

Study of dihydropteroate synthase (DHPS) gene mutations among isolates of *Pneumocystis jiroveci*

A.K. Tyagi, B.R. Mirdha, Randeep Guleria*, Anant Mohan*, Kalpana Luthra** & U.B. Singh

Departments of Microbiology, *Medicine & **Biochemistry, All India Institute of Medical Sciences
New Delhi, India

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Background & objectives: *Pneumocystis jiroveci* (also known as *P. carinii*) causes fatal pneumonia in patients with AIDS and other immunocompromised patients. Co-trimoxazole (trimethoprim + sulphamethoxazole, TMP-SMZ) is the drug of choice for treatment and prophylaxis. Widespread use of sulpha medication has raised the possible selection of resistant *P. jiroveci* strains worldwide. Non-synonymous polymorphisms associated with sulpha resistance have been observed in *P. jiroveci* dihydropteroate synthase (DHPS) gene at codons 55 and 57. In view of this, we investigated mutation at DHPS locus amongst *P. jiroveci* isolates obtained at a tertiary care hospital in north India.

Methods: Microscopic examination of *P. jiroveci* in 69 clinical samples obtained from patients suspected to have *P. carinii* pneumonia (PCP), was performed by Grocott's Gomori methenamine silver and direct fluorescent antibody staining. Molecular studies were carried out by polymerase chain reaction (PCR) using major surface glycoprotein (MSG) as the target gene. Investigations for DHPS mutations were carried at specific 55th and 57th codon using PCR-RFLP (restriction fragment length polymorphism) assay.

Results: Microscopic examination detected *P. jiroveci* in four cases and MSG gene was amplified in five cases. Further, amplification of DHPS gene was successful in four of the five cases positive by MSG gene PCR. No point mutation was observed and all four isolates presented wild-type sequences at DHPS gene by RFLP analysis.

Interpretation & conclusions: Although our findings suggest that in Indian subpopulation, point mutations in DHPS gene of *P. jiroveci* are not as common as in other parts of the developed world, further studies are needed.

Key words DHPS PCR- PCP - *Pneumocystis jiroveci* - RFLP

Pneumocystis jiroveci (formerly *P. carinii*), an opportunistic organism, is one of the causes of a severe and often fatal pneumonia in patients with acquired immunodeficiency syndrome (AIDS) and other immunocompromised patients (transplant recipients,

patients on cancer therapy or on prolonged steroid therapy and various immunodeficiency disorders)¹. Pneumonia caused by *P. jiroveci* is still termed as *Pneumocystis carinii* pneumonia (PCP). Although widespread PCP prophylaxis and use of highly active

antiretroviral therapy (HAART) have reduced the incidence of the disease in industrialized nations, it still remains a problem in many developing countries due to non adherence to prophylaxis or due to less permissibility to HAART². Use of trimethoprim-sulphamethoxazole (TMP-SMZ) which is the mainstay of PCP prophylaxis has raised concerns regarding recent development of sulpha-resistance in *P. jiroveci*^{3,4}. Sulpha medications (SMZ and dapsone, a sulphone) interfere with folate synthesis by competitively inhibiting the enzyme dihydropteroate synthase (DHPS) encoded by the folic acid synthase (FAS) gene, a multifunctional gene that also encodes dihydroneopterin aldolase and hydroxymethyldihydropterin pyrophosphokinase⁵. Point mutations, at codons 55 (Threonine to Alanine) and 57 (Proline to Serine), in the DHPS gene of *P. jiroveci* have been reported⁶⁻⁹ convincingly linking prior exposure to sulpha drug as the possible cause of emergence of sulpha drug resistance. Although similar DHPS mutations confer resistance to sulpha medications in other organisms^{10,11}, the association between DHPS mutations and clinical resistance in *P. jiroveci* infection is still unclear.

The present study was carried out to investigate the presence of *P. jiroveci* DHPS mutations by PCR-restriction fragment length polymorphism (PCR-RFLP) analysis in patients with *P. jiroveci* infection diagnosed at a tertiary care reference health centre in north India.

Material & Methods

This prospective study was conducted at the Department of Microbiology, All India Institute of Medical Sciences (AIIMS), New Delhi, a tertiary care referral and teaching hospital located in north India. The study protocol was approved by the institutional ethics committee (IEC).

Patient samples: Clinical suspicion of PCP was made in patients when the clinical and radiological picture were strongly suggestive of the findings such as persistent non-productive cough, dyspnoea, history of protracted fever of many weeks duration with radiological findings (*i.e.*, bilateral perihilar reticulonodular involvement and ground glass appearance). Patients attending both in-patients and out-patients Departments of Internal Medicine, Nephrology, Institute of Rotary Cancer Hospital (IRCH) and Paediatrics of our hospital who fulfilled our laid down inclusion criteria were included.

Based on both clinical and radiological findings, PCP was suspected and a total 69 clinical samples [bronchoalveolar lavage (BAL) (n=28); sputum (n=26); tracheal aspirate (n=5); nasopharyngeal aspirate (n=9); gastric aspirate (n=1)] from 57 patients were obtained. These patients included both HIV infected (n=25) and HIV uninfected (n=32). HIV uninfected patients consisted of patients on immunosuppressive therapy *i.e.*, post renal transplant recipients (n=11), on anti-cancer therapy (n=11), patients with autoimmune disorders (n=3), and with recurrent episodes of pneumonia (n=7). All patients were routinely examined for the presence of *P. jiroveci* in our laboratory from June 2006 to November 2006. Clinical samples which tested negative for *P. jiroveci* but tested positive for bacterial, viral and other fungal pathogens, were subsequently excluded from the study.

Identification of *P. jiroveci* was done using Grocott's Gomori methenamine silver staining (GMS)¹² and direct fluorescent antibody staining (DFA)¹³ using commercial kit (Meriflour, France) and by amplification of the major surface glycoprotein (MSG) gene by conventional polymerase chain reaction (PCR)¹⁴.

Demographical description: Of the 57 patients, 44 were males and 13 were females with an age range of 1-64 yr. None of the patients had given any previous history of pneumonia due to *P. jiroveci*. Of the 57 patients, 25 were HIV positive, had a mean CD 4 + cell counts of 155.12 cells/ μ l.

Sample processing and DNA extraction: Samples obtained from patients were immediately transported to the laboratory on ice and kept at 4°C in order to maintain the structural integrity of the cellular components, until processed. Bronchoalveolar lavage (BAL), tracheal aspirate (TA), gastric aspirate (GA), and nasopharyngeal aspirate (NPA) obtained from patients were centrifuged at 894 g at 4°C for 10 min. Induced sputum and expectorated sputum samples were liquefied with mucolytic agent (sputolysin) and were centrifuged. One part of pellet from the samples for DNA extraction was stored at -20°C until used. Other part of the pellet was used for staining. DNA from the pellet was extracted using a Qiagen kit (Qiagen, USA). Pellet obtained was first lysed in a sample specific lysis buffer and subsequently DNA was extracted using manufacturer's recommendation. The DNA pellet was dissolved in Tris buffer and was stored at -20°C until used for PCR.

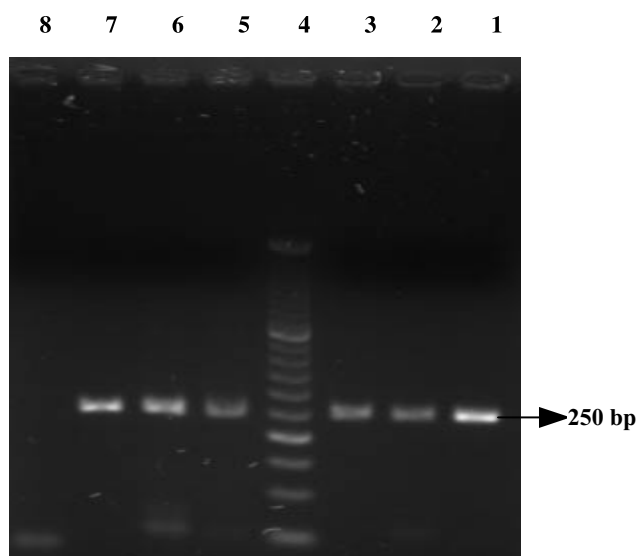


Fig. 1. Major surface glycoprotein (MSG) PCR of 5 positive samples. Lane 1: positive control (250 bp). Lanes 2, 3, 5, 6, and 7: test samples. Lane 4: 50 bp DNA Ladder. Lane 8: negative control.

Touchdown PCR for DHPS gene: DHPS gene amplification was carried out using published PCR primers Dp15 and Dp800 as described by Helweg *et al*⁶.

Dp15 (forward primer)-5'-TCTGAATTTTATAAAGC GCCTACAC -3'

Dp800 (reverse primer)-5'-ATTTCATAAACATCAT GAACCCG -3'

PCR was carried out using reagents from Fermentas life sciences (Applied Biosystems, USA). In total volume of 50 μ l, 5 μ l of 10 x PCR buffer, 2 μ l of primers (20 μ M), 0.2 μ l dNTP (10mM), 2 μ l MgCl₂ (25mM) *Taq* DNA polymerase (2.5 U) was combined with 5 μ l DNA template. PCR amplification was done with a touch-down procedure with preheating for 5 min and touchdown annealing temperatures were decreased from 68°C to 58°C, using 58°C as annealing in the last 30 cycles^{15,16}. To prevent and detect carry over contamination all pre- and post- PCR reactions were set up in different rooms with filter pipette tips and multiple negative controls were run during each PCR assay¹⁷.

Restriction fragment length polymorphism (RFLP) analysis: The 50 μ l PCR reaction mixture was divided into 3 aliquots. Wildtype/Mutation 55 was detected in the first aliquot by digesting with 1 U of *AccI* restriction enzyme (New England Biolabs, USA) at 37°C for 1 h, with cleavage of PCR product, if Wildtype at codon

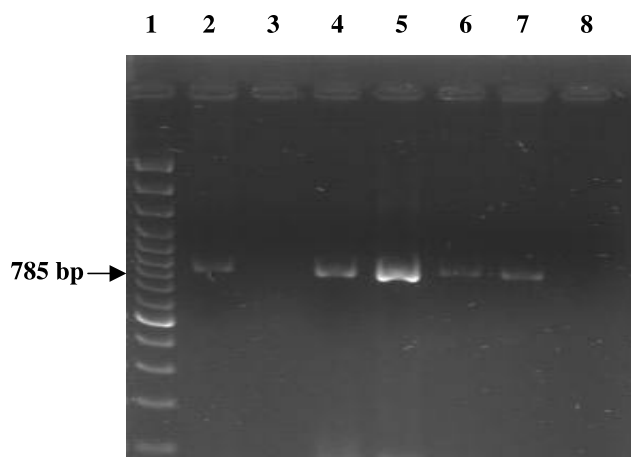


Fig. 2. Dihydropteroate synthase (DHPS PCR) of 4 positive samples. Lane 1: 100 bp DNA Ladder. Lane 2: positive control (785 bp). Lanes 3, 4, 5, 6 and 7: test samples. Lane 8: negative control.

55 was present. Wildtype/Mutation 57 was detected in the second aliquot by 1 h of digestion with *HaeIII* restriction enzyme at 37°C, with cleavage of PCR product if Wildtype at codon 57 was present. The third aliquot with 785 bp uncut PCR product was included as control. RFLP analysis was visualized on 1.5 per cent agarose gel electrophoresis. Loading of patient sample onto the gel was performed in the following order: uncut DHPS PCR product, PCR product digested with *AccI* restriction enzyme, PCR product digested with *HaeIII* restriction enzyme and PCR-negative control respectively.

Results

Out of 5 patients, which tested positive for *P. jiroveci* major surface glycoprotein (MSG) gene PCR (Fig. 1), only 4 were positive by staining methods (GMS and DFA) and successful amplification of *P. jiroveci* DHPS locus was obtained in 4 cases only (Fig. 2). In the remaining one patient, DHPS gene amplification was not successful (Fig. 2). Of the five patients positive for PCP, four were diagnosed HIV +ve at the time of presentation of PCP in our hospital and one was post renal transplant (PRT) recipient. None had received prior co-trimoxazole prophylaxis as documented from the medical records and also from personal interview with the patients and all of these patients had classical clinical symptoms and supportive radiological findings suggestive of PCP. All four HIV +ve patients were within the age group of 30-50 yr. Of the four HIV +ve patients, three had CD4 count <200 cells/ μ l (range: 13-89) and one patient had CD4 count just above 200 (238

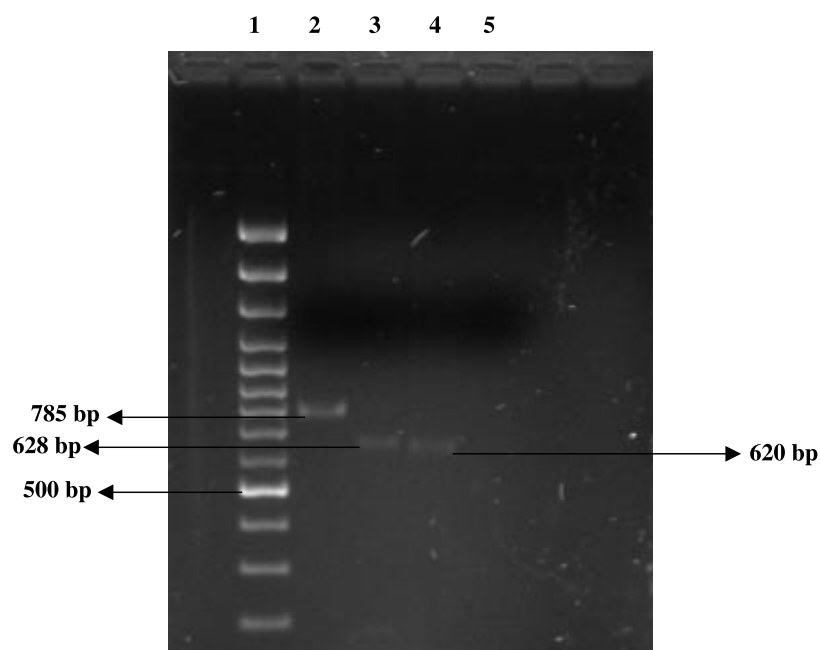


Fig. 3. Agarose gel electrophoresis of touchdown PCR- RFLP analysis of *P. jiroveci* dihydropteroate synthase (DHPS) gene. Lane 1: 100 bp DNA Ladder. Lane 2: uncut control (785 bp). Lane 3: digestion with *AccI* (wt55). Lane 4: digestion with *HaeIII* (wt57). Lane 5: PCR negative control; wt55: wild type at codon 55 (157bp, 628bp); wt57: wild type at codon 57 (165bp, 620bp).

cells/ μ l). Arterial blood gas analysis (ABG) and other relevant laboratory parameters for PCP were recorded at the time of admission and during the longitudinal clinical follow up (4-6 wk). DHPS genotypes were correctly determined by *AccI* and *Hae III* restriction enzyme cleavage in all 4 specimens. All four patients presented wild-type sequences (no point mutation either at codon 55 or at 57) at the *P. jiroveci* DHPS gene by RFLP analysis (Fig. 3). All PCP +ve patients were treated with co-trimoxazole but in one case of PRT recipient, the treatment was changed to clindamycin plus primaquine combination due to unresponsiveness to co-trimoxazole therapy.

Discussion

Sulpha resistance due to point mutations in the conserved regions of DHPS gene have been proved in various microorganisms¹⁸⁻²⁰ but sulpha susceptibility of *P. jiroveci* has not been studied probably due to lack of *in vitro* culture system. However, using molecular methods there are now increasing reports of mutations resulting into sulpha resistance in *P. jiroveci*. Lane *et al*²¹ demonstrated non synonymous polymorphism in DHPS gene from patients with PCP. DHPS mutations at codon 55 and 57 were studied previously by Kazanjian *et al*²² and by Mei *et al*²³. A CDC study of 191 isolates obtained from 4 cities in US also demonstrated point

mutations at codons 55 and 57 of *P. jiroveci* DHPS gene²⁴.

Treatment and prophylaxis failure rate of co-trimoxazole (TMP-SMZ) for *P. jiroveci* in AIDS patients ranged from 1 to 30 per cent per year and this treatment failure was associated with point mutations in the DHPS gene^{21,25}. Such point mutations have been reported at DHPS codons 23, 55, 57, 60, 111, 171, and 248²¹. The frequency of DHPS mutations at codons 55 and 57, which result in TMP-SMZ resistance²⁵, ranges from 7-25 per cent in Europe and Asia and 70 per cent in United States²⁶⁻²⁹.

In our study, *P. jiroveci* DNA was detected in 5 cases by MSG PCR. Further, amplification of the *P. jiroveci* DHPS locus was obtained in 4 out of 5 cases. All the four patients presented wild-type sequences at *P. jiroveci* DHPS gene by RFLP analysis (no point mutations either at 55th or at 57th codons) in the present study. Wissmann *et al*³⁰ also reported the absence of dihydropteroate synthase mutations in *P. jiroveci* from Brazilian AIDS patients. As none of our four patients had received prior co-trimoxazole prophylaxis, it might explain the lack of point mutations in our study. RFLP assay was used in the present study as it is a simple, rapid and cost-effective technique for detecting point mutation at codons 55 and 57 in the DHPS gene

of *P. jiroveci* in comparison to direct sequencing of PCR amplified DHPS gene. However, the limitation of RFLP assay is its inability to detect novel mutations other than those at codons 55 and 57.

In conclusion, our study was not able to show DHPS gene mutation amongst isolates of *P. jiroveci* at codons 55 and 57 in contrast to the available reports from developed countries. However, the findings do not rule out the fact that in a country like India where co-trimoxazole is used as a prophylactic agent for many opportunistic infections, the emergence of sulpha resistant *P. jiroveci* isolates that contain mutations in their DHPS gene might occur, which may possibly be acquired incidentally by other immunocompromised individuals as well. Studies involving larger number of patients would be necessary in order to validate these findings.

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Reprint requests: Dr B.R. Mirdha, Department of Microbiology, All India Institute of Medical Sciences
New Delhi 110 029, India
e-mail: mirdhabr@hotmail.com