Journal of Global Antimicrobial Resistance 17 (2019) 236–239

Contents lists available at ScienceDirect

Journal of Global Antimicrobial Resistance

journal homepage: www.elsevier.com/locate/jgar

Short Communication

Novel dihydropteroate synthase gene mutation in *Pneumocystis jirovecii* among HIV-infected patients in India: Putative association with drug resistance and mortality

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ARTICLE INFO

Article history:
Received 12 October 2018
Received in revised form 14 December 2018
Accepted 9 January 2019
Available online 15 January 2019

Keywords:
Pneumocystis pneumonia
HIV
Dihydropteroate synthase
DHPS mutation
Drug resistance
Mortality

ABSTRACT

Objectives: Pneumocystis pneumonia (PCP) remains a debilitating cause of death among HIV-infected patients. The combination trimethoprim/sulfamethoxazole (SXT) is the most effective anti-*Pneumocystis* treatment and prophylaxis. However, long-term use of this combination has raised alarms about the emergence of resistant organisms. This study was performed to investigate mutations in the dihydropteroate synthase (*DHPS*) gene and their clinical consequences in HIV-infected patients with PCP.
Methods: A total of 76 clinically suspected cases of PCP among HIV-seropositive adult patients from March 2014 to March 2017 were included. Clinical samples (bronchoalveolar lavage fluid and sputum) were investigated for the detection of *Pneumocystis jirovecii* using both microscopy and nested PCR. *DHPS* genotyping and mutational analyses were performed and the data were correlated with clinical characteristics.
Results: Among the 76 enrolled HIV-positive patients, only 17 (22.4%) were positive for *P. jirovecii*. *DHPS* gene sequencing showed a novel nucleotide substitution at position 288 (Val96Ile) in three patients (3/12; 25.0%). Patients infected with the mutant *P. jirovecii* genotype had severe episodes of PCP, did not respond to SXT and had a fatal outcome ($P = 0.005$). All three patients had a $CD4^+$ T-cell count <100 cells/μL, and two also had co-infections.
Conclusion: This study suggests that the emergence of a mutant *P. jirovecii* genotype is probably associated with drug resistance and mortality. The data also suggest that *DHPS* mutational analyses should be performed in HIV-seropositive patients to avoid treatment failure and death due to PCP. However, the role of underlying disease severity and co-morbidities should not be underestimated.

1. Introduction

Pneumocystis pneumonia (PCP) is considered a potentially life-threatening pneumonia among human immunodeficiency virus (HIV)-seropositive individuals, especially in patients with a $CD4^+$ T-cell count <200 cells/μL [1]. Although the prevalence of PCP has decreased to some extent owing to the administration of combination antiretroviral therapy (cART) [2], it still remains as an important disease among those who are either not aware of their HIV status and present in late stage with a very low $CD4^+$ T-cell count or those who do not adhere to cART treatment [3,4].

Trimethoprim and sulfamethoxazole target the dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) enzymes, respectively, and this combination is the drug of choice both for treatment and prophylaxis against PCP [4,5]. Studies on the *Pneumocystis jirovecii*-specific *DHPS* gene have shown that resistance to trimethoprim/sulfamethoxazole (SXT) occurs due to genetic mutations, and it has been reported that human-to-human

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https://doi.org/10.1016/j.jgar.2019.01.007

transmission of *P. jirovecii* infection may possibly be a reason behind the spread of mutant or resistant strains [6–8].

It has also been observed that the incidence of *DHPS* mutations varies between countries [6]. The present study was prospectively carried out among HIV-positive adult patients who presented with a first episode of PCP to our tertiary care centre in order to determine the frequency of mutations in the *DHPS* gene of *P. jirovecii* isolates.

2. Materials and methods

2.1. Clinical samples

A total of 77 respiratory samples, including bronchoalveolar lavage fluid ($n = 56$) and sputa (expectorated and/or induced) ($n = 21$) were collected from 76 HIV-seropositive adult patients with a high index of clinical suspicion of PCP between March 2014 and March 2017. Relevant demographic and clinical data were recorded from all patients. The study was approved by the Institutional Ethics Committee of the All India Institute of Medical Sciences (New Delhi, India) and informed consent was obtained from all participants.

2.2. Laboratory diagnosis of Pneumocystis *pneumonia and* DHPS *genotyping*

Clinical samples were processed for detection of *P. jirovecii* using both microscopy (Grocott–Gomori's methenamine silver staining) and nested PCR (nPCR) targeting the mitochondrial large subunit rRNA of *P. jirovecii* [9]. *DHPS* genotyping was done using nPCR [10] followed by direct sequencing using BigDye® Terminator chemistry with an Applied Biosystems® 3130xl Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA). The assays were repeated twice to ensure any possible mutations. Subsequent analyses were performed using BioEdit software v.7.2.5 and MEGA 7.0.14. Reference sequences used for the mutational analyses were **AY628435**, **U66278** and **U66281**. The GenBank accession nos. for the novel genotypes are **MH708887**, **MH708889** and **MH708890**, respectively.

2.3. Statistical analyses

Statistical analyses were performed using *t*-test and Wilcoxon rank-sum test for all variables. Clinical correlations with mutant and wild-type genotypes were determined using the Fisher's exact test, with $P < 0.05$ considered significant with 95% confidence interval.

3. Results

3.1. Clinical characteristics (Table 1)

Among the 76 enrolled patients, 61 (80.3%) were male and 15 (19.7%) were female, with a mean age of 43.5 years (range 19–68 years). Upon clinical examination, 52 patients (68.4%) had fever and also complained of dry cough and 65 patients (85.5%) complained of breathlessness on exertion. Chest radiography revealed bilateral perihilar infiltrates in 21 patients (27.6%) and another 15 patients (19.7%) had other kinds of radiological findings such as mediastinal lymphadenitis, miliary shadows, bilateral lung nodules, symmetrical consolidation or patchy consolidation. The mean $CD4^+$ T-cell count was 338 cells/μL (range 8–669 cells/μL).

Only 17 patients (22.4%) were positive for *P. jirovecii* both by microscopic examination and nPCR assay, of whom 16 (94.1%) had a $CD4^+$ T-cell count <200 cells/mm^3 (Table 1). Only 2 of the 17 PCP-positive patients were aware of their HIV status and were on antiretroviral treatment, whereas in the remaining 15 cases the HIV diagnosis was made within 1 month prior to the present and first episode of *Pneumocystis* infection. None of the patients had received anti-*Pneumocystis* prophylaxis previously. Among the 17 PCP-positive patients, only 15 had fever and 12 showed bilateral perihilar infiltrates on chest radiography, with 11 of them showing ground-glass opacity on computed tomography (CT). During the study period, all patients were treated with ART as well as SXT,

Table 1
Clinical characteristics of human immunodeficiency virus (HIV)-seropositive patients.[a]

Characteristic	HIV-positive ($n = 76$)	HIV-positive with PCP ($n = 17$)
Sex		
Male	61	14
Female	15	3
Mean age (years)	43.5	34.6
Clinical features at time of presentation		
Fever	53	15
Cough	68	16
Dyspnoea	65	16
Chest radiography		
Bilateral perihilar infiltrate	21	12
Pattern other than bilateral perihilar infiltrate[b]	15	3
Normal	19	0
CT scan (42/76)		
Ground-glass opacity	29	11
Other[c]	2	0
$CD4^+$ T-cell count		
<200 cells/μL	37	16
>200 cells/μL	39	1
On cART	7	2
Anti-*Pneumocystis* prophylaxis	2	0
Antimicrobial treatment		
Antitubercular treatment	4	2
Other antimicrobial treatment[d]	3	5

PCP, *Pneumocystis* pneumonia; CT, computed tomography; cART, combination antiretroviral therapy.
[a] Data are number of patients unless otherwise stated.
[b] Includes mediastinal lymphadenitis, miliary shadows, bilateral lung nodules, symmetrical consolidation or patchy consolidation.
[c] Includes mediastinal lymph nodes.
[d] For bacterial, viral or fungal infection.

except in one case where treatment with SXT was changed to clindamycin and primaquine combination owing to suspicion of bone marrow suppression and the patient responded well. However, three patients under sulfa (SXT) treatment did not respond to the treatment and died due to respiratory failure primarily due to PCP. Two of these patients had co-infection, one with concomitant *Mycobacterium tuberculosis* (pulmonary tuberculosis) and *Klebsiella pneumoniae* infection, and another with cytomegalovirus. All three patients had a $CD4^+$ T-cell count <100 cells/μL.

3.2. Mutational analysis of the DHPS gene target and its clinical correlation

DHPS genotyping was performed in only 12 samples, whereas in 5 samples optimal amplification could not be achieved for such analysis. *DHPS* genotyping revealed a novel nucleotide substitution at position 288 (G→A) in the conserved region in 3 samples (25.0%) resulting in a non-synonymous amino acid change from valine to isoleucine at codon 96 (Val96Ile) (Fig. 1); the other 9 samples (75.0%) had a wild-type sequence. All three patients with mutation required intensive care unit (ICU) admission ($P = 0.045$) and mechanical ventilation ($P = 0.018$) and had a higher respiratory rate (mean 31 breaths/min) compared with patients infected with wild-type ($P = 0.02$). One patient among them had a history of long-term smoking. Co-infections were reported in two of these cases ($P = 0.045$) (Table 2). All three patients died due to respiratory failure.

Table 2
Genotyping of the dihydropteroate synthase (*DHPS*) gene and its clinical significance.

Clinical characteristic	*DHPS* genotype ($n = 12$)[a]		*P*-value
	Mutant (Val96Ile)	Wild-type	
Total patients	3	9	
Mean age in years (no. male)	40.2 (3)	35.2 (7)	0.545
Mean $CD4^+$ T-cell count (cells/μL)	46	145	0.392
ICU admission	3	2	0.045
Mechanical ventilation	3	1	0.018
Weight loss	1	2	1
Loss of appetite	2	3	0.523
Alcohol intake	0	2	1
Smoking	1	2	1
Hypoxaemia (SpO_2) (mean %)	69	76	0.504
Mean respiratory rate (breaths/min)	31	23	0.02
Mean haemoglobin (g/dL)	9.3	10.6	0.193
Mean albumin (g/dL)	2.67	2.75	0.8
Co-infection[b]	2	0	0.045
HIV diagnosis			
Recent[c]	2	8	
Earlier (>1 year)	1	1	
Hospital stay			
<4 weeks	3	9	
>4 weeks	0	0	
Died	3	0	0.005

ICU, intensive care unit; HIV, human immunodeficiency virus.
[a] *DHPS* genotyping was performed in only 12 samples (in 5 samples optimal amplification could not be achieved for such analysis).
[b] Includes one with concomitant *Mycobacterium tuberculosis* and *Klebsiella pneumoniae* infection and one with cytomegalovirus infection.
[c] Presented with late-stage HIV infection (not aware of their HIV status prior to current clinical visit).

4. Discussion

DHPS gene mutations in *P. jirovecii* and their association with clinical outcome among HIV-positive patients have been reported [6,11,12]. To date, the two most common *DHPS* genotypes associated with sulfa drug resistance and treatment failure are *DHPS* genotypes 55 and 57 (at nucleotide positions 165 and 171, respectively) [11]. It has been suggested that any novel mutations or accumulation of additional mutations observed in coding regions may result in structural changes in the gene and would possibly exert some drug resistance as reported for genotypes 55 and 57 [13].

In the present study, a PCP prevalence of 22.4% (17/76) among HIV-infected adult patients was observed. An interesting observation of this study is that it reported a two times higher prevalence than that reported previously from our laboratory [14]. This finding that PCP is increasing despite the availability of cART is somewhat alarming. A possible reason for this increase could be attributed to the unawareness of enrolled cases (15/17; 88.2%) of their HIV status and thus presenting at a late stage of HIV disease with a $CD4^+$ T-cell count <200 cells/μL. In addition, 15 of the 17 HIV-seropositive patients positive for *P. jirovecii* had not received ART before presenting to us. By mutational analyses of the *DHPS* gene, a novel mutant strain was observed in 25.0% (3/12) of infected patients, whereas the remaining 9 (75.0%) had no mutations and responded well to treatment. Genotyping was not possible in five samples as optimal amplification could not be achieved, which could possibly be explained by the nature of the *DHPS* gene (single copy) and/or because of a low fungal burden in clinical samples. The mutant observed in the present study differed from that commonly reported worldwide [11–13,15] and the apparent absence of these common mutations in the current study indicates possible geographical variation or might be because none of the enrolled patients received SXT prophylaxis previously, as reported earlier [15].

This non-synonymous change occurred at nucleotide position 288 (Val96Ile) (Fig. 1), which is possibly associated with sulfonamide resistance; however, this needs to be further verified in silico, which is a limitation of this study. All three HIV-positive

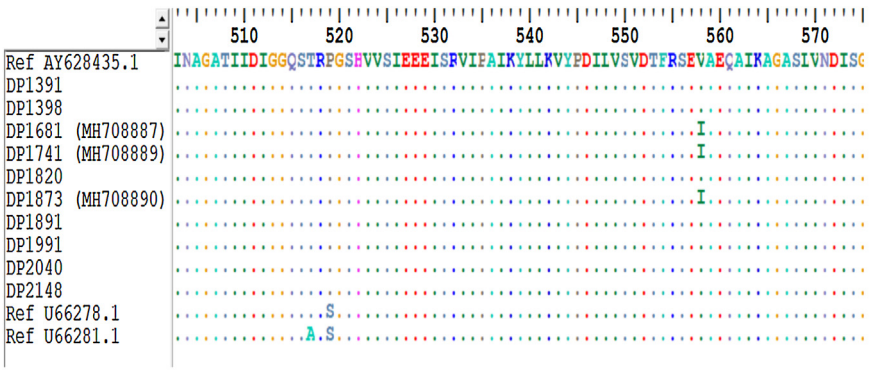

Fig. 1. Amino acid change at codon 96 (Val96Ile). GenBank accession nos.: **AY628435**, reference (wild-type); **U66278** and **U66281**, mutant genotype (codon 55/57). GenBank accession no. of novel mutations: **MH708887**, **MH708889** and **MH708890**.

patients infected with this mutant strain of *P. jirovecii* had a severe episode of PCP, had risk of ICU admission ($P = 0.045$), mechanical ventilatory support ($P = 0.018$) and had a fatal outcome ($P = 0.005$). PCP leading to respiratory failure was the cause of death in all three patients, along with co-infections in two cases. Co-infections could possibly have played an important role in disease severity and increased mortality. Studies have shown that co-existing pulmonary infections could be a possible reason behind rapid deterioration among non-survivors compared with survivors despite potentially effective antibiotic therapy [16,17].

All patients in this study had a first episode of PCP and none had received any anti-*Pneumocystis* prophylaxis, implicating possible human-to-human transmission for acquiring such mutations. Similar observations of interhuman transmission of the resistant *DHPS* gene in patients with a first episode of PCP and its clinical response to SXT have been reported previously [6,8,10]. However, the exact mechanism and selection of this resistance gene is still not clear.

In conclusion, although the number of patients included in this study was relatively small, important information observed was the increase in PCP incidence among HIV-infected patients compared with our previous study. Second, this study revealed the emergence of a novel *DHPS* genotype putatively associated with drug resistance and mortality. This information makes it imperative that disease progression should be monitored and *DHPS* genotyping should be performed to assess treatment failure in HIV-positive patients, if at all. This has also been highlighted by a recent study suggesting that mutations in the *DHPS* gene, especially in HIV-positive patients with PCP, do occur due to selective pressure of the drug [6]. However, ECIL-5 guidelines [18] do not emphasise monitoring of *DHPS* mutations in patients with haematological malignancies or in stem cell transplant recipients.

Funding

The authors thank the Science and Engineering Research Board, Department of Science and Technology, Government of India, for providing financial support for carrying out this work [grant SB/SO/HS-0046/2013].

Competing interests

None declared.

Ethical approval

This study was approved by the Institutional Ethics Committee of the All India Institute of Medical Sciences (New Delhi, India) [IESC/T-77].

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