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Biodiversity of *Pneumocystis carinii hominis*: Typing with Different DNA Regions

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The purpose of this study was to identify the most useful gene for the detection of biodiversity of *Pneumocystis carinii hominis* isolates and to compare samples from French and Italian subjects. We studied 20 bronchoalveolar lavage fluid specimens from 20 human immunodeficiency virus-infected patients (10 French and 10 Italian patients) with *Pneumocystis carinii* pneumonia by DNA sequencing of the thymidylate synthase (TS), 5S rRNA, large-subunit mitochondrial rRNA (mt LSU rRNA), and internal transcribed spacer (ITS1 and ITS2) genes. Thirteen of the 20 sequenced samples had the prototype TS gene sequence. Fourteen of the 20 samples showed the prototype sequence of the 5S rRNA gene, and 6 had variant sequences of the 5S rRNA gene. The mt LSU rRNA gene was sequenced for 18 of the 20 samples; all sequences were different from the prototype sequence and were classified into four groups. Thirteen of the 20 ITS1 and ITS2 sequences were analyzed, and all the sequences were found to be different from the prototype sequence and were classified into 10 groups. The internal transcribed spacer regions thus appear to be the most discriminatory region of DNA for analysis of the biodiversity of *P. carinii hominis* isolates.

Pneumocystis carinii remains the most frequent cause of pneumonia in immunocompromised patients, particularly those infected with human immunodeficiency virus. *P. carinii* isolates from rats, mice, ferrets, and humans differ in their reactivities with specific antisera (1, 7) and in the nucleotide sequences of certain *P. carinii* genome regions (2, 16). Genetic polymorphism has recently been observed among *P. carinii* isolates, particularly *P. carinii hominis* isolates (8, 10). Smulian et al. (17) have shown geographic variations in the humoral response to *P. carinii*. In this study, we searched for genetic diversity in *P. carinii hominis* isolates obtained from patients in France and Italy. We examined the sequences of portions of four *P. carinii* genes: the thymidylate synthase (TS) gene (3), the 5S rRNA gene (6), the large-subunit mitochondrial rRNA (mt LSU rRNA) gene (18), and internal transcribed spacers (ITSs) 1 and 2 (ITS1 and ITS2, respectively) of the rRNA genes (10).

MATERIALS AND METHODS

The samples were obtained from patients in two Paris hospitals (Hôpital Saint-Antoine and Hôpital Tenon) and one Italian hospital (Policlinico Gemelli, Rome). We examined *P. carinii* DNA extracted from 20 bronchoalveolar lavage specimens collected from 20 human immunodeficiency virus-seropositive patients (7 patients in Hôpital Saint-Antoine, 3 patients in Hôpital Tenon, and 10 patients in Policlinico Gemelli) with *P. carinii* pneumonia (PCP) proven by direct examination with standard stains (Giemsa or toluidine blue O) and indirect immunofluorescence. Eleven patients had not received prophylaxis. Among the remaining nine patients, four had received pentamidine, one had received pentamidine-sulfadiazine-pyrimethamine, two had received dapsone-pyrimethamine, one had received dapsone, and one had received co-trimoxazole.

After centrifugation of bronchalveolar lavage fluid, the pelleted samples were treated with 0.2 mg of proteinase K (Boehringer Mannheim) in the presence of 500 μl of proteinase K buffer (10 mM Tris [pH 8], 0.5% sodium dodecyl sulfate, 25 mM EDTA, 0.1 M NaCl) at 56°C for 1 h. After inactivation of the proteinase K at 94°C for 10 min, DNA was extracted with phenol-chloroform and precipitated with ethanol (11).

TS gene. A 398-bp fragment was amplified by using the following two primers: 5′-ATTTATGGGTTTCAATGG-3′ and 5′-TGCAATATTAAAGGGAAC-3′, reported by Edman (3), by PCR assay, followed by hybridization, as described by Mazars et al. (12).

5S rRNA gene. A 120-bp fragment was amplified with the primers 5′-AGT TACGGCCATACCTCA-3′ and 5′-AAAGCTACAGCACGTC-3′ under the cycle conditions reported by Kitada et al. (6).

mt LSU rRNA gene. A 340-bp fragment was amplified by using the specific primer pairs pAZ102-E (5′-GATGGCTGTTTCCAAGCCCA-3′) and pAZ102-H(5′-GTGTACGTTGCAAAGTACTC-3′) under the PCR conditions described by Wakefield et al. (18).

ITS1 and ITS2 of rRNA genes. A 772-bp fragment was amplified by nested PCR with two primer pairs, primer pair 1724F (5′-AAGTTGATCAAATTTGG TC-3′) and 3454R (5′-GAACCGGTCGATAGTGCAC-3′) and primer pair ITS1F (5′-CGTAGGTGAACCTGCGGAAGGATC-3′) and ITS2R1 (5′-GTTC AGCGGGTGATCCTGCCTG-3′), under the PCR conditions described by Lu (10).

The PCR products were electrophoresed in a 2% agarose gel (type II; Medium EEO; Sigma) with 1× Tris-borate-EDTA (0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA) and ethidium bromide (0.5 μg/ml; Sigma), blotted, and hybridized to specific radiolabelled probes (11). Then, the amplified fragments were purified by using the Wizards PCR Preps DNA Purification System kit (Promega), the Geneclean kit (Bio 101, Osyme, France), and the Qiaex kit (Qiagen Gmbh). The TS, mt LSU rRNA, ITS1, and ITS2 genes were sequenced directly from both ends, without cloning, on an automated sequencer (model 373A; Applied Biosystems) by using the Prism Ready Reaction Dye Deoxy Terminator Cycle sequencing kit (Applied Biosystems). The 5S rRNA gene was cloned by using the TA-Cloning kit (Invitrogen), and three clones from each sample were sequenced by using the Sequenase kit (Amersham).

Only samples positive on agarose gels were sequenced (those positive after hybridization were not sequenced). The sequences that were obtained were compared with each other and with the reported prototype sequences.

RESULTS

TS gene. *P. carinii* DNA was detected by the TS PCR hybridization assay in all 10 French samples and only 6 Italian samples (Table 1). A 279-bp fragment was sequenced from 13

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TABLE 1. Distribution of patients according to *P. carinii hominis* nucleotide sequence genes and geographical locations[a]

Sample	TS	5S rRNA	mtLSU rRNA	ITS1 and ITS2 type	Prophylaxis
F1	P	P	1	B1a1	None
F2	P	V6	2+3	B3b4	Cotrimo
F3	P	P	4	A1a2	None
F4	P	P	3	B2a1	Dap
F5	ND	P	2+3	ND	None
F6	ND	P	1	B1b2	Pent
F7	P	P	3	C1c1	None
F8	P	P	3	B1b1	Pent
F9	P	P	1	B1b1	Pent
F10	P	P	3	B1b3	None
I1	ND	P	3	ND	None
I2	ND	V1+V2+V3	3	ND	Pent+Sulf+Pyri
I3	ND	V3	3	ND	None
I4	P	P	ND	A2c1	None
I5	ND	P+V4+V4	2+3	ND	None
I6	P	V5	1	ND	Dap+Pyri
I7	P	P	ND	A2c1	None
I8	P	P	1	B1a2	Pent
I9	ND	P+V3+V3	3	ND	Dap+Pyri
I10	P	P	2	B1a2	None

[a] F, French; I, Italian; Cotrimo, co-trimoxazole; Dap, dapsone; Pent, pentamidine; Sulf, sulfadiazine; Pyri, pyrimethamine; P, prototype; V, variant; ND, not done because no DNA amplification was detected on the agarose gel.

samples: 8 French and 5 Italian samples (samples F1, F2, F3, F4, F7, F8, F9, F10, I4, I6, I7, I8, and I10). The nucleic acid fragment corresponds to the exons and two small introns. No variation was detected in the sequences of these 13 *P. carinii hominis* samples under the technical conditions of our study, and they are thus identical to the prototype sequence.

5S rRNA gene. All of the samples were positive for the 5S rRNA gene on agarose gels and by hybridization (Fig. 1): 14 of the 20 samples (9 French and 5 Italian samples; samples F1, F3, F4, F5, F6, F7, F8, F9, F10, I1, I4, I7, I8, and I10) had the prototype 5S rRNA sequence; 2 Italian samples (samples I5 and I9) had both the prototype sequence and identical variant

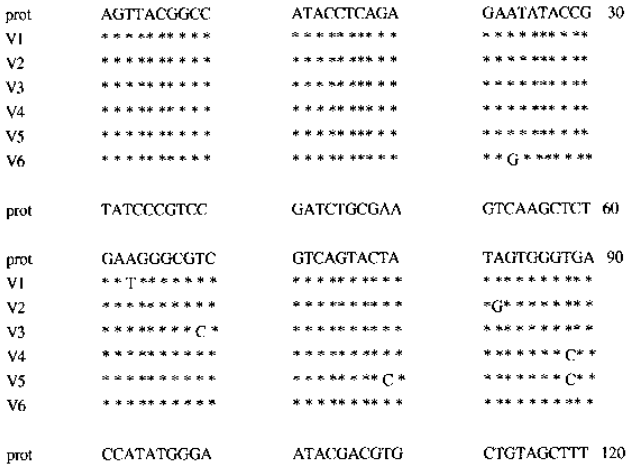

FIG. 1. Comparison of nucleotide sequences of 5S rRNA gene of *P. carinii hominis* isolates from several patients. The top line is the sequence first reported by Kidata et al. (6). The other lines represent the different variant sequences. The bases that are identical to those of the prototype strain are shown as asterisks. Prot, prototype; V, variant.

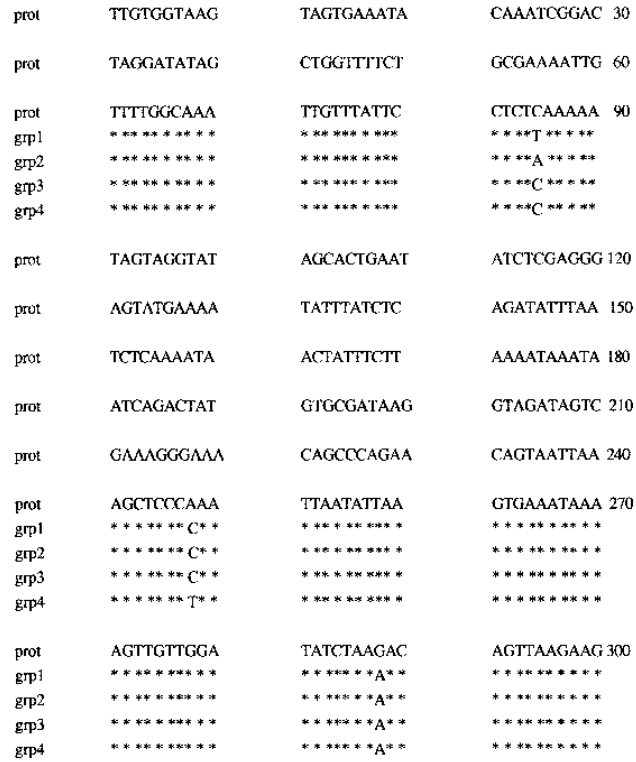

FIG. 2. Comparison of nucleotide sequences of a portion of the mt LSU rRNA gene of *P. carinii hominis* from several patients. The top line correspond to the prototype sequence reported by Sinclair et al. (16). The other lines represent the sequences of the different strains. The bases that are identical to those of the prototype strain are shown as asterisks. Prot, prototype; grp, strain group.

sequences (variants V3 and V4) in different clones; 1 Italian sample (sample I2) had different variant sequences (variants V1, V2, and V3) in each of the three clones analyzed; 3 samples (1 French and 2 Italian samples; samples F2, I3, and I6) had only one variant sequence (variant V3, V5, or V6) in all three clones. The six different variant sequences had point mutations or insertions at different positions. Notably, three Italian samples had the same variant sequence (variant V3), with a mutation at position 69 (T to C). Nine of the 11 samples (82%) from patients who had not received prophylaxis had the prototype sequence, and 4 of the 9 samples (44%) from patients who had received prophylaxis had variant sequences.

mt LSU rRNA gene. All of the samples were positive for the mt LSU rRNA gene on agarose gels with a 340-bp fragment, and only 18 of the 20 samples (10 French and 8 Italian samples) were sequenced (Fig. 2). None of the sequences were identical to the prototype sequence reported by Sinclair et al. (16).

A total of 17 of the 18 sequenced samples (9 French and 8 Italian samples) had an A-to-C change at position 248 and a G-to-A change at position 288. On the basis of a single-base polymorphism at position 85, we were able to divide the isolates into three groups. Group 1 sequences had a T (five patients; samples F1, F6, F9, I6, and I8), group 2 sequences had an A (one patient; sample I10), and group 3 sequences had a C (eight patients; samples F4, F7, F8, F10, I1, I2, I3, and I9). Three samples (2 French and 1 Italian samples; samples F2, F5, and I5) fell into two groups (groups 2 and 3). Indeed, adenine and cytosine were both found at position 85 in each sequence.

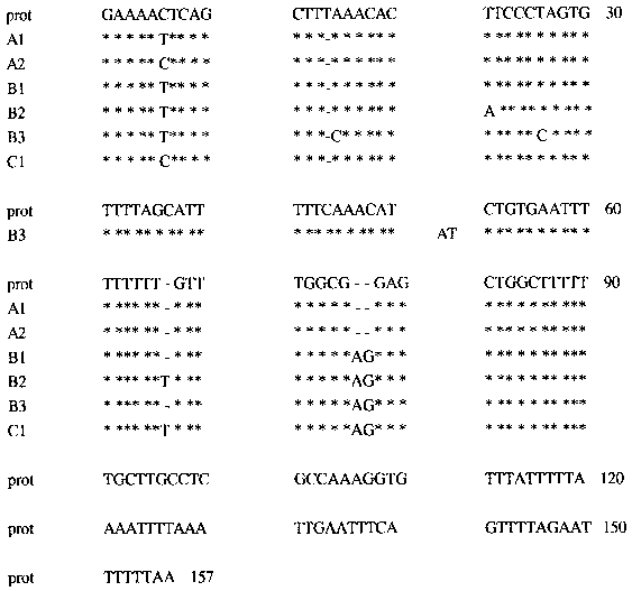

FIG. 3. Alignment of nucleotide sequences of ITS1 sequences of rRNA regions of *P. carinii hominis* from all specimens examined. The top line is the sequence for the *P. carinii hominis* isolate chosen as the prototype by Lu et al. (10). The other lines represent the sequences of the strains of different types. The bases that are identical to those of the prototype sequence are shown as asterisks. Missing bases (−) and bases that are different from those from those of the prototype sequence are indicated. Prot, prototype.

In 1 French sample (sample F3), the sequence showed a C and a G-to-A change at positions 85 and 288, respectively, and a second base polymorphism (A-to-T change) at position 248. This allowed the patient's isolate to be classified in a fourth group.

No differences were observed between the French and Italian samples.

ITS1 and ITS2 regions. All 10 French samples were positive for the ITS1 and ITS2 regions, but only 9 could be sequenced. Only 4 of the 10 Italian specimens were positive for the ITS1 and ITS2 regions, and all were sequenced. In all, 13 sequences were aligned.

The comparison revealed that *P. carinii hominis* ITS1 sequences (157-bp fragment) could be classified into three major types designated A, B, and C (Fig. 3). These types differed in sequence at positions 6, 14, 67, 76, and 77, as described by Lu et al. (10). According to this typing system, three samples (one French and two Italian samples; samples F3, I4, and I7) were classified as type A, nine samples (seven French and two Italian samples; samples F1, F2, F4, F6, F8, F9, F10, I8, and I10) were classified as type B, and one French sample (sample F7) was classified as type C. There were some sporadic variations within types A, B, and C which allowed us to subclassify the sequences into six groups (groups A1, A2, B1, B2, B3, and C1) and to identify new mutations (two C changes, at positions 15 and 26, respectively, and a 2-bp insertion between positions 50 and 51) (Fig. 3; type B3).

The alignment of the 13 *P. carinii hominis* ITS2 sequences (177-bp fragment) revealed three types (types a, b, and c) (Fig. 4). The type b sequence differed from the type a and c sequences at positions 160 and 161. The type c sequence displayed more extensive variation from the sequences of types a and b at positions 50 to 52, 63 to 67, and 165. Five samples (three French and two Italian samples; samples F1, F3, F4, I8, and I10) were classified as type a, five French samples (samples F2, F6, F8, F9, and F10) were classified as type b, and three samples (1 French and two Italian samples; samples F7, I4, and I7) were classified as type c. Similar to the ITS1 sequences, there were sporadic sequence variations among the different *P. carinii* isolates from different samples. These variations permitted us to subclassify the sequences into seven groups (groups a1, a2, b1, b2, b3, b4, and c1).

According to the types of ITS1 and ITS2, we designated the *P. carinii hominis* sequences mentioned above by a two-letter code and classified them into 10 groups, as follows: group A1a2, 1 patient (sample F3); group A2c1, two patients (samples I4 and I7); group B1a1, one patient (sample F1); group B1a2, two patients (samples I8 and I10); group B1b1, two patients (samples F8 and F9); group B1b2, one patient (sample F6); group B1b3, one patient (sample F10); group B2a1, one patient (sample F4); group B3b4, one patient (samples F2); and group C1c1, one patient (sample F7).

DISCUSSION

Not all the DNA could be amplified with the different primers, possibly because of storage or transportation conditions.

TS gene. A single copy of the TS gene is present in the *P. carinii* genome (3). This housekeeping gene codes for one of the most highly conserved enzymes (15). The sequence of this gene has been used to distinguish *P. carinii* isolates from different hosts: rat, mouse, rabbit, and human (12, 13). No difference in sequence was detected on a portion of 279 bp of the

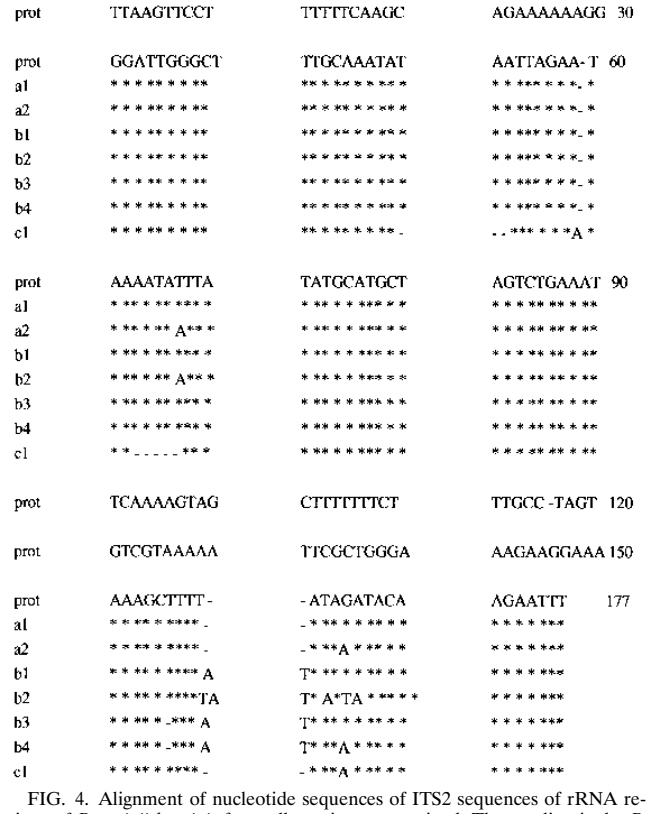

FIG. 4. Alignment of nucleotide sequences of ITS2 sequences of rRNA regions of *P. carinii hominis* from all specimens examined. The top line is the *P. carinii hominis* chosen as prototype by Lu et al. (10). The other lines represent the sequences of the strains of different types. The bases that are identical to those of the prototype sequence are shown as asterisks. Missing bases (−) and bases that are different from those of the prototype are indicated. Prot, prototype.

TS gene from among 13 *P. carinii hominis* isolates, whether the sequence was coding or noncoding, and thus submitted to pharmacological selection pressure or not. It thus appears that the TS gene could be a good marker for the host species, but it is not adapted for distinguishing isolates from the same host species (especially within *P. carinii hominis*).

5S rRNA gene. Genetic variability has been observed in *P. carinii* 5S rRNA genes derived from different rat colonies (14). The six different variant sequences of 5S rDNA analyzed in our *P. carinii hominis* samples differed from those found in *P. carinii rattus*. The 5S gene in our French samples appears to be more highly conserved (only one variant) than that in the Italian samples. Five Italian samples (samples I2, I3, I5, I6, and I9) had at least one variant sequence, and one of these samples (sample I2) had three different variant sequences and two Italian samples (samples I3 and I6) presented the same variant sequences. Interestingly, the largest number of mutations was found in the samples from patients who had received prophylaxis, while the largest number of prototype sequences was found in samples from patients who had not received prophylaxis. This points to pharmacological selection pressure but needs to be confirmed with a larger number of samples. We identified two specimens (specimens I5 and I9) with both variant and prototype sequences and one specimen (specimen I2) that contained three different 5S DNA sequences. This could be due to coinfection with different *P. carinii* strains in the same patient or to variability between different copies of the gene.

mt LSU rRNA gene. The 340-bp segment of the large subunit of the *P. carinii* mt LSU rRNA gene is known to be variable (8, 9). We found four different groups among our *P. carinii hominis* samples. Group 3 appeared to be overrepresented, but no statistical analysis was possible because of the small sample sizes. Three patients (2 French and 1 Italian patients; samples F2, F5, and I5) appeared to have two mt LSU rRNA gene sequences in the same sample. On the basis of molecular karyotyping studies, two explanations can be offered. First, if *P. carinii* is considered a diploid organism (20), these patients would be infected with the same *P. carinii* strain with two different mutant alleles (allele 1 [A85 . . . C248 . . . A288] and allele 2 [C85 . . . C248 . . . A288]). On the other hand, if *P. carinii* has a haploid genome (4), the two mutant alleles (alleles 1 and 2) could correspond to two different strains of *P. carinii*. These patients were thus simultaneously infected with two different varieties of *P. carinii*, in keeping with the findings of Hong et al. (4) and others (5, 10) who have reported coinfection with two different strains of *P. carinii* in the same host such as rats (4) and, more recently, humans (5, 10).

We found no difference in the samples from France and Italy. However, the study of the mt LSU rRNA portion of the gene indicated that genetic diversity in *P. carinii hominis* exists, given the nucleotide polymorphism at positions 85, 248, and 288. Some of these were different from those published by Sinclair et al. (16) and Lee et al. (9), especially at positions 85 and 248. Apart from these variable bases and in comparison to the high levels of diversity at this locus found by Lee et al. (9), the results of this and other studies suggest that there is a little genetic diversity among *P. carinii hominis* isolates from different regions of the world (19). Indeed, the existence of three point mutations on a 340-bp fragment indicates a mutation rate of $\approx 1\%$ and the existence of conserved sequences.

Although the differences between isolates were minimal, they did not appear to be random or technique related. The base changes were not randomly distributed, but all occurred at the same positions shown to be variable in samples from patients both who had and who had not received prophylaxis. There is no reason to think that treatment led to the genetic changes on the mitochondrial ribosomal gene.

ITS1 and ITS2 genes. The sequences of ITSs ITS1 and ITS2 (located between the 18S and 5.8S rRNA genes and between the 5.8S and 26S rRNA genes, respectively) were the most highly variable, with the identification of 10 different groups of strains, but no difference between the samples from the two geographic sites was observed.

The TS gene appears to be a good marker for characterizing *P. carinii* isolates from different host species but is not adapted for distinguishing isolates within the species *P. carinii hominis*. Due to the random distribution of mutations, the 5S rRNA gene seems to be unreliable. The ITS genes appear to be the most sensitive DNA regions for detecting biodiversity. Analysis of the mt LSU rRNA gene identified only four groups of *P. carinii* strains, but it appeared to be the region providing the most reliable results and detected the existence of coinfection. Thus, it could be used in conjunction with the ITS genes for more accurate strain identification. Identical results were obtained with a different aliquot of the same specimen, so these typing procedures were reproducible.

The typing methods used in the present study could represent a good approach for studying the epidemiology of PCP infections. Particularly during recurrent episodes of PCP, the typing methods could determine with precision whether the infection was due to reactivation of a latent *P. carinii hominis* strain from the original episode or to de novo contamination with a different *P. carinii hominis* strain. Finally, typing will also permit study of patient-to-patient transmission that has been hypothesized.

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