JIDC | THE JOURNAL OF INFECTION IN DEVELOPING COUNTRIES

Original Article

Molecular detection of DHFR gene polymorphisms in *Pneumocystis jirovecii* isolates from Indian patients

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Abstract

Introduction: *Pneumocystis* pneumonia (PCP) is an opportunistic life-threatening infection, especially for immunocompromised individuals. A trimethoprim-sulfamethoxazole (TMP-SMX) combination is commonly used for the treatment of PCP, targeting both dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) enzymes. Several studies have already shown that polymorphisms in the DHPS gene are associated with drug resistance. The present study analyzed DHFR gene polymorphisms in *Pneumocystis jirovecii* recovered from clinical samples from patients admitted to a tertiary care health center in New Delhi, India.

Methodology: Detection of *P. jirovecii* was performed using Gomori methenamine silver staining (GMS) and nested polymerase chain reaction (PCR) assay targeting the mitochondrial large subunit ribosomal RNA (mt LSU rRNA) gene. The DHFR gene was amplified using nested PCR protocol and was sequenced for detection of polymorphisms.

Results: Of 180 clinical samples, only 4% (7/180) were positive by GMS staining, and 10% (18/180) were positive by mt LSU rRNA PCR assay. Of these 18 positive samples, only 77% (14/18) were amplified by the DHFR gene PCR assay. A total of 16 nucleotide substitutions were observed in 42% (6/14) samples targeted for the DHFR gene, of which 8 nucleotide substitutions were synonymous and the rest were non-synonymous.

Conclusions: The DHFR gene mutations found in this study may possibly indicate an association of process likely to contribute to therapeutic failure or an evolutionary process, and warrant continuous monitoring.

Key words: *Pneumocystis jirovecii*; dihydrofolate reductase; trimethoprim-sulfamethoxazole; polymorphisms.

J Infect Dev Ctries 2015; 9(11):1250-1256. doi:10.3855/jidc.6810

(Received 25 February 2015 – Accepted 11 March 2015)

Introduction

Pneumocystis pneumonia (PCP), caused by *Pneumocystis jirovecii*, is an important life-threatening opportunistic fungal infection for immunocompromised individuals, including both human immunodeficiency virus (HIV)-infected and in HIV-uninfected immunocompromised patients (transplant recipients, individuals receiving prolonged immunosuppressive therapies, and patients with autoimmune disorders and malignant disorders under chemotherapy) [1]. Over the years, the incidence of PCP has relatively declined in developed countries due to the introduction of highly active antiretroviral therapy (HAART) and simultaneous administration of chemoprophylaxis; however, PCP still remains a major cause of morbidity and mortality in resource-poor countries among high-risk population [2-5].

Anti-*Pneumocystis* prophylaxis and treatment certainly decreases, to some extent, the risks of PCP as well as disease-related death, but has led to *P. jirovecii* drug resistance. The most commonly used drug for the treatment of PCP is a trimethoprim-sulfamethoxazole (TMP–SMX) combination. These therapeutic agent targets two enzymes that are involved in the biosynthesis of folic acid. SMX targets dihydropterate synthase (DHPS), while TMP targets dihydrofolate reductase (DHFR) for its action. *P. jirovecii* drug resistance emergence and/or therapeutic failure have been attributed to drug pressure-related mutations in these two enzymes. Since effective *in vitro* culture systems are unavailable for *P. jirovecii*, several

investigators have studied the putative TMP-SMX drug resistance mechanism by detecting mutations at the DHPS locus. In fact, non-synonymous DHPS point mutations have resulted in amino acid changes at positions 55 (Thr to Ala) and 57 (Pro to Ser), and many such findings have been reported from different parts of the world [6-9]. In this context, mutations at different sites conferring resistances to sulfa drugs in other organisms, including *Escherichia coli*, *Streptococcus pneumoniae*, and *Plasmodium falciparum* have also been extensively reported [10-12].

Similarly, mutations in the DHFR gene have also been reported to confer resistance in infections due to *S. pneumoniae*, *Staphylococcus aureus*, *E. coli*, *Enterococcus faecalis*, and *P. falciparum* [13-17]; however, data on the emergence of mutations in DHFR with respect to *P. jirovecii* have not been extensively studied.

Ma *et al.* (1999) had initially cloned and sequenced the DHFR gene in *P. jirovecii*; thereafter, few more studies [12,18-21] from different geographical areas have reported polymorphisms at the *P. jirovecii*-specific DHFR gene. These studies could reveal unexplained variable results in different geographical, clinical, and population settings. The present prospective study was carried out to analyze the genetic variations in DHFR gene of *P. jirovecii* in Indian patients with PCP.

Methodology

The present study was conducted in tertiary care health center at All India Institute of Medical Sciences, New Delhi, India. Demographic and clinical data of each patient were collected and recorded. The study was approved by the institutional ethical committee (IESC/T-77). Informed consent was taken from all the patients who participated in this study.

Specimens

A total of 180 clinical samples comprising of 130 bronchoalveolar lavages (BAL) and 50 induced sputa (IS) were obtained from enrolled patients with a high index of clinical suspicion of PCP (Table 1). Clinical suspicion of PCP was made in patients with symptoms such as persistent non-productive cough, dyspnea, history of protracted fever of many weeks, and with suggestive radiological findings such as bilateral perihilar reticulonodular involvement and ground-glass opacity appearance. Detection of *P. jirovecii* from clinical specimens was done using Grocott's Gomori methenamine silver (GMS) staining [22] as well as by nested PCR assays targeting mitochondrial large subunit ribosomal RNA (mt LSU rRNA) gene and DHFR [12,23].

Sample processing and DNA extraction

All BAL were spun at 4,000 rpm at 4°C for 10 minutes. IS samples were first treated with 0.0065 M dithiothreitol (DTT), a mucolytic agent and then centrifuged at 4,000 rpm on 4°C for 10 minutes. The pellet obtained was re-suspended in one-fifth of supernatant, and 200 µL of the pellet was used for DNA extraction using a Qiagen tissue kit (Qiagen, Valencia, USA).

PCR assay analyses

PCR assay targeting the *P. jirovecii*-specific mt LSU rRNA gene was performed using an equal mixture of primers PAZ102E 5'GATGGC TGTTTCCAAGCCCA-3' and PAZ102H 5'GTGTACGTTGCAAAGTACTC-3' for the external round, and PAZ 102X 5'-GTGAAATACAAATCGGACTAGG-3' and PAZ102Y 5'TCACTTA ATATTAATTGGGGAC-3 for the internal round [23]. For the external round PCR, 2.5 µL of DNA were added to the reaction mixture containing 50 mM KCL, 10 mM Tris, 1.5 mM MgCl2, 0.20 mM dNTPs, 0.4 µL M primers, and 1.8U Taq polymerase. PCR assay was carried out by an initial denaturation at 94°C for 5 minutes followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 30 seconds, and a final extension at 72°C for 5

Table 1. Distribution of the patients involved in the study.

Underlying conditions	No. of adults (n = 150)	No. of children (n = 30)
HIV seropositive (n = 30)	21	9
Transplant recipients (n = 20)	20	0
Malignant disorders (n = 33)	28	5
Autoimmune disorders (n = 7)	07	0
*Other immunocompromised conditions (n = 90)	74	16
Total (n = 180)	**150**	**30**

*Asthma (on steroids); chronic kidney disease; diabetes mellitus; chronic obstructive pulmonary disease (on steroids); pulmonary tuberculosis; diffuse proliferative glomerulonephritis

minutes. For the nested round PCR, 2 µL of undiluted external round product was added into the reaction mixture containing 50 mM KCl, 10 mM Tris, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µL M primers, and 1.8U Taq polymerase. Nested PCR assay for *P. jirovecii*-specific DHFR gene was carried out using published primers FR208-5'-GCAGAAAGTAGGTACATTATTACGAGA-3' and FR10185'-AAGCTTGCTTCAAA CCTTGTGTAACGCG-3' for the external round, and FR242 5'-GTTTGGAATAGATTATGTTCATGGTGTACG-3' and FR1038 5'GCTTCAAACCTTGT GTAACGCG-3' for the internal round [12]. The external round PCR was carried out in a 50 µL reaction mixture containing 50 mM KCl, 10 mM Tris-HCl, 0.1% Triton X-100, 3 mM $MgCl_2$, 0.2 mM concentration of each deoxynucleoside triphosphate, 1 U of Taq DNA polymerase, and 20 pmol of each primer. The nested PCR reaction was carried out using 2 µL of undiluted external round PCR product. PCR conditions for DHFR gene for both external and nested round involved initial denaturation for 10 minutes at 95°C, followed by 35 cycles of denaturation (94°C, 30 seconds), annealing (52°C, 30 seconds), and extension (72°C, 1 minute), with a final extension at 72°C for 5 minutes. All PCR mixture preparation and DNA template addition were carried out in two separate rooms in order to avoid contamination. A negative control (nuclease-free water) and a positive control (DNA of *P. jirovecii* obtained from Dr. Florent Morio, University de Nantes, France) were used to optimize the PCR assays and were included in each run of the assay. Each run of PCR assay was repeated twice in order to rule out any false-positive results. All PCR reactions were performed in a Bio-Rad C1000 thermal cycler. The PCR product obtained was visualized by gel electrophoresis using 2% agarose.

Mutation analyses

PCR products obtained were sequenced with an automated sequencer ABI prism 310 using BigDye Terminator chemistry. Sequencing of samples was repeated twice to confirm the results. DNA chromatograms were examined using BioEdit software version 7.1.3. Both forward and reverse sequences were aligned pair-wise using ClustalW software and were manually refined to obtain a better consensus sequence. Reference sequence with accession ID. AF090368 was used for mutation analyses. Sequences showing nucleotide substitution in the present study were submitted to National Center for Biotechnology Information (NCBI) GenBank and the accession IDs provided for these sequences are KM203096, KM203098, KM203099, KM203100, KM203102, and KM203105.

Results

A total of 180 patients were enrolled in the study, of whom 30 (16.7%, 30/180) were children and 150 (83.3%, 150/180) were adults. Immunocompromised patients included both HIV-seropositive and HIV-uninfected immunocompromised patients including post-renal transplant recipients and patients with malignant and other autoimmune disorders who were on prolonged immunosuppressive therapy (Table 1). Upon microscopic examination using GMS staining, only 4% (7/180) of the samples were positive, whereas

Figure 1. Standardization of nested PCR assay for mitochondrial large subunit ribosomal RNA gene of *P.jirovecii*. Lane 1:- 100 bp molecular marker; Lane 2:- Positive control; Lane 3:- Positive clinical sample; Lane 4:- Negative control.

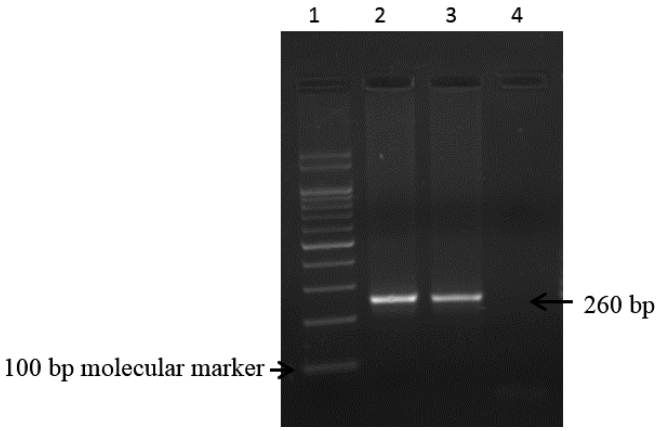
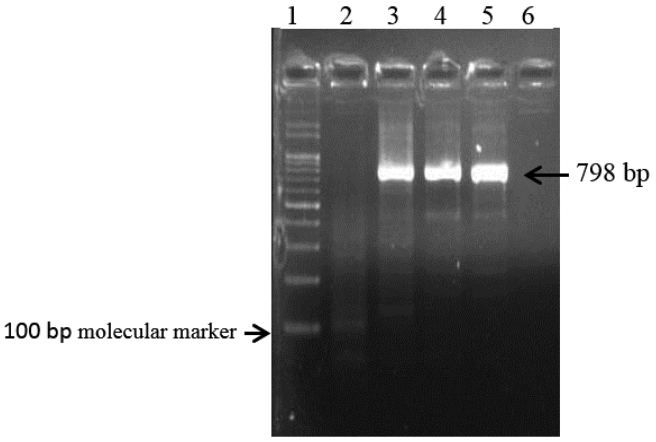


Figure 2. Standardization of Dihydrofolate reductase (DHFR) gene of *P.jirovecii*. Lane 1:- 100 bp molecular marker; Lane 2:- Negative control; Lane 3:- Positive control; Lane 4 & 5:- Positive clinical sample; Lane 6:- Blank.



18 samples (10%, 18/180) were positive by PCR assay targeting mt LSU rRNA gene with a final PCR product of *P. jirovecii*- specific 260 bp (Figure 1). Of these 18, only 14 (14/18, 77%) of a total of 180 clinical specimens (14/180, 7.7%) showed a positive amplification for the *P. jirovecii*-specific DHFR gene (Figure 2). Of these, 13 were male and 1 was female, with a median age of 40 years (range, 5 to 62 years); 5 patients had $PaO_2 \leq 90\%$, whereas the rest had $PaO_2 \geq 90\%$; 9 were on mechanical ventilation. Among HIV-positive patients, all those who were PCP positive had CD4 counts ≤ 200 cells/μL. Half of the PCP-positive patients were co-infected with other types of viral, bacterial, or fungal pneumonia such as cytomegalovirus pneumonia, *Mycobacterium* tuberculosis, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Nocardia*. None of the patients were on TMP-SMX prophylaxis. After the PCP diagnosis, all of them received full treatment. TMP-SMX treatment was successful in 12 patients (85.7%). Two patients had fatal outcomes, one with wild-type DHFR sequence, and one with a mutant DHFR strain (Table 2).

DHFR gene nucleotide polymorphisms analyses

Upon analysis of 14 DHFR gene sequences, 8 were identical to wild-type sequence available in GenBank (WT accession ID AF090368). However, 16 nucleotide substitutions were observed. Out of these 16 nucleotide substitutions, 8 were synonymous (no amino acid change) mutations, and 8 were non-synonymous mutations. Nucleotide substitutions observed in the present study did not match those reported in earlier studies of the *P. jirovecii* DHFR gene, except one at the 68th position (A→G). Only one patient having a non-synonymous mutation at position 72 (A→C) entailing an amino acid change at 24 (Thr→Pro) and two synonymous substitution at position 68 (A→G) and 71 (G→A) had a fatal outcome.

Discussion

The present study examined *P. jirovecii*-specific DHFR gene polymorphisms. Of 180 patients enrolled in this study, only 10% (18/180) of patients were positive for PCP. This correlates with the prevalence rate of PCP in India that was reported in our earlier study [24], suggesting a stable presence of the disease in the Indian population.

DHFR nucleotide sequences were aligned with the wild-type (accession ID AF090368) sequence submitted to the GenBank. Overall, 16 DHFR nucleotide substitution sites in *P. jirovecii* were found in the present study (Table 3). None of the patients included in the present study had long-term exposure to TMP-SMX treatment or were on prophylaxis before being diagnosed with PCP except one patient who was a post-renal transplant recipient and had a fatal

Table 2. Clinical and investigative features of *Pneumocystis* pneumonia (PCP)-positive patients (n = 14).

Age (years) / Sex	Underlying conditions	Fever	Dry cough	Dyspnea (on exertion)	Hypoxia (PaO_2 %)/ ventilation	CD4 count (cells/μL)	Co-infections (microbial pathogens other than PCP)	Treatment	Outcome
55/M	Hodgkin's lymphoma	Yes	Yes	Yes	< 60/Yes	NA	None	TMP-SMX	Survived
5/M	HIV	Yes	Yes	Yes	95/No	= 200	None	TMP-SMX	Survived
26/M	PRT	Yes	Yes	Yes	90/Yes	NA	CMV	TMP-SMX	Survived
48/M	PRT	Yes	Yes	Yes	98/No	NA	CMV	TMP-SMX	Survived
42/M	PRT	Yes	Yes	Yes	85/Yes	NA	*Pseudomonas*	TMP-SMX	**Expired**
57/M	ANCA	Yes	Yes	Yes	< 60/Yes	NA	*Klebsiella*	TMP-SMX	**Expired**
43/M	HIV	Yes	Yes	Yes	96/No	< 200	None	TMP-SMX	Survived
12/M	ALL	Yes	Yes	Yes	< 60/Yes	NA	CMV	TMP-SMX	Survived
38/M	DPGN/CKD ANCA	Yes	Yes	Yes	85/Yes	NA	None	TMP-SMX	Survived
62/F	PRT	Yes	Yes	Yes	93/Yes	NA	None	TMP-SMX	Survived
27/M	TB	Yes	Yes	Yes	98/No	NA	TB	TMP-SMX	Survived
21/M	PRT	Yes	Yes	Yes	95/Yes	NA	None	TMP-SMX	Survived
43/M	COPD	Yes	Yes	Yes	94/Yes	NA	*Nocardia*	TMP-SMX	Survived
11/M	HIV	Yes	Yes	Yes	95/No	< 200	None	TMP-SMX	Survived

HIV: human immunodeficiency virus; PRT: post-renal transplant; NA: not available; ANCA: anti-neutrophil cytoplasmic antibody; CKD: chronic kidney disease; DPGN: diffuse proliferative glomerulonephritis; ALL: acute lymphoblastic leukemia; TB: tuberculosis; COPD: chronic obstructive pulmonary disease; CMV: cytomegalovirus; TMP-SMX: trimethoprim-sulfamethoxazole

outcome. The observed *P. jirovecii* mutations in the DHFR region in the present study did not correspond with the findings of earlier reports except one finding at nucleotide position 68 (A→G). This nucleotide substitution (68 A→G) had already been reported by Costa *et al.* (2006) from Portugal, who observed no relationship between nucleotide change and treatment failure [20]. We observed eight non-synonymous and eight synonymous substitutions (Table 3). DHFR gene sequence from one patient with a fatal outcome showed non-synonymous mutations at position 72 (A→C), which led to an amino acid change at position 24 (Thr→Pro). This nucleotide substitution is being reported for the first time in the present study. Most of the nucleotide substitutions observed in our study are synonymous and therefore may be silent. The relationship between DHFR gene mutations and treatment failure has been reported in a study where 19 mutations were found in 18 patients among 33 PCP-positive patients studied, of which 3 were synonymous and 16 were non-synonymous mutations [19]. The authors of the study reported a relationship between *P. jirovecii* DHFR gene mutations and the use of *Pneumocystis* DHFR inhibitors, and suggested that changes in DHFR amino acids may contribute to the emergence of *Pneumocystis* drug resistance. A higher prevalence of DHFR mutations (61.1%) has been reported in Bangkok [21] than in Portugal [12] and the United States [20]. These studies are in sharp contrast with the study done by Ma *et al.* [12], in which the authors observed one synonymous DHFR mutation at position 312 (T to C) in 32 patients, of which 22 patients received TMP-SMX prophylaxis (7/22) or treatment (15/22) for a previous PCP episode. It was suggested that the mutations in the DHFR gene were not significant and that there is less selective pressure on the DHFR gene. Similar findings were also reported by Takahashi *et al.* [18]. Of 27 patients studied, 4 had mutations in the *P. jirovecii* DHFR gene, of which 2 were synonymous and 2 were non-synonymous. These 2 non-synonymous mutations were not associated with previous exposure to TMP-SMX, and the patients were treated successfully.

In our study, we observed that DHFR gene polymorphisms (found in six cases including the one which had a fatal outcome) may possibly be attributed to TMP-SMX treatment (Table 3) or to geographical segregation. It may also be possible that amino acid substitution at position 24 (Thr→Pro) could confer some level of resistance or selective pressure on the

Table 3. Variations found in the DHFR gene.

Age (years)	Sex	Underlying conditions	TMP-SMX treatment	Nucleotide changes	Amino acids	Treatment outcome
55	M	Hodgkin's lymphoma	Yes	605 T→G 676 G→A 686 T→C 689 T→C 694 G→T 704T→C	201 Ile → Met 225 Gly→Glu 228Val→ Val 229 Pro →Pro 231 Gly →Val 234 Asn→Asn	Survived
5	M	HIV CD4<200 TB	Yes	WT	WT	Survived
26	M	PRT	Yes	401 T→G	133 Gly→Gly	Survived
48	M	PRT	Yes	44 T→A 674T→A 736 G→C	14 His→Gln 224 Val→Val 245 Trp→Ser	Survived
42	M	PRT	Yes	68 A→G 71 G→A **72 A→C**	22 Leu→Leu 23 Arg →Arg **24 Thr→Pro**	Expired
57	M	ANCA	Yes	WT	WT	Expired
43	M	HIV CD4 < 200	Yes	133 T→A 134 A→T	44 Leu→Tyr 44 Leu→Tyr	Survived
12	M	ALL	Yes	WT	WT	Survived
38	M	DPGN/CKD ANCA	Yes	WT	WT	Survived
62	F	PRT	Yes	744 G→T	248 Asp→Tyr	Survived
27	M	TB	Yes	WT	WT	Survived
21	M	PRT	Yes	WT	WT	Survived
43	M	COPD	Yes	WT	WT	Survived
11	M	HIV CD4 < 200	Yes	WT	WT	Survived

WT: wild type (accession ID: AF090368); HIV: human immunodeficiency virus; PRT: post-renal transplant; ANCA: anti-neutrophil cytoplasmic antibody; CKD: chronic kidney disease; DPGN: diffuse proliferative glomerulonephritis; ALL: acute lymphoblastic leukemia; TB: tuberculosis; COPD: chronic obstructive pulmonary disease

DHFR gene, as it was unique to the particular sample's DNA sequence and absent in other sequences. However, *in vitro* study of the drug effect could not be carried out; this was the limitation of this study.

Our study predicts that point mutation found at a single position in the patient with a fatal outcome leading to an amino acid change may be an important cause of PCP treatment failure.

Conclusions

Our results suggest that the polymorphisms of DHFR may contribute to failures of TMP-SMX therapy. As acquisition of resistance is a cumulative process requiring several mutations, the silent mutations found in this study may possibly indicate the evolutionary process of *P. jirovecii*, which may lead to a change in the nucleotide sequences in the future and warrants continuous monitoring.

The present study suggests that *P. jirovecii* DHFR polymorphisms may contribute to TMP-SMX resistance. However, to prove these findings, similar studies with larger sample sizes involving multiple centers are required in order to correlate these findings with clinical outcomes and various epidemiological variants.

Acknowledgements

The authors sincerely thank Department of Science and Technology (DST), Government of India, New Delhi, for providing the financial support to conduct this research. We also thank Dr. Florent Morio, University de Nantes, France, for providing the DNA of *Pneumocystis jirovecii* to be used as a positive control for the study.

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Conflict of interests: No conflict of interests is declared.