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Dihydropteroate Synthase (DHPS) Gene Mutations in Human Pneumocystosis

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Extraordinary journey began on June 18, 1981, the Centre for Disease Control and Prevention (CDC) in United States reported a cluster of *Pneumocystis carinii* pneumonia (now known as *Pneumocystis jirovecii (P. jirovecii)* pneumonia or *Pneumocystis* pneumonia in five gay men in Los Angeles. It was known as Gay Related Immune Deficiency (GRID). Subsequently, it was confirmed that about 50% of people with the syndromes were not gay, hence, the name was changed to acquired immune deficiency Syndrome (AIDS). Following Human immunodeficiency virus infection (HIV) and AIDS, substantial increase in the number of cases of *Pneumocystis* pneumonia were reported throughout the world. Two seemingly related events emerged over the years in human pneumocystosis, one relates to the increasing use of prophylaxis in infected individuals and the other about drug resistance.

Despite decades of research in drug development trimethoprim and sulfamethoxazole (TMP-SMZ), a mixture of 1:5 ratio, remains the most effective first line regimen for both anti-*Pneumocystis* prophylaxis and therapy since 1990s. Anti-*Pneumocystis* activity of TMP-SMZ is however, almost entirely to sulfamethoxazole [1]. Many sulfa drugs (short, intermediate and long acting) are close structural analogs of para-aminobenzoic acid (*p*ABA), that exhibit its antimicrobial activities by competing with *p*ABA at the dihydropteroate synthase (DHPS) active site. This leads to critical depletion of cellular folate levels [2,3]. Folates being the crucial cofactors in the synthesis of thymidine, purines, certain amino acids and pantothenic acid, generally play critical roles in one-carbon transfer reactions in cell metabolism. Higher eukaryotes obtain folic acid and reduced folate polyglutamates from dietary sources by uptake through membrane-associated folate transport proteins. In contrast, prokaryotes and some lower eukaryotes are obligatory synthesizer of folate *de novo* [4]. DHPS and dihydrofolate reductase (DHFR) are the two key enzymes that are necessary for *P. jirovecii* to synthesize folate. These two represent the enzymatic targets for the antifolate based treatment with TMP-SMZ. In *P. jirovecii,* DHPS is part of a tri-functional protein along with two other enzymes dihydroneopterin aldolase and hydroxymethyldihydropterin pyrophosphokinase, essential in the folic acid biosynthesis pathway. In their study, Volpe et al. demonstrated that *Pneumocystis* contains DHPS activity using a new high performance liquid chromatography (HPLC) based assay. It was also observed in their study that the effects of various sulfa drugs on the DHPS were quite different than those of the *Escherichia coli* DHPS. For example, the inhibitory constant (Ki) for different organisms were different, reflecting the fact that fundamental differences in the enzyme-active site structures differs between genus and species [6].

Besides concerns about the toxicity of the TMP-SMZ treatment and low tolerance to sulfa-based drugs in some patients, there are growing evidences of the emerging resistance of the *P. jirovecii* with the acquired mutations in the targeted enzymes. Earlier studies reported about sequence variants in both DHPS and DHFR of *P. jirovecii*, suggesting the development of resistance upon exposure to the drug. Subsequently, as the number of *Pneumocystis* pneumonia (PCP) patients unresponsive to TMP-SMZ increased over the years and the corresponding strains of the pathogen were sequenced, it became possible to draw statistically significant associations to estimate possible risks of resistance upon prior exposure to the drug [7,8].

A growing number of reports identified DHPS mutations primarily at codon 55 and 57 which were associated with higher rates of treatment failure in PCP patients treated with intravenous TMP-SMZ [7,9,10]. These mutations change a single nucleotide in a base triplet. The most frequent non-synonymous single nucleotide polymorphisms (SNPs) in *P. jirovecii* DHPS have been observed at positions 165 and 171, the combination of which have led to four different

possible genetic alleles. Most common non-synonymous mutations single nucleotide polymorphisms (SNPs) in the *P. jirovecii* DHPS gene are located at nucleotide positions 165 (A–G) and 171 (C–T), causing amino acid substitutions at codon 55 (Thr to Ala) and 57 (Pro to Ser) in a highly conserved region of one of the putative active sites of the enzyme [7,11]. The presence of the most common DHPS mutations at codon 55 (Thr55Ala) and codon 57 (Pro57Ser) may be a cause of drug resistance [12]. The four different DHPS genotypes are WW (wild type in positions 165 and 171), WM (wild type in 165 and mutated in 171), MW (mutated in position 165 and wild type in 171) and MM (mutated in positions 165 and 171). Haplotypes for DHPS have been assigned based upon codons 55 and 57.

As the study of drug efficacy cannot be directly assessed in the absence of a reliable *P. jirovecii* cultivation system, use of molecular method is the only possible way of studying the prevalence of drug resistance. Interestingly, there are large geographic variations in the frequency of DHPS mutations identified in patient samples throughout the world. Several studies in high income settings have shown relatively higher mutations. Reported prevalence of DHPS codon 55 or 57 mutations was as high as 69% in the USA, whereas in European countries, the prevalence ranged from 0 to 36% [13,14]. In one of the initial US-American study, 22 out of 29 samples were positive for DHPS mutations after drug exposure [15], in contrast, two German studies showed much lower prevalence. In addition, occurrence of identical genotypes in these two German studies indicated stable transmission of few *P. jirovecii* strains with little genetic variation over time and space [16,17].

Correlation between mutation(s) in the *P. jirovecii* DHPS gene and resistance to SMZ have been reported by various studies [9,18-20]. *P. jirovecii* DHPS with the double mutations was associated with lower susceptibility to SMZ. Furthermore, double mutations resulted in threefold increase in minimum inhibitory concentrations (MICs) to that of the wild-type DHPS [21]. In Europe, the prevalence of the double mutated genotypes has been low (<3%), compared to 40% that has been reported in USA. A threefold increase in mortality rate has also been reported in patients with DHPS mutation, than those infected with wild type. Moreover, double mutated allele has been the predominant *P. jirovecii* DHPS genotype found in patients experiencing sulfa-drug prophylaxis failure [22], suggesting the existence of a selective pressure. However, some studies did not find any link between DHPS mutations and treatment failure.

Reports from low-and middle-income settings, where the burdens of AIDS and PCP are greatest, DHPS mutation in *P. jirovecii* have shown variable results. Prevalence of PCP amongst HIV-infected patients with pneumonia was 27% in some of the African countries [23]. In a cross-sectional study, HIV-infected Ugandans had low prevalence of *Pneumocystis* pneumonia, however, all *P. jirovecii* isolates harbored mutations in the DHPS gene [24]. In this study by Taylor et al., most patients individually denied prior exposure to prophylaxis with antifolate drugs. Such high prevalence of DHPS mutation despite the lack of antifolate prophylaxis may suggest two nonexclusive processes such as (i) inter-human transmission of mutant strains or (ii) population-level selective pressure exerted by antifolate use for other indications. Inter-human transmission of mutant strains has been inferred from ecological studies of mutant genotypes in which geographical residence was associated with the genotype [24]. Since the first putative description of inter-human transmission of *P. jirovecii* in 1967 [25], a large number of nosocomial outbreaks of PCP (sometimes referred to as clusters) have been reported in the literature. In a Korean study, conducted from 2007 to 2013, no DHPS mutations were observed in any of the episode of PCP in patients studied [26]. Although significant prevalence of mutations in DHPS (20–37%) have been reported from different countries [27,28], only one DHPS mutant was reported among 52 strains studied in Japan [29]. These variable findings may be due to geographical differences. The association between use of TMP-SMZ as prophylaxis and the frequency of mutations was stronger for the studies that included multiple isolates than for those that did not. These differences suggest the possibility that exposure to multiple courses of sulfa prophylaxis increases the chances of DHPS mutations. In contrast, low prevalence of DHPS mutations have been observed in many studies conducted in Latin American countries with similar usage of sulfa drugs, suggesting possible additional sources of resistant genotypes. However, Chile is the only exception, where prevalence of DHPS mutations was high, presumably acquired through inter-human transmission.

Kazanjian et al. revealed DHP gene mutation rate of 7% (1/15) in AIDS patients with PCP in Beijing. In their study among non-HIV-infected patients with *Pneumocystis* pneumonia, between January 2008 and April 2011, mutations at Thr55Ala and Pro57Ser amino acid substitutions were not observed, instead, two other nonsynonymous mutations, i.e Asp90Asn and Glu98Lys were identified from two patients [11]. In a study from India, conducted in 76 HIV-positive patients, 22.4% (17/76) were positive for *P. jirovecii*. Upon DHPS gene sequencing, a novel nucleotide substitution at position 288 (Val96Ile) was observed in three patients. All three patients infected with this particular mutant genotype had severe episodes of PCP, did not respond to TMP-SMZ treatment and had fatal outcome (P=0.005) [30].

Finally, what we observe is that although conflicting and variable reports are available pertaining to DHPS mutations in *P. jirovecii* pneumonia, the mutational surveillance study may have advantages to detect minority variants and allow for the detection of drug resistance

to generate evolving scenario that may help to develop alternative treatment algorithm/s.

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