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Pre-AIDS Era Isolates of *Pneumocystis carinii* f. sp. *hominis*: High Genotypic Similarity with Contemporary Isolates

ANTHONY G. TSOLAKI,[1] PIETER BECKERS,[2] AND ANN E. WAKEFIELD[1]*

Molecular Infectious Diseases Group, Department of Paediatrics, Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, OX3 9DS, United Kingdom,[1] and Department of Medical Microbiology, University Hospital Nijmegen, Nijmegen, The Netherlands[2]

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Isolates of *Pneumocystis carinii* f. sp. *hominis* were examined from six individuals who died of *P. carinii* pneumonia between 1968 and 1981 and who had underlying immunodeficiencies which were not due to human immunodeficiency virus infection. DNA sequence variation was analyzed in the genes encoding the mitochondrial large subunit rRNA (mt LSU rRNA), the internal transcribed spacer (ITS) regions of the nuclear rRNA, the *arom* locus, and the mitochondrial small subunit rRNA. No major variations were observed when these isolates were compared to isolates from HIV-infected individuals. A small number of minor differences were detected. A new position at which variation occurred in the mt LSU rRNA was observed in one sample. Three new ITS sequence types were identified. A total of nine different ITS sequence types were found in the six samples. Mixed infection with different ITS sequence types of *P. carinii* f. sp. *hominis* was observed in four of the six samples. The ITS locus was the most informative of the four loci for distinguishing among the isolates of *P. carinii* f. sp. *hominis*. The data suggest that isolates of *P. carinii* f. sp. *hominis* from before the AIDS pandemic are genetically very similar to those currently found in HIV-infected individuals.

The opportunistic fungus *Pneumocystis carinii* causes potentially fatal pneumonia in immunocompromised individuals. In the past, the majority of incidence of *P. carinii* pneumonia were in individuals with congenital immunodeficiencies and in patients receiving immunosuppressive therapy for organ transplantation or undergoing chemotherapy for the treatment of malignant disease. In recent years, however, there has been a large increase in the incidence of *P. carinii* pneumonia, primarily in individuals immunocompromised by infection with human immunodeficiency virus (HIV) (5, 21).

Recently developed molecular techniques have enabled the typing of *P. carinii* f. sp. *hominis* (human-derived *P. carinii*) (17). Since the organism cannot be propagated by *in vitro* culture, pure isolates of parasite cannot be obtained and standard methods of typing cannot be utilized. However, different types of *P. carinii* f. sp. *hominis* have been identified by the comparison of DNA sequences at a number of genetic loci. A portion of the gene encoding the mitochondrial large subunit rRNA (mt LSU rRNA) has been used to study genetic variation among isolates of *P. carinii* f. sp. *hominis* from HIV-infected patients in the United States (11), France, Italy (9, 10), the United Kingdom, Africa, and South America (19) and from HIV-infected patients with recurrent episodes of *P. carinii* pneumonia (7, 8).

Genetic diversity has also been reported among isolates of *P. carinii* f. sp. *hominis* at a portion of the *arom* gene, in the region encoding the 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase activity, involved in aromatic amino acid biosynthesis (1). The presence of more than one type of *P. carinii* f. sp. *hominis* in a single infected lung has been demonstrated by the typing of isolates at this single-copy-number gene (1).

The sequences of the internal transcribed spacer (ITS) regions of the nuclear rRNA operon have also been shown to vary among isolates of *P. carinii* f. sp. *hominis* (9, 12, 13). The ITS regions have been used to investigate variation among isolates of *P. carinii* f. sp. *hominis* from HIV-infected patients with recurrent episodes of *P. carinii* pneumonia (15).

In this study we have examined archival isolates of *P. carinii* f. sp. *hominis* from six individuals who had a variety of underlying immunodeficiencies but were not infected with HIV. The samples dated from the period 1968 to 1981, before the onset of the AIDS pandemic. The aim of the study was to compare the nature and extent of diversity in these samples, from a period when *P. carinii* pneumonia was a rare disease, with organisms isolated from HIV-infected individuals. Genetic diversity was examined in the genes encoding the mt LSU rRNA locus and the ITS regions. In addition, variations in the genes encoding the *arom* locus and the mitochondrial small subunit rRNA (mt SSU rRNA) were also examined in three of the samples.

MATERIALS AND METHODS

Samples. Samples of *P. carinii* f. sp. *hominis* were obtained from patients, diagnosed at the University Hospital Nijmegen, Nijmegen, The Netherlands, who died of *P. carinii* pneumonia, confirmed by histological staining, between 1968 and 1981. Samples of lung were taken post mortem and stored at −70°C until analysis. Clinical details for each sample are listed in Table 1.

DNA extraction. A small portion of lung tissue from each sample was finely minced, homogenized with a pellet mixer in a microcentrifuge tube, and treated with proteinase K (Boehringer Mannheim) at a final concentration of 1 mg/ml in the presence of 0.5% sodium dodecyl sulfate and 10 mM EDTA (pH 8.0) at 50°C overnight. Two phenol-chloroform extractions were performed, and the DNA was purified and concentrated with a DNA binding resin (Wizard DNA Clean-up System; Promega, Southampton, United Kingdom) (16). Negative buffer controls were prepared concurrently to monitor for cross-contamination.

DNA amplification. DNA amplification was carried out on the samples in a reaction mixture containing a final concentration of 10 mM Tris-HCl (pH 8.8), 10 mM KCl, 0.002% Tween 20 (vol/vol), 3 mM $MgCl_2$, 400 mM (each) deoxynucleoside triphosphate (Boehringer Mannheim), 1 mM (each) oligonucleotide primer, and 0.025 U of Ultma *Taq* polymerase (Perkin-Elmer Cetus) per μl. Nested PCR was used when the amount of *P. carinii* f. sp. *hominis* DNA in the samples was low and was insufficient to produce a visible band of amplification product on an ethidium bromide-stained gel after a single round of amplification (Table 2). Negative controls with no added DNA were included after each

* Corresponding author. Mailing address: Molecular Infectious Diseases Group, Department of Paediatrics, Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, OX3 9DS, United Kingdom. Phone: 44-1865-222344. Fax: 44-1865-222626. E-mail: wakefiel@worf.molbiol.ox.ac.uk.

TABLE 1. Clinical details

Sample	Year of acquisition	Age of patient (yr)	Underlying immunodeficiency
LT68	1968	Unknown	Unknown
US3846	1970	8[a]	Unknown (low cellular and humoral responses)
US3885	1970	25	Hypogammaglobulinemia
US4001	1971	7	Stem-cell leukemia
US4015	1971	25	None (bronchiectasis)
SP81	1981	31	Chronic myeloid leukemia

[a] Eight months.

sample. An *Eco*RI restriction endonuclease site was included at the 5′ terminus of the oligonucleotide primers to facilitate cloning of amplification products. DNA amplification was performed on the samples at the mt LSU rRNA, with a first round with the oligonucleotide primers pAZ102-H/RI and pAZ102-E/RI (18, 20) and a second round with primers pAZ102-X/RI and pAZ102-Y/RI (18) (Table 3). The conditions for the first- and second-round amplifications, respectively, were denaturation at 94°C for 1.5 min, annealing at 55°C for 1.5 min, and extension at 72°C for 2 min for 10 cycles followed by 30 cycles at 94°C for 1.5 min, 63°C for 1.5 min, and 72°C for 2 min.

DNA amplification at the ITS regions was performed with the primers ITSF3/RI and ITS2R3/RI. The conditions consisted of 10 cycles at 94°C for 1.5 min, 56°C for 1.5 min, and 72°C for 2 min and 30 cycles at 94°C for 1.5 min, 64°C for 1.5 min, and 72°C for 2 min for a total of 40 cycles. When a nested PCR was required, a first-round amplification was carried out with primer pair NITSF and NITSR, with 40 cycles consisting of 94°C for 1.5 min, 55°C for 1.5 min, and 72°C for 2 min. This was followed by a second round of amplification with the primers ITSF3/RI and ITS2R3/RI under the conditions described above (15).

DNA amplification at the EPSP synthase domain of the *arom* gene was performed by nested PCR with the first-round primers AroFunivB and AroFunivC for 40 cycles, each consisting of 94°C for 1.5 min, 50°C for 1.5 min, and 72°C for 2 min. The primer pair HsPcaro1/RI and HsPcaro2/RI was used for the second-round amplification, with 10 cycles at 94°C for 1.5 min, 52°C for 1.5 min, and 72°C for 2 min followed by a second stage of 30 cycles at 94°C for 1.5 min, 63°C for 1.5 min, and 72°C for 2 min (1).

DNA amplification at the mt SSU rRNA was performed with the first-round primers pAZ112-1OF/RI and pAZ112-1OR/RI, for 40 cycles, each consisting of 94°C for 1.5 min, 55°C for 1.5 min, and 72°C for 2 min (4). The second round of amplification was carried out with the primer pair pAZ112-13/RI and pAZ112-14/RI, with 10 cycles at 94°C for 1.5 min, 52°C for 1.5 min, and 72°C for 2 min, followed by 30 cycles at 94°C for 1.5 min, 63°C for 1.5 min, and 72°C for 2 min.

Cloning and sequencing of amplification products. The PCR products were digested with restriction endonuclease *Eco*RI, purified from 1.5% agarose gels with Geneclean II (Bio101, Stratech Scientific, Bedfordshire, United Kingdom), and cloned into the plasmid vector pUC18 at the *Eco*RI site. Recombinant plasmids were sequenced with the M13 universal primers by the dideoxy-chain termination method. Multiple clones of each sample were sequenced. Sequence analysis was performed using the University of Wisconsin Genetics Computer Group Sequence Analysis Software Package (Version 8, April 1994, Madison, Wisconsin).

RESULTS

DNA amplification with *P. carinii*-specific oligonucleotide primers was carried out on DNA extracted from post-mortem lung samples from six individuals who died between 1968 and 1981 of *P. carinii* pneumonia. The amplification products were cloned, and the sequences of the recombinants were determined.

TABLE 2. Details of DNA amplification

Sample	Type of PCR for gene encoding:			
	mt LSU rRNA	ITS	*arom* locus	mt SSU rRNA
LT68	Single	Single	Nested	Single
US3846	Nested	Nested	Nested	Nested
US3885	Nested	Nested	Nested	Nested
US4001	Nested	Nested		
US4015	Nested	Nested		
SP81	Single	Single		

TABLE 3. Oligonucleotides used

Primer	Sequence
pAZ102-H/RI	5′-GGGAATTCGTGTACGTTGCAAAGTACTC-3′
pAZ102-E/RI	5′-GGGAATTCGATGGCTGTTTCCAAGCCCA-3′
pAZ102-X/RI	5′-GGGAATTCGTGAAATACAAATCGGACTAGG-3′
pAZ102-Y/RI	5′-GGGAATTCTCACTTAATATTAATTGGGGAGC-3′
NITSF	5′-GTCGTAACAAGGTTTCCGTA-3′
NITSR	5′-CCTCCGCTTATTGATATGCT-3′
ITSF3/RI	5′-GGGAATTCCTGCGGAAGGATCATTAGAAA-3′
ITSF3/RI	5′-GGGAATTCGATTTGAGATTAAAATTCTTG-3′
pAZ112-10F/RI	5′-GGGAATTCTAGACGGTCACAGAGATCAG-3′
pAZ112-10R/RI	5′-GGGAATTCGAACGATTACTAGCAATTCC-3′
pAZ112-13/RI	5′-GGGAATTCGAAGCATGTTGTTTAATTCG-3′
pAZ112-14/RI	5′-GGGAATTCTTCAAAGAA(T,C)CGAGTT(T,C)CAG-3′
AroFunivB	5′-ATCCCACCAN(T,C)(A,C)NGGCCA-3′
AroFunivC	5′-(A,G)ATATGGA(A,G)(T,C)CAATGACNGA-3′
HsPcaro1/RI	5′-GGGAATTCTTAACAACAACAATTTTAGCC-3′
HsPcaro2/RI	5′-GGGAATTCGGTGAAATGCATGCCAAGAC-3′

Variation at the *P. carinii* f. sp. *hominis* mt LSU rRNA. A 346-bp fragment of the gene encoding the *P. carinii* f. sp. *hominis* mt LSU rRNA was amplified with a single round of amplification from samples LT68 and SP81 and a 207-bp fragment from the other samples by using a nested PCR. The samples were examined for variation at base 85 and base 248, where polymorphisms have previously been described (7–11, 14, 19). At position 85, either thymine (T) or cytosine (C) was observed (Table 4). Position 248 was spanned in only two of the samples, which were amplified by a single round of PCR, and C was observed in both samples. A new position at which variation occurred was observed in one sample, SP81, at base 81, where T was observed rather than C, as reported in the previously published sequences. To verify this new polymorphic site, 14 clones from each of three separate amplification reactions were analyzed. Thymine was observed at position 81 in all the clones.

In three samples, US3846, US3885, and US4001, two differ-

TABLE 4. Nucleotide sequence types in *P. carinii* f. sp. *hominis*[a]

Sample	ITS sequence type	Nucleotide at indicated position in gene encoding:						
		mt LSU rRNA			mt SSU rRNA		*arom* locus	
		81	85	248	160	196	121	208
LT68	B_1a_1	C	T	C	C	T	C	A
	B_2a_1							
US3846	B_1a_1	C	T	ND[d]	A	G	C	A
	B_2a_1	C	C	ND	C	T		
	B_2a_4[b]							
US3885	A_2c_1	C	T	ND	A	G	C	A
	A_2c_2[b]	C	C	ND				
	A_3c_1[b]							
US4001	B_1a_3	C	T	ND	ND	ND	ND	ND
	B_3a_3	C	C	ND				
US4015	B_1b_2	C	C	ND	ND	ND	ND	ND
SP81	B_2a_1	T[c]	C	C	ND	ND	ND	ND

[a] New sequences were compared to sequences for ITS sequence types and the genes encoding mt LSU rRNA, mt SSU rRNA, and the *arom* locus from references 15, 14, 4, and 1, respectively, used as standards.
[b] New sequence type identified in this study.
[c] New variation at this nucleotide position.
[d] ND, not determined.

TABLE 5. *P. carinii* f. sp. *hominis* ITS1 sequence types[a]

ITS1 sequence type	Nucleotide(s) at position(s):			
	2	16	74–75	111–113
A_2	C	T	—[c]	TTA
A_3[b]	T	A	—	TTA
B_1	T	T	AG	TTA
B_2	T	A	AG	TTA
B_3	C	T	AG	TTA
C	T	T	AG	—

[a] Sequence in reference 15 was used as a standard for comparison with new sequences.
[b] New sequence type identified in this study.
[c] —, nucleotide absent at this position.

ent mt LSU rRNA sequence types were observed, suggesting coinfection with more than one type of *P. carinii* f. sp. *hominis.*

Variation at the *P. carinii* f. sp. *hominis* Internal Transcribed Spacer regions. A 540-bp fragment spanning the ITS regions of the nuclear rRNA operon of *P. carinii* f. sp. *hominis* was amplified from six samples. We have previously described 5 different ITS1 types, based on polymorphisms at four different positions, and 7 different ITS2 types, based on polymorphisms at six different positions, resulting in 10 different *P. carinii* f. sp. *hominis* ITS sequence types (Tables 5 and 6) (15). Applying this method of typing to the samples in this study, one new ITS1 sequence type, A_3, was identified in sample US3885, in which thymine was observed at base position 2 (Table 5). In addition, two new ITS2 types were also observed, type a_4 in sample US3846 and type c_2 in sample US3885 (Table 6). Three new ITS sequence types, A_2c_2, A_3c_1 and B_2a_4, were identified in this study. No consistent, reproducible sequence polymorphisms were observed at positions other than those previously described. A total of nine different ITS sequences were observed in the six samples. A single ITS type was observed in two of the samples, US4015 and SP81, two different ITS types were observed in each of two samples, LT68 and US4001, and three different types were observed in each of samples US3846 and US3885 (Table 4).

Variation at the *P. carinii* f. sp. *hominis arom* locus. Three of the samples, LT68, US3846 and US3885, were also examined for variation at a 237-bp fragment of the EPSP synthase domain of the *arom* locus. We have previously described two single-base polymorphisms in this region, at base 121 and base 208 (1). One sequence type, C_{121}/A_{208}, was observed in each of the three samples investigated at this locus.

Variation at the *P. carinii* f. sp. *hominis* mt SSU rRNA. In a previous study, we described variation in a portion of the mt SSU rRNA among isolates of *P. carinii* from five different host species (4). In this paper, we report two single-base polymorphisms in a 300-bp portion of the *P. carinii* f. sp. *hominis* gene, at base 160 and base 196 (numbering according to Hunter and Wakefield [4]). Three of the samples, LT68, US3846 and US3885, were analyzed at this locus, and two different sequence types were observed, C_{160}/T_{196}, and A_{160}/G_{196}, either singly or as a coinfection (sample US3846) (Table 4).

DISCUSSION

In this study we describe the genotypic analysis of isolates of *P. carinii* f. sp. *hominis* from six patients during the period of 1968 to 1981, none of whom were immunocompromised due to infection with HIV. The six samples were analyzed at two genetic loci, and in addition, three of the samples were analyzed at a total of four different loci. The DNA sequence types determined from the samples in this study were compared with previously published sequences. No major differences in sequence types were observed. Where differences were detected, they were primarily at nucleotide positions at which variation had previously been recorded. The samples of *P. carinii* f. sp. *hominis* in this investigation differed from samples used in other typing studies in that the samples predated the AIDS pandemic and the cause of the underlying immunodeficiency of the patients was not HIV infection.

Although high levels of divergence from the published sequences were not observed, some differences were detected. At the mt LSU rRNA, a new position at which variation occurred, position 81, was found. At position 85, both C and T were observed but not A or G. In previous studies, C, T, and A have been recorded at this position (7–11, 19). At position 248, C was found, in keeping with previous studies where C or T has been observed.

Variation at the *P. carinii* f. sp. *hominis* ITS regions has been detected by DNA sequence analysis and by hybridization with type-specific oligonucleotide probes, and four different types have been identified (12, 13). Type-specific PCR and the technique of single-strand conformation polymorphism have also been used in the analysis of this locus (3, 6). We have described a method of typing at this locus by DNA sequence analysis with which 10 different types of *P. carinii* f. sp. *hominis* were identified (15). In this study, we have detected variation only at nucleotide positions which we had previously shown to be polymorphic. At the ITS1 region, we have identified one additional sequence type, A_3, which we had not detected in our previous study of samples from HIV-infected patients. This ITS1 sequence type has been reported in samples from two HIV-infected patients from Italy (9). Two new ITS2 sequence types, a_4 and c_2, were also identified in this study, which have not been reported elsewhere. Three new ITS sequence types, A_2c_2, A_3c_1 and B_2a_4 were found in this study, bringing the total identified by this methodology to 13, of which 9 were found in this investigation. Of the nine types identified in this study, the type B_2a_1 was the most common, being present in three of the six samples. In our previous study on samples from HIV-infected patients, the type B_2a_1 was also found as a single infection or as a coinfection in 54% of the samples (15).

Of the four genetic loci examined in this study, the ITS locus was the most informative. This is consistent with the results of an investigation where diversity at the ITS regions was compared with that of the mt LSU rRNA, the 5S rRNA, and a portion of the gene encoding thymidylate synthase (9). In the present study, nine different sequence types were detected in

TABLE 6. *P. carinii* f. sp. *hominis* ITS2 sequence types[a]

ITS2 sequence type	Nucleotide(s) at position(s):					
	54–56	63	67–71	122	169–172	176
a_1	TAA	—[c]	AATAT	—	—	G
a_2	TAA	—	AATAA	—	—	G
a_3	TAA	—	AATAA	—	—	A
a_4[b]	TAA	—	AATAT	—	—	A
b_1	TAA	—	AATAT	—	—AT	G
b_2	TAA	—	AATAT	C	—AT	G
c_1	—	A	—	—	—	A
c_2[b]	—	A	—	—	—	G
d	TAA	—	AATAT	—	ATAT	G

[a] Sequence in reference 15 was used as a standard for comparison with new sequences.
[b] New sequence type identified in this study.
[c] —, nucleotide absent at this position.

the samples at the ITS locus, whereas only three different types were detected at the mt LSU rRNA locus, two types were detected at the mt SSU rRNA, and one type was detected at the *arom* locus. Two of the samples, US4015 and SP81 appeared to be infected by a single type of *P. carinii* f. sp. *hominis*. In the four other samples, two or three different ITS sequence types were found. Since only one copy of the nuclear rRNA operon is thought to be present in the *P. carinii* genome (2), our data suggest coinfection with different types of *P. carinii* f. sp. *hominis* in these four samples. Mixed infection, as indicated by the identification of more than one sequence type at the ITS locus, was also reflected in most samples (three of four samples) by two different types at the mt LSU rRNA and also at the mt SSU rRNA (one of two samples). We have previously reported a high incidence (33%) of mixed infections in samples from AIDS patients (15), and mixed infections have also been found in analyses of the mt LSU rRNA and the ITS loci (7, 8, 9, 12, 13).

One new mt LSU rRNA sequence type and three new ITS sequence types have been identified in this study. It remains to be established whether these different sequence types are characteristic of samples of *P. carinii* f. sp. *hominis* from this era, or geographical location (The Netherlands) or underlying immunodeficiency (non-AIDS). Analysis of a greater number of samples from a variety of different regions of the world and with a range of underlying immunodeficiencies will help to establish the significance of these differences. The data from this study suggest that there has not been any substantial change in the type of *P. carinii* f. sp. *hominis* since the large increase in the incidence of *P. carinii* pneumonia due to the AIDS pandemic.

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