Original Article

Dihydropteroate synthase (DHPS) gene mutation study in HIV-Infected Indian patients with *Pneumocystis jirovecii* pneumonia

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Abstract

Introduction: *Pneumocystis jirovecii* dihydropteroate synthase (DHPS) gene mutations' (55th and 57th codon) association with prior sulfa prophylaxis failure has been reported from both developed and developing countries. We conducted a prospective study to determine the prevalence of *P. jirovecii* DHPS mutations from 2006 to 2009 on *P. jirovecii* isolates obtained from HIV-infected patients with a clinical diagnosis of *Pneumocystis carinii* pneumonia (PCP) admitted to our tertiary care reference health center in New Delhi, India.

Methodology: Detection of *P. jirovecii* cysts was performed by direct fluorescent antibody (DFA) staining and by Grocott's-Gomori methenamine silver staining (GMS). DNA detection was performed by polymerase chain reaction (PCR) using primers for the major surface glycoprotein (MSG) gene. *P. jirovecii* DHPS gene was amplified by nested PCR protocol and sequenced for detecting mutations at the 55th and 57th codons.

Results: Out of 147 HIV-positive patients with suspected Pneumocystis pneumonia (PCP), 16 (10.8%) PCP positive cases were detected. Of 16 cases, nine (56.2%) were positive by DFA staining, four (25%) were positive by Grocott's-Gomori methenamine silver staining, and all 16 were positive by MSG PCR. DHPS mutations at the 55th and 57th codons were observed in 6.2% of HIV patients studied, which was relatively low compared to reports from developed nations.

Conclusions: Prevalence of *Pneumocystis jirovecii* DHPS mutations associated with cotrimoxazole treatment failure may be low in the Indian subpopulation of HIV-positive patients and warrants larger studies to elucidate the true picture of *Pneumocystis jirovecii* sulfa drug resistance in India.

Key words: *P. jirovecii*; DHPS; mutation; HIV positive

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Introduction

Pneumocystis jirovecii infection causes severe pneumonia in human immunodeficiency virus (HIV) infected patients. Although the incidence of PCP dramatically declined in the industrialized nations after the introduction of Highly Active Anti Retroviral Therapy (HAART) and *Pneumocystis* pneumonia (PCP) prophylaxis, PCP still remains the most common serious opportunistic infection in HIV-infected persons [1].

The prevalence of PCP in HIV-positive patients is of great concern in developing countries [2]. According to the National AIDS Control Organization (NACO) 2008-2009 report [3], 2.3 million people are currently infected with HIV in India. Sulfa drugs act as a key agent in *P. jirovecii* treatment and prophylaxis. Cotrimoxazole is the most cost effective, the most easily available, and therefore the most widely used drug for therapy and prophylaxis of PCP. Cotrimoxazole is often used as a broad-spectrum antibiotic for treating many microbial infections; hence there is fear that its unscrupulous use may lead to the development of cotrimoxazole resistance. Cotrimoxazole is a combination of trimethoprim and sulfamethoxazole [TMP+SMX] [4] which target dihydrofolate reductase and dihydropteroate synthase (DHPS) respectively. The anti-*Pneumocystis* activity of cotrimoxazole is almost entirely due to SMX [5]. Numerous studies from developed countries have reported that mutations at codon 55 and codon 57 of the DHPS gene of *P. jirovecii* are associated with sulfa treatment and prophylaxis failure as these mutations are located in the highly conserved region of one of the active sites of the enzyme [6] and this also correlates with point mutations that cause sulfa

resistance in other microorganisms [7-9]. These studies reported the prevalence of DHPS mutations of 19% to 80% in patients with PCP who used sulfa prophylaxis [10]. Prevalence of *P. jirovecii* DHPS mutations reported from developing countries is either very low or even uncommon [11-14]. The present study was conducted to determine the prevalence of DHPS gene mutations (Condons 55 and 57) in *P. jirovecii* isolates obtained from HIV-infected Indian patients admitted to our tertiary care reference health center.

Methodology

The present prospective study on mutational analysis of *P. jirovecii* isolates obtained from HIV-infected patients between the years 2006 and 2009 was conducted at the All India Institute of Medical Sciences (AIIMS), New Delhi, India. Demographic and clinical data of each patient were obtained prospectively as well as by reviewing the medical records. Sulfa prophylaxis was considered given when there was prescription of sulfa or sulfone-containing agents at any time within the three-month period before the diagnosis of PCP.

Specimens

A total of 171 clinical respiratory samples, comprised of 63 bronchoalveolar lavage (BAL) samples, 16 tracheal aspirates (TA), 63 sputum samples, 2 gastric aspirates (GA), and 27 nasopharyngeal aspirates (NPA), were obtained from 147 HIV-positive patients with suspected PCP . Detection and identification of *P. jirovecii* was done using direct fluorescent antibody staining (DFA) [15] using a commercial kit (Meriflour, Cincinnati, USA) and Grocott's-Gomori methenamine silver (GMS) staining [16] as well as by amplification of the major surface glycoprotein (MSG) gene by conventional polymerase chain reaction (PCR) [17]. DHPS gene amplification was performed by nested PCR protocol [18,19]. The study was approved by the institute's research and ethics committees.

Sample Processing and DNA Extraction

All the samples except sputum samples were spun at 4,000 rpm at 4^{o}C for 10 minutes. Sputum samples were first treated with 0.0065 M dithiothreitol (DTT), a mucolytic agent and then centrifuged. The pellet obtained was re-suspended in 1/5th of supernatant. For DNA extraction, 200 µl of pellet was used. DNA extraction was done using the Qiagen tissue kit (Qiagen, Valencia, USA).

PCR analyses

Using an equal mixture of oligonucleotide primers JKK14 and JKK15 as forward primers, JKK17 as a reverse primer, PCR for major surface glycoprotein (MSG) gene of *Pneumocystis* was performed [17]. These primers amplified the 249 bp region of the highly conserved MSG gene of *Pneumocystis*. The reaction mixture contained 50 mM KCL, 10mM Tris, 1.5 mM $MgCl_2$, 0.25 mM dNTPs, 0.5 µM Primers and 1.5 U *Taq* polymerase. PCR was performed by an initial denaturation at 94^{o}C for 5 minutes followed by 35 cycles at 94^{o}C for 1 minute, 65^{o}C for 1 minute and 72^{o}C for 1 minute.

Nested PCR for the dihydropteroate synthase (DHPS) gene of *Pneumocystis* was done, using published primers Dp15 and Dp800 [18] in the first round (touchdown PCR) and DHPS-NF and DHPS-NR for the second round as internal primers [19]. The reaction mixture contained 10 x PCR buffer, primers (20 µM), dNTP (10mM), $MgCl_2$ (25mM), and *Taq* DNA polymerase (2.5 U) along with the DNA template. For the first round, touchdown PCR amplification was done as follows: preheating for 5 minutes then decreasing touchdown annealing temperatures from 68^{o}C to 58^{o}C, using 58^{o}C as annealing in the last 30 cycles. The nested round was done with initial denaturation at 95^{o}C for 3 minutes followed by 35 cycles at 94^{o}C for 30 seconds, 62^{o}C for 30 seconds and 72^{o}C for 45 seconds and then a final extension at 72^{o}C for 5 minutes. The external round amplified a 785 bp region while the nested round amplified a 186 bp region. All the PCR reactions were performed in an ABI 2720 thermocycler (Foster City, USA). To prevent and detect carry-over contamination, all pre- and post-PCR reactions were set up in different rooms using filter pipette tips, and multiple negative controls were run during each PCR assay [20]. PCR products were run on a 1.5 % agarose gel and were visualized in UV light.

The positive DNA band from the agarose gel was purified with the QIAquick gel extraction kit (Qiagen, Valencia, USA) according to the manufacturer's instructions. The PCR fragments after purification were subjected to sequencing with an automated DNA sequencer (model 3130xl Genetic Analyzer, Applied Biosystems, Foster City, USA) using v.3.1 BigDye Terminator cycle sequencing kit (Applied Biosystems, Foster City, USA). The primers used for sequencing were DHPS-NF and DHPS-NR. Sequence analyses were performed using Chromas

(Queensland, Australia), ClustalX 1.8 (Strasbourg, France) and GeneDoc (Boston, USA) and results were compared with wildtype sequence of *P. jirovecii* DHPS gene.

Results

Sixteen (10.8%) PCP-positive cases were detected out of 147 HIV-positive patients with suspected PCP. Clinical respiratory specimens obtained from these 16 PCP-positive cases included bronchoalveolar lavage (BAL) (n = 10), sputum (n = 5), and tracheal aspirate (n = 1). Out of 16 clinical respiratory specimens, nine (56.2%) were positive by DFA staining, four (25%) were positive by Grocott's-Gomori methenamine silver staining, and all 16 were positive by MSG PCR (Figure 1). Nested DHPS PCR successfully amplified the entire 16 samples (Figure 2) positive by MSG PCR for studying the *P. jirovecii* DHPS mutations (codons 55 and 57) in HIV-positive patients. Out of 16 HIV-positive patients, 11 were males and 5 were females with a median age of 35 years (range 14-50); median CD4+ cell count (Inter Quartile Range) was 64 cells / μl (44.7-118); median arterial oxygen pressure (PaO_2) (Inter Quartile Range) was 59.9 mm Hg (51.5- 65.9); and none of the 16 HIV patients had previous history of PCP.

Figure 1. PCR amplified products of Major Surface Glycoprotein (MSG) gene of *Pneumocystis jirovecii.* Lane 1 (50 bp DNA Ladder). Lane 2 (positive control, 249 bp). Lanes 3, 4 (test samples). Lane 5 (negative control).

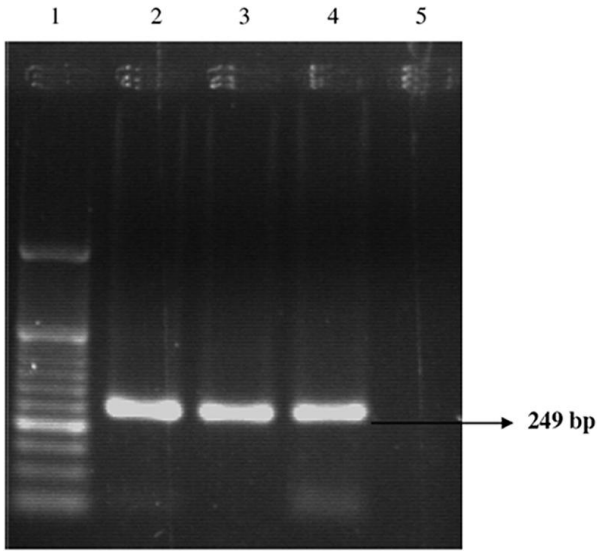
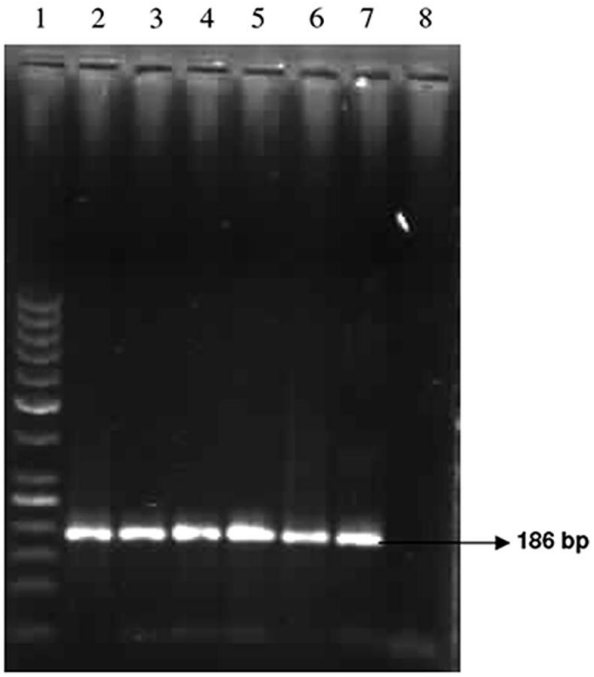


Figure 2. Nested-PCR amplified product of Dihydropteroate synthase (DHPS) gene of *Pneumocystis jirovecii*. Lane 1 (50 bp DNA Ladder). Lane 2 (positive control, 186 bp). Lanes 3, 4, 5, 6, 7 (test samples). Lane 8 (negative control).



Of the 16, only three HIV-positive patients were on cotrimoxazole (TMP-SMX) prophylaxis. In the other 13 HIV-positive patients (81.2%), PCP was the presenting illness at the time of their admission; none of them had prior sulfa prophylaxis; and during their hospital stay, all were treated with cotrimoxazole. TMP-SMX was successful in 14 patients (87.5%). One patient died during the course of TMP-SMX treatment, and in one patient the treatment was changed to clindamycin plus primaquine combination. Fifteen out of 16 (93.7%) clinical respiratory specimens studied showed the wild type DHPS genotype of *P. jirovecii* and only one clinical respiratory specimen (6.2%) showed a double mutation in the DHPS gene of *P. jirovecii* at amino acids 55 and 57 respectively (Table 1). Three out of 16 (18.7%) patients had intensive care unit admission and required mechanical ventilation (Table 1). The deaths of two patients (12.5%) were attributed to PCP but autopsies could not be performed. Of the 16 HIV-positive patients, the mortality rate among patients with wild type *P. jirovecii* was (6.6%) [1/15] and the lone mutant case also had a fatal outcome.

Table 1. DHPS mutations of *Pneumocystis jirovecii* in HIV positive Indian patients

Patient no.	Age (Yr)	Sex[a]	ICU[b] admission	TMP-SMX[c] prophylaxis	Nucleotide changes at codon 55,56,57	Amino acids[d] 55/57	Treatment outcome
1	24	M	NO	NO	ACACGGCCT	T/P	Survival
2	25	M	NO	NO	ACACGGCCT	T/P	Survival
3	26	M	NO	NO	ACACGGCCT	T/P	Survival
4	35	M	NO	YES	ACACGGCCT	T/P	Survival
5	50	M	NO	NO	ACACGGCCT	T/P	Survival
6	40	F	NO	NO	ACACGGCCT	T/P	Survival
7	40	F	NO	NO	ACACGGCCT	T/P	Survival
8	44	F	YES	NO	ACACGGCCT	T/P	Survival
9	23	F	NO	YES	ACACGGCCT	T/P	Survival
10	45	M	NO	NO	ACACGGCCT	T/P	Survival
11	39	M	NO	NO	ACACGGCCT	T/P	Survival
12	40	M	YES	YES	ACACGGCCT	T/P	Death
13	30	M	YES	NO	**G**CACGG**T**CT	**A/S**	Death
14	35	M	NO	NO	ACACGGCCT	T/P	Survival
15	35	F	NO	NO	ACACGGCCT	T/P	Survival
16	14	M	NO	NO	ACACGGCCT	T/P	Survival

[a] M, male; F, female
[b] ICU, intensive care unit
[c] TMP-SMX, trimethoprim-sulfamethoxazole
[d] A, alanine; P, proline; S, serine; T, threonine

Discussion

There is a dearth of reports from India regarding DHPS mutations in HIV patients and their associated anti-PCP treatments and prophylaxis failures. Human immunodeficiency virus (HIV) infection was reported in India in the mid-1980s, and the first AIDS case in India was reported in 1986. Cotrimoxazole remains the first drug of choice for the management of PCP as it is associated with both fewer breakthrough infections when used as a prophylactic agent and with better clinical response and improved survival when used for treatment of PCP [21,22]. Widespread use of cotrimoxazole has raised concerns regarding the emergence of sulfa resistance in *P. jirovecii* apart from various other microbial species [7-]. Studies from developed countries have reported the association between increased prevalence of *P. jirovecii* DHPS gene mutations (condons 55 and 57) with TMP-SMX prophylaxis failure [23-25]. Additionally, there are reports of the occurrence of mutant DHPS strains of *P. jirovecii* in HIV-positive patients who had not received prior TMP-SMX prophylaxis, suggesting the possibility of acquisition from a person infected with a mutant strain [25].

In this prospective study on HIV-positive Indian patients, *P. jirovecii* DHPS mutations at codons 55 and 57 were seen in only one out of 16 PCP-positive patients, while the other 15 HIV-positive patients showed the wild type DHPS alleles of *P. jirovecii*. One of the HIV-positive patients who showed double mutation in DHPS gene (Thr55Ala plus Pro57Ser) of *P. jirovecii* was a newly diagnosed case and had not received sulfa prophylaxis before the diagnosis of PCP, suggesting the possible role of acquisition. This study, which was conducted at only one tertiary care reference health center in New Delhi, showed that the DHPS mutations in *P. jirovecii* are uncommon in HIV-positive Indian patients as most of them (13 out of 16) came to know of their HIV status only at the time of PCP presentation and were not on

sulfa prophylaxis for PCP. These conditions may lead to the emergence of resistant strains of *P. jirovecii*. Apart from these factors, geographical location also appears to be an important determinant of DHPS genotype [26]. Compared to developed countries, lower rates of DHPS mutation have been reported from other developing countries [11-14] which also have similar resistance rates as ours.

Since millions of people are living with HIV in India and cotrimoxazole is widely used as a prophylactic agent against many microbial pathogens including *P. jirovecii*, sulfa-resistant *P. jirovecii* strains may emerge. Furthermore, the mutant strain that we have observed may be an indication that such resistance might already have developed in this part of the subcontinent. While this investigation suggests that *P. jirovecii* DHPS mutations may contribute to sulfa resistance, the study is limited by its small sample size. Hence, studies with larger sample sizes and the involvement of multiple health centers are warranted in India to establish the relationship between clinical outcome and infection with *P. jirovecii* DHPS mutant strains.

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