Pneumocystis jirovecii dihydropteroate synthase (DHPS) genotypes in non-HIV-immunocompromised patients: a tertiary care reference health centre study

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Studies on Pneumocystis jirovecii dihydropteroate synthase (DHPS) genotypes among non-HIV immunocompromised patients from developing countries are rare. In the present prospective investigation, 24 (11.8%) cases were found to be positive for Pneumocystis jirovecii out of 203 non-HIV patients with a clinical suspicion of Pneumocystis pneumonia (PCP). Dihydropteroate synthase (DHPS) genotype 1 (Thr55/Pro57) was noted in 95.8% P. jirovecii isolates in the present study in contrast to only 4.1% of patients with DHPS genotype 4 (Thr55Ala + Pro57Ser).

Keywords Pneumocystis jirovecii, DHPS genotypes, non-HIV patients

Introduction

Pneumocystis jirovecii (P. jirovecii) is an important opportunistic pathogen responsible for life-threatening Pneumocystis pneumonia (PCP) in immunocompromised individuals particularly in those infected with Human Immunodeficiency Virus (HIV) [1]. However, the occurrence of PCP in non-HIV immunocompromised patients is infrequent although there are a number of underlying conditions that predispose such patients to PCP, i.e., patients with malignant disorders [2], bone marrow and solid organ transplant recipients [3], autoimmune or inflammatory diseases, connective tissue disorders [4,5] and others that lead to immune system alterations [6].

In developing countries, an increasing trend of PCP has been noted in individuals with HIV/AIDS [7] as part of the concurrent AIDS epidemic. Further, it was also observed that the incidence of PCP is not rare in immunocompromised individuals without HIV/AIDS [8]. In fact, mortality rates of 30–50% due to PCP have been reported in the literature in non-HIV immunocompromised patients [9]. The lack of clear guidelines/mandates for prophylaxis for non-HIV immunocompromised individuals could be a major factor in the high mortality rates. Sulfanilamide, particularly cotrimoxazole, a synergistic combination of sulfamethoxazole and the dihydrofolate-reductase inhibitor trimethoprim (TMP+SMX) remains the drug of choice for both the treatment and prophylaxis of PCP. The anti-Pneumocystis activity is almost entirely due to sulfamethoxazole [10]. Dihydropteroate synthase (DHPS) is the enzymatic target for the sulfanilamide and the most common DHPS mutations at codon 55 (Thr55/Pro57) and codon 57 (Pro57Ser) have been correlated with resistance to this agent [11]. Presence of these non-synonymous mutations within DHPS has also been extensively studied especially in HIV-infected patients to delineate various P. jirovecii DHPS genotypes within infected populations [12]. In contrast, the frequency of P. jirovecii-specific DHPS mutations reported from developing countries is much lower [13] compared to developed countries and much less is known about DHPS genotypes in non-HIV infected patients. Thus, the aim of the present study was to determine the DHPS genotypes among P. jirovecii detected from non-HIV immunocompromised patients.

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Materials and methods

During the 3-year study period (December 2006 to December 2009), a total of 425 non-HIV immunocompromised patients attending our tertiary health care centre were screened, of which 203 patients with a high index of clinical suspicion of PCP were enrolled for further study. Demographic and necessary clinical information for each patient were collected prospectively during their visits to the hospital, as well as by reviewing their medical records. The study was approved by the institute’s research and ethics committee.

A total of 226 clinical respiratory samples were obtained from 203 patients that included 113 bronchoalveolar lavage fluid (BALF), 21 tracheal aspirates (TAs), one gastric aspirate (GA), 58 nasopharyngeal aspirates (NPAs) and samples from only one lung biopsy. All clinical samples, except sputum, were spun at 4000 rpm at 4°C for 10 minutes. Each sputum sample was treated with 0.0065 M dithiothreitol (DTT), a mucolytic agent and then centrifuged. The pellets obtained was re-suspended in 1/5th of the supernatant and 200 μl of each pellet was used for extraction of genomic DNA using commercial Qiagen tissue kit (Qiagen, USA).

Detection and identification of *P. jirovecii* was done using commercially available direct fluorescent antibody (DFA) staining kit (Meriflour, France) [14], Grocott’s methenamine silver (GMS) [15] staining, as well as by amplification of the major surface glycoprotein (MSG) as the target gene using a single round polymerase chain reaction (PCR) assay [16]. *P. jirovecii* detected from relevant respiratory clinical samples were further studied to detect DHPS gene by amplification of DHPS gene using nested PCR protocol using Dp15/Dp800 as the primary primers and DHPS-NF/DHPS-NR as the secondary primers [17,18]. The nested PCR conditions consisted of initial denaturation at 95°C for 3 min followed by 35 cycles at 94°C for 30 sec, 62°C for 30 sec and 72°C for 45 sec with a final extension at 72°C for 5 min. All the PCR reactions were performed in an ABI 2720, thermocycler (Applied Biosystems, USA). Necessary precautions were taken during the performance of each PCR reaction to prevent carry-over contamination. Both pre- and post-PCR reactions were performed in different rooms and all the reagents were handled using barrier filter pipette tips. Multiple negative controls were run during each PCR assay. Amplified products of MSG PCR and nested-DHPS PCR assays were run on 1.5% agarose gels containing ethidium bromide and the bands were visualized by UV light. MSG PCR and nested-DHPS PCR amplified products of 250 bp and 186 bp, respectively (Fig. 1) The 186 bp amplified product was further purified using QIAquick gel extraction kit (Qiagen, USA) according to the manufacturer’s instructions. Purified nested-DHPS PCR fragments were sequenced in an automated DNA sequencer (model 3130xl Genetic Analyzer, Applied Biosystems) using v.3.1 BigDye® Terminator cycle sequencing kit (Applied Biosystems, USA). The primers used for sequencing were DHPS-NF and DHPS-NR [18].

Results

Diagnosis of PCP was made in 24 (11.8%) cases of the total 203 non-HIV immunocompromised patients. Of the 24 patients, 15 were males and 9 were females with a median age of 26.5 years [range, 0.29–62 years]. Different clinical respiratory specimens obtained from these 24 PCP positive cases included 21 bronchoalveolar lavage fluid (BALF) and one sample each of sputum, nasopharyngeal aspirate and tracheal aspirates from three patients (Table 1). Fourteen (58.3%) of the 24 patients were determined to be positive by DFA staining, 7 (29.1%) were positive by Grocott’s methenamine silver staining and all 24 were found positive by single round MSG PCR assay (Table 2). The DHPS gene was amplified by nested PCR protocol in all 24 clinical specimens that were positive for *P. jirovecii* by MSG PCR assay. Sequencing analysis showed 95.8% (23/24) of the *P. jirovecii* as being DHPS genotype 1 with the nucleotide sequence ACA CGG CCT at codons 55, 56 and 57, corresponding to threonine and proline amino acids at codon 55 and 57 (Thr55+Pro57), respectively.
Antibody staining; cMSG: Major surface glycoprotein.

classification described by Beard at codon 55 and 57 (Thr55Ala nucleotide sequence GCA CGG TCT at codons 55, 56 and the remaining one (4.1%) was DHPS genotype 4 with the nucleotide sequence that differed from the wild type sequence.

Upon analysis of clinical features, dyspnoea was the predominant presenting illness in all cases followed by fever in 21 (87.5%, 21/24) and cough in 19 (79.1%, 19/24). Abnormal chest X-ray results were observed in almost all patients. Median arterial oxygen pressure (PaO₂) (Inter Quartile Range, IQR) was 53 (46.2 – 60) mm Hg. Six (25%) patients. Median arterial oxygen pressure (PaO₂) (Inter

Table 1 Underlying condition of 24 PCP positive non-HIV immunocompromised patients.

<table>
<thead>
<tr>
<th>Underlying condition (no. of patients)</th>
<th>Respiratory samples positive for PCP (no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Post renal transplant recipient (9)</td>
<td>Bronchoalveolar lavage fluid (9)</td>
</tr>
<tr>
<td>Pulmonary alveolar proteinosis (2)</td>
<td>Bronchoalveolar lavage fluid (2)</td>
</tr>
<tr>
<td>Systemic lupus erythematosus (2)</td>
<td>Bronchoalveolar lavage fluid (2)</td>
</tr>
<tr>
<td>Non-Hodgkin’s lymphoma (2)</td>
<td>Bronchoalveolar lavage fluid (2)</td>
</tr>
<tr>
<td>Metabolic liver disease (1)</td>
<td>Bronchoalveolar lavage fluid (1)</td>
</tr>
<tr>
<td>Cushing’s syndrome (1)</td>
<td>Bronchoalveolar lavage fluid (1)</td>
</tr>
<tr>
<td>Crohn’s disease (1)</td>
<td>Tracheal aspirate (1)</td>
</tr>
<tr>
<td>Myasthenia gravis (1)</td>
<td>Sputum (1)</td>
</tr>
<tr>
<td>Primary immunodeficiency (1)</td>
<td>Bronchoalveolar lavage fluid (1)</td>
</tr>
<tr>
<td>Bone marrow transplant recipient (1)</td>
<td>Bronchoalveolar lavage fluid (1)</td>
</tr>
<tr>
<td>Hyper IgM syndrome (1)</td>
<td>Bronchoalveolar lavage fluid (1)</td>
</tr>
<tr>
<td>Autoimmune hepatitis (1)</td>
<td>Nasopharyngeal aspirate (1)</td>
</tr>
<tr>
<td>On steroids (1)</td>
<td>Bronchoalveolar lavage fluid (1)</td>
</tr>
</tbody>
</table>

Table 2 Distribution of respiratory samples positive for Pneumocystis jirovecii by different screening techniques.

<table>
<thead>
<tr>
<th>Respiratory samples (no.)</th>
<th>GMS positive a</th>
<th>DFA positive b</th>
<th>MSG PCR positive c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bronchoalveolar lavage fluid (21)</td>
<td>7</td>
<td>14</td>
<td>21</td>
</tr>
<tr>
<td>Sputum (1)</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Nasopharyngeal aspirate (1)</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Tracheal aspirate (1)</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Total 24</td>
<td>7</td>
<td>14</td>
<td>24</td>
</tr>
</tbody>
</table>

© 2011 ISHAM, Medical Mycology, 49, 167–171 could not be performed and in one patient (4.1%) the treatment was changed to clindamycin plus primaquine combination for PCP.

Discussion

The distribution of P. jirovecii DHPS genotypes and its association to clinical outcome of PCP have been studied mainly in patients with HIV/AIDS primarily because P. jirovecii DHPS mutants are believed to occur in this population due to selective pressure of sulfa drugs [17]. However, Huang et al. from San Francisco General Hospital (SFGH) reported 87% (26/30) mutant DHPS genotype (DHPS genotype 4 ) in HIV-infected patients who were receiving prophylaxis compared to 40% (8/20) patients who did not receive treatment (P = 0.002) [19]. In the present study, dihydropteroate synthase locus of P. jirovecii was analyzed as it still remains the sole marker of circulation [12] especially in non-HIV immunocompromised patients. In addition, reports related to DHPS genotypes among these non-HIV populations are very limited. Our study demonstrated wild type (DHPS genotype 1) in 95.8% of cases and only one (4.1%) had DHPS genotype 4 (mutant type) of P. jirovecii. Similar results were obtained by Montes Cano et al. [20] in a study of the distribution of DHPS genotypes in 64 HIV negative subjects with different chronic pulmonary diseases and in 15 HIV patients. It was observed that DHPS genotype 1 was the most prevalent type (70%) in non-HIV patients compared to 54.5% in patients with AIDS and PCP (P = 0.63). However, the only patient (1/79) with DHPS genotype 4 (mutation at codon 55/57) had AIDS-associated PCP. A study conducted in China has shown a rate of only 7% (1/15) of DHPS mutation even in AIDS patients [21] and low rates of DHPS mutation have also been reported from developing countries like Thailand [13] and Brazil [22]. Thus, it appears that both rates of DHPS mutation, as well as the frequency of DHPS genotype 4 are much less in developing countries.

P. jirovecii DHPS genotype 4 was detected in 1 post-renal transplant (RT) recipient who had not responded to standard cotrimoxazole treatment and was treated with clindamycin plus primaquine. The single case involving DHPS genotype 4 that was associated with sulfa treatment failure in the present study is of importance in that the patient had not received any sulfa prophylaxis and this was the first episode of PCP during the present hospital admission. Takahashi et al. [23] described DHPS mutants in two patients with lymphoma (non-HIV immunocompromised individuals) who had no exposure to sulfa drugs. This demonstrates that DHPS mutations are not necessarily selected by the pressure of drug therapy and may be incidentally acquired. Such acquisition of mutant strain in predisposed
patients may pose as a potential reservoir for person-to-person transmission [24]. It is known that the prodrome duration of *P. jirovecii* infection is longer and the symptoms are milder in HIV positive patients despite a heavy microbial load. In contrast, immunocompromised HIV negative patients with *P. jirovecii* pneumonia may progress rapidly to respiratory failure. [25]. The patient with DHPS 4 genotype had a poor outcome that reinforces the fact that patients with mutant genotypes increasingly require mechanical ventilation and intensive care during clinical management of PCP [26]. In addition, this result indicates that DHPS genotype 4 may be a marker for poor clinical outcome. There are limited studies related to *P. jirovecii* DHPS genotypes in non-HIV immunocompromised patients from developing countries, which nonetheless is a very important high risk group for acquiring *P. jirovecii* infection as most of them are on intensive chemotherapy or on immunosuppressive therapy with steroids.

Various workers have indicated that the DHPS mutations and their importance in *P. jirovecii* resistance are unresolved questions. It is also of significance to note that the treatment cost, hospital cost and the difference between hospital cost and reimbursement (net cost) were found to be significantly greater in HIV negative than in HIV positive patients [27]. Since this study had the limitation of small sample size, studies with large sample size are of utmost importance and warrants further survey on *P. jirovecii* DHPS genotypes circulating among non-HIV immunocompromised patients.

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**Declaration of interest:** The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

**References**


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