Detection of *Pneumocystis jirovecii* by Two Staining Methods and Two Quantitative PCR Assays

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Abstract

Background: *Pneumocystis jirovecii* is an opportunistic pathogen that causes pneumonia, particularly in immunodeficient hosts.

Materials and Methods: We retrospectively compared the results obtained by two staining methods (toluidine blue and calcofluor white) and two quantitative (q) real time PCR assays for the detection of *P. jirovecii* in bronchoalveolar lavage (BAL) specimens. For the qPCR assays, we used newly selected probes and primers targeting the *Kex*-1 gene, which codes for a serine endoprotease, and compared the results to those from the published assay targeting the β-tubulin gene.

Results: A total of 1,843 BAL specimens were analyzed microscopically in parallel, and 74 (4.0%) were found to be positive with both stains, 23 (1.2%) were positive only with the toluidine blue stain, and six (0.3%) only with the calcofluor stain ($p = 0.003$). Of these, a selection of 186 consecutive BAL fluid samples were tested by qPCR using the respective different primer pairs. 21 of the 186 samples (11.3%) were microscopically positive with both stains as well as qPCR positive after 18–31 cycles (corresponding to 5.24×10^6 copies/ml to 640 copies/ml of native BAL) using the *Kex*-1 primer pair and between 21–33 cycles using the β-tubulin assay. A good correlation between semi-quantitative microscopy and the number of PCR cycles needed for a positive signal was noted. Of the remaining 165 samples, 153 (82%) were both microscopically and PCR negative (PCR with the two sets of primers); the remaining 12 samples (7%) were *Kex*-1-based PCR positive (from cycles 33 to 41, corresponding to 160 copies/ml of BAL or less) but microscopically negative. Of these latter samples, ten (6%) were also positive (from cycles 34 to 38) with the primers targeting the β-tubulin gene. Taking microscopy as a reference, the sensitivity of qPCR targeting the *Kex*-1 gene was 100%, and the specificity was 92.4%.

Conclusion: The sensitive qPCR analysis proved to be a rapid and reliable method to detect *P. jirovecii* in BAL.

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Introduction

Pneumocystis jirovecii (synonym *P. carinii* sp. *hominis*) [1] is a fungal opportunistic agent that causes pneumonia in patients with impaired immunity [2]. Pulmonary pneumocystosis remains one of the most frequent opportunistic infections in AIDS patients who cannot benefit from highly active antiretroviral therapy (HAART). In addition, other steadily increasing immunocompromised patients, such as those receiving immunosuppressive drugs for organ transplantation, may also develop pneumocystosis.

Since *P. jirovecii* cannot be cultured, the diagnosis is conventionally based on a direct microscopic examination of respiratory specimens; the sensitivities of such examinations range from 48% to 92% in bronchoalveolar lavage (BAL) specimens and are lower in aspirates or induced sputum [3, 4]. Nucleic acid amplification assays have been developed and described with the aim of improving the detection of *P. jirovecii* [5–8]. Many target genes, such as dihydropteroate synthase (DHPS), dihydrofolate reductase (SHFR), internal transcribed spacer regions of the rRNA (ITS), mitochondrial small subunit rRNA (mtLSUrRNA), 5S rRNA, 18S rRNA, *cdc2*, among others, have been described for the detection or typing of this micro-organism [8–12]. However, there have been frequent reports of some specimens yielding a positive conventional PCR signal when the microscopic examination was negative. The advantage of a quantita-

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tive PCR (qPCR) is that the number of target gene copies present in the specimens can be determined, which allows a quantitative comparison with the results obtained by microscopy. This information is especially useful for specimens containing low numbers of *P. jirovecii* [13, 14].

The aims of the study reported here were (1) to retrospectively quantify the differences between two staining methods for the microscopic detection of *P. jirovecii* based on a large number of routine results; (2) to establish and evaluate a new quantitative TaqMan-based PCR for the specific detection of *P. jirovecii*; (3) to determine the number of PCR cycles corresponding to the detection limit of the microscopic analysis.

Materials and Methods

In our institution BAL samples are routinely sent to different laboratories; with respect to the microscopic detection of *P. jirovecii*, samples are most often sent to the Laboratoire des Liquides Biologiques and to the Laboratoire Central de Bactériologie. We retrospectively compared the results communicated by these two laboratories between January 1999 to June 2007 for 1,843 BAL specimens analyzed in parallel. The indication for BAL specimen collection was determined by the pneumologists based on respiratory symptoms, laboratory parameters, and radiological observations. The vast majority of patients were immunocompromised, some with severe respiratory failure.

The Laboratoire des Liquides Biologiques routinely uses a toluidine blue stain [15] to detect *P. jirovecii* microscopically, while the Laboratoire Central de Bactériologie uses a calcofluor white fluorescent stain (Fungi-Fluor; Polysciences, Warrington, PA) [4]. The Laboratoire des Liquides Biologiques also determines cell distribution and their concentration. To determine the cell count found in the native fluid, the Laboratoire des Liquides Biologiques concentrated BAL samples by centrifugation at 80 *g* for 10 min. A volume of fluid (usually 100–200 μl) containing 120,000 cells was then introduced into each of two cyto-centrifuge funnels. Cyto-centrifugation at 150 *g* for 5 min produced two slides that were air-dried and stained with toluidine blue [4]. In the Laboratoire Central de Bactériologie, 200 μl of undiluted BAL samples were directly cyto-centrifuged (150 *g*). For statistical comparison, the McNemar exact test was applied.

Of the 1,843 BAL samples compared retrospectively, we selected 186 consecutive specimens collected from 143 patients (94 male, 49 female) for analysis by qPCR. All patients had signs and symptoms and/or radiological abnormalities that included *Pneumocystis* pneumonia as the differential diagnosis. According to the 130 clinical files available, 33 patients had organ transplantation (of these 23 were lung transplantations), 19 were intubated in the intensive care unit, 18 were known as HIV positive, 12 had cancer, another 21 were otherwise immunocompromised, and the remaining 27 suffered from other respiratory diseases. The BAL procedures were performed following a standardized protocol. Briefly, the flexible bronchoscope was placed in a wedged position in a sub-segmental bronchus. Two to three 50-ml aliquots of isotonic saline solution were instilled and immediately re-aspirated with a typical return of 40%–60%. Samples were processed on the same day as the microscopic examination.

For the PCR procedures, frozen (at –80 °C) BAL samples were thawed, and 500-μl aliquots were centrifuged for 10 min at 18,000 *g*. The pellet was re-suspended in the remaining 50 μl BAL liquid after the supernatant had been discarded and was then topped up to 200 μl with TE buffer (Tris 0.010 M + EDTA 0.001 M, pH 7.5). Thereafter, 30 μl lysis buffer (1.0 M Tris pH 9, 1.0 M KCl, 1% Triton X-100) containing proteinase K (3.3 mg/ml, final concentration) was added and the mixture incubated for 1 h at 56 °C, followed by 10 min at 95 °C (enzyme inactivation). 200 μl of phenol–chloroform–isoamyl alcohol (25/24/1; v/v/v) was added, mixed by vortexing (30 s), and centrifuged (2 min at 10,000 *g*); 150 μl of the aqueous DNA-containing phase was then carefully transferred into a second tube, and 600 μl of ethanol (100%) was added. The DNA was pelleted (5 min, 10,000 *g*) and the supernatant discarded. The pellet was dried for a few minutes at 56 °C (heating block) and then re-suspended in 50 μl of water (10–30 min, room temperature). For each sample, DNA was extracted in triplicate.

Primers and probes of *P. jirovecii* were designed and selected with the Primer Express Sequence Design software (Applied Biosystems, Foster City, CA). Candidate sequences were checked to be specific for *P. jirovecii* by comparing them to published sequences (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). The *Kex*-1 gene coding for a serine endoprotease was found to be unique for this organism. The probes were labeled with 5-carboxyfluorescein at the 5′-end and with 6-carboxy-*N*,*N*,*N*′,*N*′-tetramethylrhodamine at the 3′-end. The primers and probe oligonucleotides sequences were: forward primer 5′-CAACCCTGTTCCAATGCCTAA-3′, reverse primer 5′-CAACACCGATTCCACAAACAGT-3′, and probe 5′-TGCTGGTGAAGTAGCTGCCGTTCGA-3′.

Real-time PCR assays were performed in the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Samples were assayed in a 25-μl reaction mixture containing 5 μl of extracted DNA from the BAL. Primers and probes were used at a final concentration of 50 nM. The PCR procedure was programmed for one cycle consisting of 2 min at 50 °C (for the digestion of previous amplification products) and 10 min at 95 °C for inactivation of this enzyme and polymerase activation, followed by 45 cycles of 15 s at 95 °C and 60 °C for 1 min.

Positive controls were included in each run: DNA extracted from a BAL which was strongly positive based on the microscopic analysis was serially diluted. The dilution giving a positive signal after 30 cycles of amplification (threshold set at 0.02; baseline taken from cycles 3 to 18) was retained for further experiments. Aliquots were stored at 20 °C. Spiked controls were included for each BAL in order to detect PCR inhibitors.

All 186 BAL samples were tested with a second pair of published primers targeting the β-tubulin gene sequence, which has been shown to be a single copy gene in *P. jirovecii* [14]. These primers were also found to be specific for *P. jirovecii* since controls spiked with selected bacteria, fungi, viruses, as well as human DNA have been found to be negative [14]. Primers targeting the β-tubulin gene (forward: 5′-GATCCGAGACATGGTCGCTATT-3′, reverse: 5′-TTCAACCTCCTTCATGGAAACAG-3′) and probe (5′-Yakima Yellow-TGTTGCAGCGATTTTCCGCGGTA-3′-BlackAll) were used at the same concentrations and in the same conditions as for the *Kex*-1 gene. The C_t values of *Kex*-1 versus β-tubulin primers and probe for the BAL samples showed an excellent linear correlation (slope 0.9565, correlation coefficient 0.97), and two almost parallel straight lines (slopes 1.031 vs 1.058) were obtained over ten twofold serial dilutions of positive control DNA, with the *Kex*-1 probe being 2.2 cycles ahead of the β-tubulin probe under the conditions used (results not shown). This slight difference in apparent sensitivity between the *Kex*-1 and β-tubulin probe may be related to the different fluorophores used.

Table 1
Comparison of toluidine blue and calcofluor white stains for the detection of *P. jirovecii* in BAL specimens.

Detection of *P. jirovecii* by toluidine blue stain	Detection of *P. jirovecii* by calcofluor white stain					
	Positive		Negative		Total	
	n	%	n	%	n	%
Positive	74	4.0	23	1.2	97	5.3
Negative	6	0.3	1,740	94.4	1,746	94.7
Total	80	4.3	1,763	95.7	1,843	100.0

BAL, Bronchoalveolar lavage

Table 2
Comparison of microscopic and *Kex*-1 probe qPCR results for the detection of *P. jirovecii* in BAL specimens.

Detection of *P. jirovecii* by *Kex*-1 PCR	Detection of *P. jirovecii* by microscopy					
	Positive		Negative		Total	
	n	%	n	%	n	%
Positive	21	11.3	12	6.5	33	17.7
Negative	0		153	82.3	153	82.3
Total	21	11.3	165	88.7	186	100.0

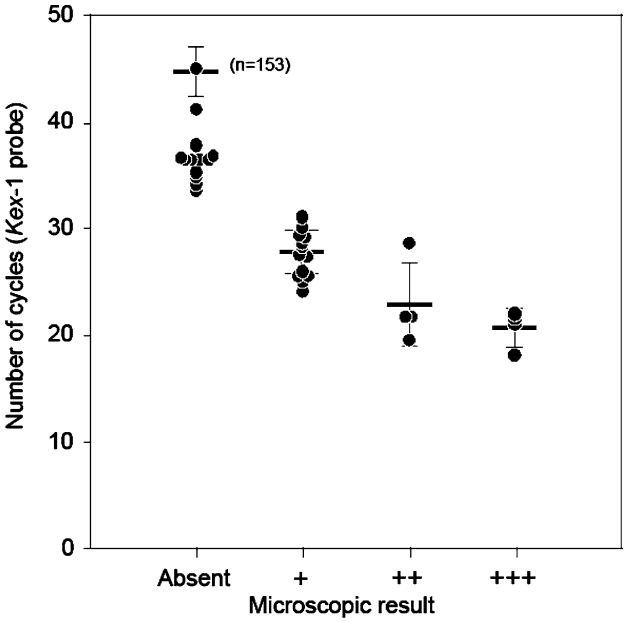

Figure 1. Correlation between semi-quantitative microscopy results and the number of *Kex*-1 qPCR cycles required for positivity. Individual results are represented by filled circles; averages and corresponding standard deviations are denoted by horizontal bars. (+ = few, ++ = many, +++ = abundant).

Results

Of the 1,843 BAL samples analyzed microscopically following staining with toluidine blue or calcofluor white, 103 (5.6%) were overall positive with one or the other method, 74 were positive with both, 23 were positive only with toluidine blue stain, and six were positive only with calcofluor white ($p = 0.003$) (Table 1). If specimens that were positive with both or either staining procedure are considered to be a truly positive specimen (i.e., the gold standard), the sensitivity of toluidine blue was 94% and of calcofluor white 78%.

Of the 186 BAL specimens analyzed by microscopy and the two PCR procedures, 21 (11.3%) were positive by both staining methods and microscopy as well as with the *Kex*-1-based PCR after 18–31 cycles (corresponding to 5.24×10^6 gene copies/ml to 640 copies/ml of BAL, calculated as described below) and the β-tubulin-based PCR after 21–33 cycles (Tables 2, 3; Figs. 1, 2). The qPCR assay obtained 33 (17.7%) positive specimens with the *Kex*-1 probe and 31 (16.7%) positive specimens with the β-tubulin probe (all 31 of the latter were also positive with the *Kex*-1 probe; Tables 2, 3).

When the results from microscopy were considered as the reference, the sensitivity of real time PCR using the *Kex*-1 primers was 100%, the specificity 92.4%, the positive predictive value was 63.6% and negative predictive value was 100%. The 12 *Kex*-1 qPCR-positive samples were detected from cycles 33 (corresponding to 160 copies/ml of BAL) to 41 (Fig. 1). Therefore, a cut-off at 32 cycles (corresponding to 320 copies/ml of native BAL) for the *Kex*-1 qPCR allowed a perfect agreement with microscopy. For the β-tubulin qPCR, this correlation was determined at a cut-off of 33 cycles (Fig. 2).

Detection limits of direct microscopy and the qPCR were furthermore determined by diluting ($\log_2$) a strongly positive BAL. The most diluted specimen that was microscopically positive was positive by both qPCR at 33 cycles.

The medical charts for the 12 patients from whom the BAL samples were microscopically negative but positive by *Kex*-1-based qPCR from cycles ≥ 33 (corresponding to ≤ 160 gene copies/ml of native BAL) were reviewed. All of these patients were immunocompromised (transplantation, HIV, or cancer). Five patients had bacterial or other fungal pneumonia diagnosed by culture, four had non-infectious acute respiratory distress syndrome, two had cardiogenic pulmonary edema, and one patient had

Table 3
Comparison of microscopy and β-tubulin probe qPCR results for the detection of *P. jirovecii* in BAL specimens.

Detection of *P. jirovecii* by β-tubulin PCR	Detection of *P. jirovecii* by microscopy					
	Positive		Negative		Total	
	n	%	n	%	n	%
Positive	21	11.3	10	5.4	31	16.7
Negative	0		155	83.3	155	83.3
Total	21	11.3	165	88.7	186	100.0

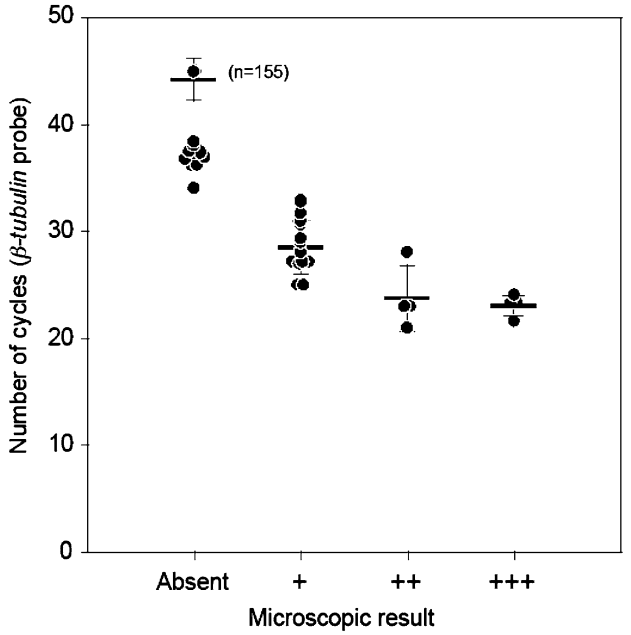

Figure 2. Correlation between semi-quantitative microscopy results and number of β-tubulin probe quantitative (q) PCR cycles required to positivity. Individual results are represented by filled circles; averages and corresponding standard deviations by denoted by horizontal bars.

sirolimus induced pneumopathy. Three patients died due to their primary disease 24 h after the BAL collection, another five had a clinical follow-up 2–16 months after the bronchoscopy and from four of these five patients a control BAL was taken. All four control BAL specimens were negative for *P. jirovecii* by microscopy and qPCR.

Discussion

Pneumocystis pneumonia in an immunocompromised host is a severe and potentially fatal disease if adequate treatment is not administered timely. We retrospectively compared two different procedures and staining methods routinely used to detect *P. jirovecii* microscopically and found that the procedure with the toluidine blue stain was significantly ($p = 0.003$) more sensitive (94%) than that with the calcofluor white stain (78%). Similar results have been previously reported, in particular in a study comparing four staining methods where the sensitivity of the calcofluor white stain was 74% [4]. The advantage of the calcofluor white stain is that in addition to detecting *P. jirovecii* cysts, it can also rapidly and reliably identify fungal elements, since it binds non-specifically to β-linked polysaccharides, such as chitin and cellulose [4]. The false negative results with the calcofluor white method were mainly due to specimens containing low numbers of *P. jirovecii* and for certain BAL specimens that did not adhere to the slide. The appropriate BAL fluid concentration based on the cell count we used for the toluidine blue stain certainly contributed to the good sensitivity achieved in our study with this stain. The exact number of false positive results could not be determined; however, we observed that such false positive results were rare because all 21 BAL specimens that were microscopically positive were confirmed with two qPCR methods of the selected consecutive 186 specimens tested in parallel.

Many publications have reported that PCR has a greater sensitivity and a better specificity than microscopic analyses [6, 8, 13, 16, 17]. The advantage of a real time PCR over classical PCR protocols is that the number of target gene copies present in the specimens can be determined. We successfully developed a quantitative real time PCR allowing the rapid detection of *P. jirovecii* in 500 μl of BAL samples. By diluting a positive specimen and determining the number of cysts visible by microscopy, we could estimate the dilution containing one cyst per 50 μl. This dilution was positive at 36 PCR cycles. From 33 cycles and more, microscopy was always negative, indicating that most specimens with very low cyst numbers remain negative by microscopic analysis. In our retrospective study we could not determine the clinical significance of specimens microscopically negative but qPCR positive between cycles 33 and 36 (corresponding to 160 copies/ml to 20 copies/ml of BAL).

In order to determine the specificity of this *Kex*-1 probe, we compared the results to those obtained with the published and well-evaluated β-tubulin probe [14]. These results were identical with the exception of two samples, which were only *Kex*-1 probe positive.

The possibility for detecting carriers by PCR analysis is of considerable interest. Studies using nested PCR for the detection of *P. jirovecii* in non-invasive samples obtained from immunocompetent adults reported that 20% were asymptomatic carriers [18] compared to 21.5% among cystic fibrosis patients [19] and 19%–40% in a population with primary pulmonary disorders [20, 21]. In a further study, the authors suggested a cut-off of ≥ 10 gene copies/μl (corresponding to ≥ 50 gene-copies per tube), as determined by serial dilutions of the cloned gene, to discriminate infection from colonization [6]. In our study, such BAL samples containing $\geq 10{,}000$ copies/ml were also microscopically positive, with the detection limit determined at 320 gene copies/ml.

The number of *Kex*-1 PCR cycles needed for a positive signal correlated well with semi-quantitative microscopy results (Fig. 1). Only for semi-quantitative results of ++ and +++ was the difference marginal. We consider qPCR to be more objective than semi-quantitative microscopy, which relies essentially on experienced and skilled personnel. In our experience, semi-quantitative positive microscopy results do not correlate with the severity of the disease; hosts factors certainly play a more important role.

We obtained promising results in our ongoing study using the same qPCR assay on non-invasive samples, such as sputum and oro-pharyngeal swabs.

The sensitive real time PCR methods provided additional information to microscopic results. Their systematic and prospective use will allow clinicians to determine the clinical significance of positive qPCR results that are negative based on microscopic analyses.

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References

1. Stringer JR, Beard CB, Miller RF, Wakefield AE: A new name (*Pneumocystis jiroveci*) for *Pneumocystis* from humans. Emerg Infect Dis 2002; 8: 891–896.
2. Frenkel JK: *Pneumocystis* pneumonia, an immunodeficiency-dependent disease (IDD): a critical historical overview. J Eukaryot Microbiol 1999; 46: 89S–92S.
3. Cregan P, Yamamoto A, Lum A, VanDerHeide T, MacDonald M, Pulliam L: Comparison of four methods for rapid detection of *Pneumocystis carinii* in respiratory specimens. J Clin Microbiol 1990; 28: 2432–2436.
4. Procop GW, Haddad S, Quinn J, Wilson ML, Henshaw NG, Reller LB, Artymyshyn RL, Katanik MT, Weinstein MP: Detection of *Pneumocystis jiroveci* in respiratory specimens by four staining methods. J Clin Microbiol 2004; 42: 3333–3335.
5. Larsen HH, Kovacs JA, Stock F, Vestereng VH, Lundgren B, Fischer SH, Gill VJ: Development of a rapid real-time PCR assay for quantitation of *Pneumocystis carinii* f. sp. *carinii*. J Clin Microbiol 2002; 40: 2989–2993.
6. Larsen HH, Masur H, Kovacs JA, Gill VJ, Silcott VA, Kogulan P, Maenza J, Smith M, Lucey DR, Fischer SH: Development and evaluation of a quantitative, touch-down, real-time PCR assay for diagnosing *Pneumocystis carinii* pneumonia. J Clin Microbiol 2002; 40: 490–494.
7. Lu JJ, Chen CH, Bartlett MS, Smith JW, Lee CH: Comparison of six different PCR methods for detection of *Pneumocystis carinii*. J Clin Microbiol 1995; 33: 2785–2788.
8. Robberts FJ, Liebowitz LD, Chalkley LJ: Polymerase chain reaction detection of *Pneumocystis jiroveci*: evaluation of 9 assays. Diagn Microbiol Infect Dis 2007; 58: 385–392.
9. Bandt D, Monecke S: Development and evaluation of a real-time PCR assay for detection of *Pneumocystis jiroveci*. Transpl Infect Dis 2007; 9: 196–202.
10. Huggett JF, Taylor MS, Kocjan G, Evans HE, Morris-Jones S, Gant V, Novak T, Costello AM, Zumla A, Miller RF: Development and evaluation of a real-time PCR assay for detection of *Pneumocystis jirovecii* DNA in bronchoalveolar lavage fluid of HIV-infected patients. Thorax 2008; 63: 154–159.
11. Tsolaki AG, Miller RF, Wakefield AE: Oropharyngeal samples for genotyping and monitoring response to treatment in AIDS patients with *Pneumocystis carinii* pneumonia. J Med Microbiol 1999; 48: 897–905.
12. Wakefield AE, Banerji S, Pixley FJ, Hopkin JM: Molecular probes for the detection of *Pneumocystis carinii*. Trans R Soc Trop Med Hyg 1990; 84: 17–18.
13. Arcenas RC, Uhl JR, Buckwalter SP, Limper AH, Crino D, Roberts GD, Wengenack NL: A real-time polymerase chain reaction assay for detection of *Pneumocystis* from bronchoalveolar lavage fluid. Diagn Microbiol Infect Dis 2006; 54: 169–175.
14. Brancart F, Rodriguez-Villalobos H, Fonteyne PA, Peres-Bota D, Liesnard C: Quantitative TaqMan PCR for detection of *Pneumocystis jiroveci*. J Microbiol Methods 2005; 61: 381–387.
15. Paradis IL, Ross C, Dekker A, Dauber J: A comparison of modified methenamine silver and toluidine blue stains for the detection of *Pneumocystis carinii* in bronchoalveolar lavage specimens from immunosuppressed patients. Acta Cytol 1990; 34: 511–516.
16. Durand-Joly I, Chabe M, Soula F, Delhaes L, Camus D, Dei-Cas E: Molecular diagnosis of *Pneumocystis* pneumonia. FEMS Immunol Med Microbiol 2005; 45: 405–410.
17. Flori P, Bellete B, Durand F, Raberin H, Cazorla C, Hafid J, Lucht F, Sung RT: Comparison between real-time PCR, conventional PCR and different staining techniques for diagnosing *Pneumocystis jiroveci* pneumonia from bronchoalveolar lavage specimens. J Med Microbiol 2004; 53: 603–607.
18. Medrano FJ, Montes-Cano M, Conde M, de la Horra C, Respaldiza N, Gasch A, Perez-Lozano MJ, Varela JM, Calderon EJ: *Pneumocystis jiroveci* in general population. Emerg Infect Dis 2005; 11: 245–250.
19. Respaldiza N, Montes-Cano MA, Dapena FJ, de la Horra C, Mateos I, Medrano FJ, Calderon E, Varela JM: Prevalence of colonisation and genotypic characterisation of *Pneumocystis jiroveci* among cystic fibrosis patients in Spain. Clin Microbiol Infect 2005; 11: 1012–1015.
20. Calderon E, de la Horra C, Medrano FJ, Lopez-Suarez A, Montes-Cano MA, Respaldiza N, Elvira-Gonzalez J, Martin-Juan J, Bascunana A, Varela JM: *Pneumocystis jiroveci* isolates with dihydropteroate synthase mutations in patients with chronic bronchitis. Eur J Clin Microbiol Infect Dis 2004; 23: 545–549.
21. Sing A, Geiger AM, Hogardt M, Heesemann J: *Pneumocystis carinii* carriage among cystic fibrosis patients, as detected by nested PCR. J Clin Microbiol 2001; 39: 2717–2718.