1 **High prevalence of *Pneumocystis jirovecii* dihydropteroate synthase gene**

2 **mutations in patients with first episode of *Pneumocystis* pneumonia in Santiago,**

3 **Chile, and their clinical response to trimethoprim-sulfamethoxazole therapy.**

4

5 Carolina A Ponce[a], Magali Chabé[b], Claudio George[a], Alejandra Cárdenas[a], Luisa

6 Duran[a], Julia Guerrero[a], Rebeca Bustamante[a], Olga Matos[c], Laurence Huang[d], Robert

7 F Miller[e] and Sergio L Vargas[a]#

8 Programa de Microbiología y Micología, Instituto de Ciencias Biomédicas, Facultad de

9 Medicina, Universidad de Chile, Santiago, Chile[a]; Center for Infection and Immunity of

10 Lille, Institut Pasteur de Lille, INSERM U1019, CNRS UMR 8204, University Lille Nord

11 de France, Lille Cedex, France[b]; Unidade de Parasitología Médica, Grupo de

12 Protozoários Oportunistas/VIH e Outros Protozoarios, Global Health and Tropical

13 Medicine, Instituto de Higiene e Medicina Tropical, Universidade NOVA de Lisboa,

14 Lisboa, Portugal[c]; Division of Pulmonary and Critical Care Medicine, and HIV, Infectious

15 Diseases, and Global Medicine Division, Department of Medicine, San Francisco

16 General Hospital, University of California, San Francisco, California, USA[d]; Research

17 Department of Infection and Population Health, Institute of Epidemiology and

18 Healthcare, University College London, London, UK[e]

19

20 Running title: *P. jirovecii* DHPS mutations on prior absence of sulfas

21

22 #Address correspondence to Sergio L Vargas, svargas@med.uchile.cl

23

ABSTRACT

Mutations in the Dihydropteroate synthase (DHPS) gene of *Pneumocystis jirovecii* associate with failure of sulfa prophylaxis. They can develop by selection in patients receiving sulfa drugs, or be acquired via person-to-person transmission. DHPS mutations raise concern about decreasing efficacy of sulfa drugs, the main available therapeutic tool for *Pneumocystis* pneumonia (PCP). The prevalence of *Pneumocystis* DHPS mutations was examined in *Pneumocystis* isolates from 56 sulfa-prophylaxis-naive adults with first-episode of PCP from 2002-2010 in Santiago, Chile. Their clinical history was reviewed to analyze the effect of these mutations on response to trimethoprim-sulfamethoxazole (TMP-SMZ) therapy and outcome. Mutant genotypes occurred in 22(48%) of 46 HIV-infected, and in 5(50%) of 10 HIV-uninfected patients. Compared to patients with wild type genotype, those with mutant genotypes were more likely to experience sulfa treatment-limiting adverse reactions, and, to have a twice-longer duration of mechanical ventilation if mechanically ventilated. Specific genotypes did not associate with death, which occurred in none of the HIV-infected, and in 50% of non-HIV-infected patients. Chile has a high prevalence of DHPS mutations presumably acquired through inter-human transmission because patients were not on sulfa prophylaxis. Results contrast with the low prevalence observed in other Latin American countries with similar usage of sulfa drugs suggesting additional sources of resistant genotypes may be possible. The twice-longer duration of mechanical ventilation in patients with mutant DHPS genotypes may suggest decreased efficacy of TMP-SMZ and warrant collaborative studies to recognize the relevance of DHPS mutations, and further research to increase therapeutic options for PCP.

INTRODUCTION

Pneumonia by the non-culturable opportunistic fungus *Pneumocystis jirovecii* (PCP), is a major cause of morbidity and mortality among human immunodeficiency virus (HIV)-infected, and other immunosuppressed patients (1). *Pneumocystis*, as other fungi, is unable to scavenge folic acid from the host and needs this gene for synthesis of folate. Prophylaxis and treatment of this infection relies mostly on the use of the trimethoprim sulfamethoxazole (TMP-SMZ) combination to inhibit folate synthesis. TMP-SMZ is widely available and has effectively reduced the incidence of PCP. Sulfa drug usage however, has been associated with mutations in the active site of the dihydropteroate synthase (DHPS) enzyme in the *fas* gene of *P. jirovecii* which also codes for dihydroneopterin aldolase and hydroxymethildihdropterin pyrophosphokinase. The other enzyme involved folic acid synthesis is dihydrofolate reductase (DHFR), which is coded in a separate gene. The trimethoprim component of TMP-SMZ selectively inhibits the DHFR enzyme activity, and the sulfamethoxazole component the activity of DHPS in *Pneumocystis* and organisms such as *Plasmodium falciparum* and *Streptococcus pneumoniae*, where DHPS and DHFR mutations have been documented as the mechanism by which sulfa resistance occurs(2-3). Furthermore, most studies have shown an association between chronic use of sulfa drugs administered as prophylaxis and presence of DHPS mutations, suggesting drug selection pressure as the mechanism by which an increase in DHPS mutants in *P. jirovecii* occur(4-6). It is not clear whether short term courses of sulfa drug in outpatient settings are sufficient for selection pressure of DHPS mutants in *P. jirovecii*. Environments like hospitals or outpatient clinics are likely more relevant for selection of mutant *Pneumocystis* as

71 patients on chronic sulfa drug use may accumulate mutant genotypes and be a

72 reservoir for transmission of resistant *P. jirovecii* strains. This is supported by the high

73 prevalence of *P. jirovecii* DHPS mutants in patients with chronic bronchitis that had not

74 received TMP-SMZ in the previous six months, and by the high transmissibility of

75 *Pneumocystis* (7-8). Germane to this work, mutations in the DHPS gene associate with

76 decreased sulfa drug efficacy *in-vitro* and with failure of anti-*Pneumocystis*

77 prophylaxis(9-10). Thus, detection of DHPS mutations in *P. jirovecii* infers that sulfa

78 resistance might be developing and therefore affecting the efficacy of the first-line agent

79 used for prophylaxis and treatment of PCP.

80 The significance of DHPS mutations in clinical response and outcome of PCP treated

81 with sulfa drugs is still controversial and data on correlation between DHPS mutations

82 and mortality from PCP is scarce and retrospective studies are conflicting (11-13). It can

83 be hypothesized however, that the speed of response to sulfa treatment might be

84 compromised in patients infected by DHPS mutant isolates, delaying clearance of

85 *Pneumocystis* and affecting interim outcomes of therapy as for example, the time

86 connected to mechanical ventilation, oxygen requirements, or others.

87 DHPS mutant genotypes are selected by sulfa drug selection pressure and have been

88 used as a marker to infer inter-human transmission of mutant *Pneumocystis* genotypes

89 from sulfa-treated patients to individuals non-treated with sulfonamides(14-16). Their

90 prevalence varies depending on geographical location, suggesting different patterns in

91 the use of sulfa drugs. Recognition of the incidence of DHPS mutations in a particular

92 region and whether mutations affect the response to anti-*P. jirovecii* therapy and

93   prophylaxis is therefore warranted. There is a need of more studies to characterize PCP

94   including the incidence of DHPS mutations of *P. jirovecii* in Chile (17-19).

95   In the present study we sought to report the incidence of DHPS mutations among adult

96   patients without history of prior use of anti-*P. jirovecii* prophylaxis, and presenting with a

97   first episode PCP in Santiago, Chile, describing their clinical presentation, response to

98   therapy, and outcome.

99

PATIENTS AND METHODS

Ethics review: The Ethics Committee for Studies in Humans of the University of Chile School of Medicine approved the study under protocol number 00267. Clinical data was reported coded to the investigators and analyzed unlinked to the identity of the subjects. Informed consent for analyses of *Pneumocystis* isolates was not required.

Patients: Respiratory specimens from adult patients presenting to two hospital clinics in Santiago, Chile between January 2002 and January 2010 who had not received TMP-SMZ or dapsone (a sulfone) as anti-*Pneumocystis* prophylaxis and who had first episode PCP were studied. Their clinical data was collected by means of retrospective hospital chart review, including patient demographics, past medical history, underlying cause of immunodeficiency (HIV infection, or other cause), receipt of anti-*Pneumocystis* prophylaxis with any agent, other drug treatments, clinical presentation, results of laboratory tests, and imaging (chest radiography, and computed tomography), clinical course (including admission to the intensive care unit [ICU] and need for mechanical ventilation), receipt of adjunctive corticosteroids, response to anti-*Pneumocystis* treatment, presence of co-pathogens, and outcome were recorded. Death attributable to PCP was defined as death caused by progressive respiratory failure.

Sample specimens: Fresh frozen respiratory specimens were sent to the University of Chile School of Medicine for diagnosis of PCP. They were processed at arrival, and extracted DNA was kept at -20°C until DHPS genotype analyses. All analyses were performed "blind" to patients' clinical details.

Diagnosis of PCP: *P. jirovecii* organisms were identified using either Grocott Gomori methenamine silver staining or direct immunofluorescence (MERIFLUOR®,

6

123 Pneumocystis, Meridian, Biosciences). In addition to staining, polymerase chain

124 reaction (PCR) with standard primers pAZ102-E and pAZ102-H (20) designed to amplify

125 the gene encoding the mitochondrial large subunit rRNA of *Pneumocystis*, and which

126 amplifies all *Pneumocystis* species, was performed on all specimens prior to

127 genotyping.

128 **Processing of specimens**: Samples were processed inside a biosafety cabinet using

129 sterile precautions to avoid contamination at all times. They were homogenized with a

130 sterile pipette and a 200 μL aliquot was used for DNA extraction. DNA was extracted

131 using the QIAamp DNA Mini kit (Qiagen, Valencia, California) as described (21), and

132 Platinum® *Pfx* DNA polymerase (Invitrogen) was used for DNA amplification. Negative

133 controls were included to monitor for cross-contamination during DNA extraction and

134 purification steps. An internal control using the human β-globin gene was used in each

135 sample to detect inhibition of the PCR reaction, i.e., false negative results. Each sample

136 was run undiluted and as a 1/5 dilution to assess for substrate inhibition. Amplification

137 products were visualized by ethidium bromide, in 2% agarose gels.

138 **Detection of mutations in the DHPS gene**: The DHPS gene binding site was amplified

139 using single Touch-down PCR using primers DHPS-3: 5'

140 GCGCCTACACATATTATGGCCATTTTAAATC 3' and DHPS-4:

141 5'GGAACTTTCAACTTGGCAACCAC3' yielding an amplification product of 370 base

142 pairs as previously described (11, 16, 22). Point mutations in positions 165 (G for T) and

143 171 (T for C) of the DHPS gene were detected using Restriction Fragment Length

144 Polymorphism analysis using restriction enzymes *AccI* (500U 10U/μL, Promega) and

145 *Hae*III (2500U, 10U/ul, Promega). Four DHPS allelic patterns were identified: wild-type

146 (genotype 1) and single mutant genotype 2 (point mutation at position 165), single

147 mutant genotype 3 (point mutation at position 171), and double mutant genotype 4

148 (point mutations at both position 165 and 171), as previously described (23-24).

149 Mutations were classified according to the pattern of band polymorphisms visualized on

150 the 2% agarose gels stained with ethidium bromide.

151 **Statistical analysis**: GraphPad Prism 5 software (San Diego, California) was used for

152 all statistical comparisons. Patient characteristics and clinical outcome were compared

153 between mutant and wild type groups in HIV-positive patients. Statistical comparisons

154 were not done in HIV-negative patients because they were too few. Qualitative

155 characteristics were described using absolute frequency, and percentages and

156 intergroup comparisons among mutant and wild type groups were performed using

157 Fisher's exact test. Non-normally distributed quantitative variables were described using

158 medians and interquartile ranges (IQR) and comparisons were made using Mann

159 Whitney Test. Normally distributed quatitative data were described using means and

160 standard deviations (SD), and comparisons were made using the unpaired t-test. A *p*

161 value of <0.05 was considered significant. Proportions of DHPS mutants overtime were

162 compared using χ^2. All comparisons were two-tailed and confidence level was set at

163 95%.

164

RESULTS

Patients and sample characteristics

A total of 56 respiratory specimens corresponding to 56 adult patients with available medical history and who had not received TMP-SMZ or dapsone as *Pneumocystis* prophylaxis and presented with a first episode of PCP were found in our respiratory specimens collection. All were immunosuppressed: 46(82%) were HIV-infected, and 10(18%) had other causes of immunosuppression: HTLV-1 associated T-cell lymphoma (n=1), meningioma (n=1), rheumatoid arthritis (n=1), dermatomyositis (n=1), psoriatic arthritis (n=1), systemic lupus erythematosus (n=1), Churg-Strauss syndrome (n=1), myasthenia gravis (n=1), chronic obstructive pulmonary disease (n=1), and chronic renal failure (n=1). The median age for HIV-infected patients was 38.5 years (range 22 - 71), and for non-HIV infected was 56.5 years (range 18 - 82). Forty five (98%) of the 46 HIV-infected patients and four (40%) of the 10 non-HIV infected patients were male. PCP was the AIDS-defining condition in 36 (78%) of the HIV-infected patients. Only one of the eight patients with HIV infection diagnosed prior to their PCP episode was receiving antiretroviral therapy. Median CD4+ T-cell count among HIV infected patients was <40 cells/µl (Table 1).

Five specimens were obtained during the period 2002 - 2004, 16 during 2005 - 2007; and 35 in 2008 - 2010. They consisted of bronchoalveolar lavage (BAL) fluid (n=26), tracheal aspirate (n=16), spontaneously expectorated sputum (n=13), and nasopharyngeal aspirate (n=1).

Parameters to characterize the degree of severity of PCP were not standardized, and individual patients' receipt of supplemental oxygen, and their arterial oxygen tension or

188 saturation measurements (obtained while breathing room air) were not recorded

189 systematically. However, all patients were admitted to the hospital, and the majority

190 54(96%) required supplemental oxygen. No significant differences were detected in

191 clinical parameters at admission (fever, cough, dyspnea, chest radiography, computed

192 tomography, platelet count, serum albumin, lactate dehydrogenase and C-reactive

193 protein levels) among HIV-infected and non-infected patients.

194 **Previous use of trimethoprim-sulfamethoxazole**

195 Hospital medical records were reviewed for use of TMP-SMZ. This sulfa-drug

196 combination is available in the Chilean therapeutic armamentarium, and occasionally

197 prescribed for respiratory and urologic conditions. Except for two patients with asthma

198 whose *P. jirovecii* isolates were DHPS genotype 1 (wild-type), only one other patient

199 had chronic lung disease. None of the patients had urologic conditions referred in the

200 medical history at hospital admission. No use of TMP-SMZ as inpatients was

201 documented. Outpatient and primary care medical records were not accessible.

202 **Prevalence of DHPS mutations**

203 Mutations in the DHPS gene were identified in 27 (48%) of 56 patients regardless of

204 their underlying diagnosis (Table 2). Mutations occurred in one (20%) of the five isolates

205 collected between 2002 - 2004, five (31%) of the 16 isolates collected between 2005-

206 2007, and 21 (60%) of the 35 isolates collected between 2008-2010 (p = 0.06).

207 Polymorphisms consisting of DHPS genotypes 2, 3, or 4 were present in 22 (48%) of

208 the 46 HIV-infected and in five (50%) of the 10 non-HIV infected patients with PCP. Co-

209 infections with wild type genotype were more frequent in the HIV-infected group, and

210 were absent in non-HIV infected patients with PCP (Table 2). No predominant pattern of

10

211 mutation polymorphism was detected. We analyzed co-infections by grouping co-

212 infections during 2002 - 2008, the first 6 years (n =13), and those during the last 3 years

213 2009 - 2012 (n = 9) of the study. Eight (61%) of 13 co-infections during the first time

214 period compare to seven (77%) of nine co-infections in the second period (p = 0.42).

215

216 **Clinical parameters and outcome**

217 Anti-PCP treatment was initiated with TMP-SMZ in 53 (95%) of the 56 patients. TMP-

218 SMZ combined with caspofungin or dapsone was used in two patients, and

219 pyrimethamine-sulphadoxine in one patient. Among HIV-infected patients,

220 discontinuation of TMP-SMZ, related to sulfa-related adverse events and not to

221 treatment failure, was needed in four (18%) of 22 patients with mutant genotypes and in

222 none of 24 with wild type isolates (p =0.045). Among HIV-infected patients requiring

223 mechanical ventilation, the duration of mechanical ventilation among those harboring

224 DHPS mutations was significantly longer, 11 days (IQR 8-56), than in those with wild

225 type isolates, 6 days (IQR 2-8); p =0.017. There was a trend towards longer

226 hospitalization in HIV-infected patients with mutations, 20 days (IQR 10-42), compared

227 with 11 days (IQR 6 - 19) in those HIV-infected patients with wild type DHPS isolates; p

228 =0.073, (Table 3). All 46 (100%) HIV infected patients survived whereas only five (50%)

229 of the ten non-HIV infected patients survived. Death was directly attributable to PCP in

230 three of them.

231

DISCUSSION

Nearly half of the patients in this study, regardless of their HIV-status, had *Pneumocystis* DHPS mutant genotypes despite no prior receipt of sulfa drugs (sulfamethoxazole or dapsone) as prophylaxis of PCP, therefore suggesting that human-to human transmission was the most likely source of acquisition of mutant isolates. Our results also, showed that mechanically ventilated patients harboring *Pneumocystis* DHPS mutant genotypes had a twice-longer duration of mechanical ventilation suggesting that these mutations might have impacted the response to anti-*Pneumocystis* treatment with sulfamethoxazole-containing sulfa combinations. In addition, adverse drug reactions to sulfa treatment of PCP, necessitating treatment change, were observed more frequently among those with mutant DHPS genotypes.

A high prevalence of DHPS mutations has also been reported in studies from other countries. For example, a recent study of AIDS related PCP in Kampala, Uganda documented that all 13 isolates of *P. jirovecii* harbored either single or double mutant DHPS genotypes, despite only two persons were receiving TMP-SMZ for PCP prophylaxis (25). This finding was ascribed to population-level selection pressure due to sulfa drug use for treatment of malaria caused by *Plasmodium falciparum* among the general population. *Pneumocystis* is not zoonotic, therefore the absence of sulfa prophylaxis for PCP in our patients suggests that they likely acquired DHPS mutations through inter-human transmission (14, 16). The mechanism and role for selection pressure at a population level is, however, not clear because sulfa drugs are the third or fourth choice of antibiotic for treatment of respiratory and urinary tract infections, in both primary and secondary care settings in Chile. They account for approximately 5% of

255 antibiotic prescriptions after synthetic penicillins, macrolides, cephalosporins, and

256 quinolones (26). Furthermore, the use of the TMP-SMZ combination has decreased

257 since 2000 from approximately 7% to 2% of total antibiotic consumption in Chile(26).

258 This consumption is similar to that reported in other Latin American countries (27)

259 where low usage of sulfa drugs parallels a low prevalence of DHPS mutations. Of note,

260 an earlier UK study showed a 36% frequency of DHPS mutations in isolates of *P.*

261 *jirovecii* in London, when there was population-level "selection pressure" from

262 widespread use of sulfa drugs both as prophylaxis against PCP, and among the general

263 population for treatment of respiratory and urinary tract infections (28).

264 Contemporaneously, a low prevalence (7.7%) of DHPS mutants were identified in

265 Zimbabwe, where sulfa drugs were rarely used. When in UK selection pressure was

266 removed a predominance (80%) of wild type genotypes was observed (28). Restriction

267 measures for use of antibiotics have been in place in Chile since 1998, however, there

268 is no indication that the frequency of detection of DHPS mutants has decreased. By

269 contrast, the possible increase in proportion of DHPS mutations overtime suggested in

270 this study becomes paradoxical when the parallel decrease in use of sulfa drugs by

271 national policies is considered. Therefore, the high frequency described in this study

272 seems too high to be explained solely by sulfa selection pressure within human

273 population.

274 Restriction of antibiotics in humans in Chile has not been accompanied by similar

275 policies in veterinary settings, and currently there is a far greater use of antibiotics

276 including sulfa drugs in the setting of pig, poultry, and fish farming than in humans in

277 Chile (29). Veterinary use of sulfa drugs may select DHPS mutants on a much larger

278   scale than in human use, and acquisition of bacterial genes generally responsible for

279   metabolic or virulence traits via horizontal gene transfer (HGT) has been documented in

280   fungal species(30-31). However, this type of acquisition of resistance has never been

281   described in *Pneumocystis*. Mutations in the DHPS gene of *P. jirovecii* have been

282   shown to arise independently among multiple *Pneumocystis* strains (32). Therefore,

283   transmission of a single resistant clone of *P. jirovecii* appears to be very unlikely.

284   The finding that adverse drug reactions to sulfa treatment were more frequent among

285   patients with mutant DHPS genotypes, whom in turn required more frequent changes in

286   anti-PCP treatment, does not have an immediately apparent explanation. Short courses

287   of sulfa drugs, for example as treatment for bronchitis or sinusitis could not be identified

288   from hospital records, and therefore, the possibility of prior exposure in a primary care

289   setting resulting in sensitization cannot be excluded. However, the two patients with

290   asthma, and one additional patient with chronic obstructive lung disease, in whom the

291   possibility of them having received undocumented sulfa-containing antibiotics in a

292   primary care was more likely, had wild type DHPS genotype 1.

293   No difference in the proportion of patients with wild type and mutant genotypes requiring

294   mechanical ventilation was detected in the present study. This observation contrasts

295   with a study by Crothers *et al*, where 14.3% of patients with PCP and mutant *P. jirovecii*

296   genotypes required mechanical ventilation, compared with 2.5% of those with wild type

297   *P. jirovecii* genotypes (p = 0.056) (33). However, mutant DHPS genotypes were

298   associated with a longer duration of mechanical ventilation in the present series and this

299   data suggests further research is needed (by way of a multi-center prospective study),

300   as data from the present study infer a reduced ability of sulfa-based regimens to clear

301 DHPS-mutant *P. jirovecii* from the lungs. Interestingly, the study by Crothers *et al* also

302 showed patients with mutant DHPS genotypes had a non-significant trend to longer

303 overall hospital stay, but data on duration of mechanical ventilation is not provided (33).

304 The inability to culture *Pneumocystis* in-vitro hinders antimicrobial sensitivity testing and

305 makes proof of a mechanistic connection difficult. Moukhlis et. al. performed functional

306 studies using a DHPS-deficient model of *Sacharomyces cerevisiae* experimentally

307 complemented with *Pneumocystis* mutant and wild type DHPS, and documented a

308 decreased susceptibility to sulfamethoxazole in those *S. cerevisiae* isolates that were

309 complemented with double mutant genotypes(34). Their results, and related earlier work

310 provide an in-vitro correlation with our findings(9-10, 34).

311 In the present study, we observed no association between mutant DHPS-genotype and

312 mortality. However this was an observational study and so was not powered to show

313 differences. Of interest, a prospective single cohort study from San Francisco General

314 Hospital consisting of 301 patients with laboratory-confirmed PCP, over a period of >10

315 years, demonstrated that although receipt of recent sulfa prophylaxis was associated

316 with mutant genotypes of *P. jirovecii*, detection of mutant DHPS genotypes was not

317 associated with mortality. This observation conflicts with findings from other authors (13,

318 35).

319 The strengths of the present study are that it is the first from Chile to describe DHPS

320 genotyping among *P. jirovecii* isolates from patients with PCP. Our results document a

321 high frequency of DHPS mutants (48%) in anti-*P. jirovecii* sulfa-prophylaxis-naive

322 patients with a first episode of PCP. This frequency is excessively high when compared

323 to the low prevalence of DHPS mutations in countries in the same continent, for

324   example Brazil (0%) and Colombia (6.6%) that report similar patterns of sulfa-drug

325   usage in humans(27, 36-37). Therefore, although our findings add further evidence to

326   support the hypothesis of inter-human transmission as a mechanism of acquisition of

327   mutant *P. jirovecii* types, additional mechanisms of acquisition may be possible.

328   Significantly, our results also suggest that DHPS mutation can diminish the efficacy of

329   sulfa-drug treatment of PCP by documenting a significantly longer duration of

330   mechanical ventilation in patients harboring mutant DHPS genotypes, and highlight the

331   need to increase the anti-*Pneumocystis* armamentarium. TMP-SMZ is the only anti-

332   *Pneumocystis* drug available in most of the world. Weaknesses of the present study are

333   the lack of prospective, systematic acquisition of clinical data, and the relatively small

334   sample size.

335   In conclusion, we describe a high frequency of DHPS mutations among adult patients

336   with first-episode of PCP who had not received sulfa drugs as PCP prophylaxis in

337   Santiago, Chile. The likely explanation being inter-human transmission and selection

338   pressure from sulfa drugs prescribed for other conditions. Additionally, the potential role

339   of veterinary use of sulfa drugs in selection of DHPS mutations for transmission to

340   humans deserves further study. Patients with PCP and mutant DHPS genotypes were

341   more likely to experience treatment-limiting adverse reactions to sulfa-drug treatment,

342   and to require a longer duration of mechanical ventilation, thus inferring a decrease on

343   treatment efficacy. The final treatment outcome was not affected, as patients harboring

344   mutant DHPS genotypes were no more likely to die. The prevalence of DHPS mutations

345   in clinical isolates of *Pneumocystis* should be monitored, and their significance in

346   delaying response to therapy needs to be confirmed in larger collaborative studies.

347

356 **REFERENCES**

357 1. **Miller RF, Huang L, Walzer PD.** 2013. *Pneumocystis* pneumonia associated

358   with human immunodeficiency virus. Clin Chest Med. **34**:229-41.

359 2. **Brooks DR, Wang P, Read M, Watkins WM, Sims PF, Hyde JE.** 1994.

360   Sequence variation of the hydroxymethyldihydropterin pyrophosphokinase:

361   dihydropteroate synthase gene in lines of the human malaria parasite,

362   *Plasmodium falciparum*, with differing resistance to sulfadoxine. Eur J Biochem.

363   **224**:397-405.

364 3. **Haasum Y, Strom K, Wehelie R, Luna V, Roberts MC, Maskell JP, Hall LM,**

365   **Swedberg G.** 2001. Amino acid repetitions in the dihydropteroate synthase of

366   *Streptococcus pneumoniae* lead to sulfonamide resistance with limited effects on

367   substrate K(m). Antimicrob Agents Chemother. **45**:805-9.

368 4. **Stein CR, Poole C, Kazanjian P, Meshnick SR.** 2004. Sulfa use,

369   dihydropteroate synthase mutations, and *Pneumocystis jirovecii* pneumonia.

370   Emerg Infect Dis. **10**:1760-5.

17

371   5.   **Matos O, Esteves F.** 2010. Epidemiology and clinical relevance of *Pneumocystis*

372   *jirovecii* Frenkel, 1976 dihydropteroate synthase gene mutations. Parasite.

373   **17**:219-32.

374   6.   **Kazanjian P, Armstrong W, Hossler PA, Burman W, Richardson J, Lee CH,**

375   **Crane L, Katz J, Meshnick SR.** 2000. *Pneumocystis carinii* mutations are

376   associated with duration of sulfa or sulfone prophylaxis exposure in AIDS

377   patients. J Infect Dis. **182**:551-7.

378   7.   **Calderon E, de la Horra C, Medrano FJ, Lopez-Suarez A, Montes-Cano MA,**

379   **Respaldiza N, Elvira-González J, Martín-Juan J, Bascuñana A, Varela JM.**

380   2004*. Pneumocystis jiroveci* isolates with dihydropteroate synthase mutations in

381   patients with chronic bronchitis. Eur J Clin Microbiol Infect Dis. **23**:545-9.

382   8.   **Rabodonirina M, Vanhems P, Couray-Targe S, Gillibert RP, Ganne C, Nizard**

383   **N, Colin C, Fabry J, Touraine JL, van Melle G, Nahimana A, Francioli P,**

384   **Hauser PM.** 2004. Molecular evidence of interhuman transmission of

385   *Pneumocystis* pneumonia among renal transplant recipients hospitalized with

386   HIV-infected patients. Emerg Infect Dis. **10**:1766-73.

387   9.   **Iliades P, Meshnick SR, Macreadie IG.** 2004. Dihydropteroate synthase

388   mutations in *Pneumocystis jiroveci* can affect sulfamethoxazole resistance in a

389   Saccharomyces cerevisiae model. Antimicrob Agents Chemother. **48**:2617-23.

390   10. **Meneau I, Sanglard D, Bille J, Hauser PM.** 2004. *Pneumocystis jiroveci*

391   dihydropteroate synthase polymorphisms confer resistance to sulfadoxine and

392   sulfanilamide in *Saccharomyces cerevisiae*. Antimicrob Agents Chemother.

393   **48**:2610-6.

18

394   11.   **Helweg-Larsen J, Benfield TL, Eugen-Olsen J, Lundgren JD, Lundgren B.**

395   1999. Effects of mutations in *Pneumocystis carinii* dihydropteroate synthase

396   gene on outcome of AIDS-associated *P. carinii* pneumonia. Lancet. **354**:1347-51.

397   12.   **Navin TR, Beard CB, Huang L, del Rio C, Lee S, Pieniazek NJ, Carter JL, Le**

398   **T, Hightower A, Rimland D.** 2001. Effect of mutations in *Pneumocystis carinii*

399   dihydropteroate synthase gene on outcome of *P. carinii* pneumonia in patients

400   with HIV-1: a prospective study. Lancet. **358**:545-9.

401   13.   **Yoon C, Subramanian A, Chi A, Crothers K, Meshnick SR, Taylor SM, Beard**

402   **CB, Jarlsberg LG, Lawrence GG, Avery M, Swartzman A, Fong S, Roth B,**

403   **Huang L.** 2013. Dihydropteroate synthase mutations in *Pneumocystis*

404   pneumonia: impact of applying different definitions of prophylaxis, mortality

405   endpoints and mutant in a single cohort. Med Mycol. **51**:568-75.

406   14.   **Hauser PM, Nahimana A, Taffe P, Weber R, Francioli P, Bille J,**

407   **Rabodonirina M.** 2010. Interhuman transmission as a potential key parameter

408   for geographical variation in the prevalence of *Pneumocystis jirovecii*

409   dihydropteroate synthase mutations. Clin Infect Dis. **51**:e28-33. doi:

410   10.1086/655145.

411   15.   **Huang L, Beard CB, Creasman J, Levy D, Duchin JS, Lee S, Pieniazek N,**

412   **Carter JL, del Rio C, Rimland D, Navin TR.** 2000. Sulfa or sulfone prophylaxis

413   and geographic region predict mutations in the *Pneumocystis carinii*

414   dihydropteroate synthase gene. J Infect Dis. **182**:1192-8.

415   16.   **Totet A, Latouche S, Lacube P, Pautard JC, Jounieaux V, Raccurt C, Roux**

416   **P, Nevez G.** 2004. *Pneumocystis jirovecii* dihydropteroate synthase genotypes in

417   immunocompetent infants and immunosuppressed adults, Amiens, France.

418   Emerg Infect Dis. **10**:667-73.

419   17.   **Chernilo S, Trujillo S, Kahn M, Paredes M, Echevarria G, Sepulveda C.** 2005.

420   [Lung diseases among HIV infected patients admitted to the "Instituto Nacional

421   del Torax" in Santiago, Chile]. Rev Med Chil. **133**:517-24.

422   18.   **Bahamondes ML, Villar ZM, Orellana CC, Gonzalez RJ, Montenegro UC.**

423   2006. [*Pneumocystis jiroveci* pneumonia characteristics in adults with AIDS with

424   or without antiretroviral therapy]. Rev Chilena Infectol. **23**(3):215-9.

425   19.   **Perez C, Garcia P, Calvo M, Labarca J, Bustos M, Beroiza T, Gaete P,**

426   **Moreno R, Acuña G, Vial P.** 2011. [Etiology of pneumonia in chilean HIV-

427   infected adult patients]. Rev Chilena Infectol. **28**(4):343-8.

428   20.   **Wakefield AE, Miller RF, Guiver LA, Hopkin JM.** 1993. Oropharyngeal

429   samples for detection of *Pneumocystis carinii* by DNA amplification. Q J Med.

430   86:401-6.

431   21.   **Ponce CA, Gallo M, Bustamante R, Vargas SL.** 2010. *Pneumocystis*

432   colonization is highly prevalent in the autopsied lungs of the general population.

433   Clin Infect Dis. **50**:347-53.

434   22.   **Helweg-Larsen J, Eugen-Olsen J, Lundgren B.** 2000. Rapid detection of

435   dihydropteroate polymorphism in AIDS-related *Pneumocystis carinii* pneumonia

436   by restriction fragment length polymorphism. Scand J Infect Dis. **32**:481-3.

437   23.   **Beard CB, Carter JL, Keely SP, Huang L, Pieniazek NJ, Moura IN, Roberts**

438   **JM, Hightower AW, Bens MS, Freeman AR, Lee S, Stringer JR, Duchin JS,**

439   **del Rio C, Rimland D, Baughman RP, Levy DA, Dietz VJ, Simon P, Navin**

440   **TR.** 2000. Genetic variation in *Pneumocystis carinii* isolates from different

441   geographic regions: implications for transmission. Emerg Infect Dis. **6**:265-72.

442   24.   **Huang L, Crothers K, Atzori C, Benfield T, Miller R, Rabodonirina M,**

443   **Helweg-Larsen J.** 2004. Dihydropteroate synthase gene mutations in

444   *Pneumocystis* and sulfa resistance. Emerg Infect Dis. **10**:1721-8.

445   25.   **Taylor SM, Meshnick SR, Worodria W, Andama A, Cattamanchi A, Davis JL,**

446   **Yoo SD, Byanyima P, Kaswabuli S, Goodman CD, Huang L.** 2012. Low

447   prevalence of *Pneumocystis* pneumonia (PCP) but high prevalence of

448   *Pneumocystis* dihydropteroate synthase (dhps) gene mutations in HIV-infected

449   persons in Uganda. PLoS One.**7**:e49991. doi: 10.1371/journal.pone.0049991.

450   26.   **Bavestrello FL, Cabello MA.** 2011. [Community antibiotic consumption in Chile,

451   2000-2008]. Rev Chilena Infectol. **28**:107-12.

452   27.   **Wirtz VJ, Dreser A, Gonzales R.** 2010. Trends in antibiotic utilization in eight

453   Latin American countries, 1997-2007. Rev Panam Salud Publica. **27**(3):219-25.

454   28.   **Miller RF, Lindley AR, Ambrose HE, Malin AS, Wakefield AE.** 2003.

455   Genotypes of *Pneumocystis jiroveci* isolates obtained in Harare, Zimbabwe, and

456   London, United Kingdom. Antimicrob Agents Chemother. **47**:3979-81.

457   29.   **Cabello FC.** 2004. [Antibiotics and aquaculture in Chile: implications for human

458   and animal health]. Rev Med Chil. **132**:1001-6.

459   30.   **Bruto M, Prigent-Combaret C, Luis P, Moenne-Loccoz Y, Muller D.** 2014.

460   Frequent, independent transfers of a catabolic gene from bacteria to contrasted

461   filamentous eukaryotes. Proc Biol Sci. 281:20140848.

31. **Jaramillo VD, Sukno SA, Thon MR.** 2015. Identification of horizontally transferred genes in the genus Colletotrichum reveals a steady tempo of bacterial to fungal gene transfer. BMC Genomics.**16**:2.

32. **Ma L, Kovacs JA.** 2001. Genetic analysis of multiple loci suggests that mutations in the *Pneumocystis carinii* f. sp. *hominis* dihydropteroate synthase gene arose independently in multiple strains. Antimicrob Agents Chemother. **45**:3213-5.

33. **Crothers K, Beard CB, Turner J, Groner G, Fox M, Morris A, Eiser S, Huang L.** 2005. Severity and outcome of HIV-associated *Pneumocysti*s pneumonia containing *Pneumocystis jirovecii* dihydropteroate synthase gene mutations. AIDS. **19**:801-5.

34. **Moukhlis R, Boyer J, Lacube P, Bolognini J, Roux P, Hennequin C.** 2010. Linking *Pneumocystis jiroveci* sulfamethoxazole resistance to the alleles of the DHPS gene using functional complementation in *Saccharomyces cerevisiae*. Clin Microbiol Infect. **16**:501-7.

35. **Rabodonirina M, Vaillant L, Taffe P, Nahimana A, Gillibert RP, Vanhems P, Hauser PM.** 2013. *Pneumocystis jirovecii* genotype associated with increased death rate of HIV-infected patients with pneumonia. Emerg Infect Dis. **19**:21-8; quiz 186.

36. **Muñoz C, Zuluaga A, Restrepo A, Tobon A, Cano LE, Gonzalez A.** 2012. Molecular diagnosis and detection of *Pneumocystis jirovecii* DHPS and DHFR genotypes in respiratory specimens from Colombian patients. Diagn Microbiol Infect Dis. **72**:204-13.

485   37.   **Wissmann G, Alvarez-Martinez MJ, Meshnick SR, Dihel AR, Prolla JC.** 2006.

486   Absence of dihydropteroate synthase mutations in *Pneumocystis jirovecii* from

487   Brazilian AIDS patients. J Eukaryot Microbiol. **53**:305-7.

488

Table 1. Demographic and clinical characteristics of 56 patients with first episode of *Pneumocystis* pneumonia (PCP).

	HIV-infected n=46			Non-HIV-infected n=10		
	n/n total (%)			n/n total (%)		
	Wild-type DHPS genotypes 24/46 (52)	Mutant DHPS genotypes 22/46 (48)	p value	Wild-type DHPS genotypes 5/10 (50)	Mutant DHPS genotypes 5/10 (50)	p value
Mean age [years] (SD)	40 (12.2)	40.1 (9.3)	0.977	48.5 (24.7)	55.6 (23.3)	0.671
Male gender	24/24 (100)	21/22 (95)	0.468	2/5 (40)	2/5 (40)	1
New HIV diagnosis	20/24 (83)	18/22 (82)	0.699	NA	NA	
Receipt of PCP prophylaxis	0/24 (0)	0/22 (0)	1	0/5	0/5	NA
Not receiving antiretroviral Therapy	24/24 (100)	21/22 (96)	0.478	NA	NA	
Fever	15/24 (63)	12/22 (55)	0.765	3/5 (60)	2/3 (67)	1
Mean temperature [ºC] (SD)	38.1 (0.85)	38.2 (0.98)	0.786			
Cough	18/24 (75)	19/22 (86)	0.464	3/5 (60)	3/4 (75)	1
Dyspnoea	21/24 (88)	19/21 (91)	1	4/5 (80)	3/4 (75)	1
Chest radiograph	22/24	21/22		5/5	5/5	
Abnormal	21/22 (96)	21/21 (100)	1	5/5 (100)	5/5 (100)	
Bilateral interstitial infiltrates	18/22 (82)	16/21 (76)		1/5 (20)	1/5 (20)	NA
Another pattern	3/22 (14)[a]	5/21 (24)[b]		2/5 (40)[c]	4/5 (80)[d]	
Not determined				2/5 (40)		
Thoracic CT scan	15/25	15/22		3/5	3/5	
Abnormal	15/15 (100)	15/15 (100)	1	3/3 (100)	3/3 (100)	NA
Ground glass infiltrates	12/15 (80)	14/15 (93)		1/3 (33)	1/3 (33)	
Another pattern	3/15 (20)[e]	1/15 (7)[f]		2/3 (67)[g]	2/3 (67)[h]	
Median CD4 cell count [cell/µl] (IQR) n	27 (11–57.5) (20/24)	37 (18-64) (19/22)	0.465	ND	ND	
Median serum albumin [g/l] (IQR) n	2.95 (2.37-3.40) (18/24)	2.90 (2.40-3.30) (13/22)	0.794	3.15 (2.35-3.43) (4/5)	ND	
Median serum LDH [IU/l] (IQR) n	854.5 (568.8-1290) (22/24)	863 (665-1089) n=19	0.927	875 (558,8-3210) (4/5)	1099 (840-1405) (5/5)	0.730
Mean haematocrit [%] (SD)] n	39.9 (6.39) (23/24)	36.8 (4.72) (21/22)	0.076	30.1 (8.84) (5/5)	31.5 (9.54) (4/5)	0.782
Median C-reactive Protein [mg/l] (IQR) n	19.6 (4.8-99.9) (18/24)	8.5 (1.1-86.2) (19/22)	0.475	73 (3.40-217.0) (5/5)	5,0 (1.90-50.25) (4/5)	0.191
Other lung pathology	2/24(8)[i]	1/22 (5)[j]	1	3/5(60)[k]	1/5 (20)[l]	0.524

Key: DHPS = dyhydropteroate synthase; LDH = Lactate dehydrogenase. NA = not applicable; ND = not determined
[a] Bilateral interstitial infiltrates plus bilateral lobar consolidation =1; bilateral interstitial infiltrates plus bilateral alveolar consolidation =1; diffuse basal infiltrates =1
[b] Bilateral interstitial infiltrates plus air bronchogram and consolidation =1; diffuse alveolar- interstitial pattern =2; diffuse alveolar- interstitial pattern with apical cavitation =1; right lobar consolidation =1
[c] Alveolar haemorrhage =1; left pulmonary nodules =1
[d] Bilateral peri-bronchovascular thickening =1; Right basal consolidation =1; bilateral alveolar consolidation plus air bronchogram =1; right middle lobe consolidation =1
[e] Bilateral ground- glass infiltrates and bilateral consolidation =1; non- characteristic interstitial infiltrates =1; diffuse infiltrates =1
[f] Bilateral ground-glass infiltrates and four cavitation- suggestive nodules (1)
[g] Alveolar Hemorrhage (1); pneumomediastinum (1)
[h] Bilateral ground glass pattern and unilateral consolidation (1); unilateral consolidation (1)
[i] Bronchial asthma (2)
[j] Mycobacterium tuberculosis
[k] Dermatomyositis (1); COPD plus Cystic fibrosis (1); Bronchial asthma (1)
[l] Myasthenia gravis

490 **Table 2:** *Pneumocystis jirovecii* Dihydropteroate Synthase genotypes in respiratory specimens from 56 adult patients with newly diagnosed
491 *Pneumocystis* pneumonia.

DHPS genotype*	HIV-infected (n = 46) [n positive/n total (%)]	Non-HIV-infected (n = 10) [n positive/n total (%)]
Wild-type		
G1	24/46 (52)	5/10 (50)
Mutant	22/46 (48)	5/10 (50)
Single mutant	2/22 (9)	2/5 (40)
G2 (position 165)	1/22 (5)	2/5 (40)
G3 (position 171)	1/22 (5)	0
Double mutant		
G4 (position 165 + 171)	5/22 (23)	3/5 (60)
Co-infection with wild-type	15/22 (68)	0
G5 (1 + 2)	9/22 (41)	0
G6 (1 + 3)	0	0
G7 (1 + 4)	6/22 (27)	0
Mixed single + double mutant		
G8 (2 + 4)	0	0

492 *Genotypes (G1 - G8) as described in ref. 24.

493

25

Table 3: Dihydropteroate Synthase genotypes and clinical outcomes among 56 patients with first episode of *Pneumocystis* pneumonia.

Treatment and outcome [n (%)]	HIV-infected n=46			Non-HIV-infected n=10		
	Wild-type DHPS genotypes (n=24)	Mutant DHPS genotypes (n=22)	*p* value	Wild-type DHPS genotypes (n=5)	Mutant DHPS genotypes (n=5)	*p* value
Initial treatment with trimethoprim-sulfamethoxazole	23 (96)*i*	20 (91)*ii*	0.600	5 (100)	5 (100)	ND
Adverse effect	2 (8)*iii*	4 (18) *iv*	0.452	0 (0)	0 (0)	ND
Need for treatment change	0 (0)	4 (18)*v*	0.045	1 (17)*vi*	0 (0)	ND
Sulfa allergies	1 (4)	4 (18)	0.178	0 (0)	0 (0)	ND
Adjunctive corticosteroids	21 (84)	15 (70)	0.159	4 (83)	4 (80)	ND
Duration of hospitalization, days						
Median (IQR)	11 (6-19)	20 (10-42)	0.073	30 (14-40)	25 (8-37)	ND
n with available data / n	19/24	16/22		5	5	
Required supplemental Oxygen	24 (100)	20 (91)		5 (100)	5 (100)	
Median (days [IQR])	11.5 (6.7-19.5)	14.5 (7.0-20)	0.203	34 (14-40)	17 (10-24)	ND
n with available data / n	22/24	20/22		5	5	
Need for early ICU admission	8 (36)	7 (35)	0.763	1 (20)	3/4 (75)	ND
n with available data / n	22/24	20/22		5	4	
Mechanical ventilation	9 (38)	7 (32)		4 (80)	5(100)	
Median (days [IQR])	6 (2.25-7.75)	11 (8-56)	0.017	25 (10-30)	14 (8-35)	ND
Pulmonary co-infection	7 (29)	5 (23)		3 (60)	2 (40)	
Bacterial	1*i*	3*l*		3*n*	2*o*	
Viral	4*j*	0		0	0	
Bacterial + Viral	2*k*	2*m*		0	0	
Treatment outcome						
Survived	24 (100)	22 (100)		2 (40)	3 (60)	
Died	0 (0)	0 (0)		3 (60)	2 (40)	
Death due to PCP	0 (0)	0 (0)		2 (40)	1 (20)	

(i) pyrimethamine + sulfadoxine (1)
(ii) caspofungin + trimethoprim-sulfamethoxazole (TMS) (1), dapsone +TMS (1)
(iii) rash (2)
(iv) rash (2), acute kidney injury (1), rash, interstitial nephritis, hepatitis (1)
(v) change TMS to: dapsone (2), dapsone + clindamycin (1)
(vi)treatment failure change to iv pentamidine

[i] *Acinetobacter baumannii* =1,
[j] Cytomegalovirus (CMV)
[k] *Acinetobacter baumannii* + CMV =1, *Enterobacter cloacae* + CMV =1
[l] *Streptococcus pneumoniae* =1, *Klebsiella pneumoniae* =2, *Stenotrophomonas maltophilia* =1
[m] β-haemolytic Streptococcus + CMV =1, atypical Mycobacteria + CMV =1
[n] *Pseudomonas aeruginosa* =1, *Klebsiella pneumoniae* =1, *Proteus mirallis* =1
[o] *Enterobacter cloacae* =2

494