Original Report

Biomolecular Techniques to Detect *Pneumocystis carinii* f. sp. *hominis* Pneumonia in Patients with Acquired Immunodeficiency Syndrome

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ABSTRACT

Objectives: To verify the clinical value of two different polymerase chain reactions (PCRs) for noninvasive diagnosis and follow-up during *Pneumocystis carinii* f. sp. *hominis* pneumonia (PCP) and to analyze the *P. carinii* f. sp. *hominis* genotypes involved.

Methods: Internal transcribed spacers (ITSs) nested PCR was applied to 630 samples (bronchoalveolar lavage, sera, peripheral blood mononuclear cells, and oropharyngeal samples) from 122 patients with acquired immunodeficiency syndrome and pneumonia and 40 control samples from 20 subjects seronegative for human immunodeficiency virus. One hundred and eighty samples also were examined by mt-rRNA PCR. Bronchoalveolar lavage samples and 33 sera were analyzed by type-specific oligonucleotide hybridization.

Results: On bronchoalveolar lavage samples, the two PCRs consistently confirmed the morphologic diagnosis of PCP. The sensitivity of ITSs nested PCR versus mt-rRNA PCR was 57.3% versus 14.3% on sera, 32.3% versus 22.8% on peripheral blood mononuclear cells, and 69.1% versus 48.6% on oropharyngeal samples (garglings). Both PCRs had 100% specificity. Type-specific oligonucleotide hybridization revealed in 72.2% of bronchoalveolar lavage samples a single *P. carinii* f. sp. *hominis* genotype, whereas in 27.8% co-infection with more than one strain was detected.

Conclusion: On noninvasive samples, ITSs nested PCR was more sensitive than mt-rRNA PCR, and it confirmed the diagnosis in all patients with PCP. For each patient with PCP at least one noninvasive sample was positive for *P. carinii* f. sp. *hominis* DNA.

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In absence of lung biopsy, the gold standard for *Pneumocystis carinii* f. sp. *hominis* pneumonia (PCP) diagnosis is the direct demonstration of the microorganism in induced sputum or in bronchoalveolar lavage (BAL). Sampling patients by either induced sputum or BAL can be difficult, especially in patients with severe respiratory distress. Several authors have used biomolecular techniques to detect the presence of *P. carinii* f. sp. *hominis* DNA by the polymerase chain reaction (PCR) of specific genomic regions.[1,2,3] The internal transcribed spacers (ITSs) nested PCR followed by type specific oligonucleotide (TSO) hybridization of *P. carinii* f. sp. *hominis* isolates derived from BAL and blood samples represents a further tool to study *P. carinii* f. sp. *hominis* virulence and drug susceptibility related to various genotypes.[4] The purpose of this study was to compare the sensitivity and specificity of two different PCR assays to detect *P. carinii* f. sp. *hominis* in samples taken from patients via several methods.

MATERIAL AND METHODS

From April 1995 to September 1997, 122 patients seropositive for human immunodeficiency virus (HIV), from the 2nd Department of Infectious Diseases at L. Sacco Hospital in Milan, Italy, were enrolled in the study. Human immunodeficiency virus-seropositive patients were clinically evaluated for acute respiratory disease, characterized by at least two of the following features: cough, dyspnea, elevated serum lactate dehydrogenase level, arterial hypoxia, and chest x-ray abnormalities showing interstitial pneumonia. Patients with respiratory disease were examined by bronchoscopy for evidence of *Pneumocystis carinii* f. sp. *hominis* organisms. Blood (for serum and peripheral blood mononuclear cells [PBMC]) and oropharyngeal washes were collected on the same day before bronchoscopy. For each patient with BAL morphologically positive for *P. carinii* f. sp. *hominis*, addi-

tional blood samples were collected during follow-up. Clinical data, concomitant opportunistic infections (with special regard to fungi), and use of primary PCP prophylaxis, were determined by reviewing hospital records. Samples collected from 20 subjects working as health care personnel in the ward were used as controls.

Samples were collected and processed as follows:

- Serum. From 7-mL blood samples, withdrawn by sterile Vacutainer (Becton Dickinson Vacutainer Systems) venipuncture into tubes containing clotting gelatin, serum was aliquoted in cryovials and stored at -80°C within 4 hours from collection.
- Peripheral Blood Mononuclear Cells. Cells were separated from 7-mL blood samples, collected into Vacutainer heparinized tubes, by a Ficoll-Paque PLUS (Amersham Pharmacia Biotech, Uppsala, Sweden) gradient in RPMI-1640 with 20 mM Hepes (HyQ, HyClone Europe Ltd., Cramlington, United Kingdom). Cells were washed twice in RPMI, resuspended in 1 mL of phosphate buffered saline, aliquoted in cryopreservation Nalgene vials and stored at -80°C until used.
- Oropharyngeal Samples (Garglings). Ten milliliters of sterile normal saline were gargled in the morning for about 2 minutes, aliquoted, and stored at -80°C until used.
- Bronchoalveolar Lavage. At least 5 mL of BAL were collected and separated within 12 hours from bronchoscopy and stored at -80°C until used.

For DNA preparation, samples were processed as follows: 250 μL of each sample were digested in 250 μL of proteinase K buffer (50 mM KCl; 15 mM Tris-HCl, pH 8.3; and 0.5% NP40) containing 0.2 mg/mL proteinase K (Boehringer Mannheim GmbH, Mannheim, Germany) at 56°C for 2 hours; the enzyme was then inactivated by boiling for 10 minutes. DNA was extracted with phenol-chloroform, precipitated in ethanol, resuspended in 50 μL TE buffer, and purified through columns (MicroSpin S-200HR columns, Amersham Pharmacia Biotech). Sterile water was used as extraction control.

Ten microliters of each sample were amplified with nested PCR using specific primers for ITSs of rRNA[5]: 1724F (5′-AAGTTGATCAAATTTGGTC-3′) and ITS2R (5′-CTCGGACGAGGATCCTCGCC-3′) for the first step (35 cycles, denaturation at 94°C for 1 minute, annealing at 47°C for 1 minute, and extension at 72°C for 1 minute); ITS1F (5′-CGTAGGTGAACCTGCGGAAGGATC-3′) and ITS2R1 (5′-GTTCAGCGGGTGATCCTGCCTG-3′) for the second step (35 cycles, denaturation at 94°C for 1 minute, annealing at 58°C for 1 minute, extension at 72°C for 2 minutes). One hundred and eighty samples were also amplified with single-step PCR, using specific primers for mt-rRNA[6]: pAZ102-E (5′-GATGGCTGTTTCCAAGCCCA-3′) and pAZ102-H (5′-GTGTACGTTGCAAAGTACTC-3′) (40 cycles, denaturation at 94°C for 1 minute 30 seconds, annealing at 50°C for 1 minute 30 seconds, and extension at 72°C for 2 minutes). Polymerase chain reaction controls were run with each clinical sample, by using deionized water as negative and a well-characterized *P. carinii* DNA as positive control. Amplified products were electrophoresed on 6% acrylamide gel and stained with ethidium bromide. The expected size of PCR products was 550 base pairs (bp) for ITSs and 346 bp for mt-rRNA (PhiX174 DNA HAE II marker, Amersham Pharmacia Biotech).

Polymerase chain reaction products were hybridized (dot blot) as follows: denaturation with 0.4 N NaOH; blotting on Nytran paper; prehybridization overnight at 37°C with 100 μg/mL salmon sperm DNA (SIGMA Chemicals, St. Louis, MO) in 6X SCC, 5X Denhart's solution, 0.5% SDS, 0.05% $Na_4P_2O_7$; hybridization with pAZ 102-L2 probe for mt-rRNA PCR,[7] and 1A, 1B, 2a, 2b, 2c probes for ITSs nested PCR, according to TSO typing as detailed by Lu et al,[4] at high stringency conditions. Either ^{32}P-radiolabelled or biotinylated probes were used, with signal detection obtained by using the Phototop® Detection Kit (New England BioLabs Inc., Beverly, ME). To read the results, the membranes were exposed to Kodak autoradiography film.

RESULTS

After bronchoscopy, 68 of 122 (55.7%) patients were found to have PCP, defined by the clinical presentation and the presence of trophic or cystic forms of *P. carinii* f. sp. *hominis* on BAL after Giemsa and toluidine-blue staining. A total of 54 (44.3%) of the patients had other AIDS-related opportunistic diseases: recurrent bacterial pneumonia (principally due to *Klebsiella pneumoniae, Staphylococcus aureus, Pseudomonas aeruginosa, Xanthomonas maltophilia, Haemophilus influenzae, Streptococcus pneumoniae*), tuberculosis, atypical mycobacteriosis, cytomegalovirus infection, aspergillosis, and non-Hodgkin lymphoma. *Candida* spp oropharyngeal co-infection was present in 51 of 68 (75%) patients with PCP and in 43 of 54 (79.6%) patients with other opportunistic diseases. The authors examined, by ITSs nested PCR, 630 samples (122 BALs, 220 sera, 126 PBMC, and 162 garglings): 374 samples (68 BALs, 146 sera, 81 PBMC, and 79 garglings) were collected from 68 patients with AIDS (mean age, 35.6 y, range, 26–48 y; mean CD4+,

Table 1. Main Clinical Features of Patients Included in the Study

Patients (n = 122)	*Male (n)*	*Female (n)*	*CD4+ (cells/mm³)*	*Age (y)*	*Oropharyngeal Candidiasis n (%)*
PCP+* (68)	60	8	49.2	35.6	51 (75.0)
PCP–† (54)	44	10	107.3	34.6	43 (79.6)

PCP = *Pneumocystis carinii* f. sp. *hominis* pneumonia.
*HIV+ patients with bronchoalveolar lavage = proven PCP; †HIV+ patients with other pulmonary diseases.

Table 2. Sensitivity and Specificity of Internal Transcribed Spacers (ITSs) Nested vs. mt-rRNA Polymerase Chain Reaction (PCR)

	ITSs Nested PCR		*mt-rRNA PCR*	
	PCP+ (n = 68) (%)*	*PCP–† (n = 54) (%)*	*PCP+* (n = 35) (%)*	*PCP–† (n = 10)*
BAL+	68 (100.0)	5 (9.2)‡	35 (100.0)	0
Sera+	39 (57.3)	0	5 (14.3)	0
PBMC+	22 (32.3)	0	8 (22.8)	0
Garglings+	47 (69.1)	0	17 (48.6)	0

PCP = *Pneumocystis carinii* f. sp. *hominis* pneumonia; BAL = bronchoalveolar lavage; PBMC = peripheral blood mononuclear cells. *HIV+ patients with BAL proven PCP; †HIV+ patients with other opportunistic pulmonary diseases; ‡PCR positivity only after the second step of amplification.

49.2/mm^3, range, 2-220 mm^3) with morphologic diagnosis of PCP, 216 control samples (54 BALs, 54 sera, 45 PBMC, and 63 garglings) from 54 patients with AIDS (mean age, 34.6 y, range 23-46 y; mean CD4+, 107.3/mm^3, range 2-500 mm^3) with morphologically negative BAL for *P. carinii* f. sp. *hominis* (Table 1), and another 40 control samples (20 sera and 20 garglings) from 20 HIV-negative subjects working as health care personnel in the ward. The authors also examined, by mt-rRNA PCR, 180 samples (45 BALs, 45 sera, 45 PBMC, and 45 garglings) collected from 35 of 68 patients affected by PCP and from 10 of 54 PCP-negative patients. Internal transcribed spacers nested PCR demonstrated the presence of *P. carinii* f. sp. *hominis* DNA in 73 of 122 (59.8%) BALs: in 68 patients the results concurred with the direct morphologic diagnosis. In contrast, BALs from five patients yielded detectable levels of *Pneumocystis carinii* f. sp. *hominis* DNA even though the samples were negative by microscopic examination. Since these patients, during 3 months of follow-up, did not develop PCP in absence of *P. carinii* therapy and prophylaxis, it is possible that these were asymptomatic carriers.

The ITSs nested PCR detected the presence of *P. carinii* f. sp. *hominis* DNA in 191 of 306 (62.4%) non-BAL samples collected from patients with PCP: 59 of 79 (74.7%) garglings, 91 of 146 (62.3%) sera, and 41 of 81 (50.6%) PBMC were positive. At least one positive serum was noted in 39 of 68 (57.3%) patients with PCP: 22 of 68 (32.3%) had positive PBMC and 47 of 68 (69.1%) had positive oropharyngeal washes. All noninvasive samples from 54 HIV-positive patients with morphologically negative BAL for *P. carinii* f. sp. *hominis* and from 20 healthy subjects were negative (specificity 100%). At the same time, the application of mt-rRNA PCR on 35 positive BALs confirmed, in all the cases, the morphologic diagnosis of PCP. In contrast, after mt-rRNA PCR, 5 of 35 (14.3%) patients with PCP had positive serum, 8 of 35 (22.8%) positive PBMC, and 17 of 35 (48.6%) positive oropharyngeal washes, whereas control samples from 10 PCP patients were negative (Table 2).

Among patients with positive serum for *P. carinii* f. sp. *hominis* DNA, 10 received aerosol pentamidine (300 mg/mo) as primary prophylaxis and four received cotrimoxazole (960 mg/d). Twenty-five patients did not receive any prophylaxis during 6 months prior to the acute episode of PCP. Prophylaxis against *P. carinii* of all patients involved in the study is detailed in Table 3.

Serum samples collected during the follow-up showed that the presence of *P. carinii* f. sp. *hominis* DNA was limited to the first 8 to 12 days after the initiation of chemotherapy. During antipneumocystis chemotherapy, a rapid disappearance (less than 5 days) of serum *P. carinii* f. sp. *hominis*-DNA was observed in patients promptly recovering from PCP. These data suggest a possible relation between detection of *P. carinii* f. sp. *hominis* DNA in serum and severity of PCP (Table 4).

Type-specific oligonucleotide hybridization revealed the presence of 69 *P. carinii* f. sp. *hominis* isolates in 54 of 68 BAL samples (Table 5). In 14 of 68 BALs it was not possible to define *P. carinii* f. sp. *hominis* genotype because of the lack of hybridization at ITS1 or ITS2 sequences. Nine of 14 samples were then analyzed by cloning and sequencing and revealed new ITS types, as previously reported.[8] In 15 of 54 (27.8%) patients with PCP, TSO hybridization demonstrated the presence of more than one *P. carinii* f. sp. *hominis* genotype, suggesting a co-infection.

Comparing the results obtained after TSO hybridization on 28 BALs and 33 sera collected from 25 patients during 28 episodes of PCP (25 prime episodes and 3 relapses), the authors detected in 16 of 28 BALs a single *P. carinii* f. sp. *hominis* genotype and in 12 of 28 a co-infection with more than one *P. carinii* f. sp. *hominis* genotype. Besides, in 4 of 12 co-infections, perfect correspondence was noted between the *P. carinii* f. sp. *hominis* genotype detected on BAL and the one in serum. In contrast, for 8 of 12 patients the *P. carinii* f. sp. *hominis* genotype detected in serum only partially matched the one detected on BAL (Table 6). Genotype

Table 3. Prophylaxis in 122 Patients, Scored according to Bronchoalveolar Lavage Morphology Examination and *Pneumocystis carinii* f. sp. *hominis* DNA Serum Detection

	BAL Morphology and DNA Serum Detection†*		
Prophylaxis (n = 122) (100%)	*BAL+; DNA Serum+ (n = 39) (32%)*	*BAL+; DNA Serum– (n = 29) (23.8%)*	*BAL–; DNA Serum– (n = 54) (44.3%)*
Cotrimoxazole (960 mg/d) (n = 51) (41.8)	4 (3.3)	18 (14.8)	29 (23.7)
Pentamidine aerosol (300 mg/mo) (n = 38) (31.1)	10 (8.2)	7 (5.7)	21 (17.2)
None	25 (20.5)	4 (3.3)	4 (3.3)

BAL = bronchoalveolar lavage. *By Giemsa and toluidine blue; †by ITSs nested PCR.

Table 4. Clearance of Serum *Pneumocystis carinii* f. sp. *hominis* DNA Correlated to the Beginning of Chemotherapy in 39 Patients

Days from Beginning of Chemotherapy	Number of Patients with PCP		
	Mild (n = 26) (66.7%)	Severe (n = 13) (33.3%)	Total (n = 39) (100.0%)
<5	20 (51.3)	3 (7.7)	23 (59.0)
6–10	5 (12.8)	4 (10.2)	9 (23.1)
>10	1 (2.6)	6 (15.4)	7 (17.9)

PCP = *Pneumocystis carinii f.* sp. *hominis* pneumonia.
Mild PCP: 450 UI > LDH >1000 UI ; arterial pO_2 > 70 mmHg at diagnosis.
Severe PCP: LDH > 1000 UI; arterial PaO_2 < 70 mmHg at diagnosis.
LDH = lactate dehydrogenase.

analysis performed during three relapses revealed the presence of either the same genotype or a different one (Table 7).

DISCUSSION

Several authors have reported the possible application of various PCR techniques for the detection of *P. carinii* f. sp. *hominis* on various biologic samples collected from patients affected by PCP.[1,2,3] The purpose of the present study was to compare the sensitivity and specificity of two different PCRs (ITSs vs. mt-rRNA PCR) applied on BAL and noninvasive samples (blood and oropharyngeal washes) collected from AIDS patients with PCP. When applied to BALs, the sensitivity of the two techniques gave similar results, as expected after the experimental detection of two versus three *P. carinii* nuclei for mt-rRNA (multicopy gene) PCR and ITSs (single or double copy gene) nested PCR, respectively.[9,10]

All cases of PCP confirmed by microscopic diagnosis were observed to be positive by ITSs nested PCR and mt-rRNA PCR of BAL samples. Five morphologically negative BALs had detectable levels of *P. carinii* f. sp. *hominis* DNA, confirmed by TSO typing, in repeated controlled experiments. The authors suggest that asymptomatic carriers of *P. carinii* f. sp. *hominis* exist, as confirmed in patients recovering from actual pneumonia with cephotaxime or tobramycin therapy, the absence of overt PCP during 3-month follow-up, in the absence of antipneumocystis chemotherapy and prophylaxis, and the presence of a detectable level of *P. carinii* f. sp. *hominis* DNA (after the second step of PCR). This finding underscores the importance of evaluating these data. The hypothesis of the existence of asymptomatic carriers of *P. carinii* f. sp. *hominis* among AIDS patients has been suggested by other authors also.[11,12] Further research is needed to define the necessity of new adequate chemoprophylactic or therapeutic approaches.

Results obtained from serum samples revealed that ITSs nested PCR was more sensitive than mt-rRNA PCR (57.3% vs. 14.3%) for the detection of *P. carinii* f. sp. *hominis* DNA in patients with PCP. Various hypotheses can be proposed to explain these different results,[9,10] such as the presence of a low number of DNA copies in blood, or a peculiar susceptibility (or lability) to serum DNAse of the specific target sequence, or possibly, the number of PCR cycles (70 vs. 40). In fact, nested PCR is estimated to be 30 to 50 times more sensitive than single-step PCR.[13] The proper collection and storage of biologic samples is essential to obtain amplification of *P. carinii* f. sp. *hominis* DNA from serum; a delay in freezing or storage at 4°C can cause a reduction of PCR positive results. Examination of samples of positive serum, stored either at 4°C or at room temperature, 1, 2, 4, 6, or 10 days after storing, verified a rapid disappearance of *P. carinii* f. sp. *hominis* DNA, suggesting its peculiar lability. The possible presence of *P. carinii* f. sp. *hominis* DNA

Table 6. Results after Internal Transcribed Spacers (ITSs) Nested Polymerase Chain Reaction (PCR) and Type-Specific Oligonucleotide Typing in Case of Single *P. carinii* f. sp. *hominis* Infection (I,II,III) and Co-infection (IV, V, VI)

Patient Number	Serum Specimen	Day of Therapy	ITSs Nested PCR	P. carinii f. sp. hominis Types
I	Serum	0	+	Bc
	BAL	0	+	Bc
	Serum	+2	–	
II	Serum	–5	+	Ac
	BAL	0	+	Ac
	Serum	+11	–	
III	Serum	–2	+	Ba
	BAL	–1	+	Ba
	Serum	+8	–	
	Serum	+15	–	
IV	BAL	0	+	ABabc
	Serum	+11	+	Ba
V	BAL	0	+	ABac
	Serum	+2	+	Ba
	Serum	+14	–	
VI	BAL	0	+	ABc
	Serum	+3	+	Ac
	Serum	+6	–	

BAL = bronchoalveolar lavage.

Table 5. Type-Specific Oligonucleotide Hybridization: Typing of 69 *Pneumocystis carinii* f. sp. *hominis* Isolates in 54 Bronchoalveolar Lavage Samples

Number of Isolates	Isolate (n = 69) (%)				
	Ab	Ac	Ba	Bb	Bc
69 (100%)	5 (7.2)	16 (23.2)	9 (13.1)	18 (26.1)	21 (30.4)

Table 7. Genotypes Involved in Three Episodes of *Pneumocystis carinii* Pneumonia (PCP) Relapses: Either Reinfection (Genetic Switch) or Reactivation (Same Genotype)

Patient	First PCP Episode	Relapse
I	Ac	Ac
II	Bc	Ab
III	Bb	Bbc

in serum as already fragmented free-floating DNA was demonstrated when researchers were unable to detect whole microorganisms in centrifuged serum and by the inhibitory effect of serum pretreatment with DNAse on subsequent amplification steps (data not presented).

Data obtained from serum samples collected during follow-up of patients with PCP showed a strict relation between the acute phase of the disease and the presence of *P. carinii* f. sp. *hominis* DNA in serum. Since blood can be easily collected and stored, ITSs nested PCR applied for the detection of *P. carinii* f. sp. *hominis* DNA on serum samples before the beginning of specific therapy makes this a highly specific (100%), even if moderately sensitive (57.3%) diagnostic tool, not just a useful means to follow the course of the disease. The detection of *P. carinii* f. sp. *hominis* DNA in PBMC as well as in serum, both in patients undergoing primary specific prophylaxis and in naive patients, could suggest that the occurrence of extrapulmonary pneumocystosis (not observed in patients in the present study, at least by clinical and echographic examination) is attributable to preliminary blood-stream dissemination. Although the presence of *P. carinii* f. sp. *hominis* DNA in respiratory samples (BAL) could, in some cases, demonstrate a subclinical colonization during other pulmonary infections, the positivity of serum seems to be exclusively related to acute PCP, suggesting greater damage to the interstitial-alveolar barrier. This would concur with the findings of Miyawaki et al,[3] but it is in contrast with the observations of other authors.[9,13] These contradictory findings need further investigations for researchers to understand the real predictive or diagnostic value of the presence of *P. carinii* f. sp. *hominis* DNA in blood. The presence of *P. carinii* f. sp. *hominis* DNA in blood samples (serum and PBMC) collected either from patients undergoing pentamidine aerosol as primary prophylaxis or from naive patients needs to be more carefully studied to determine whether this phenomenon spontaneously occurs during the disease or if it could be favored by iatrogenic procedures.

The application of PCR on oropharyngeal washes, originally proposed with mt-rRNA primers by Wakefield and colleagues,[14] showed a sensitivity ranging from 56%; for PCR alone to 78% for PCR followed by oligoblotting: using ITSs nested PCR alone on oropharyngeal samples the present authors had similar results (69.1%). The high specificity of ITSs primers for *P. carinii* f. sp. *hominis* was further demonstrated by the absence of cross-reactions in the presence of oropharyngeal candidosis, in patients affected either by PCP or by other opportunistic pulmonary infections.

The negativity for *P. carinii* f. sp. *hominis* DNA on oropharyngeal washes collected from HIV-negative controls demonstrates an epidemiologic value, confirming the fact, described by other authors,[15,16] that subjects working as health care personnel are not involved as respiratory carriers for *P. carinii* f. sp. *hominis*.

Combined analysis of the results obtained by ITSs nested PCR on serum, PBMC, and garglings demonstrated the presence of *P. carinii* f. sp. *hominis* DNA in all patients with PCP, since each of them had at least one positive noninvasive sample collected before bronchoscopy. These results underline the possibility that ITSs nested PCR could have a clinical application for noninvasive diagnosis of PCP in AIDS patients.

The possibility of typing different *P. carinii* f. sp. *hominis* genotypes by TSO hybridization, indeed, represents a promising means to obtain more information for further studies about virulence and drug susceptibility. Preliminary results obtained after TSO hybridization on BAL and serum samples collected at different times suggest either a different genotype-related blood dissemination or a subsequent clearance from blood during specific chemotherapy. The lack of hybridization observed by the authors could be explained by the recent finding of further variation among ITSs sequences, underlining the necessity of sequencing for *P. carinii* f. sp. *hominis* typing, at least in "missed" isolates that cannot be properly typed by the TSO technique originally described by Lu et al.[4] Typing of *P. carinii* f. sp. *hominis* isolates, after all, could be a useful epidemiologic tool to investigate the dynamics of relapses occurrence, demonstrating the possibility of either endogenous reactivation (same genotype) or reinfection (genetic switch), as described by other authors.[17,18]

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