Clinical Significance and Phylogenetic Relationship of Novel Australian *Pneumocystis jirovecii* Genotypes[▽]

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Pneumocystis jirovecii is an important opportunistic pathogen in immunocompromised patients. Molecular typing is employed to study this pathogen, as no culture system exists. No Australian *P. jirovecii* strains have been previously studied. Direct sequencing, targeting the internal transcribed spacer (ITS) regions of the nuclear rRNA operon, the mitochondrial large-subunit rRNA (mt LSU rRNA), and the dihydropteroate synthase (DHPS) gene, was performed on 68 Australian samples, collected between 2001 and 2007. Seven novel Australian ITS haplotypes (a composite of the ITS1 and ITS2 regions) were identified (SYD1m, SYD1g, Isyd2, Eysd3, Osyd4, Ag, and Hc). A dendrogram of published ITS haplotypes revealed that of the seven novel haplotypes, three (SYD1m, SYD1g, and Osyd4) are closely related to the haplotype Eg. Applying statistical parsimony, an Australian haplotype network was constructed which identified Eg as the ancestral haplotype, with two unresolved loops encountered. This suggests that the ITS lacks the resolution required for evolutionary analysis. Only two mt LSU rRNA genotypes were detected, with genotype 1 predominating. Mutant DHPS genotypes were present in 13% (8/60) of the samples. The novel haplotype Isyd2 was associated with less severe disease than the other Australian haplotypes. In contrast, patients with mutant DHPS genotypes were more likely to have severe disease, require invasive ventilation, and have a poor outcome than patients with wild-type DHPS genotypes. In conclusion, genetic clinical correlates continue to be found for *Pneumocystis pneumonia*; however, they remain controversial and warrant further study.

Pneumocystis jirovecii is an important opportunistic pathogen in immunocompromised patients and presents predominantly as *Pneumocystis pneumonia* (PcP). Although the incidence has declined in human immunodeficiency virus (HIV)-infected patients since the advent of highly active antiretroviral therapy and institution of cotrimoxazole prophylaxis, it remains the most common AIDS-defining illness in Australia (21). In contrast, rates in other patient groups have increased (e.g., organ transplant patients and patients prescribed long-term corticosteroid therapy), so that PcP prophylaxis has been advocated in these patients (16, 25, 33).

The study of PcP is hampered by the lack of a culture-based system, and therefore, molecular methods are employed for typing and determining drug-resistant genotypes. Although numerous methods have been described, direct DNA sequencing remains the most commonly employed method. The targets sequenced include the internal transcribed spacer (ITS) regions of the nuclear rRNA operon (ITS1/2), the mitochondrial large-subunit rRNA (mt LSU rRNA) locus, and the dihydropteroate synthase (DHPS) gene (2). The subsequent information obtained is dependent on the stability and amount of variation at the targeted site. The mt LSU rRNA is stable and highly conserved, with only six unique genotypes, and thus has been used to address various epidemiological questions (17,

30). In contrast, ITS haplotypes (a composite of ITS1 and ITS2 alleles) have assisted in understanding patterns of transmission and phylogenetic relatedness. Thus, the ITS remains the most common target used to identify links between PcP genes and clinical characteristics and/or outcomes (14, 18). Several studies have documented that more severe disease is correlated with specific ITS haplotypes (14, 18). These findings are by no means universal, since other studies have found no such correlation (10). DHPS gene sequencing has been used to answer specific clinical questions related to treatment and/or prophylaxis failures (9, 11, 15).

Molecular typing of PcP strains from Africa (26), Asia (27), Europe (13, 32), and North America (13) has been described. To our knowledge, no Australian PcP strains have been studied. Thus, we undertook this study to determine the circulating genotypes, the intragenotype relationships and the potential correlations between the identified genotypes and clinical disease severity and/or outcomes.

MATERIALS AND METHODS

Specimens. Aliquots of the original specimens (65 induced sputa and three bronchoalveolar lavage samples) was stored at -80°C after they tested positive for *P. jirovecii* by immunofluorescence (Monofluo kit *P. jirovecii* [Bio-Rad]). All specimens collected between 2001 and 2007 at St. Vincents Hospital (Sydney, Australia) were included in the study.

DNA extraction, amplification protocols, and sequencing. The specimens underwent DNA extraction using the Qiagen BioRobot EZ1 (Qiagen, Hilden, Germany) tissue protocol after an initial concentration (centrifugation at 13,000 rpm for 1 min) and digestion step (the supernatant was resuspended in 180 μl of ATL buffer (Qiagen, Hilden, Germany) with 20 μl of proteinase K and incubated at 56°C for 2 h). Subsequent PCRs were performed using PureTaq Ready-To-Go

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PCR beads (GE Healthcare). DNA amplification of the mt LSU rRNA and DHPS genes and the ITS1 and ITS 2 regions was performed using previously published primers and protocols (9, 11, 28, 32). Several PCR modifications were adopted for the ITS amplification, as suggested by Beser et al. (3). They included increasing the primer concentration, the elongation time, and the denaturing temperature, as well as decreasing the number of cycles in the first and second PCR steps. The subsequent positive products were purified with a Qiaquick PCR purification kit (Qiagen) and commercially sequenced at the SUPAMAC facility (Camperdown, Sydney, Australia).

Sequence analysis. Sequence data were analyzed by means of the Chromas Lite software version 2.01 (Technelysium Pty Ltd.) and aligned with known genotypes using the multiple-alignment program CLUSTAL W version 2.0, which is incorporated in the BioEdit software package (version 7.0.9) (8). *P. jirovecii* ITS genotypes were aligned with previously published and unpublished genotypes (GenBank accession no. AF013806 to AF013834, AF374238 to AF374265, AF498265, AF135711 to AF135712, AY328043 to AY328066, AY550105 to AY550109, and AF013835 to AF013840) (13, 23, 24, 26, 27, 29, 31). The mt LSU rRNA genotypes were characterized based on the polymorphic sites at codons 85 and 248: genotypes 1 (85:C/248:C), 2 (85:A/248:C), 3 (85:T/248:C), and 4 (85:C/248:T) (12). The DHPS gene was aligned with the two previously published drug resistance sites (codon 55 and codon 57) (22).

Phylogenetic analysis. The concatenated sequences of ITS1 and ITS2 were used in the phylogenetic analyses, with regions of sequences with ambiguous alignments (i.e., at nucleotide positions 28 and 215 to 217) excluded from all samples. These regions occur when more than one equally plausible alignment exists.

The phylogenetic relationships between Australian and global PcP haplotypes were established using the program PAUP* 4.03b (28), utilizing the unweighted pair group method with arithmetic average (UPGMA). Sequence gaps (caused by insertions and deletions in the alignment) are considered missing data, thereby greatly reducing the probability of incorrect and inaccurate phylogenies in the tree and hence faulty interpretations of global haplotype relationships. In addition, several ITS1 ($n = 12$) and ITS2 ($n = 18$) types were not included in this analysis, as the haplotypes formed by these ITS genotypes have not been detailed to allow inclusion (24, 29).

Construction of an Australian PcP haplotype network was performed with the aid of the software TCS version 1.21 (5). Using statistical parsimony with a 95% confidence interval (CI) to connect the different haplotypes with the fewest possible mutational events, the intragenotype relationships between the Australian PcP haplotypes were represented. The most likely ancestral haplotype was determined by the same program. For this analysis to be accurate, greater sequence certainty is required, including confidence in the real presence of the gaps in the sequences. Thus, for this analysis, the gaps were included and were treated as mutations.

Clinical data. Patient demographics, age, and underlying risk factors (HIV status, immune suppression, and/or corticosteroid usage) for PcP were retrospectively retrieved from the medical records. Clinical presentation; mode, type, and duration of therapy; outcome; and follow-up were obtained from the medical records, with severe disease defined as an arterial partial arterial oxygen pressure of <70 mm Hg or an oxygen saturation of <94% on room air. In HIV-infected patients, highly active antiretroviral therapy, the HIV load, and the CD4 count within 1 month of presentation were also obtained. Use of PcP prophylaxis within the preceding month was collected from outpatient scripts.

Ethics approval was obtained from the St. Vincents Hospital research office (reference number 07/SVH/96).

Statistical analysis. Categorical data were analyzed using the chi-square or Fischer's exact test, while nonparametric continuous data were compared using the Kruskal-Wallis test with SPSS version 10.0 (SPSS Inc., Chicago, IL).

Nucleotide sequence accession numbers. The novel ITS genotypes identified (SYD1, syd2, syd3, and syd4) were submitted to GenBank and assigned the following accession numbers: EU442879 to EU442882.

RESULTS

ITS1 and ITS2 genotypes. Direct sequencing identified a total of eight ITS1 genotypes: seven known (A, E, H, I, K, N, and O) and a single novel genotype (SYD1) that occurred in 10% ($n = 6$) of the samples. Nine ITS2 genotypes were detected; six of them (c, e, f, g, m, and o) have been described previously, while the remaining three (syd2, syd3, and syd4) are novel genotypes. Of these, syd2 was the most frequent, occur-

TABLE 1. Australian *P. jirovecii* ITS region haplotypes detected

ITS haplotype ^a	No. (%) of samples
Eg.....	13 (22)
Isyd2.....	11 (18)
Ec.....	8 (13)
SYD1g.....	5 (8)
Gg.....	5 (8)
Ne.....	3 (5)
Og.....	3 (5)
Esyd3.....	3 (5)
No.....	2 (3)
Ee, Eo, Ag, Hc, Kf, SYD1m, Osyd4.....	1 (2) (each)

^a ITS haplotypes are formed by the combination of ITS1 and ITS2 regions.

ring in 18% ($n = 11$) of the samples, followed by syd3 and syd4 in 5% ($n = 3$) and 2% ($n = 1$) of the samples, respectively. Sixteen *P. jirovecii* ITS haplotypes were detected when ITS1 and ITS2 genotypes were combined. The three most common haplotypes were Eg ($n = 13$; 22%), Isyd2 ($n = 11$; 18%), and Ec ($n = 8$; 13%), with the remaining haplotypes occurring at frequencies of less than 9% (Table 1). Two new combinations were detected: Ag and Hc. Of the novel ITS genotypes, SYD1 clustered with ITS2 genotype g in all but one sample. All novel ITS2 genotypes, in combination with a single ITS1 genotype, formed solitary new haplotypes, Isyd2, Esyd3, and Osyd4, respectively.

The UPGMA dendrogram, based on the alignment of 129 haplotypes of *P. jirovecii*, is shown in Fig. 1. Provided that all gaps were treated as missing data, three of the novel Australian haplotypes (SYD1m, SYD1g, and Osyd4) were indistinguishable from Eg. In contrast, Isyd2 was distinct from Eg and was identical to the European haplotype, which has been documented from only a single sample (13). Other haplotypes closely related to Isyd2 (On [$n = 2$], Gn [$n = 1$], and Cn [$n = 2$]) have similarly occurred in small numbers in European samples (13, 32). Within the more distant but closely related cluster (i.e., Ai, Ci, Di, Bi, Bl, and Bk), only Bi and Ai have not been restricted to Europe (13, 32). However, the majority of Bi (94%; 81/86) and Ai (89%; 42/47) have occurred in European samples. Of the haplotypes occurring in Australian, only Hc, Ee, and Ec clustered closely, but not exclusively, with the novel Asian haplotypes Ir, Ic, and Gb (27). Thus, no clear relationship between Asian and Australian haplotypes was found.

The 95% parsimony network is shown in Fig. 2. The parsimony cladogram was not fully resolved, with two loops encountered, involving haplotypes No, Ne, Eo, and Ee. The most common haplotype, Eg, was considered by TCS analysis as the most probable ancestral haplotype. Based on the network, the novel genotype Isyd2 was the most distant haplotype, separated from the ancestral haplotype by seven mutational steps and missing samples.

Mt LSU and DHPS genotyping. Mt LSU rRNA sequences showed two genotypes: genotype 1 (87%; 52/60) and genotype 3 (13%; 8/60). There was no clustering with a specific ITS haplotype. Similarly, mutant DHPS genotypes were not restricted to any ITS haplotype and occurred in 13% (8/60) of samples, with both mutant forms detected (at codons 55 and 57) in three and five samples, respectively.

FIG. 1. UPGMA dendrogram for 129 *P. jirovecii* haplotypes, treating the sequence gaps as missing data. The two components of the dendrogram come together at point X to form the complete dendrogram. Australian haplotypes are indicated with boldface type.

FIG. 2. ITS haplotype network of Australian strains. The size of each oval is proportional to the number of individuals. Each line represents a single mutational step connecting two haplotypes. Empty nodes indicate haplotype states that were absent in the samples.

Clinical characteristics and correlation. The 68 samples corresponded to 60 patients; 5 had duplicate samples (the same genotypes) collected on alternate days, and 3 had a relapse or reinfection with the same genotypes 40, 46, and 160 days after their initial PcP episodes. No mixed infections were detected.

The median age of patients with PcP was 41 years. The majority (90%; 53/60) of cases occurred in severely immunocompromised HIV-infected individuals (median CD4 count, 71 cells/ μ l) (Table 2). The remaining seven (10%) patients were at risk for PcP secondary to corticosteroid therapy. Most patients had severe disease, with six patients requiring intubation. The median length of stay was 13.7 days. All patients received cotrimoxazole therapy, with 90% of the patients surviving their PcP episodes.

The novel Australian ITS haplotype, Isyd2, was associated with less severe disease than all other Australian haplotypes ($P < 0.05$; odds ratio [OR], 4; 95% CI, 1.03 to 15). However, there was no difference in treatment outcomes between the ITS haplotypes. In contrast, mutant DHPS genotypes were associated with severe disease ($P < 0.05$; OR, 10; 95% CI, 1.2

to 90), the need for invasive ventilation ($P < 0.01$; OR, 31; 95% CI, 2.7 to 352), and poorer outcomes (death) ($P < 0.05$; OR, 15; 95% CI, 2 to 63) than patients with wild-type DHPS genotypes (Table 3). There was no difference detected in the choice of agent, route, and dose of administration that could account for the disparity in outcomes between the DHPS genotypes. In addition, no clinical correlation with mt LSU rRNA genotypes was detected.

DISCUSSION

The most prevalent haplotype worldwide, including Australia, is Eg, accounting for 23% of all published combinations (10, 13, 24, 26, 27, 30). Unlike previous studies, a novel genotype (Isyd2) was found to be the second most common Australian haplotype, occurring in 18% of our samples. Six other unique haplotypes were detected (SYD1g, SYD1m, Esyd3, Osyd4, Ag, and Hc), but at much lower frequencies, between 2% and 8%. All the remaining combinations (61%; 37/60) have been previously described.

The parsimony network determined that Eg was the ances-

TABLE 2. Demographic and clinical characteristics of patients with PcP

Characteristic	Value
Median age (range) (yr)	41 (28–73)
Male [no. (%)]	55 (92)
Non-HIV infected ^a [no. (%)]	7 (13)
HIV-infected [no. (%)]	53 (88)
Median (range) CD4 count (cells/ μ l)	71 (3–258)
Median HIV load (copies/ml)	>100,000
Patients taking HAART [no. (%)]	8 (15)
Clinical	
Admitted [no. (%)]	50 (83)
Median (range) length of stay (days)	13.7 (2–50)
Severe disease ^b [no. (%)]	43 (72)
Steroid use [no. (%)]	43 (72)
Intubated [no. (%)]	6 (10)
Treatment outcome	
Recovered [no. (%)]	54 (90)
Died [no. (%)]	6 (10)

^a Patients' underlying risks included corticosteroid use for a hematological malignancy ($n = 1$), solid-organ transplantation ($n = 3$), chronic lung disease ($n = 1$), and metastatic carcinoma ($n = 2$).

^b Severe disease was defined by the presence of an arterial oxygen pressure of <70 mm Hg and/or oxygen saturation of <94% on room air.

tral haplotype, which is similar to a previous study (26). This suggests that the novel Australian haplotypes have evolved from Eg. What remains unclear is whether they are imported haplotypes or have evolved in Australia. Similarly, given its close clustering with the European combination In, Isyd2 may likewise be an imported haplotype. However, the frequency of Isyd2 detection ($n = 11$) was greater than that of In ($n = 1$). In addition, In may merely reflect an in vitro recombination event, as amplification protocol modifications to minimize these events (3) were not implemented by Lee et al. (13). Thus, it remains more plausible that Isyd2 has been present in Australia for longer and has evolved in Australia.

An unresolved loop involving haplotypes No, Ne, Eo, and Ee was detected, similar to a previous study (26). The number of unresolved loops increased when all the published haplotypes were included (data not shown). This suggests that the ITS regions are prone to frequent homoplastic and/or in vivo recombination events. Thus, the ITS region lacks the resolution to resolve intraspecies phylogenetic patterns. To obtain this greater evolutionary clarity, additional studies are warranted, but they should focus on alternative targets, possibly adopting those typing methods used successfully for other pathogenic fungi, such as *Candida albicans* (4) and *Aspergillus fumigatus* (1).

Clinical correlation with ITS haplotypes has been conflicting. Several studies have found a correlation between genotype Ai and moderate/severe disease, while Ne has been associated with mild disease (18). In contrast, a Portuguese study, established Ne to be associated with poorer outcomes (14). In our study, Ai was not detected, while Ne occurred in only three samples (5%) and was not associated with severe disease. However, the novel haplotype Isyd2 was associated with less severe disease. No other associations were documented be-

TABLE 3. Clinical characteristics of patients with DHPS gene mutations compared to patients with wild-type DHPS gene

Characteristic	Value for DHPS genotype:	
	Mutant ^a	Wild type
No. of patients (%)	8 (13)	52 (87)
Median age; range (yr)	44 (27–53)	42 (15–66)
HIV infected [no. (%)]	7 (88)	46 (89)
Median CD4 count; range cells/ μ l	50 (32–149)	108 (3–175)
Previous prophylaxis [no. (%)]	2 (25)	6 (12)
Corticosteroids [no. (%)]	7 (88)	26 (50)
Severe disease ^b [no. (%)]	7 (88)	21 (40)
Intubated ^b [no. (%)]	3 (38)	1 (2)
Died ^b [no. (%)]	3 (38)	3 (6)

^a Three patients had the codon 57 mutant (m57), and five patients had the codon 55 mutant (m55).

^b Significant association ($P < 0.05$).

tween ITS haplotypes, clinical presentation, treatment failure, and/or outcomes (10, 32). These contradictory findings suggest that there are probably other, as yet unresolved genetic factors that contribute to pathogen virulence. This is supported by the genetic similarity of the ITS regions of Ai or Bi to Isyd2 but contrasting clinical associations.

Only two mt LSU rRNA genotypes were detected, with genotype 1 predominating, similar to previous studies. However, unlike other studies, genotype 3 occurred in samples from non-HIV-infected patients (2, 19).

Mutant DHPS genotypes were present in 13% (8/60) of the samples. While this prevalence is lower than previously documented rates (between 20% and 82%) (6, 9, 11, 20), it is similar to a recent Italian study, where these genotypes were present in 8.4% of the samples (32). Our data did not find an association between DHPS mutants and cotrimoxazole prophylaxis. This may be secondary to the low prevalence of DHPS mutants in the study but may also be related to patients being noncompliant with prophylaxis. Despite this, our data support previously reported associations between mutant DHPS genotypes and severe disease, the need for invasive ventilation, and poor outcomes compared to patients with wild-type DHPS genotypes (9, 32).

There are several limitations to our single-center study. The number of specimens in our study was relatively small, as reflected by the wide 95% CIs of the significant associations. However, previous reports have examined even fewer samples than our study and have detected multiple genotypes from a single patient's sample as a result of cloning experiments (26, 27). The cloning methodology is prone to in vitro recombination events, resulting in a spuriously high number of ITS haplotypes (3). Although the novel Australian genotypes differ little from previously published haplotypes, the frequency at which they occurred and the genetic variation remain consistent with the published novel haplotypes. Our data suggest that patients with strains harboring mutant DHPS genotypes have more severe disease and poorer outcomes. The clinical relevance of mutant DHPS genotypes remains unclear and controversial, since cotrimoxazole remains the treatment of choice in these patients (7, 22) and therefore is not associated with frank clinical resistance. There are probably other, as yet unidentified host- or pathogen-specific factors that give rise to

poorer outcomes in these patients, and they merit further study.

In conclusion, seven novel Australian *P. jirovecii* ITS haplotypes were identified. Isyd2 was associated with less severe disease than the other observed haplotypes. In contrast, DHPS mutations were associated with more severe disease and poorer outcomes. The resolution of phylogenetic patterns using ITS haplotypes is probably inadequate for intraspecies separation. Further studies are therefore warranted to not only address evolutionary patterns, but also resolve clinical genotype correlations.

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