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Heterogeneity and Compartmentalization of *Pneumocystis carinii* f. sp. *hominis* Genotypes in Autopsy Lungs

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The extent and importance of genotype heterogeneity of *Pneumocystis carinii* f. sp. *hominis* within lungs have not previously been investigated. Two hundred forty PCR clones obtained from respiratory specimens and lung segments from three patients with fatal *P. carinii* pneumonia were investigated to detect genetic diversity in the internal transcribed spacer (ITS) region of the nuclear rRNA operon, the mitochondrial large-subunit (mtLSU) rRNA gene, and the dihydropteroate synthase-encoding gene. For two of the three examined patients, a mixture of different mtLSU rRNA and ITS genotypes was observed. Not all genotypes present in the lungs at autopsy were detected in the diagnostic respiratory samples. Compartmentalization of specific ITS and mtLSU rRNA sequence types was observed in different lung segments. In conclusion, the interpretation of genotype data and in particular ITS sequence types in the assessment of epidemiological questions should be cautious since genotyping done on respiratory samples cannot a priori be assumed to represent all genotypes present within the lung.

The interpretation of molecular typing results in epidemiological studies of *Pneumocystis carinii* is complicated by the existence of coinfections. Several studies have reported coinfection with more than one genotype present in specimens from single patients (5). However, the majority of studies have relied on DNA extraction from diagnostic respiratory samples, which yield only a limited number of organisms. Very little is known of the dynamics of genotypes within the lungs, and whether genotype variation could result from compartmentalization of genotypes within the lungs has not previously been investigated.

To address these questions, we studied polymorphisms at three genetic loci by a detailed investigation of DNA obtained from different segments of autopsy lungs. The mitochondrial large-subunit (mtLSU) rRNA and internal transcribed spacer (ITS) loci were chosen for analysis since these genes are genes most frequently used for genotype analysis and the dihydropteroate synthase (DHPS) locus, the target of sulfone and sulfonamide drugs, was chosen because of its association with sulfa resistance (9, 16).

Specimens were obtained at diagnosis and postmortem from three patients who died of *P. carinii* pneumonia (PCP) between 1994 and 1997. PCP was diagnosed by microscopy and confirmed by histology at autopsy. Two patients with AIDS were included. Patient AIDS-1 was a 56-year-old male who died after 3 days of therapy, and patient AIDS-2 was a 45-year-old male who died after 17 days of therapy. Patient HL (age, 31 years) had a recurrence of Hodgkin's lymphoma and died 14 days after diagnosis of PCP.

After autopsy the whole right lungs from patients AIDS-1 and AIDS-2 were stored at −80°C. After thawing, five biopsies were cut from five different areas of each lung. Two- by three-cm biopsies were selected from the apex and from the superficial and central areas of the middle lobe and from the base and central part of the inferior lobe of the right lungs. From patient HL large biopsies were taken from five different segments of the right lung at autopsy and stored at −80°C; however, information on the exact segment from which the biopsies were taken was not recorded. Bronchoalveolar lavages (BAL) from the two human immunodeficiency virus (HIV)-positive patients were processed as reported previously (3). A diagnostic BAL specimen was unavailable from patient HL, but an oral wash specimen was obtained at diagnosis and the ITS sequence type was determined as previously described (8).

DNA was extracted from lung tissue and BAL was collected with a Qiagen kit (Qiagen, Hilden, Germany). All PCR setups were done with reagents from the AmpliTaq Gold kit (Applied Biosystems, Naerum, Denmark) using primers obtained from DNA Technology ApS, Aarhus, Denmark. Every nine samples were flanked by positive controls (from a patient with PCP) and negative buffer controls.

DNA amplification of the mtLSU rRNA region was done with primers Paz102E and Paz102H as reported previously (10). PCR amplification of the ITS gene was done with primers ITSF3 and ITS2R3 (30) and the DHPS gene was amplified with primers Dp15 and Dp800 (9). PCR amplification products were cloned using the Topo TA Cloning Kit (Invitrogen). From each PCR product, five recombinant clones were selected for further analysis. Recombinant plasmids were purified using the Promega Wizard Miniprep kit (Promega). DNA sequencing was done by simultaneous bidirectional sequencing with universal M13 primers on a Licor 4200 IR^2 DNA sequencer. Sequence types were determined only if base-calling accuracy was greater than a quality value of 30, corresponding to an error rate of less than 1 base per 1,000 bp. ITS typing followed previously published nomenclature (19). To validate sequencing results, DNA from three different lung segments

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TABLE 1. *P. carinii* f. sp. *hominis* mtLSU, ITS1, and ITS2 sequence types for two patients with heterogeneity of genotypes[a]

Origin of sample clone or clone name	mtLSU rRNA nucleotide at position:		ITS type
	85	248	
Patient AIDS-2			
BAL	T	C	Eg
	T	C	Eg
	T/C	C	Eg
	C	C	Eg
	C	C	Eg
Apex			
A	C	C	Eg
B	C	C	Eg
C	C	C	Eg
D	C	C	Eg
E	C	C	Eg
Middle lobe			
1A	C	C	Eg
1B	C	C	Eg
1C	C	C	Eg
1D	C	C	Eg
1E	C	C	EFR004[c]
2A	C	C	Eg
2B	C	C	Ec
2C	C	C	Ec
2D	C	C	Ec
2E	T	C	Ec
Inferior lobe			
1A	ND[b]	C	Eg
1B	C	C	Eg
1C	C	C	Ec
1D	C	C	Eg
1E	T	C	Eg
2A	C	C	Eg
2B	C	C	Eg
2C	C	C	Eg
2D	C	C	Eg
2E	C	C	Eg
Patient HL			
Oral wash	ND	ND	Eg
	ND	ND	Eg
	ND	ND	Eg
	ND	ND	Ne
	ND	ND	Na6[d]
Lung segment			
1A	C	C	Bl
1B	C	C	Eg
1C	C	C	Bl
1D	C	C	Gl
1E	C	C	Gl
2A	A	C	Cg
2B	C	C	Cg
2C	ND	ND	Bg
2D	C	C	Bg
2E	C	C	Eg
3A	C	C	Be
3B	C	C	Ei
3C	C/T	C	Ei
3D	C	C	Bg
3E	C/T	C	Eg
4A	C	C	Eg
4B	T	C	Eg
4C	C	C	Eg
4D	T	C	Eg
4E	C	T	Ee
5A	C	C	Nd
5B	A	C	Cg
5C	C	C	Ei
5D	C	C	Eg
5E	C	C	Bi

[a] Results from patient AIDS-2 are not shown as this patient had the same mtLSU (type TC), ITS (type Ai), and DHPS (wild-type) types in all subclones.
[b] ND, not done.
[c] EFR004, ITS2 sequence previously described in a French isolate (25).
[d] Na6, ITS2 type a6 (G at position 54 of the ITS2 sequence) (12).

was tested twice by PCR, cloning, and sequencing. In addition, another 32 different PCR clones were resequenced. The sequences were identical for all duplicate samples.

To investigate if sequencing of recombinant PCR clones had preferentially amplified or missed sequence types of mtLSU rRNA, we performed allele-specific PCR (AS-PCR) for detecting a polymorphism at position 85 of the mtLSU rRNA. Primers pAA, pAC, and pAT, detecting an A, C, or T at position 85, were used in a seminested PCR with primer pAZ102E as the downstream primer (13, 24). The AS-PCR was performed with an annealing temperature of 63°C for primer pAC and an annealing temperature of 60°C for primers pAA and pAT. The lower limit of detection was 1.6 pg for control DNA of types C, T, and A. Unspecific signals were generated when using an excess of 80 pg of control DNA. All steps in the AS-PCR experiments, including DNA extraction, were repeated at least twice.

In the mtLSU rRNA gene, five different sequence types have been described based on nucleotide polymorphisms at positions 81, 85, and 248 (17, 20, 30, 33). In the present study, polymorphisms were observed only at positions 85 and 248 (Table 1). For patient AIDS-1, a single mtLSU rRNA sequence type with T at position 85 and C at position 248 was observed in the diagnostic BAL and in all autopsy lung recombinant clones. However, a more complex picture was observed in the BAL and lung biopsies from patients AIDS-2 and HL. In the BAL samples from patient AIDS-2, the mtLSU rRNA type of three clones was type TC, one clone was type T/CC, and one clone was type CC. In contrast, type CC was predominant in the lung biopsies investigated, since all clones, except two that were type TC, obtained from a segment of the middle lobe were type CC. For the HL patient, four different mtLSU rRNA genotypes were observed in the lungs. All clones obtained from lung segment 1 were type CC; however, mixtures of types CC, AC, TC, and CT were found in the other lung segments. Type CT was observed only in a single clone. To explore if the observed heterogeneity of mtLSU rRNA was caused by insufficient sensitivity to detect minor type populations or random PCR-mediated selection of specific genotypes, AS-PCR detecting polymorphisms at position 85 of the mtLSU rRNA was done. Since a type T at position 85 was detected in only two clones obtained from the middle and inferior lobes of patient

AIDS-2, with the remaining 22 clones being type C, AS-PCR was set up with primers pAT and pAC to test whether detectable amounts of type T were present but had been missed by cloning and sequencing. In the BAL sample, types C and T, but not type A, at position 85 were detected by the AS-PCR, confirming the results of cloning and sequencing. By testing DNA from the apex and the middle and inferior lobes, type C was detected in all segments. In contrast, type T was detected only in the middle and inferior lobes and not in the DNA obtained from the apex, confirming that type T was below the level of detection in the DNA obtained from these lung segments. Similarly, AS-PCR performed on DNA from patient HL supported heterogeneity of mtLSU rRNA sequence types. In all lung samples, type C at position 85 was detected by AS-PCR, but type A was detected only in lung segments 3 and 9, in which cloning and sequencing had previously detected these types.

ITS1 and ITS2 genotypes were determined by sequencing of diagnostic respiratory samples and autopsy samples from all three patients (Table 1). Heterogeneity of ITS types was observed for patients AIDS-2 and HL, but not for patient AIDS-1, for whom sequence type Ai was detected in all clones. For patient AIDS-2, all clones derived from the BAL specimen were ITS type Eg. However, the following two more ITS types were observed in the lung samples: type Ec, which was found in four clones from middle lobe 2, and type EFR004, previously described in a specimen from France (19), which occurred in a single clone from middle lobe 1. The most pronounced ITS heterogeneity was observed for patient HL, for whom a total of 11 different ITS genotypes were observed. In the oral wash sample, three different ITS types, types Eg, Ne, and Na6 (which differs from type Ne by a G at position 54 of the ITS2 gene instead of a T), were found. Genotype Eg was the most frequently detected sequence type in both the oral wash sample (3 of 5 clones) and in the lung (8 of 24 clones), while types Ne and Na6 were detected only in the oral wash sample and not in the autopsy lung samples. In addition, types Bl, Gl, Cg, Bg, Be, Ei, Ee, and Bi were detected in autopsy lung samples but not in the oral wash sample.

Four different genotypes in the DHPS gene have been consistently observed (2, 9, 22). For the present study only a single sequence type was found for each patient, although mixed infections have been observed in other patients. The wild-type DHPS genotype was observed in all clones from patients AIDS-2 and HL and a mutation at codon 55 was observed in all clones from patient AIDS-1.

In summary, as expected, the most predominant genotypes detected in the diagnostic respiratory samples were also the most commonly detected in the lung segments; however, minor populations of ITS genotypes which were not found in the respiratory samples were detected in the lung segments from two of three patients. Interestingly, our study suggests that mtLSU rRNA sequence types were compartmentalized in different lung segments, since specific mtLSU rRNA sequence types were detected in different lung segments, a finding that was confirmed by AS-PCR.

The presence of coinfections with more than one genomic type of *P. carinii* has previously been described in diagnostic respiratory samples. For humans, mixed infection has been documented through direct sequencing of PCR products (2), sequencing of PCR clones (30), type-specific oligonucleotide (TSO) hybridization (21), and single-strand conformation polymorphism (SSCP) (7) analysis. Depending on the number and variability of genes assessed, coinfection rates between 10 and 30% have been reported by use of sequencing, TSO hybridization, and subcloning (2, 5). By SSCP analysis of four genomic regions, 77% of patients carried a mixed infection with two or more genotypes (6). Apart from coinfection, the presence of multiple genotypes could possibly indicate (i) heterozygosity of polyploid organisms, (ii) heterozygosity of diploid organisms, and/or (iii) multicopy genes with genotype variation between copies. However, since different alleles of a genomic region are most often present in different proportions in a given specimen (23) and since the genome of *P. carinii* is thought to contain only a single copy of the rRNA operon (4, 28), it is most likely that two or more sequence types reflect infection with multiple *P. carinii* f. sp. *hominis* types.

A key epidemiological question is whether infection results from reactivation of latent infection or from de novo infection (14, 18, 31). In approximately half of HIV patients with recurrent episodes of PCP, changes in mtLSU rRNA and ITS sequence types are observed (13, 18, 31). However, the significance of these switches is unclear. One possibility is that patients are cleared of infection but are reinfected. The alternative is that *P. carinii* is not eliminated by treatment and infection eventually relapses. Although genotype switching would seem to indicate reinfection, different genotypes might be detected during different episodes if mixed infections were present due to sampling artifacts or low sensitivity. Keely et al. attempted to address these issues by concurrent mtLSU rRNA and ITS typing and AS-PCR on samples obtained from HIV patients with recurrent PCP (13, 14). They observed concurrent genotype switches in both the ITS and mtLSU rRNA loci and were unable to detect the second-episode genotype by AS-PCR in the first PCP episode in four of five patients with mtLSU rRNA genotype switches, suggesting that recurrent PCP is caused by reinfection.

Our results cast doubt on this interpretation. Since heterogeneity of ITS sequence types and mtLSU rRNA types was observed within lungs, it is difficult to exclude the possibility that heterogeneity or sampling artifacts may be involved in genotype switches between recurrent episodes. In support of this, we recently published evidence that although half of patients who experienced recurrent PCP had ITS genotype changes, ITS genotype changes were also observed in 10 of 19 patients who had repeated bronchoscopies performed during the same episode of PCP (11). The finding of marked ITS heterogeneity between autopsy lung segments suggests that sampling artifacts could be one explanation for the ITS changes observed.

Presently, evidence that primary PCP is caused by reinfection rather than reactivation is based on animal experiments, in which primary infection is completely cleared (27, 32), and autopsy studies, in which *P. carinii* DNA is not detected in immunocompetent adults (25). However, whether reactivation or reinfection causes recurrent PCP in HIV-infected patients may be more complex. Most recurrent PCP cases were reported in the pre-highly active antiretroviral therapy era in patients with absence or failure of secondary chemoprophylaxis in the setting of a continuous low CD4 count. Whether secondary prophylaxis eventually sterilizes these patients in the

absence of immune reconstitution or if latent forms are carried until reactivation is not definitely known.

The large number of different sequence types observed within one patient is intriguing. In theory, the presence of multiple genotypes in a patient may reflect the fact that infection was acquired by airborne challenge with multiple different "strains." However, this interpretation requires that the reproduction of *P. carinii* is clonal (29) and that sequence types are stable during infection. In many fungi there is evidence of multiple species and multiple strain carriage, but there is also evidence that colonizing strains may undergo microevolution (26). Although most cyst and trophic forms of *P. carinii* are haploid (34), a minority of cysts may exhibit diploid forms (1) and sexual conjugation may exist (12, 15). If sexual reproduction occurs, then part of the genetic variation may result from genetic recombination.

Our study is the first to document heterogeneity of *P. carinii* sequence types within the lung which is not present in diagnostic respiratory specimens. These data have potentially important implications for the genotyping of *P. carinii* f. sp. *hominis.* The interpretation of genotype data, and in particular ITS sequence types, should be done cautiously since genotyping done on respiratory samples cannot a priori be assumed to represent all genotypes present within the lung. In future, the development of new genotype methods will have to examine the degree of genotype variation within human lungs and should preferably be based on multilocus sequencing in order to reliably distinguish strain differences.

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