MOLECULAR GENETICS

Partial Sequence of the rRNA operon of *Pneumocystis carinii* isolates from Puerto Rico.

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ABSTRACT. Several reports indicate geographic variation of isolates of *Pneumocystis carinii hominis*. We have sequenced the internal transcribed spacer (ITS) region and large subunit Group I intron of rRNA genes from *P. carinii* DNA obtained from two patients from Puerto Rico. Both can be subclassified as Type II, according to the sequence of the ITS region. A system capable of identifying individual isolates will be an essential tool for epidemiological studies of the organism. The amplification of DNA from fixed tissues may facilitate the processing of a large number of samples. *Key Words: Pneumocystis carinii, Ribosomal RNA, Group I intron, Ribozyme, Internal transcribed spacer, Puerto Rico.*

Pneumocystis carinii is a ubiquitous opportunistic fungal pathogen that causes life-threatening pneumonia in patients with immune deficiencies. *P. carinii* pneumonitis or PCP is the most common clinical presentation in patients with Acquired Immune Deficiency Syndrome (AIDS), although it has also been found in other immunosuppression states (1). In the Caribbean, the incidence of PCP has been documented for both Jamaica and Trinidad (2). Although there is a lack of literature describing the situation of PCP in the island of Puerto Rico, this disease is known to infect up to 80% of the AIDS patients in the United States. Studies performed on the mainland have demonstrated that geographic variation might exist. Antigenic and genetic variation among strains has been observed (3-18). This variation does not entirely correlate to host specificity (19).

One of the main obstacles in the study of this organism is the lack of a long-term culture method (20). Therefore, different molecular techniques have been developed both to identify and subclassify *P. carinii* isolates. Several groups have approached the problem of the classification of the organism by studying its morphological features, metabolic products and gene sequence (1). In this work, we report the sequence of the ribosomal RNA internal transcribed spacer (ITS) and the 26S rRNA group I intron of samples obtained from fixed tissues from patients in Puerto Rico. The ITS region as well as the location and sequence of rRNA Group I self-splicing introns, have previously been used to define sequevars or subspecies (7, 8, 16). The subspecies termed *P. carinii* sp. f. *hominis* (21), which has been isolated mostly in clinical isolates, can further be subtyped by the sequence of the ITS highly variable region (17).

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Materials and Methods

Sample Preparation. Pathological samples (paraffin-embedded lung tissue) were kindly provided by Dr. A.A. Roman-Franco from the Department of Pathology of the School of Medicine-UPR Medical Sciences Campus. Samples A-185-92 and A-244-93 were obtained from a 60-year-old immunosuppressed female cancer patient and a one-year old female AIDS patient, respectively.

Several sections (7 μm thick) were prepared using sterile technique. The microtome blade, tweezers, and all materials used were cleaned with xylene between each tissue block. Sections were deparaffinized by adding 1 ml of xylene to each and incubated at room temperature for 30 min. Tissue and residual paraffin were pelleted by

centrifugation. Samples were washed twice with 500 µl of 100% ethanol and lyophilized. Pellets were resuspended in 200 µl digestion buffer (50 mM Tris-HCl, pH8.0, µl mM EDTA, 0.5% Tween 20) containing Proteinase K (200 µg/ml) and incubated at 37°C overnight, followed by incubation at 95°C for 10 min to inactivate the proteinase. Samples were centrifuged to remove debris and an aliquot of the supernatant used for PCR amplification. DNA extracts were stored at -20°C.

PCR Amplification of rRNA genes. Previously described protocols were used for the amplification of the ITS region (17). Amplification of the 26S rRNA intron was performed in reactions (50 µl) containing 3 µl template DNA, 5 µl 10X Taq extender buffer (Stratagene, La Jolla, CA), 0.4 µM of each primer, 0.2 µM deoxynucleoside triphosphates, 2.5 mM $MgCl_2$, and 2.5 units each of *Taq* polymerase (Boehringer Mannheim, Indianapolis, IN) and *Taq* Extender PCR Additive (Stratagene), overlaid with 50 µl light mineral oil. Primary PCR amplification was done using primers #4359 (7) and #4358 (16). An initial step of two cycles of 2 min at 94°C, 1 min at 59°C and 1 min at 72°C was followed by 30 cycles of 1 min at 94°C, 1 min at 60°C and 1 min at 72°C and a final 5 min extension cycle at 72°C. Nested PCR was done as follows: Four µl from the primary PCR were used to amplify a smaller fragment using primers #7721 (5'-CGGCGGGAGTAACTATGAC-3') and #5981 (5'-TAAGAGCACCTCCCCTCC-3'). Cycles were as described above except that the annealing temperature during the first step was 56°C and during the following 30 cycles was 57°C. Cloning of the PCR products and subsequent sequencing was as previously described (16, 18).

Results and Discussion

No visible bands were observed after a primary PCR, but single bands corresponding to the expected size were observed for all samples after semi-nested PCR (ITS region) or nested PCR (26S rRNA group I intron) (data not shown). Sequences of the 26S rRNA intron are shown in Figure 1, and include a portion of the sequence of the

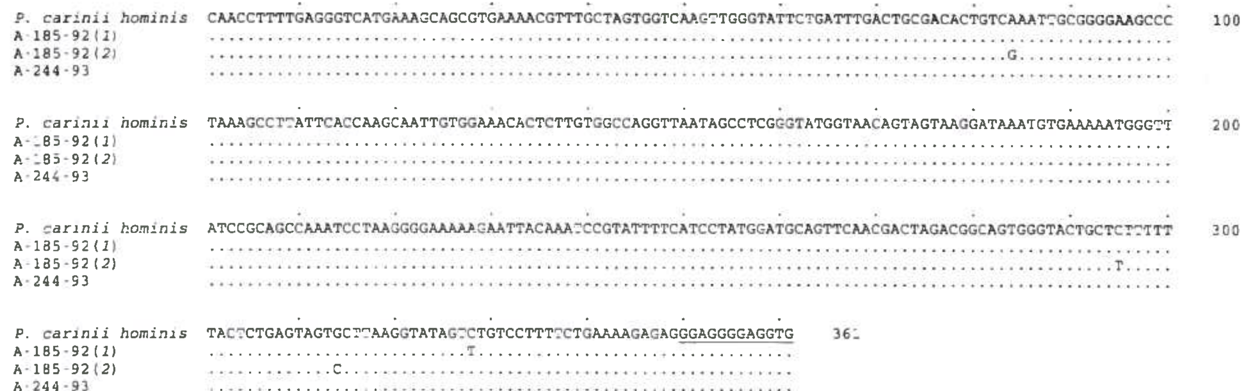

Figure 1. Comparison of sequences determined from clones obtained after amplification of the 26S group I intron from pathological tissue. The sequence of *P. carinii* sp. f. *hominis* has previously been described (16). The 5' end of PCR primer #7721 corresponds to base number 2222 of a conserved region of the 26S rRNA gene, while in primer #5981, the 5' end corresponds to base number 2247 of the 26S rRNA gene [base numbering according to the sequence of type Pc1 genomic DNA (7)] and the 3' end (sequence underlined) corresponds to the sequence of the *P. carinii* sp. f. *hominis* Group 1 intron. Dots indicate identity.

downstream primer used for amplification. For sample A-185-92, two distinct sequences observed in two sequenced clones indicated by *1* and *2*, differing at positions 84 (A/G), 295 (C/T), 314 (G/C) and 328 (T/C). Sample A-244-93 shows the same sequence as A-185-92(*1*), except at position 328 where its sequence agrees with that of A-185-92(*2*), and is the same as the intron of four patients from the mainland (18).

Some polymorphism was also found in the sequences of the ITS region for these samples (figure 2). Clone A-244-93(B) differs from A-244-93(A) only by two bases deleted at positions 79 and 80 of the ITS1 region, and also varies in the sequence of the 5.8S gene by one base change in position 193 and a deletion at position 212. All four clones had a run of five rather than 4 t's starting at position 60 (17). In the ITS2 region, clone A-185-92(*b*) differs from the other three clones in a base change at position 240 and two deletions at positions 274 and 277. At position 189, clone A-244-93(*b*) has a base change.

Amplification of DNA from paraffin tissue has been

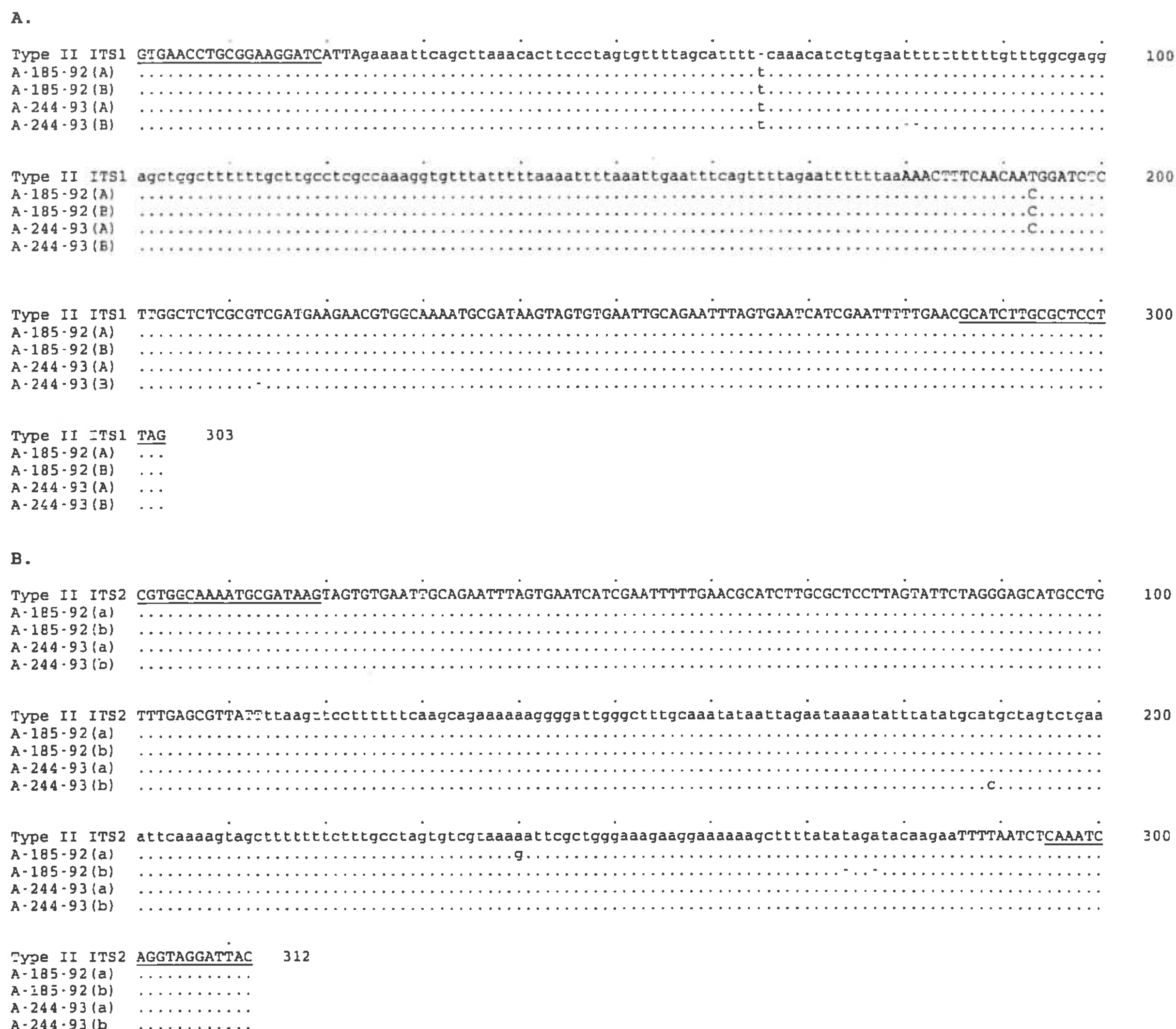

Figure 2. Sequence of the Internal Transcribed Spacer Regions. Fig. 2A shows the sequence obtained for the ITS 1 region and Fig. 2B show the sequence for the ITS 2 region. Underlined are the primers used for the semi-nested PCR. The sequence of the Type II ITS region has previously been described (17). Shown in uppercase are the sequences of the 5.8S gene, as well as of flanking regions of the 16S (Fig. 2A) and 26S (Fig. 2B) genes; ITS regions are shown in lowercase. Dots indicate identity, dashes represent missing bases.

successfully used (22) although it has been suggested that fragments no larger that 400 base pairs be used as targets because of the possibility of DNA degradation occurring during the fixation procedure (23). The size expected for the PCR product obtained for the primary reaction was 535 bp. for the ITS region and approximately 500 bp. for the intron, while the final amplification products were about 300 bp. for the ITS1 and ITS2 regions and 361 bp. for the 26S rRNA intron. Amplification products could be easily visualized as a single band of the expected size (data not shown).

Previously, clinical isolates have been subtyped according to the sequence of the ITS1 and ITS2 regions (17). According to this subclassification scheme, the samples used in this study can be assigned to Group II where the ITS types coincide with type B (ITS1) and b (ITS2). When compared with other Group II isolates and each other, A-185-92 and A-244-93 show minor polymorphisms, which could possibly be due to DNA damage occurring during fixation or to mixed infections, which have previously been noted to occur (17).

The ability to distinguish between subtypes of *P. carinii* according to the ITS region has been suggested as a possible way to study the epidemiology of this organism

(16, 18). This work demonstrates that *P. carinii* strains can be recognized by sequence polymorphisms in DNA amplified from fixed specimens. Analysis of ITS and intron sequences of the rRNA operon reveal that patients on Puerto Rico may be infected with similar strains to those encountered on the mainland, and that infections with mixed strains may occur.

Acknowledgement

We thank Dr. A. A. Roman-Franco (University of Puerto Rico, Medical Sciences Campus) for pathological specimens essential to this work, and R. Felder for assistance with automated DNA sequencing. This work was supported in part by NIH Training Grant 1T32 GM08339 (María Ortiz-Rivera) and NIH Grant 1R01 GM53815 (Michael J. Leibowitz).

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