Distribution of DHPS Mutations Among ITS Subtypes of *P. carinii*

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Sulfa drugs are widely used in the prophylaxis and treatment of *Pneumocystis carinii* pneumonia. Co-trimoxasole, the first-line agent, is a combination of sulfamethoxazole plus trimethoprim but functions as sulfa monotherapy since trimethoprim appears to be inactive. Dapsone, a sulfone, is a commonly used second line agent. Both sulfamethoxazole and dapsone act by binding to the dihydropteroate synthase (DHPS) (reviewed in ref [2, 16] (Fig. 1).

Sulfa-resistant *Pneumocystis carinii* may be an emerging public health problem. However, since in vitro cultivation of human-derived organisms is not possible, molecular tools for determining resistance are needed. Genetic polymorphisms in the *P. carinii* DHPS were first reported by us in 1997 [10]. Two of the mutations, at positions 55 and 57, appear to be in the enzyme active site (Fig. 1, based on the E. coli structure [1]). These mutations have been associated with prophylaxis break-through in a number of studies [4, 6, 8, 10, 12-15].

These mutations appear to have occurred recently, and then to have spread over time. In several studies, the mutations were infrequent in patient samples obtained before 1992-4, and then became more frequent as the sulfa drugs became used more widely in prophylaxis [4, 8, 9]. Also, the prevalence of these mutations varies geographically, with higher prevalences at sites with older epidemics [6, 9].

How did the mutations evolve? There are two possibilities. The mutations might have arisen only once and then spread as a result of selection on a population level. Alternatively, Mutations could arise independently in each treated patient and increase as a result of selection within host. Obviously, intermediate scenarios (where the mutations arose independently but infrequently) are also possible.

In order to understand how these mutations arose, we asked the question of whether DHPS mutations were associated with individual strains of human-derived *P. carinii*. Strains were defined by sequencing the Internally Transcribed Spacer (ITS) regions. Human-derived *P. carinii* contain >15 ITS1 types (A-O) and >14 ITS2 types (a-n) More than 50 different combinations have been identified [5, 7, 11].

We postulated that, if mutations have arisen once or only a few times, then DHPS mutations should be associated with specific ITS types. However, we did not anticipate that the associations would be perfect, since ITS and DHPS genes are probably unlinked. And there may be horizontal genetic transfer (sexual, parasexual, etc.) in *P. carinii*. 
We obtained ITS and DHPS genotyping data from 57 patient isolates retrospectively and prospectively. These patients were from Indiana, Atlanta, San Francisco, Los Angeles, Seattle and were a subset of patients used in previous studies [3, 6, 8-10]. We then selected all 37 isolates with monoclonal infections (only single ITS type and DHPS type).

Characteristics of 37 monoclonal patient isolates are shown in Table 2. There were 22 WT, 14 double mutants and 1 single mutant (position 55). Mutations were more common in Atlanta and the West Coast than Indiana, and more common in samples obtained more recently, but these differences were not statistically significant.

One ITS type, Eg, was common in all 3 sites (Table 3). Three other types, Ea, Eb, and Ne, were found in two of the 3 sites. The other 6 ITS types were only found in single sites.

The distribution of DHPS mutations among ITS types is shown in Figure 2. Mutations are very common in some ITS types (such as Ne), but not found in others.

We next asked the question of whether the ITS types that contained mutant DHPS genes were related evolutionarily. A cladogram (Fig. 3) were constructed using the consensus sequences for ITS 1 and ITS2, combined in the same manner as the isolates used in our study. Monkey-derived *P. carinii* ITS types were used as outgroups. The cladogram was bootstrapped, with values < 50 not well supported. No evolutionary relatedness among ITS types containing mutations was observed.

We then asked post-hoc, whether certain groups of ITS types contained statistically higher numbers of DHPS mutants than other ITS types using the Fishers’ exact test. Among isolates from Indiana, Ee isolates were significantly more likely to be DHPS mutants than all others. Among all isolates, Ne, Eg and Ee isolates were more likely to contain DHPS mutants than all others. This is not an artefact associated with date of acquisition, since Ee and Eg are among the oldest of the isolates collected. These data are consistent with the possibility that DHPS mutations arose infrequently.

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