

Genetic Polymorphisms of 3 Cases *Pneumocystis jirovecii* in Shanxi Province PR China

Ting Xue (✉ beyondtinger@126.com)

First Hospital of Shanxi Medical University

Wei-Qin Du

11th affiliated hospital of shanxi medical university

Wenjuan Dai

1st hospital of shanxi medical university

Yishan Li

1st hospital of shanxi medical university

Shu-Feng WANG

1st hospital of shanxi medical university

Xinri Zhang

1st hospital of shanxi medical university

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Abstract

Background *Pneumocystis jirovecii* (*P. jirovecii*) is an opportunistic fungus and is a well-known cause of potentially fatal *Pneumocystis jirovecii* pneumonia (PJP). However, the epidemiology and biology of *P. jirovecii* is limited because of the lack of a continuous and reliable culture system in vitro. This study was performed with the objectives of investigating the genetic polymorphisms of *P. jirovecii* from 3 cases in Shanxi Province PR China with the utilizations of molecular biological technique.

Methods Analysis of genetic polymorphisms in *P. jirovecii* including the internal transcribed spacer regions of rRNA operon (ITS), superoxide dismutase (SOD), dihydropteroate synthase (DHPS) and dihydrofolate reductase (DHFR), cytochrome b (CYB) and the mitochondrial large-subunit rRNA (mt LSU rRNA) genes were performed by Nested-PCR amplification, TA clone, sequencing and genotyping. We assessed its correlation with clinical characteristics of patients with *P. jirovecii* pneumonia (PJP).

Results Of three patients with PJP included in the present study, the genetic diversities and polymorphic combinations of SOD 1, DHFR 312C, DHPS WT, mt3 genotypes separately occurred in the two HIV-positive patients, while coinfections with two genotypes of *P. jirovecii* in the same patient without HIV were detected for different genetic loci including ITS, SOD, CYB and mt LSU rRNA in this study. The pulmonary concurrent infection with other pathogens was detected in this HIV-negative patient.

Conclusions The present study is the first analyzed and assessed the genetic polymorphisms of 3 cases *P. jirovecii* in Shanxi province PR China suggesting that genetic diversities and concurrent infection with other pathogens were possibly associated with the clinical characteristics and outcomes in PJP patients especially those without HIV.

Trial registration Not applicable.

1. Background

Pneumocystis, a typical opportunistic fungus, has obviously specificities for host species. *Pneumocystis jirovecii* (*P. jirovecii*), the species that infects humans, can lead to life-threatening *P. jirovecii* pneumonia (PJP) or *Pneumocystis* pneumonia (PCP), most commonly in immunodeficient people such as those infected with human immunodeficiency virus (HIV), solid organ transplant recipients, and patients with malignant tumors[1]. Recent numerous studies indicated that the high prevalence of *P. jirovecii* colonization and infection is found in pulmonary diseases particularly in chronic obstructive pulmonary disease (COPD)[2–5]. However, it is difficult to clearly understand the biology and epidemiology of *P. jirovecii* infection and colonization, since there is no reliable and continuous cultivable system in vitro. The primary transmission route has yet to be further proven. The development and application of molecular biological techniques has provided powerful assays for the epidemiology of *P. jirovecii*. So far, the case reports from Shanxi province were limited. In this study, we retrospectively reviewed three cases, including two HIV-positive patients and an HIV-negative patient, confirmed as PJP in our department from Shanxi Province, and analyzed the polymorphic genotypes from multiple genomic regions of *P. jirovecii*

isolated from those three patients by sequencing. Meanwhile, we assessed the correlation between genetic polymorphisms and clinical characteristics of these PJP patients.

2. Methods

2.1 Patients

Three PJP patients, two HIV-positive patients and one HIV-negative patient, admitted to the department of respiratory internal medicine of the First Affiliated Hospital of Shanxi Medical University, PR China, from August 2019 to June 2020, were included in this study. The diagnosis of PJP was confirmed by clinical manifestations, clinical laboratory data, hypoxemia combined with high-resolution computed tomography (HRCT), especially based on observing the morphological character of pathogen by modified Giemsa staining and Gomori's methenamine silver nitrate staining (GMS) assays of respiratory samples respectively. The two HIV-positive patients had been definitely diagnosed with AIDS (Acquired Immune Deficiency Syndrome) but without the highly active antiretroviral therapy (HART) at that time. The only one HIV-negative patient was seronegative for HIV-1 and 2 antibody detection based on anti-HIV-1 and 2 antibody enzyme-linked immunosorbent assay (ELISA). All the laboratory operating procedures were executed according to the relevant international standards.

This retrospective study was approved by the Medical Ethics Committee of the First Affiliated Hospital of Shanxi Medical University (reference number 2019-K051). The three patients involved in this study provided the written informed consent.

2.2 Sample collection

Bronchoscopic examination was performed according to the patients' condition and bronchoalveolar lavage fluid (BALF) specimens were collected. 2-layered nylon gauze was used for removing the mucus of BALF. Strict aseptic technique was performed in the whole operation procedure following standard bronchoscopy procedures. The filtered BALF was respectively used for DNA extraction (described below) and making smears on slides. The slide smears were stained by Giemsa staining and GMS to detect *P. jirovecii* cysts.

2.3 DNA extraction

BALF specimens were centrifuged at 1,500 rpm for 15 minutes and cell pellets were collected, washed with saline solution approximately three times, and then centrifuged at 12,000 rpm for 10 minutes. After centrifugation, the supernatant was discarded. The DNA of cell pellets from BALFs was extracted following the conventional phenol-chloroform extraction method protocol. The DNA extracts were quantitated using the NanoDrop UV-Vis spectrophotometer (Thermo Fisher Scientific, USA) and stored at -80°C before use.

2.4. DNA amplification, cloning and sequencing

We amplified 6 different loci of the *P. jirovecii* genome including mt LSU rRNA, CYB, SOD, DHPS, DHFR and the complete ITS1, 5.8S rRNA, and ITS2 regions (ITS1-5.8S-ITS2) by nested PCR with a Premix Taq PCR kit (TaKaRa Biotechnology Co., Ltd., Dalian, China). The primers used in this study were listed in Table 1. The first PCR reactions contained 12.5 µL Premix Taq™ (Takara Biotechnology Co., LTD., Dalian, China), 2–5 µl DNA samples, 1 µl 10 µM forward primers and reverse primers, and RNase-free water (Takara Biotechnology Co., LTD., Dalian, China) up to a final volume of 25 µl. The PCR amplified conditions for the first round included denaturation at 94 °C for 5 min; followed by 35 cycles of denaturation at 94 °C for 60 s, annealing at 47 °C for 60 s and extension at 72 °C for 90 s; with a final extension at 72 °C for 10 min. The second PCR mixture solution composed of 12.5 µl Premix Taq™ (Takara Biotechnology Co., LTD., Dalian, China), 3 µl the first-round PCR products as DNA template, 1 µl 10 µM ITS1F/ITS2R1 and 7.5 µl RNase-free water (Takara Biotechnology Co., LTD., Dalian, China) up to a final volume of 25 µl.

Table 1
Primer sequences used for nested PCR

Primers	Sequence (5'-3')	Size of nested PCR products (bp)
ITS [6]	1724F 5'-AAG TTG ATC AAA TTT GGTC-3' ITS2R 5'-CTC GGA CGA GGA TCC TCG CC-3' ITS1F 5'-CGT AGG TGA ACC TGC GGA AGG ATC-3' ITS2R1 5'-GTT CAG CGG GTG ATC CTG CCT G-3'	578
SOD[7]	MnSOD-Fw 5'-GGG TTT AAT TAG TCT TTT TAG GCA C-3' MnSOD-Rw 5'-CAT GTT CCC ACG CAT CCT AT-3' SODF3 5'-AGT CTT TTT AGG CAC TTG AAC CT-3' SODR4 5'-TCC AAG AAT AAC TTT GCC TTG AGT-3'	560
DHFR[8]	FR208 5'-GCA GAA AGT AGG TAC ATT ATT ACG AGA-3' FR1018 5'-AAG CTT GCT TCA AAC CTT GTG TAA CGC G-3' FR242 5'-GTT TGG AAT AGA TTA TGT TCA TGG TGT ACG-3' FR1038 5'-GCT TCA AAC CTT GTG TAA CGC G-3'	798
DHPS[9]	DHPS1 5'-CAA ATT AGC GTA TCG AAT GAC C-3' DHPS2 5'-GCA AAA TTA CAA TCA ACC AAA GTA-3' DHPS3 5'-AGC GCC TAC ACA TAT TAT GG-3' DHPS4 5'-GTT CTG CAA CCT CAG AAC G-3'	278
CYB[10]	CytbFw 5'-CCC AGA ATT CTC GTT TGG TCT ATT-3' CytbRw 5'-AAG AGG TCT AAA AGC AGA ACC TCA A-3' CytbF3 5'-TCT CGT TTG GTC TAT TGG TG-3' CytbR4 5'-AAG CAG AAC CTC AAA TTC AAG ATA-3'	590
mt LSU rRNA[11]	pAZ102-E 5'-GAT GGC TGT TTC CAA GCC CA-3' pAZ102-H 5'-GTG TAC GTT GCA AAG TAC TC-3' PAZ102-X 5'-GTG AAA TAC AAA TCG GAC TAG G-3' PAZ102-Y 5'-TCA CTT AAT ATT AAT TGG GGA CC-3'	252

The second round of PCR was performed at 94 °C for 5 min; 35 cycles at 94 °C for 30 s, 61 °C for 30 s and 72 °C for 60 s; with a final extension at 72 °C for 10 min.

DNAs from *P. jirovecii*-positive patient specimen stored in our laboratory previously were employed as positive controls. A no-template control with ultrapure distilled water was included in each PCR run. To prevent cross-contamination of samples, separate rooms were used and 25 µl PCR mixture systems of each step of the nested PCR were covered with 40 µl sterile liquid paraffin. Nested 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 by UV irradiation. The expected specific amplified DNA bands were excised from the gel and extracted with the agarose gel extraction kit (Tiangen Biotech Co., LTD., Beijing, China). The extracted DNA fragment was ligated into TA cloning vector pMD18-T (Takara, Biotechnology Co., LTD., Dalian, China) with TA cloning technique followed by transforming in competent *Escherichia coli* JM109 strains according to the manufacturer's instructions. The correct recombinant plasmid was constructed by selecting Ampicillin-resistant transformants. 8 to 13 plasmid clones were randomly selected for Sanger sequencing with ABI 3730xl DNA Analyzer (Thermo Fisher Scientific, USA) for each PCR product.

2.5. Sequence analysis and genotyping

The nucleotide sequences obtained in the present study were analyzed using the ClustalW software. Reference sequences with the accession numbers of the loci sequence of ITS, SOD, DHFR, DHPS, CYB and mt LSU rRNA used for alignment in this study are MK300654, M58605, AF320344, AF146753, AF090368 and AY628435, respectively. The genotypes were named based on the previously published nomenclature. The mutations in the sequences were confirmed with sequencing of plasmid clones and there were at least 2 clones for each patient.

3. Results

3.1 General Information of patients with PJP

BALF specimens from 3 patients with imaging manifestations suspected PJP were obtained from Shanxi province, PR China were obtained, and general clinical information were listed in Table 2. The typical cysts of *P. jirovecii* in BALF-smear slides using pathogenic identification included Giemsa and GMS staining methods were visualized (Fig. 1). All these 3 patients were confirmed as PJP, and were positive for *P. jirovecii* DNA using mt LSU rRNA as the gene target in PCR assay.

Table 2
Clinical characteristics of PJP patients

Clinical information	SX_0001	SX_0002	SX_0003
Age (years)	65	51	65
Sex	Male	Male	Male
Underlying conditions	NA ^a	hepatic cysts	ILD ^b
Thoracic HRCT findings ^c	GGO ^d +	GGO +	GGO +
Presence of HIV 1/2 antibody ^e	+/- ^f	+/-	-
CD ₄ T lymphocytes count (cells/ μl)	232	176	NA
Serum parameters			
BDG (pg/ml) ^g	> 600	NA	> 600
LDH (U/L) ^h	432	699	9734
CRP (mg/L) ⁱ	73.63	129.17	340.00
PCT (ng/ml) ^g	0.975	0.161	11.26
PO ₂ (mmHg) ^k	80	65	100
ESR (mm/h) ^l	61.10	60.80	47.30
Concurrent infection with other pathogens	-	-	Candida norvegensis and Burkholderia cepacia
Anti-PJP Therapy ^m	-	-	-
HAART before PJP ⁿ	-	-	-
Clinical outcomes	survivor	survivor	Non-survivor
^a NA, not available.			
^b ILD, Interstitial lung disease.			
^c HRCT, high-resolution computed tomography.			
^d GGO, ground-glass opacity.			
^e +, positive; -, negative.			

Clinical information	SX_0001	SX_0002	SX_0003
^f HIV, human immunodeficiency virus.			
^g BDG, 1,3-β-D-glucan. Positive (> 10 pg/ml) and Negative (< 10 pg/ml).			
^h LDH, lactate dehydrogenase. The normal range of LDH is 120–250 U/L.			
ⁱ CRP, C-reactive protein. The normal range of CRP is 0–6 mg/L.			
^g PCT, pro-calcitonin. The normal range of PCT is 0-0.05 ng/ml.			
^k PO ₂ , Partial pressure of oxygen. 80–110 mmHg is the normal range of PO ₂ .			
^l ESR, erythrocyte sedimentation rate. 0–15 mm/h for men is the normal range of ESR.			
^m Anti-PJP therapy, TMP-SMZ prophylaxis against <i>P. jirovecii</i> pneumonia.			
ⁿ HAART, Highly Active Anti-Retroviral Therapy.			

3.2 Analysis of multilocus genotyping

The mt LSU rRNA gene amplification was successful among the 3 BALF specimens mentioned above which were positive for other genes including ITS, SOD, DHFR, DHPS, CYB and mt LSU rRNA. Genotyping result and the numbers of genotypic sequences in each specimen were respectively summarized in Tables 3 and 4. The analysis of multilocus genotyping in this study suggested genetic diversities of *P. jirovecii* isolates in Shanxi Province, PR China.

Table 3
Genotypes and SNPs of *P. jirovecii* detected at five distinct loci

PJP patients No.	Sample type	Genotypes and SNPs of <i>P. jirovecii</i> identified in each locus					
		ITS	SOD	DHFR	DHPS	CYB	mtLSU rRNA
SX 0001	BALF	ITS 2 + ITS 59	SOD 1	DHFR312	WT	CYB 7	mt 3
SX 0002	BALF	ITS 4	SOD 1	DHFR312	WT	CYB 2	mt 3
SX 0003	BALF	ITS 10 + ITS 16	SOD1 + SOD 2	DHFR312	WT	CYB 1 + CYB 8	mt 1 + mt 2
Abbreviations: BALF, bronchoalveolar lavage fluid; SNPs, single nucleotide polymorphisms; 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 ribosomal subunit.							

Table 4

Nucleotide variation position and the number of sequence reads in five distinct loci of *P. jirovecii*

Locus	Genotypes ^a	Location ^b	No. of sequence reads in specimen		
			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	sod1	110C/215T	11	13	8
	sod2	110T/215C	0	0	2
DHPS	DHPS WT	165A (55Thr) / 171C (57Pro)	12	12	12
DHFR	DHFR 312	312C (117Gly)	12	11	11
CYB	cyb1	279C/348A/516C/547C/566C/838C	0	0	6
	cyb2	279C/348A/516C/547C/566C/838T	0	8	0
	cyb7	279C/348A/516C/547C/566T/838C	9	0	0
	cyb8	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

Genotyping results for ITS regions in two of these 3 patients were mixed genotypes such as ITS 2 and ITS 59 for patient SX_0001 and ITS10 and ITS 16 for patient SX_0003. There was only one genotype of ITS gene ITS 4 for patient SX_0002.

In the result of analysis of SOD gene, SOD1 (110C/215T) was presented in all these 3 patients. There were mixed genotypes containing SOD1 (110C/215T) and SOD2(110T/215C) in sample of the HIV-negative patient.

DHFR 312 (312C) and DHPS WT were the most frequent genotypes in the genotyping analysis using DHFR and DHPS as the gene targets among these 3 patients complicating PJP, respectively.

Similarly, genotyping using CYB as the gene target among these 3 patients were CYB 7 for SX_0001, CYB 2 for SX_0002, and mixed genotypes including CYB 1 and CYB 8 for SX_0003.

The genotyping analysis of mt LSU rRNA as the target gene amplification showed that genotype mt3 (mt85T/248C) were presented in the two HIV-positive patients with PJP. However, mt LSU-rRNA genotype in the *P. jirovecii* strain isolated from the HIV-negative patient was containing more than one nucleotide sequence type including mt1 (mt85C/248C) and mt2 (mt85A/248C).

The different combinations of genotypes and genetic diversities of *P. jirovecii* isolates were observed in these 3 patients from Shanxi Province, PR China in this present study.

4. Discussion

ITS, SOD, DHFR, DHPS, CYB and mt LSU rRNA genes of *P. jirovecii* in this study were amplified, cloned, sequenced and classified respectively. Meanwhile, we analyzed the combinations of the multilocus genotype and assessed the association between the genotypes and clinical conditions of patients. The correlations of the clinical characteristics such as the severity of the diseases and geographical origins with the multilocus gene sequences of the *P. jirovecii* were reported in the previous studies [2, 10, 12, 13]. However, it is difficult to accurately determining their association mentioned above based on the small sample sizes in this study.

The ITS region of *P. jirovecii* is the most polymorphic loci and widely used for genotyping [1]. In this study, we assessed and analyzed the *P. jirovecii* ITS genotype of 3 clinical samples from Shanxi province according to the new, simplified genotype nomenclature system [2]. We found ITS 4, ITS10 and ITS 59 genotypes, which were also identified in our previous study [2]. Furthermore, ITS 59, the GenBank accession number is MK300661, was the second occurrence of ITS genotypes in China. ITS 2, ITS10 and ITS 16 were also found in this study. There were two BALF specimens were detected with more than one ITS genotypes, while only single ITS genotype was found in the remaining one patient. These findings showed that there were mixed infections with two different *P. jirovecii* strains in the 2 patients mentioned above, similar to the previous reports of the ITS genotyping of *P. jirovecii* in China [14, 15]. This study suggested that a PJP individual might be co-infected with more than one genotype of *P. jirovecii*. Furthermore, the genotypes of *P. jirovecii* ITS region plays important roles in understanding its epidemiology, transmission and pathogenesis [2, 15–17]. So far, the epidemiological studies on *P. jirovecii* ITS genotypes in Europe, Africa, America and other Asian countries showed that there have been at least 62 ITS genotypes reported in GenBank [1]. Genotype 1 was the most common ITS genotypes based on worldwide studies [18–23]. The most prevalent genotype of ITS in Japan was Genotype 10 [24], which was observed in our study. In previous studies in China, Genotype 22 was most common in Beijing, Tianjin and Liaoning [2, 15], whereas, Genotype 1 in Guangzhou [14]. However, it is difficult to accurately assess and statistically analyze the correlation between geographic diversity and *P. jirovecii* ITS genotypes based on the small sample size in this study. Notwithstanding the foregoing, our study identified and analyzed the ITS genotype of *P. jirovecii* isolates from Shanxi province in PR China for the

first time. Meanwhile, the results in this study provided the partial epidemiological data of *P. jirovecii* ITS genotypes in China and further demonstrated the new ITS genotype nomenclature system previously reported is a simpler and useful tool to analyze and interpret.

SOD mutations of *P. jirovecii* were commonly observed at two different positions of nucleotide bases (i.e., 110 and 215) and genotypes SOD1 and SOD2 were detected most frequently in previously studies [12, 25–28]. Genotypes SOD3 and SOD4 were the second most common [7]. In the present study, both SOD1 and SOD2 were observed, but other SOD genotypes were not. Mixed genotypes containing SOD1 and SOD2 were found in the isolate from the HIV-negative patient. Previous studies showed that the clinical outcomes of the patients infected with *P. jirovecii* SOD1 genotype were poor, indicating that the virulence of *P. jirovecii* might be closely correlated with genotype SOD1 [10, 12, 29, 30].

The combination of trimethoprim and sulfamethoxazole (TMP-SMX), the first-line drug for treatment against PJP, is well tolerated and significantly effective [1]. DHFR and DHPS are respectively targets of TMP and SMX. With the widespread use of TMP-SMX, the development of drug resistance by *P. jirovecii* has been emerged as a concerning problem. Several previous studies have demonstrated that treatment failures of PJP are closely correlated with the high mutation rates of DHPS and DHFR gene and the frequencies of mutations in DHPS gene are greatly higher than that in DHFR, indicating that the DHPS mutations are caused by the pressure of drug selection but not random occurrences [31, 32]. The DHPS wild-type (WT) sequence was more frequent displaying in *P. jirovecii* DHPS genotype, while WT and DHFR 312 synonymous mutations (nucleotides at position 312C) were common in DHFR genotype [7, 33, 34]. The synonymous mutations in DHFR genotypes are probably based on polymorphisms rather than the pressure for drug selection [1, 34]. Furthermore, the rates of DHPS and DHFR mutations were still very low [12, 35–37]. In this study, DHPS WT and DHFR 312C were observed at the DHPS and DHFR locus and no other mutations were detected, consistent with the prior studies reported in PR China [12, 34, 37]. This low prevalence of DHPS and DHFR mutations was not only demonstrated in China but also in other developing countries such as Brazil, South Africa and Thailand [38–40]. The reason for no mutations in DHPS and DHFR genes in this study were was probably due to the PJP subjects included without TMP-SMZ prophylaxis.

Atovaquone, a ubiquinone analog that inhibits electron transport at the cytochrome bc₁ complex, targets the mitochondrial CYB gene. Atovaquone is effective for PJP prophylaxis in at-risk groups. Previous studies supported the point that CYB mutations in Q0 region (i.e., coenzyme Q binding site) are significantly associated with atovaquone [41–43]. In this present work, CYB mutations were not only including genotype CYB 1 and genotype CYB 2, which are the common genotypes documented in previous studies [7, 12], but also containing genotype CYB 7 and genotype CYB 8. However, the patients collected in our study had not received atovaquone for treatment or prophylaxis against PJP, implying a negative pressure for drug selection. Therefore, CYB mutations had no correlation with atovaquone in this study, but *P. jirovecii* mutations were detected, consistent with previous studies [12]. Additionally, genotype CYB 1 and genotype CYB 8 were observed together in the patient without HIV, suggesting the presence of mixed infection.

We found that the Genotype 3 of mt LSU-rRNA gene was observed in the PJP patients with HIV in this study. A previous study in China suggested that the most common mt LSU-rRNA genotype was Genotype 3 (15/30) in AIDS-PJP patients^[12], which were consistent with our study. Medrano F.J. reported that Genotype 3 was most commonly detected in AIDS-PJP patients, while Genotype 2 in the groups without HIV in Spain [44]. In previous studies, Genotype 2 was most common in patients with chronic respiratory diseases and autoimmune diseases, similar to this, Genotype 2 was also observed in the patients with ILD in our study. This patient was co-infected with genotypes 1 and 2. These studies indicated that the virulence of Genotype 3 was stronger than other genotypes of mt LSU-rRNA gene and would be more likely to invade and damage the lung tissues among immunocompromised patients. Further studies are required to confirm the above findings. Additionally, unlike the studies mentioned above, Genotype 2 was the highest frequent genotype in patients with HIV, but Genotype 3 was the most common genotype in HIV-negative patients with malignant diseases in India [30]. The predominant genotype of mt LSU-rRNA gene was Genotype 1 in the patients from Portugal, Spain and some other regions [22, 45]. Genotype 3 was the most common genotype in patients with HIV in eastern China [12] and in this study. These previous epidemiological surveys suggested that the distribution of *P. jirovecii* mt LSU-rRNA genotypes might be associated with geographical origins.

Moreover, coinfections with two genotypes of *P. jirovecii* in the same patient without HIV were detected for different genetic loci including ITS, SOD, CYB and mt LSU rRNA in this study, while coinfections in HIV positive patients were uncommon. These mixed-genotypes were confirmed by sequencing of plasmid clones and the suspected mutations were detected in at least 2 clones for each patient. The pulmonary concurrent infection with other fungi and bacteria was detected in the HIV-negative patient included in this work who was co-infected with multiple strains of *P. jirovecii*. Combined with some serum biochemical parameters especially 1, 3- β -D-Glucan (BDG) and lactate dehydrogenase (LDH) and clinical as well as laboratory characteristics and of the patients, it seems that the HIV-negative patient was more serious, suggesting that coinfections with multiple strains of *P. jirovecii* and other pulmonary pathogens were correlated with the severities of the diseases. Severe hypoxemia was presented in this non-HIV patient with PJP. According to the data of clinical studies, the clinical symptoms the PJP patients without HIV are commonly atypical, rapid and much more serious than the AIDS-PJP patients, with the poorer clinical outcomes even death [46–49]. However, it is unknown the varieties of clinical characteristics whether caused by the mixed genotypes based on the small sample sizes. Furthermore, despite the relatively high prevalence of coinfection with multiple *P. jirovecii* strains in humans were reported in vast majority of the strain variation studies [1, 2, 22, 24, 40, 45, 50], it is also unacknowledged that the occurrence mechanism, potential clinical outcomes and influences on *P. jirovecii* virulence and transmission. Further studies are needed to elucidate these unknown fields on coinfection.

We assessed the combinations of *P. jirovecii* genotype at ITS, mt LSU-rRNA, DHFR, DHPS, CYB, and SOD loci. This is the first time to analyze the multilocus genotypes of *P. jirovecii* isolated from Shanxi. The combination of SOD 1, DHFR 312C, DHPS WT, mt3 genotypes separately occurred in the two HIV-positive patients. A recent similar study indicated combination of SOD1, DHFR312T, DHPS WT, mt3, CYB1

genotypes is associated with the poorest prognosis [12]. Except the DHFR 312 synonymous mutations and CYB1, the combination of SOD 1, DHFR 312C or DHFR 312T, DHPS WT, mt3 are possible to be far more common among HIV-positive populations. Polymorphic combinations of mt85A, SOD110C/215T, CYB C and mt85T, SOD110C/215T, CYB C in *P. jirovecii* were the most common genetic types previously reported, obviously correlated with more severe PJP cases and with high rates of mortality [30]. The combination type of mt85A, SOD110C/215T, CYB C was observed in the HIV-negative-PJP case with a more serious clinical consequence, whereas, it was also detected in one AIDS-PJP patient in our study. Therefore, the larger-scale and more intensive studies on the genetic polymorphisms of *P. jirovecii* were needed to reveal its associations with clinical characteristics and outcomes.

5. Conclusions

In conclusion, we performed the genetic polymorphisms analysis of the ITS, SOD, DHFR, DHPS, CYB and mt LSU rRNA gene sequences in *P. jirovecii* and assessed its correlation with clinical characteristics of PJP patients. The present study would provide useful epidemiological information on the multilocus sequence typing and strain variations of *P. jirovecii* in Shanxi Province, PR China. A larger-scale collection of *P. jirovecii* clinical isolates with the data on epidemiological parameters are required for further study to investigate the correlation between *P. jirovecii* virulence, transmission, drug resistance and clinical outcomes.

6. List Of Abbreviations

Pneumocystis jirovecii: *P. jirovecii*

Pneumocystis jirovecii pneumonia: PJP

Chronic obstructive pulmonary disease: COPD

Human Immunodeficiency Virus: HIV

internal transcribed spacer regions of rRNA operon: ITS

superoxide dismutase: SOD

dihydropteroate synthase: DHPS

dihydrofolate reductase: DHFR

cytochrome b: CYB

mitochondrial large-subunit rRNA: mt LSU rRNA

high-resolution computed tomography: HRCT

Gomori's methenamine silver: GMS

Acquired Immune Deficiency Syndrome: AIDS

highly active antiretroviral therapy: HART

trimethoprim and sulfamethoxazole: TMP-SMX

1, 3- β -D-Glucan: BDG

lactate dehydrogenase: LDH

Declarations

Ethics approval and consent to participate

The protocol for the experimentation was approved by the Medical Ethics Committee of the First Affiliated Hospital of Shanxi Medical University (Shanxi, PR China) (reference number 2019-K051).

Patient consent for publication

Applicable. The three patients involved in this study provided the written informed consent.

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Declaration of Competing Interest

There is no conflict of interest.

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Authors' contributions

This present study was conceived and designed by TX, and performed and analyzed by WQD and TX. The analysis of statistical genetic polymorphisms and manuscript preparation and revision were performed by TX. WQD, WJD, YSL, SFW HL and XRZ contributed to the collection of BALF specimens and patients' information as well as the clinical diagnosis. All authors read and approved the final manuscript and are responsible for all aspects of this study.

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Figures

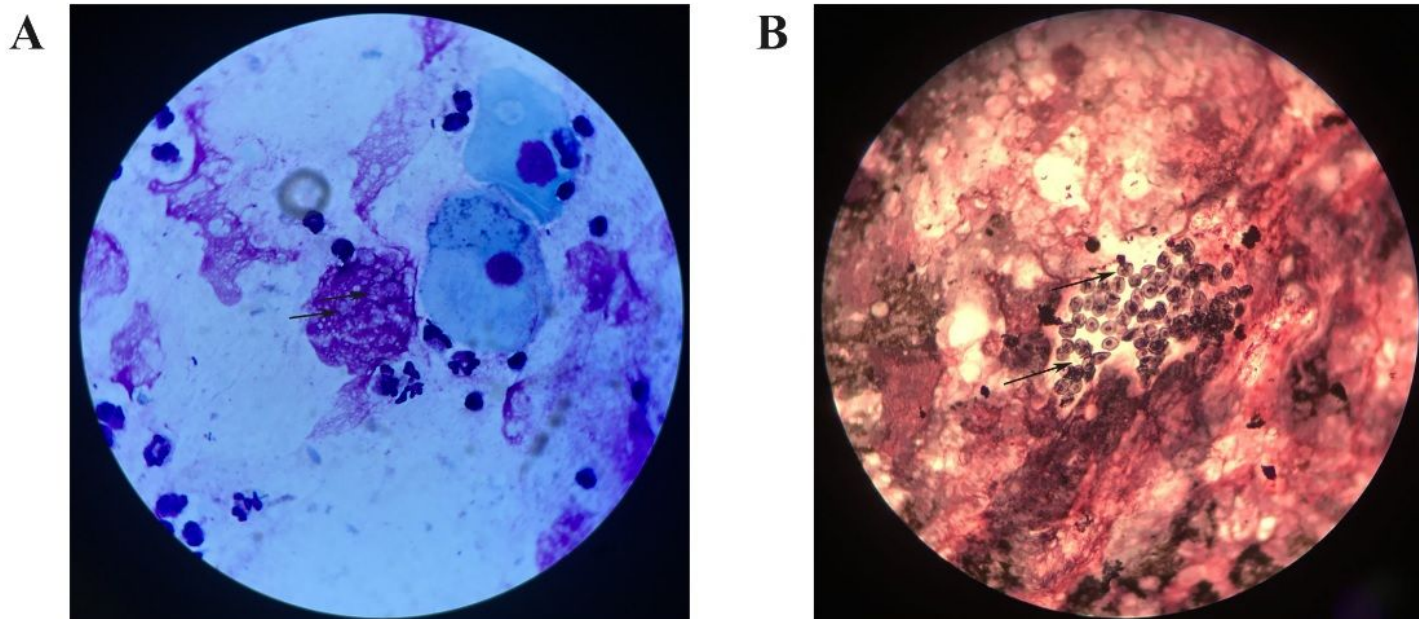


Figure 1

Pathogenic identification of *P. jirovecii* infection was assessed by Giemsa and GMS (100 ×). A. Result of Giemsa staining. The cyst wall of *P. jirovecii* formed a circle transparent zone due to not being stained with Giemsa assay. 2-8 intracystic bodies in cyst were colored in blue, arranged like a ring (arrows). Typical *Pneumocystis* cysts were assuming as crescent or irregular sphere. B. Result of GMS staining. *P. jirovecii* cysts, brown or puce sphere or ovoid with a black small stick in the middle, were visualized under Olympus microscope (arrows).