MOLECULAR DIAGNOSIS AND EPIDEMIOLOGY

Oropharyngeal samples for genotyping and monitoring response to treatment in AIDS patients with *Pneumocystis carinii* pneumonia

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A nested PCR, amplifying a portion of the gene encoding the mitochondrial large subunit ribosomal RNA (mt LSU rRNA) of *Pneumocystis carinii* sp. f. *hominis* was applied to oropharyngeal samples obtained on repeated occasions from 12 HIV-infected patients with *P. carinii* pneumonia (PCP) to monitor response to anti-*P. carinii* treatment. Genotyping of *P. carinii* sp. f. *hominis* was also performed on paired samples of oropharyngeal and broncho-alveolar lavage samples before the start of treatment, and on oropharyngeal samples during the course of treatment, by analysis of sequence variation at the internal transcribed spacer (ITS) regions of the nuclear rRNA operon. When a simple dilutional method was used, a reduction in the amount of amplification product was observed in samples from all patients during the course of treatment. In eight of the 12 patients, a single ITS sequence type was found in the oropharyngeal samples and also in the paired broncho-alveolar lavage sample. A mixed infection was identified in the samples from three patients. In eight patients, the ITS sequence types identified in the oropharyngeal sample were the same as in the broncho-alveolar lavage sample. Nested PCR amplifying the mt LSU rRNA on oropharyngeal samples provides a non-invasive method of monitoring response to treatment of PCP. ITS sequence typing of *P. carinii* sp. f. *hominis* from oropharyngeal samples appears to be a reliable alternative to broncho-alveolar lavage samples and provides a non-invasive tool for further epidemiological studies.

Introduction

The opportunist fungus *Pneumocystis carinii* sp. f. *hominis* is an important cause of pneumonia in immunocompromised individuals, patients receiving immunosuppressive therapy for organ transplantation and patients receiving chemotherapy for the treatment of malignant disease. Despite the widespread availability of highly active antiretroviral therapy and effective regimens of anti-*P. carinii* prophylaxis, *P. carinii* pneumonia (PCP) remains an important cause of respiratory morbidity in individuals with HIV infection, especially in those who are unaware of their HIV serostatus or who are intolerant of, or non-compliant with *P. carinii* prophylaxis or antiretroviral therapy. As immunosuppressed patients with PCP

rarely produce sputum, and the diagnostic yield from spontaneously expectorated sputum is low for *P. carinii* [1], diagnosis has relied on histochemical or immunofluorescence staining of respiratory secretions obtained by the invasive techniques of hypertonic saline induced sputum, or bronchoscopic alveolar lavage (BAL) [2, 3].

The application of molecular diagnostic techniques to BAL fluid [4] or induced sputum [5] has demonstrated that DNA amplification has a higher sensitivity than conventional staining for detection of *P. carinii* sp. f. *hominis*. Oropharyngeal (OP) samples, obtained by simple rinsing of the mouth, provide an alternative non-invasive sample. A preliminary study has shown that *P. carinii* sp. f. *hominis* DNA can be detected in 78% of OP samples from HIV-infected persons with PCP by the PCR with oligoblotting, with primers designed to the mitochondrial large subunit ribosomal RNA (mt LSU rRNA) gene [6]. A single round of touchdown PCR at the same locus has also been shown to be effective in the diagnosis of PCP in OP samples

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[7, 8]. A nested PCR at the mt LSU rRNA has now been developed, based on the original primers pAZ102-H and pAZ102-E [4–6, 9] with internal primers pAZ102-X and pAZ102-Y, which has an improved sensitivity for the detection of *P. carinii* sp. f. *hominis*. This is able to detect one or two organisms [10, 11], and has been applied successfully to OP samples [12].

Differences among isolates of *P. carinii* sp. f. *hominis* have been demonstrated by identification of DNA polymorphisms at a number of loci. At a portion of the mt LSU rRNA gene, two single base polymorphisms have been identified with five different types of *P. carinii* sp. f. *hominis* [11, 13–15]; at the 5-enolpyruvyl shikimate-3-phosphate synthase domain of the *arom* locus, two single-base polymorphisms have been observed, resulting in four different types [11, 16]. Variations at the internal transcribed spacer (ITS) regions of the nuclear RNA operon of *P. carinii* sp. f. *hominis* have been found to be more informative and have been used to develop a method of typing isolates of *P. carinii* sp. f. *hominis* [11, 17–24]. To date, BAL fluid or lung biopsy material has been used to obtain isolates of *P. carinii* sp. f. *hominis* for typing.

In this study, a nested PCR with primers to the mt LSU RNA was applied to OP samples, obtained on repeated occasions from HIV-infected persons with PCP to monitor response to treatment. The *P. carinii* sp. f. *hominis* detected in the OP samples were typed by analysis of sequence variations at the ITS regions.

Materials and methods

Patients

A total of 12 patients was studied prospectively; all were HIV-1 antibody-positive homosexual men admitted to the specialist HIV/AIDS Unit, Middlesex Hospital Site, University College London Hospitals Trust, for investigation of respiratory symptoms. A diagnosis of PCP was made on the basis of typical clinical presentation and positive histochemical staining of BAL fluid from 11 patients (patients 1–3 and 5–12) and hypertonic saline-induced sputum from one patient (patient 4), with Grocott's methenamine silver stain. All fiberoptic bronchoscopies were done by one respiratory physician (RFM) by a technique described previously [25]. Sputum induction was as described previously [25]. At the time of bronchoscopy a 10-ml portion of BAL fluid was saved, frozen immediately after collection and stored at -20°C before analysis.

The CD4 lymphocyte count and previous prophylaxis against *P. carinii* for each patient were recorded at presentation. The disease severity (Table 1) was assessed by means of the arterial oxygen tension (measured with the patient breathing room air; the lower the arterial oxygen tension, the more severe the pneumonia) and the degree of chest radiographic abnormalities by a scoring system as described previously [6]. Co-pathogens were identified in the BAL fluid from two patients, patient 6 had *Streptococcus pneumoniae* which was treated with oral

Table 1. Patient characteristics, disease severity and treatment of PCP

Patient no.	Sample code	Prophylaxis	Episode of PCP	CD4 count ($\times 10^6/\text{L}$)	Disease severity		Treatment drug/route/duration
					PaO ₂ (kPa)	Chest X-ray score	
1	H32	Nil	1	30	9.5	2	TMP/SMX iv (d1–d13) → TMP/SMX po (d14–d21)
2	H15	Dapsone	1	100	9.6	2	Pent iv (d1–d8) → pent neb (d9–d21)
3	H33	Nil	1	90	6.9	3	TMP/SMX iv (d1–d9) → clin/prim po (d10–d14) → atovaquone on (d15–d21) methylprednisolone iv (d1–d5) → prednisolone po (d6–d15)
4	H27	Nil	1	20	11.9	2	Pent iv (d1–d5) → clin/prim po (d6–d21)
5	H10	Nil	1	310	8.6	3	TMP/SMX iv (d1–d8) → clin/prim iv/po (d9–d14) → iv neb (d15–d21) methylprednisolone iv (d1–d5) → prednisolone po (d6–d15)
6	H28	Dapsone	1	80	11.0	2	Pent iv (d1–d5) → clin/prim po (d6–d16) → atovaquone po (d17–d21)
7	H22	Neb pent	2	70	10.2	2	Clin/prim iv/po (d1–d14) → clin/prim po (d15–d21) methylprednisolone iv (d4–d8) → prednisolone po (d9–d15)
8	H23	Neb pent	3	60	11.3	2	Atovaquone po (d1–d21)
9	H6	TMP/SMX	1	20	8.6	3	TMP/SMX iv (d1–d14) → TMP/SMX po (d15–d21)
10	H43	Nil	1	30	8.3	2	TMP/SMX iv (d1–d11) → TMP/SMX po (d12–d21) methylprednisolone iv (d1–d3)
11	H44	Neb pent	1	10	8.1	3	Clin/prim iv/po (d1–d10) → clin/prim po (d11–d21) methylprednisolone iv (d1–d5) → prednisolone po (d6–d15)
12	H34	Dapsone	1	50	9.2	2	Pent iv (d1–d8) → clin/prim po (d9–d21)

→, treatment changed (because of adverse reaction, not failure to respond); iv, intravenous; po, by mouth; TMP/SMX, trimethoprim/sulphamethoxazole; pent, pentamidine; neb, nebulised; clin, clindamycin; prim, primaquine; d, day. Chest X-ray score: 0, normal; 1, subtle increased perihilar interstitial markings; 2, more prominent interstitial markings, extending laterally, without evidence of alveolar infiltrates; 3, gross interstitial infiltrates with mild alveolar infiltrates; 4, extensive interstitial and alveolar infiltrates.

erythromycin and patient 8 had *Haemophilus influenzae* which was treated with oral amoxycillin. Cotrimoxazole (trimethoprim/sulphamethoxazole, TMP/SMX) was the preferred treatment unless patients had a previous history of adverse reactions to these agents, in which case alternative therapies were used according to a unit protocol [26]. In patients with an arterial oxygen tension <9.3 kPa breathing room air, adjuvant glucocorticoids were given, following published guidelines [27]. Response to treatment was assessed by defervescence and improvements in tachypnoea, dyspnoea and arterial oxygenation. When treatment was changed, it was because of adverse reaction to therapy and not because of treatment failure. Finally, use of secondary prophylaxis was recorded and patients were followed up for 6 months after their recovery from PCP.

Before treatment began, OP samples were obtained from each patient as described previously [6]. In summary, patients gargled and rinsed out their mouth with 10 ml of sterile saline 0.9%. Further OP samples were obtained from each patient, where possible on a daily basis, until they were discharged from hospital. Consecutive daily samples were not obtained in all patients for several reasons. Some patients declined repeat sampling on some days but not on others, some patients were temporarily absent from the ward (e.g., for weekend leave) and others were well enough to be managed as outpatients and, therefore, returned to the ward for monitoring only intermittently or when they developed adverse drug reactions and required change of therapy and close monitoring. On each occasion, samples were obtained in the morning, where possible soon after the patient had awoken and before they had brushed their teeth and eaten breakfast. The OP samples were frozen immediately and stored at -20°C. OP samples were obtained before bronchoscopy or sputum induction on the days these procedures were performed. The BAL fluid and OP samples were coded and all analyses were done blind to the clinical details.

PCR detection of *P. carinii*

The OP samples (4 ml) were centrifuged at 12 000 *g* for 5 min and the pellets were recovered. DNA was extracted from the OP pellets and the BAL samples (0.5 ml) [12]. The presence of *P. carinii* in the OP samples was detected with a nested PCR at the mt LSU rRNA, with primers pAZ102-H and pAZ102-E in the first round amplification followed by pAZ102-X and pAZ102-Y in the second round amplification as described previously [10, 11]. Taq DNA polymerase (Promega, Southampton) was used throughout the study. Serial five-fold dilutions of the sample DNA were used in each PCR experiment and an approximate estimation of the *P. carinii* sp. f. *hominis* load in each sample was obtained. Negative controls were included in each experiment in both DNA extraction and

amplification, to monitor for possible contamination. DNA extraction and PCR were performed in a laminar flow cabinet and disposable tips, tubes and reagent aliquots were used to avoid contamination. A sample of *P. carinii* sp. f. *hominis* DNA, obtained from a patient with histologically confirmed PCP, was used as a positive control in each experiment.

P. carinii ITS sequence typing

DNA amplification at the ITS regions was performed on OP and BAL samples by a nested PCR, with NITSF and NITSR in the first round amplification and ITSF3/RI and ITS2R3/RI in the second round amplification, as described previously [11, 20]. The primer pair N18SF (5'-GGTCTTCGACTGGCAGC-3') and N26SRX (5'-TTACTAAGGGAATCCTTGTTA-3') were subsequently used as first round primers in 10 samples and showed greater specificity for *P. carinii* sp. f. *hominis*. Amplification products were either sequenced directly by cycle sequencing with radiolabelled terminators (Thermo Sequenase cycle sequencing kit, Amersham), or cloned and sequenced by conventional or automated sequencing. Amplification products were sequenced directly by cycle sequencing with the primers 5.8S-ITS1RX (5'-CAATT-CACACTACTTATCGCA-3'), 5.8S-ITS2FX (5'-GCA-GAATTTAGTGAATCATCG-3') and ITS1HPT (5'-AAAAAAAAAATTCACAGATGTTTGA-3'). When an unambiguous sequence was obtained, the ITS type was assigned to the sample. In samples in which the sequence was not definitive, suggesting the presence of more than one type of *P. carinii* sp. f. *hominis*, the amplification products were cloned and the sequence of four clones was determined by either conventional or automated sequencing. Some samples were analysed by both methods to confirm the validity of the methods. The BAL samples from patients 3, 6 and 12 were sequenced directly, those from patients 2, 5, 7 and 9 were cloned and then sequenced, and those from patients 1 and 10 were analysed by both methods. The OP samples from patient 4 were sequenced directly, those from patients 1, 2, 3, 5 and 11 were cloned and then sequenced, and those from patients 6, 7, 8, 9, 10 and 12 were analysed by both methods.

Results

P. carinii sp. f. *hominis* load in patients receiving anti-*P. carinii* therapy

Sequential OP samples were obtained from 12 HIV-infected patients with confirmed PCP who were receiving treatment for pneumonia. An OP sample was taken from each patient before the start of treatment and before bronchoscopy (day 1). A BAL sample was obtained from 11 of the 12 patients 1–3 days later. Further OP samples were taken from each patient at intervals for a period of 8–24 days. The

number of OP samples analysed for each patient ranged from six to 14 (Fig. 1).

Nested PCR at the mt LSU rRNA, with serial dilutions of each sample was used to assess the *P. carinii* load in each sample. This PCR has previously been shown to be specific for *P. carinii* and to have a high level of sensitivity because of the presence of multiple copies of the mitochondrial genome/organism [4–6, 28–35]. *P. carinii* DNA was detected in the samples taken before the start of treatment (day 1) from all the patients (Fig. 1). The approximate amount of *P. carinii* DNA in each OP sample was estimated, and showed a high level of amplification product in the samples from all the patients at day 1, which was still present at day 3 (Fig. 1). Fluctuations in the amount of amplification product, resulting in an overall reduction, were observed in samples from all patients during the course of their treatment. An overall reduction was detected by day 7 in one of the patients (patient 1), by day 14 in a further five (patients 2, 3, 4, 5 and 6) and in all patients by day 21 (Fig. 1 and Table 2). In four of the samples (from patients 4, 8, 9 and 10), *P. carinii* DNA was detected in the final OP sample collected, on days 9, 15, 16 and 17, respectively. Therefore, in these patients it was not possible to assess the time point at which *P. carinii* could no longer be detected. After recovery from PCP, all patients began secondary prophylaxis. No patient developed recurrent PCP during the 6-months follow-up period.

P. carinii sp. f. *hominis* ITS sequence types from paired OP and BAL samples

The ITS sequence type of *P. carinii* sp. f. *hominis* extracted from paired OP and BAL samples was determined in 11 of the 12 patients (bronchoscopy was not performed on patient 4). The OP sample was taken before the start of treatment (day 1) and before the BAL sample. A single BAL sample was taken from 11 patients (an IS sample was taken from patient 4) 1–3 days later for diagnostic purposes, and subsequently used for ITS sequence typing (Table 2). Previous studies have reported 13 different ITS sequence types [11, 20]. In this study, a total of five ITS1 types and nine ITS2 types were observed, resulting in a total of 15 ITS sequence types, of which 10 are new to this study (Miller, Tsolaki and Wakefield, unpublished observations). In eight of the patients (patients 3, 5–8, 10–12) a single ITS sequence type was observed in the OP sample at day 1. This same ITS sequence type was also detected in the BAL sample, as a single type

(Table 2). In patient 9, two ITS sequence types were found in the OP sample (B_{1a3} , B_{1c1}) and only one of these types (B_{1a3}) was found in the BAL sample taken 3 days later. In two patients (patients 1 and 2) more than one ITS sequence type was detected in the BAL and the OP samples. In patient 1, two ITS types (B_{1a1} and B_{2a3}) were observed in the OP sample at day 1, and on day 3 of treatment, a mixture of ITS types was observed in both the OP and BAL samples. In patient 2, four ITS types (B_{1e1} , B_{1a1} , B_{2a1} , B_{2a3}) were observed in the OP sample on day 1, and four ITS types (B_{1e1} , B_{1e3} , B_{1a3} , B_{2a2}) in the BAL sample taken on day 2 of treatment. ITS type B_{1e1} was present in both these samples (Table 2).

P. carinii sp. f. *hominis* ITS sequence types in sequential OP samples during anti-*P. carinii* therapy

The ITS sequence type was determined in the OP sample at the final time point for which PCR at the ITS locus yielded a positive amplification product. In four of the 12 patients, the ITS sequence type was determined in the final OP sample in which *P. carinii* DNA was detected by PCR at the mtLSU rRNA locus. However, in eight of the patients the study failed to amplify *P. carinii* DNA by the ITS PCR in samples in which PCR at the mt LSU rRNA gene was positive. PCR at the mt LSU rRNA was more sensitive than at the ITS regions. ITS PCR was attempted on multiple OP samples from these patients, and the latest sample during the course of treatment at which an amplification product was obtained was used for typing. This ranged from day 3 (patient 1) to day 15 (patient 12) (Fig. 1 and Table 2).

A single ITS sequence type was found at the final time point for which PCR at the ITS locus yielded a positive amplification product in the sample from 10 of the patients. In nine of these (patients 3–8, 10–12), a single ITS sequence type was also found in the OP sample before the start of treatment (day 1). In the samples from these nine patients, the same ITS sequence type was found at day 1, in the sample taken before the start of treatment, and during the course of treatment. In three of the patients (patients 1, 2 and 9) more than one ITS sequence type was found in the OP sample taken before the start of treatment. In patient 9, two types were found in the OP sample (B_{1a3} , B_{1c1}) taken before the start of treatment, and only one of these (B_{1a3}) was found in the BAL sample taken 3

Fig. 1. *P. carinii* sp. f. *hominis* amplification product in sequential OP samples, taken from day 1 up to day 25, from 12 patients receiving anti-*P. carinii* therapy. +++, *P. carinii* sp. f. *hominis* amplification product detectable at neat, 5- and –25-fold dilution of sample; ++, *P. carinii* sp. f. *hominis* amplification product detectable at neat and 5-fold dilution of sample; +, *P. carinii* sp. f. *hominis* amplification product detectable in neat sample; 0, no detectable *P. carinii* sp. f. *hominis* amplification product. ■, days on which *P. carinii* sp. f. *hominis* amplification product was detected; □, days on which samples were tested but no *P. carinii* sp. f. *hominis* amplification product was detected; ▨, days on which samples also underwent ITS sequence typing.

Table 2. ITS sequence typing of *P. carinii* sp. f. *hominis* from OP and BAL samples

Patient no.	Sample code	Last detectable DNA signal after start of treatment (days)	OP sample at start of episode		BAL sample		OP sample during treatment	
			Day	ITS type	Day	ITS type	Day	ITS type
1	H32	6	1	B ₁ a ₁ B ₂ a ₃	3	B ₁ a ₁ B ₁ a ₃ B ₂ a ₁	3	B ₁ a ₁ B ₁ a ₃ B ₁ a ₂ B ₄ a ₃
2	H15	9	1	B ₁ e ₁ B ₁ a ₁ B ₂ a ₁ B ₂ a ₃	2	B ₁ e ₁ B ₁ e ₃ B ₁ a ₃ B ₂ a ₂	9	B ₁ e ₁ B ₁ e ₂ B ₅ a ₃
3	H33	9	1	B ₁ b ₁	4	B ₁ b ₁	9	B ₁ b ₁
4	H27	9	1	B ₁ b ₁	—	—	9	B ₁ b ₁
5	H10	12	1	B ₂ a ₅	4	B ₂ a ₅	5	B ₂ a ₅
6	H28	13	1	B ₂ a ₁	3	B ₂ a ₁	4	B ₂ a ₁
7	H22	15	1	B ₂ a ₁	2	B ₂ a ₁	6	B ₂ a ₁
8	H23	15	1	B ₂ a ₁	2	B ₂ a ₁	12	B ₂ a ₁
9	H6	16	1	B ₁ a ₃ B ₁ c ₁	4	B ₁ a ₃	16	B ₁ a ₃
10*	H43	17	1	B ₁ a ₃	3	B ₁ a ₃	10	B ₁ a ₃
11	H44	17	1	B ₁ a ₃	2	B ₁ a ₃	6	B ₁ a ₃
12	H34	21	1	A ₄ a ₃	2	A ₄ a ₃	15	A ₄ a ₃

*Patient 10 – additional OP samples during treatment: day 2, ITS type B₁a₃; day 3, ITS type B₁a₃.

days later, and in the OP sample taken after treatment for 16 days.

In patient 1, two ITS types (B₁a₁, B₂a₃) were observed in the OP sample at day 1. After treatment for 3 days, a mixture of ITS types was detected in both the OP and BAL samples; four ITS types were detected in the OP sample (B₁a₁, B₁a₃, B₁a₂, B₄a₃) whilst three ITS types were observed in the BAL sample (B₁a₁, B₁a₃, B₂a₁). One ITS type (B₁a₁) was common to all three samples from this patient, whilst two ITS types (B₁a₃, B₁a₁) were common to the OP and BAL samples collected on day 3. More than one ITS sequence type was also found in the samples from patient 2. Four ITS types (B₁e₁, B₁a₁B₂a₁, B₂a₃) were detected in the OP sample taken before the start of treatment and four ITS types (B₁e₁, B₁e₃, B₁a₃, B₂a₂) were detected in the BAL sample taken on day 2 of treatment. ITS type B₁e₁ was present in both these samples and again on day 9, when three ITS types were observed (B₁e₁, B₁e₂, B₅a₃) (Table 2).

Discussion

An earlier study demonstrated the utility of OP samples in the detection of *P. carinii* sp. f. *hominis*, by a single round of PCR at the mt LSU rRNA followed by probing with a radiolabelled oligonucleotide, and showed a sensitivity of 78% [6]. The present study used a nested PCR at the mt LSU rRNA and obtained *P. carinii*-specific amplification product with all the samples from patients with confirmed PCP, taken before the start of anti-*P. carinii* treatment. OP samples have been shown to be effective for the diagnosis of PCP [6–8, 12]. This study demonstrated the value of these samples in epidemiological studies, as multiple, sequential samples can be obtained from patients

without the need for potentially dangerous invasive procedures. A simple method was used to obtain an approximate estimation of the *P. carinii* sp. f. *hominis* load in these samples, by PCR assay of serial dilutions of extracted DNA. A diminution of amplification signal with time during the course of therapy was demonstrated, which was an indication of the relative amounts of *P. carinii* sp. f. *hominis* in the samples. The earlier study showed that there are fewer *P. carinii* sp. f. *hominis* organisms in an OP sample than a BAL sample [6]. Therefore, it is assumed that the *P. carinii* sp. f. *hominis* burden in the OP sample reflects the load in the BAL sample, but that the number of *P. carinii* is lower. *P. carinii* sp. f. *hominis* primarily infects the alveolar spaces, with only low numbers of organisms in upper respiratory tract samples (particularly OP samples), undetectable by microscopy but detectable by PCR.

Earlier studies showed an inverse relationship between the strength of the amplification signal and the duration of anti-*P. carinii* treatment [6, 30]. In the present study, *P. carinii* sp. f. *hominis* DNA was detected in samples from each of the 12 patients, who had a range of disease presentations and severity, up to day 6 of treatment. The reduction in the *P. carinii* sp. f. *hominis* load in the OP samples correlated with the clinical improvements of the patients. This is consistent with findings from a previous study in which *P. carinii* load in HIV-infected patients with PCP was monitored by methenamine silver staining of repeat BAL samples taken after 21 days of anti-*P. carinii* treatment. A reduction in *P. carinii* count was seen in the majority of patients who responded to treatment and who did not relapse. In contrast, no reduction in *P. carinii* count was observed in some clinical responders. The majority of these patients had a poor outcome, with a relapse of *P. carinii* pneumonia within 6 months [36].

Determination of the *P. carinii* load by DNA amplification rather than methenamine silver staining may result in a more accurate estimate of the number of viable *P. carinii* sp. f. *hominis* organisms in a sample. DNA from all life-cycle stages of the organism is detected by PCR, whereas methenamine silver staining only visualises the cystic phase of the organism, and does not distinguish between viable organisms and the cell wall of empty, non-viable cysts. However, the results of the present study suggest that the quality of the OP samples may fluctuate to some extent. This is to be expected, as *P. carinii* sp. f. *hominis* is primarily present as an alveolar infection and many factors such as variability in cough and the effectiveness of the muco-ciliary escalator will influence the number of organisms in an OP sample. In a few patients, consecutive samples showed fluctuations between positive and negative results, before being consistently negative for *P. carinii* sp. f. *hominis*. The inclusion of numerous controls in each experiment ensured that this fluctuation was not due to contamination, but was a true reflection of the parasite load in these upper respiratory tract samples. Despite these technical complications, the results show that OP samples provide useful non-invasive specimens for epidemiological studies on *P. carinii* sp. f. *hominis*.

Although *P. carinii* sp. f. *hominis* DNA was detected in the last available sample in four of the 12 patients in this study, all the patients recovered from their pneumonia and none had another episode of PCP in the following 6 months. Patient 11 had a further episode of PCP 7 months later. Further studies are now in progress, applying this methodology to samples from patients who failed to respond to treatment, patients who initially responded and then deteriorated towards the end of their course of treatment, and patients who responded yet underwent an early relapse.

These data demonstrate that detection of *P. carinii* sp. f. *hominis* by nested PCR at the ITS regions was less sensitive than nested PCR at the mt LSU rRNA. ITS sequence genotyping was possible in only four of the 12 patients at the final time point at which the mt LSU rRNA PCR was positive. The most plausible explanation for this finding is the copy number of the two genes, the mitochondrial genome being present in many copies within each organism, whereas the nuclear-encoded ITS regions of the rRNA operon are thought to be present in only one copy per *P. carinii* genome [37].

In nine of the patients, a single *P. carinii* sp. f. *hominis* ITS sequence type was observed in the OP sample taken before the start of treatment. In eight of these patients, the same type was observed in the BAL sample taken 1–3 days later (there was no BAL sample for patient 4), showing a good correlation between ITS sequence type observed in the BAL and OP samples. In OP samples from three patients (patients 1, 2 and 9) at

day 1 in which more than one ITS sequence type was detected, a more complex picture was observed. Two-to-four different ITS sequence types were observed in the OP sample before the start of treatment and in the BAL sample from these three patients. In each case, one of the types was present in both the OP and BAL samples.

In the majority of patients (9 of 12), the ITS sequence type identified before the start of treatment was the same as at the final time point. In these nine patients, a single *P. carinii* sp. f. *hominis* type was identified in each sample; this suggested *P. carinii* sp. f. *hominis* strain stability over this time period and a lack of ITS genotype switching during the course of treatment in these patients. In patient 10, ITS sequence typing was performed on four different OP samples during the treatment (days 1, 2, 3 and 10). The same type (B₁a₃) was found as a single type in all the samples. In the three patients in whom mixed infections were observed, the data were more complex. In patient 2, a total of nine different ITS types was found throughout the treatment period. In each patient, one of the ITS sequence types was common to all three samples, taken before the start of treatment, during treatment and at the final time point. Other studies have also reported the lack of clonality in *P. carinii* sp. f. *hominis* infection showing mixed infection with multiple types of *P. carinii* sp. f. *hominis*, as demonstrated by polymorphisms at a number of genetic loci [11, 15, 18, 20, 21, 23, 24]. A total of seven different ITS sequence types has been reported in a sample from Denmark [24].

The ITS sequence types B₂a₁ and B₁a₃ were the most common types found in this study, each observed in five of the 12 patients. These *P. carinii* sp. f. *hominis* types have also been reported as frequent types in samples from Denmark and the USA (types Ne and Eg respectively) [24]. Two patients in this study had had a previous episode of PCP (patients 7 and 8). The ITS sequence type B₂a₁ was found in the samples from both these patients, giving further support to the original observation that B₂a₁ was frequently found in second and subsequent episodes of PCP, and may represent a more persistent or more transmissible type of *P. carinii* sp. f. *hominis* [20].

This study demonstrated that ITS sequence typing of *P. carinii* sp. f. *hominis* can be done on non-invasive OP samples. Studies are now in progress to utilise these non-invasive samples for typing at other genetic loci, particularly those that encode the targets of anti-*P. carinii* therapies. The possible emergence of drug-resistant strains of *P. carinii* sp. f. *hominis* has been suggested by the identification of isolates with polymorphisms in the gene encoding dihydropteroate synthase (DHPS), the target of sulphonamide drugs [38–40] and in the gene encoding cytochrome b, the target of atovaquone [41]. The use of OP samples for

sequence typing at these gene targets will greatly facilitate studies on the identification of drug resistance in *P. carinii* sp. f. *hominis*.

Nested PCR amplifying the mt LSU rRNA from OP samples provides a non-invasive method for monitoring response to treatment of PCP in HIV-infected patients, and permits the analysis of the duration of the infection with different anti-*P. carinii* treatment regimens. ITS sequence typing of *P. carinii* sp. f. *hominis* from OP samples appears to be a reliable alternative to typing from BAL fluid. This will provide a non-invasive tool for further epidemiological studies, and in particular the correlation of different *P. carinii* sp. f. *hominis* ITS sequence types with duration of the infection, the association of different ITS sequence types with different treatment regimens and the identification of organisms that demonstrate resistance to anti-*P. carinii* drugs.

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