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Genetic Polymorphisms of *Pneumocystis jirovecii* in HIV-Positive and HIV-Negative Patients in Northern China

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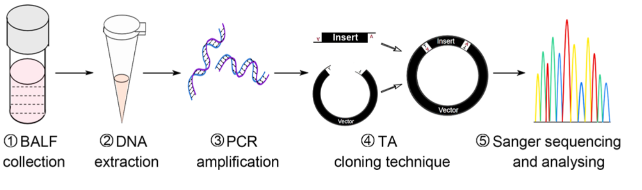
Abstract

Pneumocystis jirovecii is an opportunistic fungus that can cause severe and potentially fatal *Pneumocystis* pneumonia (PCP) in immunodeficient patients. In this study, we investigated the genetic polymorphisms of *P. jirovecii* at eight different loci, including six nuclear genes (ITS, 26S rRNA, *sod*, *dhps*, *dhfr* and β-Tub) and two mitochondrial genes (mtLSU-rRNA and *cyb*) in three PCP cases, including two patients with HIV infection and one without HIV infection in Shanxi Province, P.R. China. The gene targets were amplified by PCR followed by sequencing of plasmid clones. The HIV-negative patient showed a coinfection with two genotypes of *P. jirovecii* at six of the eight loci sequenced. Of the two HIV-positive patients, one showed a coinfection with two genotypes of *P. jirovecii* at the same two of the six loci as in the HIV-negative patient, while the other showed a single infection at all eight loci sequenced. None of the three drug target genes (*dhfr, dhps* and *cyb*) showed mutations known to be potentially associated with drug resistance. This is the first report of genetic polymorphisms of *P. jirovecii* in PCP patients in Shanxi Province, China. Our findings expand our understanding of the genetic diversity of *P. jirovecii* in China.



Keywords: *Pneumocystis jirovecii*, genetic polymorphisms, genotypes, multilocus, epidemiology

Introduction

Pneumocystis is a genus of atypical fungi demonstrating different degrees of genetic diversity between and within different species that infect mammals with high host specificity. The human-specific species, *Pneumocystis jirovecii,* causes life-threatening *Pneumocystis* pneumonia (PCP) in immunodeficient individuals, especially those with human immunodeficiency virus (HIV) infection (Ma et al. 2018). Recent studies have indicated a high prevalence of *P. jirovecii* colonization and infection in individuals with chronic obstructive pulmonary disease (COPD) (Wang et al. 2015; Cañas-Arboleda et al. 2019; Xue et al. 2020). However, the epidemiology and genetic diversity of *P. jirovecii* in different patient populations remain poorly understood.

Although genetic diversity of *P. jirovecii* has been reported in multiple studies from different regions in China (Li et al. 2013; Deng et al. 2014; Sun et al. 2015; Wang et al. 2019), all these studies are limited to only a few loci, and there is no such report from Shanxi Province in Northern China. In this study, we retrospectively investigated three confirmed cases of PCP, including two in HIV-positive patients and one in the HIV-negative patient from our hospital in Shanxi Province. Genetic polymorphisms of *P. jirovecii* in these patients were determined at eight different loci.

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Experimental

Materials and Methods

Patients and samples. Three patients with PCP were included in this study, including two positive and one negative for HIV-1. Patients were admitted to the Department of Respiratory and Critical Care Medicine of First Affiliated Hospital of Shanxi Medical University between August 2019 and June 2020. The diagnosis of PCP was confirmed based on clinical manifestations and laboratory tests, including hematology, high-resolution computed tomography (HRCT), modified Gomori methenamine silver nitrate staining (GMS) of bronchoalveolar lavage fluid (BALF) samples. The two HIV-positive patients had a confirmed diagnosis of the acquired immune deficiency syndrome (AIDS) but did not receive highly active antiretroviral therapy. Based on the ELISA results, the HIV-negative patient was seronegative for HIV-1 and HIV-2 antibodies.

The Medical Ethics Committee approved this retrospective study of our hospital (2019-K051). In addition, written informed consent was obtained from all three patients.

DNA extraction. The BALF specimens were centrifuged at 350 g for 15 min, followed by washing the cell pellets with saline solution three times. DNA was extracted from washed cell pellets using the conventional phenol-chloroform extraction method. DNA extracts were quantified using a NanoDrop-UV-Vis spectrophotometer (Thermo Fisher Scientific, USA) and stored at −80°C until use.

DNA amplification, cloning, and sequencing. We amplified eight different loci of the *P. jirovecii* genome using nested PCR with the Premix-Taq PCR kit (TaKaRa Biotechnology Co., Ltd., Dalian, China) following the manufacturer's instructions. The loci included mitochondrial large-subunit rRNA (mtLSU-rRNA), cytochrome b (*cyb*), nuclear large rRNA subunit (26S), and the complete internal transcribed spacers 1 and 2 (ITS1 and ITS2) along with the 5.8S rRNA of the nuclear rRNA operon (referred to as ITS hereafter), superoxide dismutase (*sod*), dihydropteroate synthase (*dhps*), dihydrofolate reductase (*dhfr*), and β-tubulin (β-Tub). The primers used in this study are listed in Table I. The PCR amplification conditions for β-Tub and 26S were the same as those previously reported

Table I
PCR primers used in this study.

Genes (reference)	Primer names and sequences (5'-3')	Size of nested PCR products (bp)
ITS (Lee et al. 1998)	1724F 5'-AAGTTGATCAAATTTGGTC-3' ITS2R 5'-CTCGGACGAGGATCCTCGCC-3' ITS1F 5'-CGTAGGTGAACCTGCGGAAGGATC-3' ITS2R1 5'-GTTCAGCGGGTGATCCTGCCTG-3'	578
sod (Esteves et al. 2010b)	MnSOD_Fw 5'-GGGTTTAATTAGTCTTTTTAGGCAC-3' MnSOD_Rw 5'-CATGTTCCCACGCATCCTAT-3' SODF3 5'-AGTCTTTTTAGGCACTTGAACCT-3' SODR4 5'-TCCAAGAATAACTTTGCCTTGAGT-3'	560
dhfr (Lane et al. 1997)	FR208 5'-GCAGAAAGTAGGTACATTATTACGAGA-3' FR1018 5'-AAGCTTGCTTCAAACCTTGTGTAACGCG-3' FR242 5'-GTTTGGAATAGATTATGTTCATGGTGTACG-3' FR1038 5'-GCTTCAAACCTTGTGTAACGCG-3'	798
dhps (Ma et al. 1999)	DHPS1 5'-CAAATTAGCGTATCGAATGACC-3' DHPS2 5'-GCAAAATTACAATCAACCAAAGTA-3' DHPS3 5'-AGCGCCTACACATATTATGG-3' DHPS4 5'-GTTCTGCAACCTCAGAACG-3'	278
cyb (Esteves et al. 2010a)	CytbFw 5'-CCCAGAATTCTCGTTTGGTCTATT-3' CytbRw 5'-AAGAGGTCTAAAAGCAGAACCTCAA-3' CytbF3 5'-TCTCGTTTGGTCTATTGGTG-3' CytbR4 5'-AAGCAGAACCTCAAATTCAAGATA-3'	590
mtLSU rRNA (Wakefield 1996)	pAZ102_E 5'-GATGGCTGTTTCCAAGCCCA-3' pAZ102_H 5'-GTGTACGTTGCAAAGTACTC-3' pAZ102_X 5'-GTGAAATACAAATCGGACTAGG-3' pAZ102_Y 5'-TCACTTAATATTAATTGGGGACC-3'	252
β-Tub (Pasic et al. 2020)	Pneumo Tub_F 5'-TCATTAGGTGGTGGAACGGG-3' Pneumo Tub_R 5'-ATCACCATATCCTGGATCCG-3'	303
26S rRNA (Pasic et al. 2020)	PneumoLSU_F 5'-TCAGGTCGAACTGGTGTACG-3' PneumoLSU_R 5'-TGTTCCAAGCCCACTTCTT-3'	297

(Pasic et al. 2020), and the conditions for other genes were the same as described in previous studies (Lee et al. 1998; Wang et al. 2019; Xue et al. 2019). DNA from *P. jirovecii*-positive specimens stored in our laboratory was used as the positive control. A non-template control with ultrapure-distilled water was included in each PCR run. To prevent cross-contamination of the samples, separate rooms were used, and the PCR mixture from each step of nested PCR was covered with 40 μl of sterile liquid paraffin. All PCR products were separated by electrophoresis on 2% agarose gels, stained with 4S Green Plus Nucleic Acid Stain (Sangon Biotech Co., Ltd. Shanghai, China), and visualized under UV irradiation. The amplified DNA bands of the expected sizes were excised from the gel and extracted using an agarose gel DNA extraction kit (Tiangen Biotech Co., Ltd., Beijing, China). Following the manufacturer's instructions, the extracted DNA fragment was cloned into the TA cloning vector pMD18-T (TaKaRa Biotechnology Co., Ltd., Dalian, China). Recombinant plasmid clones were selected by blue-white screening on agar plates containing ampicillin. For each PCR product, 8 to 13 plasmid clones were randomly selected for Sanger sequencing in the ABI 3730xl DNA analyzer (Thermo Fisher Scientific, USA).

Sequence analysis and genotyping. The nucleotide sequences obtained in this study were analyzed and aligned using ClustalW software (https://www.genome.jp/tools-bin/clustalw). At least two plasmid clones are required to define a nucleotide polymorphism. The genotypes were named based on previously published nomenclature (Table II). The reference sequence for each gene was obtained from GenBank, with its accession number listed as follows: *ITS*, MK300654; *mtLSU-rRNA,* M58605; *cyb*, AF320344; *sod*, AF146753; *dhfr*, AF090368; *dhps*, AF139132; β-Tub, MG208106 and 26S KT272445. Known *P. jirovecii* multi-locus sequence type (MLST) profiles at β-Tub, *cyb*, *26S*, and *sod* genes were retrieved from the Fungal MLST Database at http://mlst.mycologylab.org.

Table II
Nucleotide polymorphic sites and number of plasmid clones sequenced at eight distinct loci of *Pneumocystis jirovecii*.

Locus	Genotypes[a]	Location[b]	No. of plasmid clones sequenced		
			SX_0001	SX_0002	SX_0003
ITS	ITS 4	KC470776	0	12	0
	ITS 10	JQ365725	0	0	4
	ITS 16	AB469817	0	0	8
	ITS 22	KC470795	6	0	0
	ITS 59	MK300661	10	0	0
sod	*sod 1*	110C/215T	11	13	8
	sod 2	110T/215C	0	0	2
dhps	*dhps WT*	165A (55Thr) / 171C (57Pro)	12	12	12
dhps	*dhfr312*	312C (117Gly)	12	11	11
cyb	*cyb 1*	279C/348A/516C/547C/566C/838C	0	0	6
	cyb 2	279C/348A/516C/547C/566C/838T	0	8	0
	cyb 7	279C/348A/516C/547C/566T/838C	9	0	0
	cyb 8	279T/348A/516C/547C/566C/838C	0	0	3
mt LSU rRNA	mt1	85C/248C	0	0	2
	mt2	85A/248C	0	0	8
	mt3	85T/248C	10	10	0
β-Tub	β-Tub 1	86G/281A	8	0	6
	β-Tub 2	86G/281G	4	12	5
26S rRNA	26S 2	86T/290A	12	11	0
	26S 3	86C/290A	0	0	5
	26S 4	86A/290A	0	0	6

ITS – internal transcribed spacer regions of rRNA operon, *sod* – superoxide dismutase, *dhfr* – dihydrofolate reductase, *dhp* – dihydropteroate synthase, WT – wild-type, *cyb* – cytochrome b, mt – mitochondrial large rRNA subunit, β-Tub – β-tubulin, 26S rRNA – 26S ribosomal RNA gene

[a] – the genotype nomenclature based on previously published studies and [b] – the genotype locations according to the studies previously reported (Walker et al. 1998; Ma et al. 1999; Beard et al. 2000; Denis et al. 2000; Takahashi et al. 2002; Esteves et al. 2010b; Maitte et al. 2013; Xue et al. 2019; Pasic et al. 2020)

Results

General information on PCP patients. Clinical information of the patients involved in this study is summarized in Table III. The presence of *P. jirovecii* in all patients was confirmed by microscopic observation of *P. jirovecii* cysts in BALF samples stained with GMS (Fig. 1).

Multilocus sequence genotyping. All eight genetic loci *P. jirovecii* were successfully amplified and sequenced in the BALF specimen from all three patients. Table II shows the polymorphic nucleotide sites, and the number of plasmid clones sequenced for each PCR product from 8 loci. Genotype profiles are summarized in Table IV.

The HIV-negative patient (SX_0003) showed a co-infection with two genotypes of *P. jirovecii* at six of the eight loci sequenced. Of the two HIV-positive patients, one (SX_0001) showed a co-infection with two genotypes of *P. jirovecii* at two loci, while the other (SX_0002) showed a single infection at all eight loci sequenced.

Of note, the *dhps* gene (the target of sulfa drugs) in all three *P. jirovecii* specimens was present as a wild-type sequence. The *dhfr* gene (the target of trimethoprim) in all three *P. jirovecii* specimens showed a single synonymous change in the same position (from

Table III
Clinical characteristics of patients with *Pneumocystis jirovecii* pneumonia.

Clinical information	Patient No.		
	SX_0001	SX_0002	SX_0003
Age (years)	65	51	65
Sex	Male	Male	Male
Underlying conditions	NA[a]	Hepatic cysts	ILD
Thoracic HRCT findings	GGO [d] +	GGO +	GGO +
HIV 1/2 antibody	+/–	+/–	–/–
CD_4 T-lymphocyte count (cells/μl)	232	176	NA
Serum parameters			
1,3-β-D-glucan, normal < 10 pg/ml	> 600	NA	> 600
Lactate dehydrogenase, normal 120–250 U/l	432	699	9,734
C-reactive protein, normal 0–6 mg/l	73.63	129.17	340.00
Procalcitonin, normal 0–0.05 ng/ml	0.975	0.161	11.26
Partial pressure of oxygen, normal 80–110 mmHg	80	65	59.70
Erythrocyte sedimentation rate, normal 0–15 mm/h	61.10	60.80	47.30
Concurrent infection	–	–	*C.n.* and *B.c.*[b]
Anti-PCP therapy[a]	–	–	–
HAART before PCP	–	–	–
Clinical outcomes	survived	survived	died

NA – not available; ILD – interstitial lung disease; HRCT – high-resolution computed tomography;
GGO – ground-glass opacity; HIV – human immunodeficiency virus;
HAART – highly active antiretroviral therapy
+– positive, – – negative
[a] – Anti-PCP therapy, TMP-SMZ prophylaxis for *P. jirovecii* pneumonia
[b] – *Candida norvegensis* and *Burkholderia cepacia*

Table IV
Genotypes of *Pneumocystis jirovecii* detected at eight genetic loci.

Patient No.	HIV1/2 anti-body	Genotypes at 8 loci							
		ITS	*sod*	*dhfr*	*dhps*	*cyb*	mtLSU rRNA	β-Tub	26S rRNA
SX_0001	+/–	ITS2 + ITS59	*sod1*	*dhfr312*	*WT*	*cyb7*	mt3	β-Tub1 + β-Tub2	26S2
SX_0002	+/–	ITS4	*sod1*	*dhfr312*	*WT*	*cyb2*	mt3	β-Tub2	26S2
SX_0003	–/–	ITS10 + ITS16	*sod1* + *sod2*	*dhfr312*	*WT*	*cyb1* + *cyb8*	mt1 + mt 2	β-Tub1 + β-Tub2	26S3 + 26S4

ITS – internal transcribed spacer regions of rRNA operon, *sod* – superoxide dismutase, *dhfr* – dihydrofolate reductase, *dhps* – dihydropteroate synthase, WT – wild-type, *cyb* – cytochrome b, mt – mitochondrial large rRNA subunit, β-Tub – β-tubulin, 26S rRNA – 26S rRNA gene

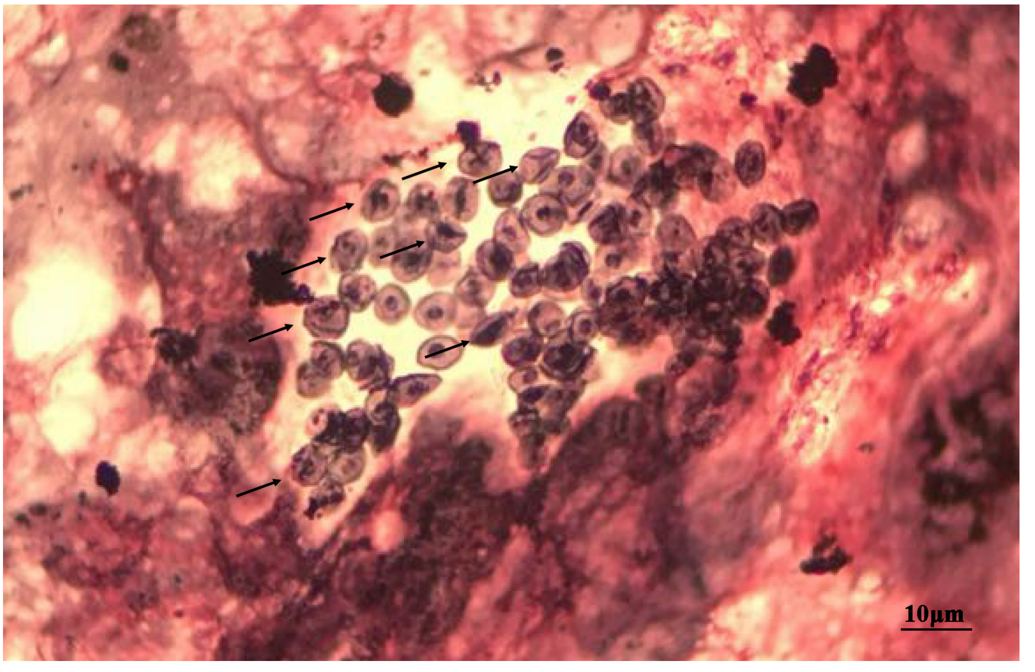

Fig. 1. Identification of *Pneumocystis jirovecii* using GMS staining methods. Cysts appear as brown or puce spheres or ovoids with a small black stick inside (arrows). The reddish background instead of the typical greenish background is most likely due to periodic acid treatment and without light green counterstaining in our staining method.

T to C at nucleotide 312). The *cyb* gene (the target of atovaquone) in the three *P. jirovecii* specimens showed polymorphisms in three nucleotide positions (at 279, 566 and 838), resulting in 4 genotypes including *cyb 1, cyb 2, cyb 7* and *cyb 8* based on the nomenclature system described by Esteves and Maitte (Esteves et al. 2010b; Maitte et al. 2013). Genotypes *cyb 2* and *cyb 7* were presented only in patients SX_0002 and SX_0001, respectively, while genotypes *cyb 1* and *cyb 8* were present as a mixture in the patient SX_003. Of the three polymorphisms, one is synonymous (at 279 in genotype *cyb 8*) and the other two are nonsynonymous (at 566 in genotype *cyb 7* and 838 in genotype *cyb 2*).

Due to the presence of coinfection with two genotypes at 2 or 6 loci in two of the three patients (SX_0001 and SX_0003), we could not determine the exact MLST types in either patient (Table V).

Table V
Multi-locus sequence type (MLST) profiles of *P. jirovecii* from PCP patients in this study in comparison with known *P. jirovecii* MLST profiles in Fungal MLST Database.

MLST types*	β-Tub	*cyb*	26S rRNA	*sod*	Patient no.
3	1	1	4	2	SX_0003
8	2	8	4	1	SX_0003
13	1	1	4	1	SX_0003
15	1	8	4	1	SX_0003
19	1	8	3	2	SX_0003
21	2	1	3	1	SX_0003
22	2	1	3	2	SX_0003
23	2	1	4	1	SX_0003
35	2	7	2	1	SX_0001
51	1	7	2	1	SX_0001
52	2	2	2	1	SX_0002
NA	1	1	3	2	SX_0003
NA	2	8	3	1	SX_0003
NA	2	8	3	2	SX_0003
NA	2	1	4	2	SX_0003
NA	2	8	4	2	SX_0003
NA	1	1	3	1	SX_0003
NA	1	8	3	1	SX_0003
NA	1	8	4	2	SX_0003

* – The first 11 MLST types (numbered 3 to 52) identified in this study correspond to those in the Fungal MLST Database at http://mlst.mycologylab.org
NA – types identified in this study and not available from the Fungal MLST Database
In both patients SX_0001 and SX_0003 (with co-infection of two genotypes at 2 or 6 loci, respectively), there were a total of four and 64 potential MLST profiles, respectively. Only two and 16 of those profiles are listed in this table while the true profiles could not be determined in this study.

Discussion

Despite having been recognized as an important human pathogen for many years, strain variation of *P. jirovecii* remains poorly understood due largely to the absence of a reliable in vitro culture system. To date, *P. jirovecii* strain typing has relied primarily on analyzing genetic markers after PCR amplification. While there have been about a dozen genetic markers reported (Ma et al. 2018), most studies have used only a small number of genetic markers in epidemiological investigations, potentially limiting the discriminatory power for strain differentiation. In this study, we performed strain typing of *P. jirovecii* using a total of eight genetic markers, including six nuclear genes (ITS, 26S rRNA, *sod*, *dhps*, *dhfr* and β-Tub) and two mitochondrial genes (mtLSU-rRNA and *cyb*).

While only three clinical specimens were examined including two from HIV-infected patients and one from a non-HIV patient, we identified complex genotype profiles (Table II). Multiple unique genotypes (from 2 to 5) were identified at all these eight loci except for two (*dhps* and *dhfr*), which showed a single genotype. Two of three clinical specimens showed a mixture of multiple genotypes at two or six loci, suggesting a coinfection with multiple *P. jirovecii* strains, without any strains shared between the three patients. This represents the first report of genetic polymorphisms in PCP patients in Shanxi Province, China. Our findings expand our understanding of the genetic diversity of *P. jirovecii* in China.

The ITS locus involved in this study includes ITS1 and ITS2, and 5.8S rRNA of the nuclear rRNA operon was amplified in one fragment of approximately 490 bp and is also known as ITS1-5.8S-ITS2 (Xue et al. 2019). Sequence analysis of this locus in this study identified five unique genotypes (nos. 4, 10, 16, 22, and 59) based on the genotype nomenclature system in our earlier report (Xue et al. 2019), which is more than genotypes identified from all other seven loci examined. This is consistent with many previous studies showing this locus to be the most polymorphic genetic marker for *P. jirovecii* genotyping (Ma et al. 2018). All ITS genotypes identified in this study have also been reported from previous studies conducted by our group (Xue et al. 2019) and others in China (Li et al. 2013; Sun et al. 2015) as well as studies from other countries (Atzori et al. 1998; Miller and Wakefield 1999; Matsumura et al. 2011).

In this study, we examined genetic polymorphisms of three drug target genes, including *dhfr*, *dhps* and *cyb*, which are the targets of trimethoprim, sulfa, and atovaquone drugs, respectively. No nonsynonymous mutation was found at *dhfr* or *dhps* in any specimens in this study, while a single synonymous change in the same position at *dhfr* (from T to C at nucleotide 312) was present in all three specimens. This change has been reported in previous studies from China (Deng et al. 2014; Wang et al. 2019) and other countries (Esteves et al. 2010b; Muñoz et al. 2012; Suárez et al. 2017; Singh et al. 2019). As for the *cyb* gene, we identified nucleotide changes at three positions (at 279, 566 and 838), which gave rise to 4 unique genotypes (*cyb 1, cyb 2*, *cyb 7* and *cyb 8*). All these genotypes have also been reported from China (Deng et al. 2016; Wang et al. 2019) and other countries (Esteves et al. 2010b; Maitte et al. 2013; Sokulska et al. 2018; Szydlowicz et al. 2019; Le Gal et al. 2020; Goterris et al. 2022). The nucleotide changes at two positions (566 and 838) are synonymous (S189L and L180F) but do not correspond to any of the seven mutations that are suggested to be associated with atovaquone resistance in previous studies (Kessl et al. 2004). The absence of mutations in all these three drug targets potentially associated with resistance is consistent with no known use of the respective drugs in the history of the patients.

The major limitation of this study is the small sample size, which precludes the generalization of the results to a larger population and the assessment of correlation of genotypes with clinical characteristics and treatment outcomes. Further studies are required using more samples from different patient populations.

Conclusions

In conclusion, we assessed and analyzed the genetic polymorphisms of *P. jirovecii* genotypes at eight loci and identified complex genotype profiles, including the presence of coinfection with up to 5 genotypes at six loci. This is the first report of genetic polymorphisms in PCP patients in Shanxi Province, China. Our findings expand our understanding of the genetic diversity of *P. jirovecii* in China. However, a large-scale collection of clinical isolates of *P. jirovecii* from different patient populations is required for more detailed studies and the correlation of genotypes with clinical characteristics and outcomes.

Acknowledgments

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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