Genetic characterization of the dihydrofolate reductase gene of *Pneumocystis jirovecii* isolates from Portugal

Marina C. Costa¹, Francisco Esteves¹, Francisco Antunes² and Olga Matos¹*

¹Unidade de Protozoários Oportunistas/VIH e outras Protozooses, Unidade de Parasitologia e Microbiologia Médicas (UPMM), Instituto de Higiene e Medicina Tropical, Rua da Junqueira 96, 1349-008 Lisboa, Portugal; ²Clínica das Doenças Infecciosas, Hospital de Santa Maria, Avenida Prof. Egas Moniz, 1649-028 Lisboa, Portugal

Received 24 April 2006; returned 24 July 2006; revised and accepted 14 September 2006

**Objectives:** The aim of the present study was to evaluate the genetic variation of *Pneumocystis jirovecii* dihydrofolate reductase (DHFR) gene in an immunocompromised Portuguese population and to investigate the possible association between DHFR genotypes and *P. jirovecii* pneumonia (PcP) prophylaxis with co-trimoxazole.

**Methods:** One hundred and thirty-eight *P. jirovecii* isolates were submitted to DHFR genetic characterization by PCR and sequencing.

**Results:** In the studied population, 72.7% of the patients presented sequences identical to the wild-type sequence of the *P. jirovecii* DHFR gene and 27.3% presented point substitutions. A total of nine substitution sites were identified; four synonymous substitutions at nucleotide positions 201, 272, 312 and 381 were detected in 31 patients. Five non-synonymous substitutions were observed, leading to the DHFR mutations Leu-13 → Ser, Asn-23 → Ser, Ser-31 → Phe, Met-52 → Leu and Ala-67 → Val. With the exception of the polymorphism at position 312 and the mutation at codon 52, all polymorphisms were reported in this study for the first time.

**Conclusions:** Our results suggest that DHFR gene polymorphisms are frequent in the Portuguese immunocompromised population but do not seem to be associated with PcP prophylaxis failure ($p = 0.748$ and $p = 0.730$).

Keywords: polymorphisms, mutations, co-trimoxazole, drug resistance

**Introduction**

Pneumonia caused by *Pneumocystis jirovecii* (PcP) is an important opportunistic infection in AIDS and other immunocompromised patients, although widespread PcP chemoprophylaxis and highly active antiretroviral therapy (HAART) have reduced the incidence of this infection.

Co-trimoxazole, a combination of sulfamethoxazole and trimethoprim, is the key agent for treatment and prophylaxis of PcP. Sulfamethoxazole inhibits the enzyme dihydropteroate synthase (DHPS) while trimethoprim targets the enzyme dihydrofolate reductase (DHFR), both components of the folic acid pathway.

Over the last few years, emergence of *P. jirovecii* sulfa resistance related with mutations at codons 55 and 57 of the DHPS gene has been suggested and demonstrated.¹,² Alteration of DHFR enzyme is also a common resistance mechanism in clinically important microbial pathogens, such as *Plasmodium falciparum*,³ *Staphylococcus aureus*⁴ and *Streptococcus pneumoniae*.⁵ However, information about the genetic variation of the *P. jirovecii* DHFR gene is scarce. Until now, few studies have addressed the genetic heterogeneity of the *P. jirovecii* DHFR gene,⁶,⁷ but only in one study have the authors established an association between DHFR non-synonymous polymorphisms and failure of PcP prophylaxis with DHFR inhibitors, namely with pyrimethamine.⁷

In this study, we intended to characterize the sequence variation of the DHFR gene from *P. jirovecii* isolated from Portuguese immunocompromised patients and to investigate the association of DHFR polymorphisms and failure of PcP prophylaxis.

*Corresponding author. Tel: +351-21-3652638; Fax: +351-21-3632105; E-mail: omatos@ihmt.unl.pt

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Materials and methods

Specimens and patients

One hundred and thirty-eight pulmonary specimens (74 induced sputa, 62 bronchoalveolar lavage fluids and two oral washes) obtained between May 1995 and October 2004, from 128 immuno-compromised patients with respiratory symptoms, were studied. Three patients had three pulmonary specimens collected during the PcP episode and four patients had two specimens collected. One hundred and nine patients were HIV-infected and eleven were HIV-negative (four with leukaemia, two with bone marrow aplasia, one transplant recipient and four unknown). In the remaining eight patients the cause of immunosuppression was not known. Data on anti-*P. jirovecii* prophylaxis were available for 114 patients: 89 patients were not receiving anti-*P. jirovecii* prophylaxis, and to our knowledge were not exposed to trimethoprim; and 25 were receiving anti-*P. jirovecii* prophylaxis (21 with co-trimoxazole and four with pentamidine).

The identification of *P. jirovecii* organisms was performed by indirect immunofluorescence with monoclonal antibodies (MonoFluo Kit *Pneumocystis carinii*, Sanofi Diagnostics Pasteur) and by the computer program CLUSTAL W (version 1.82), available at the European Bioinformatics Institute website (www.ebi.ac.uk), and by the clone sequences available at the GenBank database.

Statistical analysis

Statistical analysis was performed using SPSS version 13.0, for WINDOWS. Associations between failure of PcP prophylaxis and DHFR polymorphisms were investigated using $\chi^2$ analysis. A $P$ value < 0.05 was considered statistically significant. PcP prophylaxis was defined as adherence to co-trimoxazole chemoprophylaxis for a minimum of 2 months preceding PcP diagnosis. A failure of prophylaxis was defined as the development of PcP in patients who received anti-*Pneumocystis* prophylaxis.

Nucleotide sequence accession numbers

The accession numbers of the new DHFR sequences obtained in this study are DQ417355 through DQ417360.

Results

In the present study, a 798 bp fragment of the *P. jirovecii* DHFR gene was sequenced and compared with the wild-type sequence present in GenBank (accession number AF90368).

Of the 128 PcP episodes studied, 93 (72.7%) presented sequences identical to the wild-type sequence of the *P. jirovecii* DHFR gene and in 35 (27.3%) point substitutions were identified (Table 1). A total of nine substitution sites were identified; four synonymous (silent) substitutions at nucleotide positions 201 (T to A), 272 (T to C), 312 (T to C) and 381 (C to T) were identified in 31 patients. Five non-synonymous substitutions at nucleotide positions 38 (T to C), 68 (A to G), 92 (C to T), 154 (A to T) and 200 (C to T), which lead to amino acid alterations at codons 13 (leucine to serine), 23 (asparagine to serine), 31 (serine to phenylalanine), 52 (methionine to leucine) and 67 (alanine to valine), respectively, were identified in five different patients. One patient presented simultaneously one synonymous polymorphism, at position 272, and one non-synonymous polymorphism, at codon 31.

Ten randomly selected specimens were cloned and four to five clones of each specimen were sequenced. In this study, all clones, from each specimen presented identical sequences. We did not detect a mixture of wild-type and polymorphic DHFR sequences.

Five specimens with cloned sequences presented synonymous polymorphisms at position 312, two cloned specimens had synonymous polymorphisms at position 381 and the remaining three cloned specimens presented wild-type sequences.

Table 1. Genetic variation of dihydrofolate reductase gene in 128 *Pneumocystis jirovecii* pneumonia episodes

<table>
<thead>
<tr>
<th><em>P. jirovecii</em> DHFR sequences</th>
<th>No. of patients (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Wild type</strong></td>
<td></td>
</tr>
<tr>
<td>Polymorphisms</td>
<td></td>
</tr>
<tr>
<td>synonymous</td>
<td></td>
</tr>
<tr>
<td>201 (T to A)</td>
<td>1</td>
</tr>
<tr>
<td>272 (T to C)</td>
<td>1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>312 (T to C)</td>
<td>27</td>
</tr>
<tr>
<td>381 (C to T)</td>
<td>2</td>
</tr>
<tr>
<td>non-synonymous</td>
<td></td>
</tr>
<tr>
<td>38 (T to C) Leu-13—Ser</td>
<td>1</td>
</tr>
<tr>
<td>68 (A to G) Asn-23—Ser</td>
<td>1</td>
</tr>
<tr>
<td>92 (C to T) Ser-31—Phe</td>
<td>1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>154 (A to T) Met-52—Leu</td>
<td>1</td>
</tr>
<tr>
<td>200 (C to T) Ala-67—Val</td>
<td>1</td>
</tr>
</tbody>
</table>

<sup>a</sup>Detected in the same patient.
In the present study, involving 138 immunocompromised patients and PcP prophylaxis with co-trimoxazole in 114 Portuguese isolates, we detected an alteration of a methionine for a leucine, as a result of a nucleotide substitution at position 154. However, mutations described here do not match those known to cause trimethoprim or pyrimethamine resistance in other microorganisms.3–5

In our study, there was no statistically significant association between PcP prophylaxis failure and the occurrence of DHFR mutations. The majority of patients presenting the amino acid changes described here, only one was receiving co-trimoxazole for PcP prophylaxis. The remaining patients, according to the clinical charts, had not been exposed to co-trimoxazole. In Portugal, this is a drug specially used for the prophylaxis of PcP and of toxoplasmic encephalitis, in HIV-positive patients. It is not commonly used in other situations. Also, it is not a common drug used in the general population for treatment of bacterial infections. Therefore, the mutations observed in this study may not be selected by trimethoprim use.

Nahimana et al.7 have reported a significant association between DHFR mutations and anti-
P. jirovecii prophylaxis, however the majority of polymorphisms were detected in patients receiving pyrimethamine as a DHFR inhibitor, combined with sulfadoxine or atovaquone (all second-line drugs for PcP prophylaxis, rarely used). These authors suggested that pyrimethamine, rather than trimethoprim, could exert a higher pressure on the DHFR locus. In fact, in vitro kinetics studies demonstrated that pyrimethamine is a stronger inhibitor of the P. jirovecii DHFR than trimethoprim.12

Further studies on P. jirovecii DHFR genetic heterogeneity, in a larger patient population, need to be conducted in order to better characterize the role of this gene in the development of resistance to co-trimoxazole.

### Acknowledgements
We thank Professor Celso Cunha (Unit of Molecular Biology, Institute of Hygiene and Tropical Medicine, Lisboa, Portugal) for his helpful assistance with cloning. This work was supported in part by ‘Associação para a investigação e o desenvolvimento da Faculdade de Medicina de Lisboa’.

### Transparency declarations
None to declare.

### References
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