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Apparent Absence of Selective Pressure on *Pneumocystis jirovecii* Organisms in Patients with Prior Methotrexate Exposure

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ABSTRACT *Pneumocystis jirovecii* infections occur in patients treated with methotrexate (MTX) because of immunosuppressive effects of this highly potent dihydrofolate reductase (DHFR) inhibitor. Conversely, MTX may act as an anti-*P. jirovecii* drug and consequently may exert a selective pressure on this fungus. In this context, we compared the sequences of the *dhfr* gene of *P. jirovecii* isolates obtained from two groups of patients with *P. jirovecii* infections. The first group, with systemic diseases or malignancies, had prior exposure to MTX (21 patients), whereas the second group (22 patients), the control group, did not. Three single nucleotide polymorphisms (SNPs) were observed at positions 278, 312, and 381. The first one was located in the intronic region and the two others were synonymous. Based on these SNPs, three *P. jirovecii dhfr* alleles, named A, B, and C, were specified. Allele A was the most frequent, as it was observed in 18 patients (85.7%) and in 16 patients (72.7%) of the first and second groups, respectively. No significant difference in *P. jirovecii dhfr* gene diversity in the two patient groups was observed. In conclusion, these original results suggest that MTX does not exert an overt selective pressure on *P. jirovecii* organisms.

KEYWORDS *Pneumocystis jirovecii*, *dhfr*, dihydrofolate reductase, genetic diversity, methotrexate

Pneumocystis jirovecii is an opportunistic and transmissible fungus responsible for severe pneumonia, *Pneumocystis pneumonia* (PCP), in immunocompromised patients. PCP remains the most frequent AIDS-defining illness in human immunodeficiency virus (HIV)-infected patients in metropolitan France (1). PCP also occurs in other immunosuppressed patients not infected with HIV, such as those treated with immunosuppressive and/or cytostatic therapies (2). In this context, cases of PCP in patients treated with methotrexate (MTX) monotherapy or MTX associated with other immunosuppressive drugs have been reported (3). Using analysis of databases on [ncbi.nlm.nih.gov](https://pubmed.ncbi.nlm.nih.gov/) (PubMed), we identified about 180 citations comprising *Pneumocystis* and MTX (key words “*pneumocystis* [Title/Abstract]” and “methotrexate [Title/Abstract]”) during the period from 1976 to 2021, mostly related to patients with systemic diseases, specifically rheumatoid arthritis (75 citations from 1983 to 2021).

In other respects, *P. jirovecii* infection prophylaxis and treatment are based on the wide use of sulfamethoxazole and trimethoprim (SMX-TMP), which target dihydropteroate

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synthase (DHPS) and dihydrofolate reductase (DHFR), respectively, two enzymes involved in folate biosynthesis. Two major nonsynonymous mutations have been described at nucleotide positions 165 and 171 of the *dhps* locus of *P. jirovecii* (4). Variants harboring these mutations have been associated with failure of sulfonamide prophylaxis and putative resistance of *P. jirovecii* organisms to these drugs (reviewed in reference 5). Currently, prior exposure to sulfonamides and the city of patient residence have been identified as the two main predictors of *P. jirovecii* DHPS mutants worldwide (6, 7). Nonsynonymous mutations have also been described in the *dhfr* gene of *P. jirovecii* (8–12). Some variants harboring nonsynonymous mutations have been associated with failure of prophylaxis by trimethoprim and trimethoprim analogues (9) and putative resistance of *P. jirovecii* organisms to these drugs (13). *In vitro* experiments have also shown resistance to trimethoprim inhibition in recombinant enzymes harboring nonsynonymous mutations (14, 15). Considering that MTX is a highly potent DHFR inhibitor in mammalian cells and in *P. carinii* organisms (16), we hypothesized that beyond its cytotoxic activity, the drug may exert selective pressure on *P. jirovecii* organisms. To test this hypothesis, genetic diversities of *dhfr* gene of *P. jirovecii* organisms obtained from two patient groups were compared. One group had prior exposure to MTX, whereas the second one did not, at the time of *P. jirovecii* infection diagnosis.

RESULTS

Taken together, no *P. jirovecii dhfr* single nucleotide polymorphisms (SNPs) were observed in 31 patients (17 from the first group and 14 from the control group) in comparison to the reference sequence AF090368 described by Ma and colleagues (17). Three *P. jirovecii dhfr* SNPs were observed among the 12 other patients (four from the first group and eight from the control group). The first SNP, at nucleotide position 312 (T312C), was observed in patients B4, B10 and B18 of the first group and in patients B22, B25, B29, B31, B32, B35, B37, and B43 of the control group. The second SNP, at nucleotide position 381 (C381T), was observed only in patient B37 of the control group. These two SNPs correspond to synonymous mutations previously reported in references 17 and 11, respectively. The third SNP, previously described (10) and located in the unique intronic region of *dhfr* gene, was observed at nucleotide position 278 (C278T) only in patient B15 of the first group. Four cases of mixed infections were detected. Specifically, a C residue and a T residue were identified at nucleotide position 312 in patients B18, B32, and B43. In patient B37, a C residue and a T residue were identified at nucleotide positions 312 and 381 (Table 1 and Table 2).

Three *P. jirovecii dhfr* alleles, named A, B, and C, were determined in relation to the three aforementioned SNPs (Table 1 and Table 2). Allele A was the most frequent allele and was observed in 18 patients of the first group and in 16 patients of the second group. Allele B was observed in three patients of the first group and in seven patients of the control group. Allele C was observed only in one patient of the first group. The allele mix was not determined in patient B37 because of the presence of several different alleles that could not be identified (Table 1 and Table 2). No significant difference in allele distribution was shown between the two groups of patients (Fisher's exact test, $P = 0.28$).

DISCUSSION

In this study, we provided the first data on *P. jirovecii dhfr* gene diversity in patients with prior exposure to MTX, compared to patients without prior exposure to MTX. Three different SNPs whose association made it possible to describe three putative different alleles, A, B, and C, were detected. The results pointed out the diversity of the *P. jirovecii dhfr* gene for all patients taken together. Nonetheless, this genetic diversity does not differ in patients with or without prior exposure to MTX, considering the absence of any significant difference in allele distribution effectively observed in these two patient groups.

Nahimana and colleagues have reported a significant association between nonsynonymous mutations on the *dhfr* gene of *P. jirovecii* and failure of PCP prophylaxis

TABLE 1 Characteristics of 21 patients with prior exposure to methotrexate at the time of *Pneumocystis jirovecii* infection diagnosis and the identified SNPs and alleles of the *P. jirovecii dhfr* gene

Patient code ^e	Underlying condition(s)	Patient still on MTX at time of <i>Pneumocystis jirovecii</i> diagnosis	Duration of MTX treatment before <i>Pneumocystis jirovecii</i> diagnosis (days)	Sample	Presentation of <i>Pneumocystis jirovecii</i> infection (alternative diagnosis of PCP)	Treatment of <i>P. jirovecii</i> infection	Residue at position:					
							Outcome	278	312	381	Allele	
B1	Horton's disease	Yes	>90	BAL ^d	PCP ^e	SMX-TMP ^g	Improvement	C	T	C	C	A ^f
B2	Rheumatoid arthritis, glioblastoma	No ^c	>90	Sputum	PCP	SMX-TMP	Improvement	C	T	C	C	A
B3	Rheumatoid arthritis	Yes	15	BAL	Colonization (bacterial pneumonia) ^f	— ^h	Improvement	C	T	C	C	A
B4	Still's disease	Yes	>90	BAL	Colonization (autoimmune lung disease) ^f	—	Improvement	C	C	C	C	B
B5	Rheumatoid arthritis	Yes	>90	BAL	Colonization (iatrogenic lung disease) ^f	—	Improvement	C	T	C	C	A
B6	Rheumatoid arthritis	Yes	>90	BAL	PCP	SMX-TMP	Deterioration	C	T	C	C	A
B7	Rheumatoid arthritis	Yes	>90	BAL	PCP	SMX-TMP	Improvement	C	T	C	C	A
B8	Wegener's granulomatosis	Yes	>90	BAL	PCP	Atovaquone	Improvement	C	T	C	C	A
B9	Acute myeloid leukemia	Yes	18	BAL	PCP	SMX-TMP	Improvement	C	T	C	C	A
B10	Polyarthritis nodosa	Yes	44	BAL	PCP	SMX-TMP	Improvement	C	C	C	C	B
B11	Rheumatoid arthritis, bronchial carcinoma	Yes	68	Sputum	PCP	SMX-TMP	Deterioration	C	T	C	C	A
B12	Psoriasis	Yes	>90	BAL	PCP	SMX-TMP	Improvement	C	T	C	C	A
B13	Rheumatoid arthritis	Yes	>90	BAL	PCP	SMX-TMP	Improvement	C	T	C	C	A
B14	Rheumatoid arthritis	Yes	>90	BAL	PCP	SMX-TMP	Improvement	C	T	C	C	A
B15	Psoriasis	No ^c	>90	Sputum	Colonization (autoimmune lung disease) ^f	—	Improvement	T	T	C	C	C
B16	Rheumatoid arthritis	Yes	>90	BAL	Colonization (autoimmune lung disease) ^f	—	Improvement	C	T	C	C	A
B17	Rheumatoid arthritis	Yes	>90	BAL	PCP	SMX-TMP	Deterioration	C	T	C	C	A
B18 ^b	Breast carcinoma	Yes	>90	BAL	PCP	SMX-TMP	Deterioration	C	C/T ⁱ	C	C	A and B
B19	Polymyalgia rheumatica	Yes	>90	Sputum	Colonization (iatrogenic lung disease) ^f	—	Improvement	C	T	C	C	A
B20	Polymyalgia rheumatica	No ^c	>90	BAL	Colonization (bacterial pneumonia) ^f	—	Improvement	C	T	C	C	A
B21	Horton's disease	Yes	41	Sputum	PCP	SMX-TMP, atovaquone	Improvement	C	T	C	C	A

^aPatients numbered from B1 to B21 were monitored at Brest University Hospital, Brest, France. Age, sex, and dates were not indicated *in extenso*, since they could be identifiable patient information.

^bMixed infection in patient B18.

^cFor patients B2, B15, and B20, methotrexate treatment was stopped 97, 4, and 17 days, respectively, before *P. jirovecii* infection diagnoses.

^dBAL, bronchoalveolar lavage fluid.

^ePCP, *Pneumocystis pneumonia*.

^f*P. jirovecii* was not detected by microscopic examination. The fungus was only detected by PCR assay. The patients presented alternative diagnoses of PCP, and they improved despite the absence of specific treatment against *P. jirovecii*.

^gSMX-TMP, sulfamethoxazole-trimethoprim.

^h—, patients B3, B4, B5, B15, B16, B19, B20 did not receive any specific treatment against *P. jirovecii* infection.

ⁱTwo nucleotides were identified at this position on sequence electrophoregrams.

^jAllele A corresponds to the reference sequence (GenBank accession no. AF090368) (17). It harbors C, T, and C residues at nucleotide positions 278, 312, and 381, respectively.

TABLE 2 Characteristics of 21 patients without known prior exposure to methotrexate at the time of *Pneumocystis jirovecii* infection diagnoses and the identified SNPs and alleles of the *P. jirovecii dhfr* gene

Patient code ^a	Underlying condition(s)	Sample	Presentation of <i>P. jirovecii</i> infection (alternative diagnosis of PCP)	Treatment of <i>P. jirovecii</i> infection	Outcome	Residue at position:			Allele
						278	312	381	
B22	HIV infection	BAL ^d	PCP ^f	SMX-TMP ^h	Improvement	C	C	C	B
B23	HIV infection	BAL	PCP	SMX-TMP	Improvement	C	T	C	A ^k
B24	Esophageal carcinoma, COPD ^c	BAL	Colonization (iatrogenic lung disease) ^g	— ⁱ	Improvement	C	T	C	A
B25	Kidney transplant	BAL	PCP	SMX-TMP, atovaquone	Deterioration	C	C	C	B
B26	Myeloma	BAL	PCP	SMX-TMP	Deterioration	C	T	C	A
B27	Cholangiocarcinoma	BAL	PCP	SMX-TMP	Deterioration	C	T	C	A
B28	Prostatic carcinoma	BAL	PCP	SMX-TMP	Deterioration	C	T	C	A
B29	Kidney transplant	BAL	PCP	SMX-TMP	Improvement	C	C	C	B
B30	HIV infection	BAL	Colonization (bacterial pneumonia) ^g	— ⁱ	Improvement	C	T	C	A
B31	Esophageal carcinoma	BAL	PCP	SMX-TMP	Deterioration	C	C	C	B
B32 ^b	Lymphoma	BAL	PCP	SMX-TMP	Deterioration	C	C/T ^j	C	A and B
B33	HIV infection	Sputum	PCP	Atovaquone	Deterioration	C	T	C	A
B34	Glioblastoma	Sputum	PCP	SMX-TMP	Deterioration	C	T	C	A
B35	Kidney transplant	BAL	PCP	SMX-TMP	Deterioration	C	C	C	B
B36	HIV infection	BAL	PCP	SMX-TMP	Improvement	C	T	C	A
B37 ^b	Lung carcinoma	Sputum	PCP	SMX-TMP	Deterioration	C	C/T ^j	C/T ^j	Mixed ^l
B38	Kidney-liver transplant	Sputum	PCP	SMX-TMP	Deterioration	C	T	C	A
B39	Acute myeloid leukemia	BAL	PCP	SMX-TMP, atovaquone	Deterioration	C	T	C	A
B40	Lymphoma	ETA ^e	PCP	SMX-TMP	Improvement	C	T	C	A
B41	Lung carcinoma	BAL	PCP	SMX-TMP	Improvement	C	T	C	A
B42	Lymphoma	Sputum	PCP	Atovaquone, caspofungin, pentamidine	Deterioration	C	T	C	A
B43 ^b	HIV infection	BAL	PCP	SMX-TMP	Improvement	C	C/T ^j	C	A and B

^aPatients numbered from B22 to B43 were monitored at Brest University Hospital, Brest, France. Age, sex, and dates were not indicated *in extenso*, since it could be identifiable patient information.

^bMixed infections in patients B32, B37, and B43.

^cCOPD, chronic obstructive pulmonary disease.

^dBAL, bronchoalveolar lavage fluid.

^eETA, endotracheal aspiration.

^fPCP, *Pneumocystis pneumonia*.

^g*P. jirovecii* was not detected by microscopic examination. The fungus was only detected by PCR assay. The patients presented alternative diagnoses of PCP, and they improved despite the absence of specific treatment against *P. jirovecii*.

^hSMX-TMP, sulfamethoxazole-trimethoprim.

ⁱ—, patients B24 and B30 did not receive any specific treatment against *P. jirovecii* infection.

^jTwo nucleotides were identified at this position on sequence electropherograms.

^kAllele A corresponds to reference sequence (GenBank accession no. AF090368) (17). It harbors C, T, and C residues at nucleotide positions 278, 312, and 381, respectively.

^lAllele mix was not determined because of the presence of several different alleles that could not be identified. Considering previous description of SNP C381T by Costa et al. (11), the association of allele B with another putative allele harboring 278C, 312T, and 381T is the most likely hypothesis.

based on DHFR inhibitors (9). These inhibitors were mainly pyrimethamine, rarely trimethoprim, and not MTX. Obviously, MTX is not used for PCP prophylaxis or treatment because of its cytotoxic activity, while it is mainly used as a long-term treatment for systemic diseases or malignancies. This cytotoxic activity is partly due to the fact that mammalian cells harbor a high-affinity transmembrane transport system, the reduced folate carrier (RFC), which is the major route for MTX uptake (18).

In the present study, 18 out of 21 patients with prior exposure to MTX were still treated with MTX when *P. jirovecii* infections were diagnosed. Occurrence of these infections in this context suggested ineffectiveness of the drug against the fungus and consequently in PCP prevention. Conversely, MTX is known to have a strong *in vitro* competitive inhibitor effect on *Pneumocystis* DHFR (16). These discrepant data between *in vitro* and *in vivo* observations can be explained by the fact that *Pneumocystis* organisms may not have the aforementioned transmembrane transport system, the absence of which reduces MTX uptake (16). Indeed, no significant similarity was found in genomes and proteomes of *P. jirovecii* (BioProject no. PRJEA68827 from Cisse and colleagues [19]; BioProject no. PRJNA223510 from Ma and colleagues [20]), *Pneumocystis carinii* (BioProject no. PRJNA223511 [20]), *Pneumocystis murina* (BioProject no. PRJNA70803 [20]), and other closely related fungi, i.e., *Saccharomyces cerevisiae* (BioProject no. PRJNA393501 [21]) and *Schizosaccharomyces pombe* (BioProject no. PRJNA527756 [22]), using the RFC protein of *Homo sapiens*, *Pan troglodytes* (chimpanzee), *Bos taurus* (cattle), *Rattus norvegicus* (Norway rat), and *Mus musculus* (mouse) (GenBank accession no. AAC50180, XP_001157360, NP_001069921, NP_001030309, and NP_112473, respectively) as query sequences in tblastn and blastp searches (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). This decreased MTX uptake may explain the apparent low selective pressure exerted by MTX. Moreover, the absence of nonsynonymous mutation in the *dhfr* gene of *P. jirovecii* in the 16 patients with long-term MTX exposure (more than 90 days) is consistent with a low selective pressure exerted by MTX. Our approach was essentially based on detection and identification of nonsynonymous mutations as recently reviewed and published by Leidner and colleagues (15). However, it has been suggested that synonymous mutations may affect protein translational kinetics and drug-target interactions (23–25). This may represent a new field of investigation for studies on *P. jirovecii*. Be that as it may, in the present study, no significant difference in SNP distributions was shown between patients with and without prior MTX exposure.

The average/low number of patients enrolled in this retrospective monocentric study may explain the absence of any significant difference of *P. jirovecii dhfr* gene diversity in patients with or without prior exposure to MTX. Indeed, despite the 14-year period of inclusion, only 21 patients met the criteria for being enrolled in the first patient group of this study. In conclusion, these original results show that MTX does not exert an overt selective pressure on *P. jirovecii* organisms so far.

MATERIALS AND METHODS

A request was made with the collaboration of the Clinical Data Center regarding patients who tested positive for *P. jirovecii* infection at Brest University Hospital during the period from 31 March 2008 to 30 March 2022 on the basis of the patients' prior exposure to MTX during the year before *P. jirovecii* infection diagnosis, whatever the MTX dosage and its duration. Among the 43 patients initially selected, only 21 were retained on the basis of the availability and quality of patients' archival *P. jirovecii* DNA specimens. In this first group of 21 patients, the median age was 74 years (range, 42 to 89 years); the male/female ratio was 10/11. Underlying conditions were autoimmune diseases (17 patients), glioblastoma and rheumatoid arthritis (1 patient), bronchial carcinoma and rheumatoid arthritis (1 patient), acute myeloid leukemia (1 patient), and breast carcinoma (1 patient) (Table 1). A second group of patients contemporaneously diagnosed with *P. jirovecii* infection served as a control group, consisting of 22 patients without known prior exposure to MTX at the time of *P. jirovecii* infection diagnosis. The median age was 64 years (range, 30 to 81 years); the male/female ratio was 18/4. Underlying conditions included cancer (6 patients), HIV infection (6 patients), hematological malignancy (5 patients), organ transplantation (4 patients), and esophageal carcinoma with chronic obstructive pulmonary disease (COPD) (1 patient) (Table 2). None of the patients of the first and second groups had sulfonamide PCP prophylaxis. Only one patient of the second group (patient B33) had prior exposure to sulfonamides related to one PCP treatment 2 years earlier.

The diagnosis of PCP was initially made in the parasitology and mycology laboratory using direct

microscopy on bronchoalveolar lavage (BAL) specimens (calcofluor white and Wright-Giemsa stains) and a real-time PCR assay targeting the *P. jirovecii* mitochondrial large subunit rRNA gene (*mtLSUrRNA*) (26, 27) on BAL, endotracheal aspirate, or induced sputum specimens. The PCR assays were performed after a DNA extraction procedure using NucliSens easyMag (bioMérieux, Marcy l'Etoile, France). Seven patients of the first group and two patients of the second group were considered to be colonized by *P. jirovecii*, since they presented alternative diagnoses of PCP and/or did not receive specific treatment against *P. jirovecii*. *P. jirovecii* was only detected by the PCR assay because of the low fungal burdens in these patients. Thirty-four patients, including 14 patients of the first group and 20 of the second group, had overt PCP, with diagnoses based on clinical and radiological criteria combined with *P. jirovecii* detection (Table 1 and Table 2). The corresponding 43 *P. jirovecii* isolates had been stored at -80°C for further examination.

The primer pair FR208 and FR1018 was used to amplify the full length of the *dhfr* gene as described elsewhere (17). Primer specificity was checked using the Basic Local Alignment Search Tool (BLAST) (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). PCRs were carried out in a final volume of 50 μL , containing 200 μM each deoxynucleoside triphosphate (deoxynucleoside triphosphate set; Eurogentec, Seraing, Belgium), 0.5 μM forward and reverse primers, 2 U of DNA polymerase (HGS Diamond *Taq* DNA polymerase, Eurogentec) and MgCl_2 at a final concentration of 3 mM. Amplification was conducted under the following conditions: an activation step at 95°C for 10 min followed by 45 cycles, including denaturation at 94°C for 30 s, annealing at 60°C for 45 s and extension at 72°C for 1 min, and then a final extension step at 72°C for 10 min. The PCR products were electrophoresed on a 1.5% agarose gel containing ethidium bromide to visualize the expected bands of 832 bp. To avoid contamination, each step was performed in different areas with different sets of micropipettes. The reagents used in the PCR mixtures were prepared in a laminar flow cabinet. To monitor for possible contamination, negative controls were included. PCR products were purified using ExoSAP-IT (Affymetrix, Inc., USA) and sequenced from the two strands using the dideoxy chain termination method (BigDye Terminator version 1.1 cycle sequencing kit; Applied Biosystems, Foster City, CA) on the ABI Prism 3130xl DNA sequencer (Applied Biosystems, Courtaboeuf, France).

DNA sequences of the *dhfr* gene were edited using Pregap and Gap software (Staden package version 2.0.0b11-2016; Staden Group) (28). Consensus sequences were aligned with the reference sequence (GenBank accession no. AF090368 [17]) using the BioEdit software with the ClustalW program.

Statistical analyses were performed using Prism software (version 7.0; GraphPad Software, San Diego, CA, USA). Qualitative variables were compared using Fisher's exact test. A two-sided *P* value of 0.05 was considered significant.

The study was noninterventional and did not require informed consent and ethical approval according to French laws and regulations (CSP Art L1121e1.1).

Accession number(s). The nucleotide sequences of the *dhfr* gene obtained in the present study have been deposited in GenBank under accession no. ON932600 to ON932642.

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