Isoenzyme Diversity in *Pneumocystis carinii* from Rats, Mice, and Rabbits

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Pneumocystis carinii is an opportunistic pathogen that causes pneumonia in immunocompromised patients. To investigate the genetic diversity of *P. carinii* populations, multilocus enzyme electrophoresis was used to analyze five enzyme systems (malate dehydrogenase, glucose phosphate isomerase, leucine aminopeptidase, malic enzyme, and 6-phosphogluconate dehydrogenase). Only five different multilocus associations (zymodemes) were recorded for the 70 isolates studied. While only one multilocus combination was found in mice and rabbits, three different multilocus associations were recorded in rats. Population genetic tests and phylogenetic analysis strongly suggest that *P. carinii* genotypes are host-specific, in agreement with molecular study results, and that no genetic exchange occurs between genotypes from different host species. This hypothesis could be verified only by the evolutionary genetic approach, which relies here on multilocus analysis.

Pneumocystis carinii is an opportunistic pathogen that causes severe pneumonia in immunocompromised patients, especially in persons infected with human immunodeficiency virus [1, 2] or treated with immunosuppressive drugs [3–5]. *P. carinii* is found in the lungs of other mammals [6], and rats [7], mice [8], ferrets [9], rabbits at weaning [10], and piglets [11] are the most useful animal models of *P. carinii* pneumonia [12]. Evidence of genetic variability has been reported among *P. carinii* isolates from different host species and even from the same species. Genetic variability has been recorded for antigens [13–15], karyotypes [16–18], and molecular levels [19–25]. There are still few conflicting data on whether parasites that infect 1 mammalian host species [26, 29] are infectious to others [30].

Population genetic approaches have been useful in clarifying the population structure of various pathogens [31–34]. The same methods could improve our knowledge of *P. carinii* epidemiology by exploring whether *P. carinii* populations from different host species are genetically isolated from each other and by exploring whether strains from a given host reproduce clonally or sexually, a question epidemiologically relevant. If a species is clonal, its multilocus genotypes are stable in space and time and can be used as epidemiologic markers. This is not true if frequent recombination renders these genotypes unstable [34].

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In microorganisms, population genetic methods rely mainly on the analysis of linkage disequilibrium (nonrandom association of genotypes at different loci). Because these require the survey of several loci, polymerase chain reaction amplification of isolated genes cannot be used. If several different primers are used on the same isolate and the isolate is composed of several genotypes (mixed stock), different primers may not amplify the same genotype. Moreover, random-amplified polymorphic DNA methods [35] cannot be used for *P. carinii* analysis because of the presence of host DNA in the parasite sample. Thus, at present, multilocus enzyme electrophoresis (MLEE) is the only method available for analysis of several *P. carinii* loci. Here we describe an MLEE analysis that assessed 5 different genetic loci of 70 *P. carinii* isolates from 3 different hosts.

Materials and Methods

P. carinii sources. Seventy parasite stocks were isolated over 3 years from 3 mammalian hosts: rat, mouse, and rabbit. Twenty-eight Wistar rats (Iffa Credo, Lyon, France), 2 Lou rats (CNRS, Orléans, France), 1 Ofa rat (Iffa Credo), 4 outbred U42 white mice (our laboratory), and 13 BALB/c/U42 (BU) hybrid white mice (our laboratory) were used as sources of *P. carinii.* Rats and mice were given corticosteroids in drinking water: Rats were given dexamethasone (2 mg/L; Merck, Chibret, France) for 9–12 weeks, and outbred white mice were given prednisolone methasulfobenzoate (40 mg/L; Houdé Laboratories, Puteaux, France) for 13–14 weeks. All mice and rats were housed in one room of our laboratory animal facility.

We purchased 22 weanling rabbits of different strains: 4 California/New Zealand hybrids from Vasseur (Prouzel, France) and 3 from Charles River (Rouen, France); 1 Dutch (Harlan, Oxon, UK); and 1 chinchilla (Harlan, Zeist, Netherlands). The remaining 13 rabbits were from French breeding facilities: By region or city, we obtained 2 Hollandais and 1 each brun marron Lorraine, Feunoir, and Rex (east); 1 Blanc de Bouscat (Verdun); 1 Nains de Couleur and 1 Polonais aux yeux roses (Var); 1 Argentés de Cham-

Table 1. Acetate gel electrophoresis procedures for *P. carinii* enzyme detection.

Enzyme	Buffer	Dilution		Time (min)
		Soaking	Migration	
GPI	A	0.25	1	20
MDH	B	0.7	0.9	25
LAP	C	1	1	20
6-PGD	A	0.5	0.75	20
ME	A*	0.25	0.75	25

NOTE. All procedures were at 200 V, except for ME (malic enzyme), which was at 160 V. Buffer indicates stock solutions used to prepare soaking and migration buffers. Time is time for electrophoretic migration. Buffers: A, 0.5 *M* TRIS-versene-borate, pH 8; A*, 50 m*M* $MgCl_2$ added to stock buffer A before dilution; B, TRIS-barbital–sodium barbital, pH 9; C, 30 m*M* TRIS–10 m*M* EDTA–20 m*M* $MgCl_2$–10 m*M* boric acid, pH 7.8. GPI, glucose phosphate isomerase; MDH, malate dehydrogenase; LAP, leucine aminopeptidase; 6-PGD, 6-phosphogluconate dehydrogenase.

pagne (Saone et Loire), 1 California (Dordogne), 1 Sablés des Vosges (Bas-Rhin), and 2 outbred (Boulogne and Rodez). Weanling rabbit is a useful animal model, because it develops spontaneous *P. carinii* pneumonia [10].

We used uninfected rats, mice, and rabbits as negative controls: 8 rats (strains, Wistar, Nude, and Lou), 6 mice (strains, U42, BU, and SCID), and 8 rabbits (strains, California/New Zealand hybrid, Sauteur, and Bélier).

Extraction and quantification of P. carinii. Experiments were done under sterile conditions at 4°C to preserve enzyme activity. Lungs were quickly removed, and parasites were extracted as previously described [36]. Briefly, the lungs were cut into small pieces in sterile Hanks' medium and stirred in an Erlenmeyer flask for 1–2 h at 4°C. The pellet was filtered through gauze, centrifuged, resuspended in a buffered hemolytic solution, incubated for 10 min at 4°C, centrifuged again, and resuspended in Hanks' medium. The suspension was filtered successively through 250- and 63-μm stainless steel and then through 10- and 8-μm filters (Nuclepore, Serlabo, France). Total parasite counts were made as previously described [36] with use of toluidine blue O [37] and RAL-555 (Réactifs RAL, Paris), a Giemsa-like stain [38]. No other microorganisms were detected in parasite extracts. Lungs from control animals were processed similarly. In the control samples, we verified that parasites were absent or present only in very small amounts.

MLEE. Soluble enzyme extractions and electrophoresis were done as described [39] with slight modifications. The soluble enzyme extract was obtained by mixing the parasite sample with water. The enzyme extract was divided into equal aliquots and maintained at −80°C until use.

Enzyme systems. Five enzyme systems were used to characterize *P. carinii* isolates: glucose phosphate isomerase (GPI; EC 5.3.1.9), leucine aminopeptidase (LAP; EC 3.4.11), malate dehydrogenase NAD^+ (MDH; EC 1.1.1.37), malate dehydrogenase $NADP^+$ or malic enzyme (ME; EC 1.1.1.40), and 6-phosphogluconate dehydrogenase (6-PGD; EC 1.1.1.44). Electrophoresis was done on cellulose acetate plates. Migration conditions are listed in table 1.

Genetic variability and taxonomic clustering. Each distinguishable and reproducible zymogram for a given enzyme system was equated to a distinct genotype [34]. To evaluate the level of genetic isolation in the population under study, segregation of alleles at a given locus and recombination of genotypes occurring at different

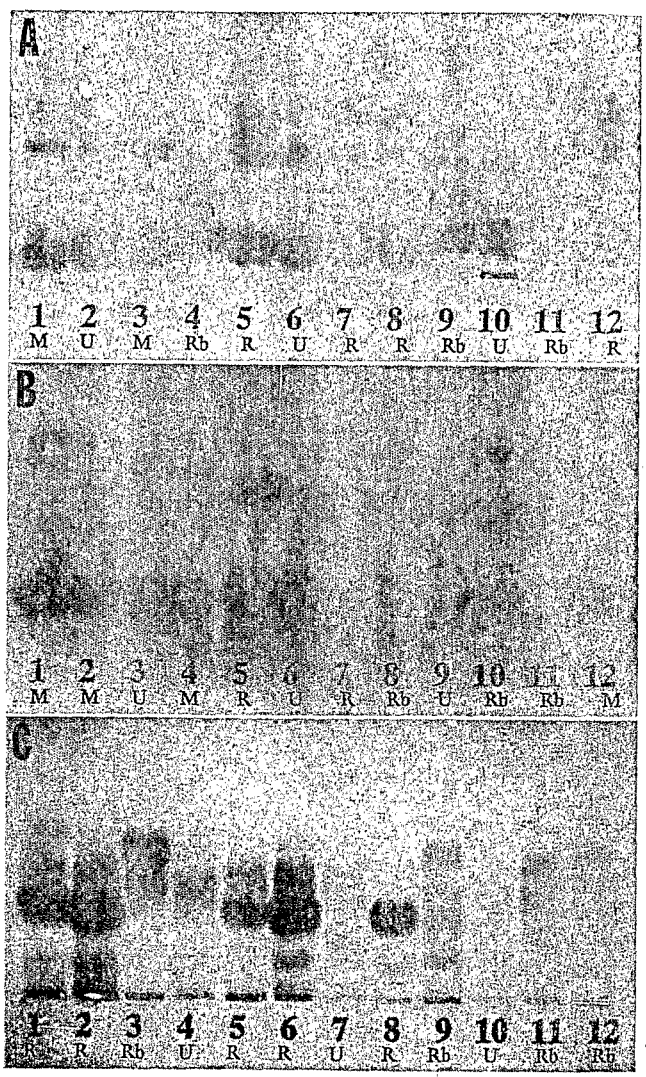

Figure 1. Malate dehydrogenase (MDH), glucose phosphate isomerase (GPI), and leucine aminopeptidase (LAP) zymograms of *P. carinii* isolates from rats (R), mice (M), rabbits (Rb), and lungs of uninfected controls (U). **A**, MDH patterns of isolates from U42 mouse (lane 1), BU mouse (lane 3), California rabbit (lane 4), Wistar rats (lanes 5, 7, 8), Sablés des Vosges rabbit (lane 9), outbred rabbit from Rodez (lane 11), Ofa rat (lane 12), and control samples from U42 mouse (lane 2), Wistar rat (lane 6), and California/New Zealand hybrid rabbit (lane 10). **B**, GPI patterns of isolates from U42 mouse (lane 1), BU mice (lanes 2, 4, 12), Wistar rats (lanes 5, 7), California/New Zealand hybrid rabbits (lanes 8, 10, 11), and control samples from BU mouse (lane 3), Wistar rat (lane 6), and California/New Zealand hybrid rabbit (lane 9). **C**, LAP patterns of isolates from Wistar rats (lanes 1, 2, 5, 6, 8), California/New Zealand hybrid rabbits (lanes 3, 9, 11, 12), and control samples from nude rat (lane 7) and California/New Zealand hybrid rabbits (lanes 4, 10).

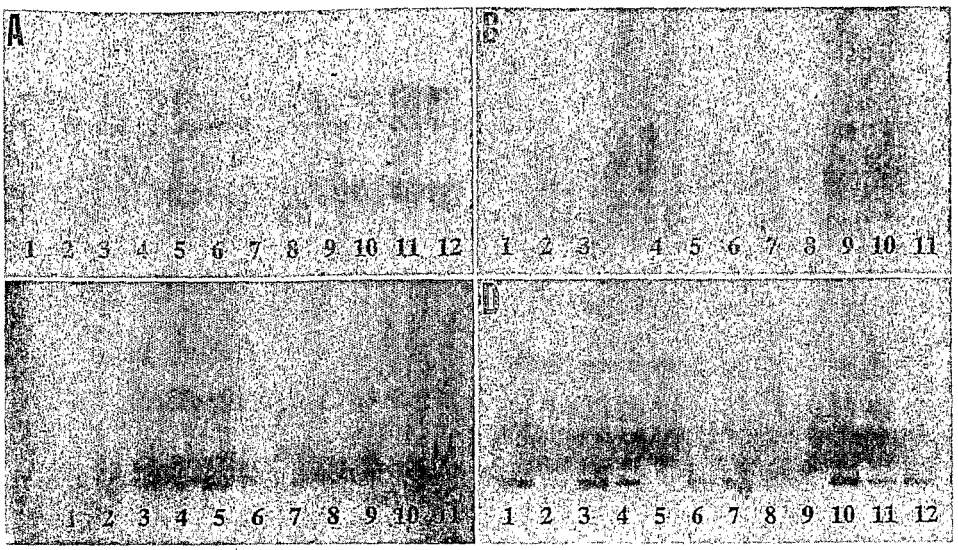

Figure 2. Malate dehydrogenase (MDH) and glucose phosphate isomerase (GPI) zymograms of *P. carinii* isolates from rats and rabbits. **A**, MDH patterns in rat isolates: Wistar (lanes 1–5, 7, 9, 11, 12), Lou (lane 8), Ofa (lane 10), and lung control sample from nude rat (lane 6). **B**, MDH patterns in rabbit isolates: Argentés de Champagne (lane 1), California/New Zealand hybrids (lanes 2, 7), chinchilla (lane 3), outbred from Happe and Rodez (lanes 4, 11), California (lane 6), Polonais aux yeux roses (lane 8), Sablés des Vosges (lane 10), and lung control samples from sauteur (lane 5) and bélier (lane 9). **C**, GPI patterns in rat isolates: Wistar (lanes 3–9), Lou (lane 10), Ofa (lane 11), and lung control samples from Wistar (lane 1) and nude rats (lane 2). **D**, GPI patterns of rabbit isolates: California/New Zealand hybrid (lane 1), Sablés des Vosges (lane 2), Argentés de Champagne (lane 3), Feu noir (lane 4), California (lane 5), outbred from Happe and Rodez (lanes 7, 8), brun marron de Lorraine (lane 10), Hollandais (lanes 11, 12), and lung control samples from California/New Zealand hybrids (lanes 6, 9).

loci (enzyme systems) were explored by Hardy-Weinberg and linkage disequilibrium analysis, respectively, as described [34]. Genetic distances among *P. carinii* isolates were estimated by Jaccard's distance [40], which measures the ratio of band mismatches between pairs of stocks according to the following formula: $D = 1 - [a/(a + b + c)]$, in which a = the number of bands common to the 2 compared genotypes, b = the number of bands present in the first genotype and absent in the second, and c = the number of bands absent in the first genotype and present in the second.

A dendrogram was computed from the distance matrix by the unweighted pair-group method with the arithmetic averages (UPGMA) method [41]. Robustness of the phylogenetic clustering shown on the dendrogram was estimated by bootstrap analysis [42].

Results

No isoenzyme polymorphism was detected in control samples with uninfected lung cells. Specific parasite enzyme activities were easily identified by comparing zymograms of parasite isolates with those of control isolates. Host cell isoenzymes were constantly different from those of parasites (figure 1).

For two enzyme systems (6-PGD and ME), parasite enzyme activities were detected only with $>200 \times 10^6$ parasites. For the other enzyme systems (GPI, MDH, and LAP), enzyme activities were seen independently from the number of parasites. In 1 case, an additional MDH band was detected in a rabbit isolate that contained twice the number of parasites seen in other rabbit *P. carinii* stocks (figures 1, 2).

All five enzyme systems were polymorphic (figure 3). Variability in the five systems made it possible to distinguish five different isoenzyme combinations or zymodemes (figure 3, table 2). Isolates from different host species exhibited clearly distinct isoenzyme patterns (figure 1). However, for the MDH system, one common band was shared by rat and mouse isolates (figure 3). Another MDH band was shared by the most enriched rabbit isolate and by 19 rat isolates (figure 1). Because this band was not constant in the rabbit isolates, it was not taken into account for computation of genetic distances.

Variability within a given host species was detected only in rat isolates and only by the MDH system, which showed three distinct profiles (figure 3). In both mice and rabbits, all isolates were genetically identical (figure 2). In rat isolates, there was variability within isolates from the same rat strain, and some isolates from distinct rat strains were genetically identical (figure 2). The dendrogram constructed from Jaccard distances reflects the inferred phylogenetic relationships among the 5 multilocus genotypes recorded: The 3 rat genotypes were closely related to each other; genotypes from mice and rabbits branched at 0.94 and 1.0, respectively (figure 4).

Data from several loci obtained by the isoenzyme method, the only method possible for *P. carinii*, enabled us to evaluate

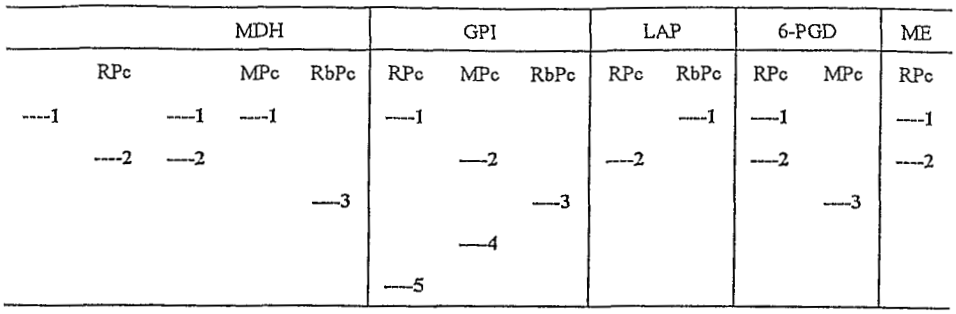

Figure 3. Representation of *P. carinii* patterns by malate dehydrogenase (MDH), glucose phosphate isomerase (GPI), leucine aminopeptidase (LAP), 6-phosphogluconate dehydrogenase (6-PGD), and malic enzyme (ME) systems. Pc, *P. carinii;* R, rat; M, mouse; Rb, rabbit; dashed line, representation of 1 isoenzyme. Nos. 1–5 associated with isoenzymes: 1 indicates fastest isoenzyme migrating on gel. Proportions not applied.

the degree of genetic isolation between genotypes. By use of the null hypothesis that isolates from the 3 different host species are panmictic and therefore exchange genes randomly (pertaining to a common gene pool), one can test the working hypothesis that they actually represent three distinct gene pools. All linkage disequilibrium tests showed considerable departures from panmictic expectation. If all isolates pertained to the same gene pool (null hypothesis), the probability of observing the multilocus genotypes Z1, Z3, Z4, and Z5 at levels as high or higher than actually observed (test d1 [34]) is 1.2×10^{-9}, 1.9×10^{-15}, 2.9×10^{-15}, and 2×10^{-15}, respectively. The probability of observing any genotype with a frequency as high or higher (or for observing as few or fewer genotypes) than actually observed for the more frequent genotype (test d2 [34]) is <.0001. Likewise, the probability of observing a linkage disequilibrium as great or greater than observed (test f [34]) is <.0001.

We performed a Hardy-Weinberg analysis [34] on the MDH locus of rat isolates by using the working hypothesis that *P. carinii* is diploid and that the 3 MDH genotypes represent 2 homozygous and 1 heterozygous genotype. The result was an apparent excess of heterozygotes (not statistically significant, $\chi^2 = 2.65$ with 1 *df*; $P > .05$). It was not possible to perform a linkage disequilibrium analysis on rat isolates considered separately, since only the MDH locus is variable. No statistical tests (Hardy-Weinberg or linkage disequilibrium analysis) were possible on rabbit and mouse isolates considered separately, since no genetic variability was observed.

Table 2. *P. carinii* multilocus genotypes identified with 5 enzyme systems.

Zymodeme	MDH	GPI	LAP	6-PGD	ME	Host
1	1	1/5	2	1/2	1/2	Rat
2	2	1/5	2	1/2	1/2	Rat
3	1/2	1/5	2	1/2	1/2	Rat
4	1	2/4	0	3	0	Mouse
5	3	3	1	0	0	Rabbit

NOTE. 0 indicates not detected. MDH, malate dehydrogenase; GPI, glucose phosphate isomerase; LAP, leucine aminopeptidase; 6-PGD, 6-phosphogluconate dehydrogenase; ME, malic enzyme.

Discussion

The UPGMA dendrogram (figure 4) suggests that the 3 *P. carinii* rat genotypes are closely related and that the other genotypes cluster apart. This phylogenetic picture must be considered cautiously. Genetic distances inferred from only 5 different loci are subject to notable SDs. If a broader range of loci were considered, it is possible that the genetic distances would be somewhat modified. Nevertheless, the considerable genetic distances found (near the maximum possible; 1.0 indicates no alleles in common) for isolates from different hosts are in agreement with data obtained by other methods [43].

The considerable genetic distances found for *P. carinii* genotypes from different hosts are remarkable, since genetic variability in isolates from a given host is either weak (rat isolates) or null (mice and rabbit isolates), even though the populations of rats, mice, and rabbits that we surveyed had diverse origins. Bootstrap analysis suggests that the nodes that separate rat from mouse genotypes and rabbit genotypes from rat or mouse genotypes are robust (verified on 100% of 100,000 assays). Isoenzyme analysis therefore corroborates results obtained from other methods and strongly suggests that different hosts harbor dramatically distinct *P. carinii* genotypes. This level of linkage disequilibrium suggests that genotypes from different hosts have been genetically isolated from each other for a very long time, although for our study, rats and mice have been

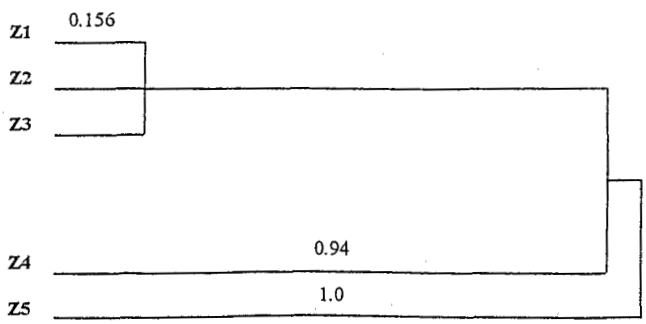

Figure 4. Unweighted pair-group with arithmetic averages method [41] elaborated from matrix of Jaccard's genetic distances [40] among representative *P. carinii* isolates from rats (Z1–Z3), mice (Z4), and rabbits (Z5).

reared in the same facility for 7 years. The most parsimonious hypothesis to account for this result is to consider that genotypes from rats, mice, and rabbits represent 3 distinct gene pools.

Provided that genotypes from the 3 different hosts are apparently genetically isolated from each other, if one could show that each of the 3 *Pneumocystis* populations taken from rats, mice, and rabbits represents a common gene pool (exchanges genes freely), it would be possible to equate these 3 categories with classical biologic species [44]. This is difficult with the available data. Indeed, for the mouse and rabbit isolates, no genetic variability was found, which renders any population genetic test impossible or meaningless.

The situation is hardly different with rat isolates, which showed variability only at the MDH locus. This variability produced negative results by Hardy-Weinberg statistical analysis. Nevertheless it would be premature to conclude that rat isolates represent a common gene pool for two reasons: First, it was impossible to reject the null hypothesis (isolates were from a common gene pool: panmyctic situation) and second, Hardy-Weinberg analysis, which differs from linkage disequilibrium statistics, remains tentative in most microorganism species [45]. In our study, it is not certain that all of the two-banded patterns correspond to heterozygous individuals. Some exhibited a typical pattern with two equally stained bands, but others had asymmetric profiles (figure 2). Alternative explanations for these two-banded profiles are gene duplication or mixtures of genotypes that correspond to zymodemes Z1 and Z2. In this last case, zymodeme Z3 would not correspond to a distinct genotype and should be discarded from the analyses. This would not change any of the major conclusions of the present work. Zymodemes Z1 and Z2 could correspond to the variant and prototype forms of rat *P. carinii* previously described on the basis of karyotype data [17, 21, 46]. In these previous studies, these forms were considered distinct stable strains. Additional data are required before it is possible to know whether *P. carinii* from a host species represent clonal lines or biologic species.

By MLEE, we confirmed that *P. carinii* isolates from different host species represent distinct genotypes and that these genotypes are probably genetically isolated from each other. This conclusion has obvious epidemiologic implications: It supports the view that *P. carinii* from different animals cannot be infective to each other. Available data do not permit us to know whether these host-specific populations correspond to biologic species. Therefore, giving them the status of distinct species remains a matter of convenience at present [45].

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