymptomatic carriage and PCP. A BAL specimen with <10³ copies per capillary of *Pneumocystis*-specific DNA is more likely to indicate a chronic carrier state, but in such cases follow-up is required to ensure that the patient is not in the early stage of an active PCP.

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INTRODUCTION

Pneumocystis jiroveci, formerly known as *Pneumocystis carinii* f. sp. *hominis*, is a fungal pathogen that causes *Pneumocystis* pneumonia (PCP), a common and serious opportunistic infection in immunocompromised patients. Diagnosis of PCP in the laboratory was, until a few years ago, dependent on visualization of *Pneumocystis* organisms in stained preparations of appropriate respiratory specimens using the Giemsa and Gomori–Grocott techniques. Sensitivity of the staining techniques, although acceptable (70–92%) for bronchoalveolar lavage (BAL) specimens (Wakefield *et al.*, 1990; Cregan *et al.*, 1990), was low (35–78%) for aspirates and induced sputum samples (Zaman *et al.*, 1988; Wakefield *et al.*, 1991; Lipschik *et al.*, 1992). In the last 10 years, PCR (Wakefield *et al.*, 1990) has considerably increased sensitivity of detection of *Pneumocystis*, which is now 86–100% in BAL, aspirates and induced sputum specimens (Roux *et al.*, 1994; Cartwright *et al.*, 1994; Caliendo *et al.*, 1998). However, one problem with this technique is that it is frequently

positive in patients with asymptomatic carriage, with a rate of 2–21% (Sing *et al.*, 1999, 2000; Nevez *et al.*, 1999; Helweg-Larsen *et al.*, 2002; Takahashi *et al.*, 2002; Maskell *et al.*, 2003). Real-time PCR, recently described by Larsen *et al.* (2002), could have a role in distinguishing between colonization and clinical disease. The aim of this study was to compare sensitivity and specificity of different techniques (standard staining, conventional PCR and real-time PCR) used in diagnosis of PCP and, as suggested by Larsen *et al.* (2002), to propose an adapted cut-off value for differentiating between carriage and PCP using real-time PCR.

METHODS

Patients and clinical samples. Samples for this study came from all patients seen in the University Hospital of Saint Etienne between January 2002 and July 2003 presenting with clinical symptoms of pulmonary infection associated with immunosuppression, justifying a search for *Pneumocystis* in BAL.

From 150 patients with immunosuppression due to different diseases and/or immunosuppressive treatments, 173 BAL samples were obtained. Samples comprised 138 specimens retrieved by washing with

Abbreviations: BAL, bronchoalveolar lavage; PCP, *Pneumocystis* pneumonia.

50 ml 0.9% NaCl (BAL-50) and 35 retrieved by washing with 150 ml 0.9% NaCl (BAL-150). Of these 150 patients, 19 were HIV seropositive and the rest were seronegative.

Sample preparation and classical staining. All BAL samples containing mucous material had twice their volume of 0.9% NaCl added and they were then mixed vigorously for 5 min. To avoid non-specific inhibition of PCR, no detergents or mucolytic agents were used in this step. Samples were centrifuged at 3000 g for 10 min and pellets were resuspended in 1/10 of the starting volume. A portion of the resuspended pellet (100 µl) was used to prepare smears for Giemsa and Gomori–Grocott staining and smears were examined by two microscopists experienced in *Pneumocystis* diagnosis. Additional samples of 200 µl of the resuspended pellets were stored at 4 °C until used for DNA extraction and amplification.

DNA extraction, conventional PCR and real-time PCR. To detect *Pneumocystis* DNA in BAL samples, 200 µl of each resuspended pellet was used for DNA extraction with the QIAamp DNA Mini kit (Qiagen) according to the manufacturer's recommendations.

Conventional PCR. PCR was performed according to Wakefield *et al.* (1990). The PCR mixture (50 µl) contained 10 pmol of each primer, pAZI02E and pAZI02H, derived from the mitochondrial large subunit rRNA (*mtLSUrRNA*) gene, 200 µM dNTPs (dATP, dCTP, dGTP and 3 × dUTP), 3 mM MgCl₂, 5 µl 10 × PCR buffer, 1.25 U *Taq* DNA polymerase, 1 IU uracyl DNA glycosylase (PCR Core Kit Plus; Roche Molecular Biochemicals) and 10 µl purified DNA. Denaturing, annealing and extension times were 1 min each, at 95, 58 and 72 °C, respectively. DNA samples were amplified for 40 cycles. The specific product (364-bp fragment) was separated on a 3% agarose gel (Metaphor agarose; Tebu) and detected after staining with ethidium bromide under UV illumination.

Real-time PCR with the LightCycler. Real-time PCR was performed with fluorescence resonance energy transfer (FRET) hybridization probes using the LightCycler. PCR was performed with LC FastStart DNA Master hybridization probes (Roche Molecular Biochemicals) in a quantitative touch-down real-time PCR as previously described by Larsen *et al.* (2002). Briefly, 20 µl of a mixture containing Fast Start *Taq* DNA polymerase, dNTPs (with dUTP instead of dTTP), 5 mM MgCl₂, 0.5 IU heat-labile uracyl-DNA glycosylase (Roche Molecular Biochemicals), 10 pg mouse DNA, 5 µl PCR extract, 1 µM of each selected primer and 0.20 µM of each hybridization probe was used. Internal control was the mouse galactose-1-phosphate uridyl transferase (*GALT*) gene (Costa *et al.*, 2001). Specific primers and probes were used for co-amplification of the internal control (Costa *et al.*, 2001) and the *MSG* gene of *Pneumocystis* (Larsen *et al.*, 2002). A double fluorescence reading for each sample was taken at the annealing step: one for LCRed 640 used for the *Pneumocystis* probe (channel 2) and the other for LCRed 705 used for the internal control probe (channel 3).

Standards and external controls. To obtain standards for real-time PCR, *P. jiroveci*-positive BAL samples with Gomori–Grocott smear were used, and 10-fold dilutions were made to obtain 10¹–10⁴ copies per capillary (Larsen *et al.*, 2002).

Data analysis. In the absence of a sensitive gold standard, diagnosis of PCP is difficult in certain cases. The criteria that we used for confirming an ongoing pneumocystosis were clinical findings of PCP with characteristic X-ray findings (diffuse interstitial infiltrates), dyspnoea with partial pressure of arterial oxygen <70 mmHg and response to anti-*Pneumocystis* treatment but resistance to other antibiotics. Based on these criteria for diagnosis, sensitivity and specificity of each laboratory technique were estimated.

Statistical assessment of these characteristics was performed using the χ^2

test ($P < 0.05$ was considered significant). Statistical assessment of differences of the mean of *Pneumocystis* DNA concentration [log (copies) per capillary] from PCP or non-PCP cases was performed by unpaired Student's *t*-test ($P < 0.05$ was considered significant).

RESULTS AND DISCUSSION

Of 150 patients examined, 11 (7.33%) developed PCP. In the remaining 139, diagnosis of PCP was not confirmed. Of 11 confirmed cases, seven were HIV seropositive and four were seronegative.

Comparison of different techniques

Concerning sensitivity and specificity of the diagnostic techniques, results obtained for the two classical staining methods (where either one or both were positive) were 60% [6/10 (the volume was insufficient for one test)] and 100% (139/139), respectively. For conventional PCR, sensitivity and specificity were 100% (11/11) and 87% (121/139) and, for real-time PCR, 100% (11/11) and 84.9% (118/139), respectively. There was a significant difference ($P < 0.01$) in both sensitivity and specificity between PCR and staining techniques; staining gave excellent specificity but lacked sensitivity, while the two PCR tests were much more sensitive but could give false positives.

False-positive cases with the molecular techniques

In this study, false-positive cases using the molecular biology techniques were frequent compared with clinical diagnosis, and were similar to data published elsewhere (see Introduction). There were 18/139 (12.9%) false positives with conventional PCR; these were also positive with real-time PCR, and there were a further three positives, giving a total of 21/139 (14.5%) positives with real-time PCR. With the further three positives detected by real-time PCR, quantities of DNA detected were very small (1.3, 1.8 and 3.8 copies per capillary), indicating a slightly higher sensitivity of approximately 1 DNA copy per capillary. The coherence in both PCR techniques confirms the presence of *P. jiroveci*-specific DNA in asymptomatic patients and their probable carrier state.

False-negative cases with standard staining techniques

Of cases that had different results from the clinical and laboratory diagnoses, false-negative cases were frequent with the Giemsa and Gomori–Grocott staining techniques. It is important to note that false negativity using the staining techniques was rare (1/6) in PCP cases with HIV (Table 1), but more frequent (3/4) in PCP non-HIV cases (Table 2). This difference between HIV-positive and -negative cases has also been found in previous studies, which have shown the difficulty of confirming diagnosis in non-HIV-infected cases owing to lack of sensitivity (25–67%), even in BAL specimens (Roux *et al.*, 1994; Weig *et al.*, 1997; Sing *et al.*, 2000). This is worrying because, as in this study, there was a large

Table 1. Comparison of different diagnostic methods in seven HIV-positive patients who developed pneumocystosis

Dates are given in the form dd/mm/yy. ND, Not done.

Case	Date of sample	Death (date)	CD4 (mm ⁻³)	BAL*	Gomori–Grocott	Giemsa	Conventional PCR	Quantitative PCR (copies per capillary)
1	21/01/02	Yes (01/03/02)	29	150	+	+	+	1.159 × 10 ⁶
	15/02/02			150	–	–	+	2.29 × 10 ⁵
	28/02/02			150	–	–	+	1.46 × 10 ²
2	08/04/03	No	33	50	+	+	+	4.91 × 10 ⁵
3	06/01/03	No	27	50†	ND	ND	+	2.08 × 10 ⁵
4	04/12/02	No	10	50	+	+	+	1.40 × 10 ⁵
5	05/08/02	No	29	50	–	+	+	2.09 × 10 ⁴
	24/09/02			50	–	–	–	–
6	10/01/02	Yes (25/01/02)	24	50	+	–	+	1.06 × 10 ⁴
	24/01/02			50	–	–	+	5.35 × 10 ⁴
7	27/11/02	No	83	50	–	–	+	1.07 × 10 ³

*50, BAL fluid specimen after washing with 50 ml 0.9% NaCl; 150, BAL fluid specimen after washing with 150 ml 0.9% NaCl.

†Volume of sample insufficient (<2 ml) for conventional staining.

Table 2. Comparison of different diagnostic methods in four HIV-negative patients who developed pneumocystosis

See Table 1 for details. CLL, Chronic lymphocytic leukaemia.

Case	Date of sample	Death (date)	Associated disease	BAL	Gomori–Grocott	Giemsa	Conventional PCR	Quantitative PCR (copies per capillary)
1	02/04/02	Yes (20/04/02)	CLL	150	–	–	+	2.38 × 10 ⁵
	15/04/02			150	–	–	+	1.11 × 10 ⁴
2	22/04/02	No	CLL	150	–	–	+	1.52 × 10 ⁵
3	14/12/02	Yes (18/12/02)	Renal transplantation, astrocytoma	150	+	+	+	5.85 × 10 ⁴
4	27/12/02	No	Wegener's disease	50	–	–	+	1.14 × 10 ³

number of PCP cases among seronegative patients and, as described by Ninin *et al.* (1998), deterioration is rapid, and death results in 35.4% of cases.

DNA concentration in relation to clinical findings

In this study, one of the objectives was to try to determine a cut-off value that could discriminate between cases of PCP and asymptomatic *Pneumocystis* carriers. For this, it is important to have the same type of specimen for all cases. In the present study we had 173 BAL specimens divided into two groups, BAL-50 and BAL-150. No difference in sensitivity or specificity between the two groups regarding diagnosis of PCP was demonstrated, and they were therefore analysed together. However, clinically, BAL-50 was much better tolerated by patients who were severely hypoxaemic.

As shown in Fig. 1, concentrations of DNA detected in PCP patients were very high ($P < 0.0001$). All cases had $>10^3$

copies of DNA per capillary, whereas all cases that did not have PCP but had detectable *Pneumocystis*-specific DNA had $>10^4$ copies of DNA per capillary (Table 3). Using 10^3 copies of DNA per capillary of BAL as a cut-off, determined by real-time PCR, increased specificity from 84.9 (118/139) to 98.6% (137/139) without reducing the sensitivity of the technique.

In addition, the limit of detection of the staining techniques was well below that of real-time PCR. Thus, all BAL specimens that were positive by the staining techniques had DNA concentrations $>10^4$ copies per capillary. This quantitative difference in sensitivity (a factor of 10^4) has already been noted in different studies. Leigh *et al.* (1993) defined a factor between 10^4 and 10^6 using serial dilution, while Ribes *et al.* (1997) found a factor of at least 10^2 .

As regards a cut-off to differentiate asymptomatic carriage from PCP, there was an overlap between the two groups (Fig. 1) in spite of a significant difference. That is the reason why a

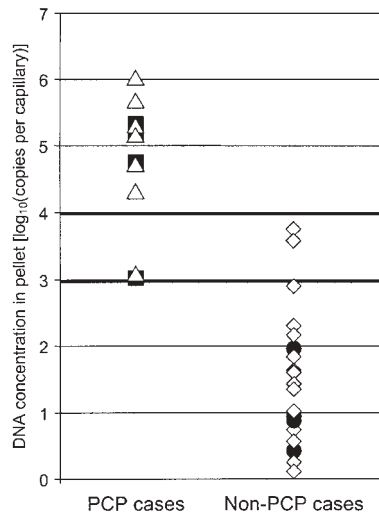


Fig. 1. Quantity of DNA by clinical category. DNA concentrations in PCP cases [$n = 11$: Δ , HIV-positive ($n = 7$); \blacksquare , HIV-negative ($n = 4$)] and non-PCP cases in BAL [$n = 21$: \bullet , HIV-positive ($n = 3$); \diamond , HIV-negative ($n = 18$)] are shown. Mean concentrations: $\log[\text{mean PCP (copies per capillary)}] = 4.72 \pm 1.01$; $\log[\text{mean non-PCP (copies per capillary)}] = 1.50 \pm 1.01$; $P < 0.0001$.

'grey zone' between 10^3 and 10^4 copies per capillary must be proposed. This also allows for possible minor inter-laboratory differences in performing the techniques. A BAL sample with $<10^3$ copies of DNA per capillary is likely to be a chronic carrier state but, in such cases, follow-up is necessary, as such patients could be in the early stage of an active PCP.

To conclude, real-time PCR is a rapid technique (<3 h including extraction, amplification and visualization). Having a high sensitivity as with conventional PCR, it has the added advantage of quantification to determine a cut-off that permits differentiation between carriage and disease in the majority of cases.

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Table 3. Distribution of clinical and biological parameters according to *Pneumocystis* DNA concentration by quantitative real-time PCR in BAL fluid specimens of 150 patients (173 specimens)

Pellet quantitative PCR (copies per capillary)	Number of patients	Clinical symptoms of PCP	Grocott- or Giemsa-positive	Conventional PCR-positive
$>10^4$	9	9	6/8*	9
$<10^4$ and $>10^3$	4	2	0	4
$<10^3$ and $>10^2$	3	0	0	3
$<10^2$ and $>10^1$	8	0	0	8
$<10^1$ and $>10^0$	8	0	0	5
Negative	118	0	0	0

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