─────────────── **Original Article** ───────────────

Molecular detection of *Pneumocystis jirovecii* using polymerase chain reaction in immunocompromised patients with pulmonary disorders in northeast of Iran

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Abstract

Background and Purpose: Pneumocystis pneumonia, caused by *Pneumocystis jirovecii*, is a fatal disease threatening patients with AIDS or immunosuppression. Assessment of colonization in these patients is of great significance, since it can lead to severe pulmonary disorders. Considering the scarcity of published reports on *Pneumocystis jirovecii* isolates from patients in Mashhad, Iran, we aimed to evaluate pneumocystis colonization in individuals with different pulmonary disorders.

Materials and Methods: We used nested polymerase chain reaction (PCR) method to amplify mitochondrial large subunit-ribosomal ribonucleic acid (mtLSU-rRNA) gene in 60 bronchoalveolar lavage (BAL) samples, obtained from patients, referring to the Department of Internal Medicine (Pulmonary Diseases Section) at Imam Reza Hospital, affiliated to Mashhad University of Medical Sciences, Mashhad, Iran.

Results: DNA of *Pneumocystis jirovecii* was detected in 10 out of 60 BAL samples (16.66%) via nested PCR method.

Conclusion: According to the present findings, the colonization rate of *Pneumocystis jirovecii* was similar to the rates reported in other similar studies in Iran.

Keywords: Nested PCR , P. jirovecii

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Introduction

*P*neumocystis jirovecii (abbreviated as *P. jirovecii*) is an opportunistic fungus, responsible for a severe pulmonary disease, known as pneumocystis pneumonia (PCP) (1). PCP, caused by *P. jirovecii,* is regarded as one of the most frequent and serious complications, affecting immunocompromised patients such as those with HIV infection [2].

PCP is an important cause of morbidity and mortality among immunocompromised patients [3, 4]. Patients with malignancies, solid organ transplant recipients and patients treated by immunosuppressive agents are susceptible to this infection [5, 6, 7-13]. In addition, some other conditions such as diabetes and severe malnutrition increase the risk of PCP [14, 15].

Over the past decades, despite the decline in the number of PCP cases due to the use of highly active antiretroviral therapy (HAART) in HIV-infected patients, the incidence of this infection has remained high among patients with other conditions. Since colonization of the pulmonary tract with this fungus may promote the risk of infection, evaluation of colonization rate in immunocompromised patients is essential [14, 15].

Today, PCP can be diagnosed via different methods, depending on laboratory conditions, available resources, and type and quality of samples obtained from patients [16-18]. Specific staining methods such as methenamine silver, Giemsa, hematoxylin & eosin and fluorescence staining can be applied for the diagnosis of PCP [19-24]. Based on previous studies, the sensitivity of staining techniques, used for the diagnosis of PCP, varies between 70% and 90% in bronchoalveolar lavage (BAL) [22-26].

Today, molecular methods such as polymerase chain reaction (PCR) are being increasingly applied for the diagnosis of PCP

[27]. To detect *P. jirovecii* in clinical samples, many studies have been conducted, using different gene targets and PCR techniques [28, 29]. However, since 1979, no studies have evaluated *P. jirovecii* isolates from patients with pulmonary disorders in Mashhad, Iran. The aim of this study was to isolate P. *jirovecii* from patients with pulmonary disorders by nested PCR method.

Material and Methods
Patient population
In the present study, 75 immunosuppressed patients, suspected of pneumonia, who were admitted to the Department of Internal Medicine (Pulmonary Diseases Section) at Imam Reza Hospital, affiliated to Mashhad University of Medical Sciences, Mashhad, Iran were recruited during September 2012 and March 2014.

60 patients were included in this study with at least three of the following symptoms: dry cough, dyspnea, fever, abnormal auscultation sound and new pulmonary infiltrates. The patients were considered to be immunosuppress-ed if they met one of the following criteria: bone marrow/solid organ transplantation, hematological malignancy, uncontrolled diabetes, malnutrition and receiving immunosuppressive treatment.

Laboratory processing of specimens
The obtained BAL samples were centrifuged at 4000 rpm for 10 min. Afterwards, the supernatant was removed and the pellet was resuspended in one-fifth of the supernatant. The final product was stored at a temperature of -20 °C for DNA extraction.

DNA extraction and PCR amplification
DNA extraction was performed, using Prime Prep Genomic DNA Isolation Kit (GenetBio, Korea), according to manufacturer's instructions. DNA was eluted in 50 μL of elution buffer, which came with the kit, and was maintained at -20 °C.

Primers
PCR was performed for mitochondrial large subunit (mtLSU) ribosomal ribonucleic acid (rRNA) gene, using previously published primers (30). The used primers were as follows: pAZ102-E (5'-GAT GGC TGT TTC CAA GCC CA-3') and pAZ102-H (5'-GTG TAC GTT GCA AAG TAC TC-3') for external round PCR and pAZ102-X (5'-GTG AAA TAC AAA TCG GAC TAG G-3') and pAZ102-Y (5'-TCA CTT AAT ATT AAT TGG GGA GC-3') for nested round PCR.

PCR and sequencing
For first-round PCR, 2 μL of DNA was added to the reaction mixture, containing 2.5 mM $MgCl_2$, 1 mM deoxyribonucleotide triphosphates (dNTPs), 1 mM primers and 1.8U Taq polymerase. PCR assay was carried out by an initial denaturation at 94 °C for 5 min, followed by 20 cycles of denaturation at 94 °C for 1 min, 56 °C for 1 min and 72 °C for 1 min in an ABI 2720 thermal cycler (Applied Biosystems, Foster City, CA).

For the nested round PCR, 2μL of 10-time diluted external-round product was added into the reaction mixture, containing 2.5 mM $MgCl_2$, 0.5 mM dNTPs, 1 mM primers and 1.8 U Taq polymerase. PCR conditions for nested round PCR were as follows: initial denaturation at 94 °C for 5 min, followed by 20 cycles of denaturation at 94 °C for 1 min, at 59 °C for 1 min and at 72 °C for 1 min.

PCR amplified products were run on 1.5% agarose gel, stained with ethidium bromide, and were visualized under an ultraviolet transilluminator. Positive and negative controls were included in each experiment. The positive control was the DNA extracted from BAL smears, which were positive, based on Giemsa staining and PCR experiments. On the other hand, sterile distilled water was used as the negative control in each run of the test. All positive PCR products of mtLSU-rRNA gene were sequenced. Sequences in this study were imported to MEGA software version 5 (http://www.megasoftware.net). Afterwards, they were adjusted and exported to Basic Local Alignment Search Tool (BLAST) of National Center for Biotechnology Information (NCBI) database for species recognition. For statistical analysis, Fisher's exact test and independent samples t-test were performed, using SPSS version 16.

Results

Among 99 samples transferred to the mycology laboratory, 39 samples were excluded due to positive culture for *Mycobacterium tuberculosis*, fungal contamination, and low concentration of DNA or incomplete information provided in the questionnaire. Among 60 patients enrolled in this study, 36 and 24 cases were male and female, respectively. It should be mentioned that two patients were from Turkmenistan and one originated from Afghanistan.

Regarding the risk factors, 16 patients had diabetes, 12 cases had been under long-term antibiotic therapy, 9 cases suffered from cancer, 8 cases had received chemotherapy and 8 subjects had undergone major surgeries. Congenital immunodeficiency, hypereosinophilia syndrome, use of immunosuppressive drugs and an unknown form of immunodeficiency were the risks reported in four patients (Table 1).

Two primers, i.e., pAZ102-E and pAZ102-H in external round PCR and two primers, i.e., pAZ102-X and pAZ102-Y, in nested round PCR successfully amplified 346bp and 267bp fragments of *P. jirovecii*, related to 10 positive cases, respectively.

In terms of clinical symptoms, 44 patients (73.33%) had fever, and 55 cases (91.66%) presented with persistent dry coughs and wheezing. The molecular test (PCR) was performed on all samples, and 10 samples showed positive results for *P. jirovecii* (Figure 1).

The risk factors found in 10 confirmed cases of *P.jirovecii* were diabetes (n=3), chemotherapy

(n=2), malignancy (n=2), extensive surgery (n=1), immunosuppressive drugs (n=1) and congenital immunodeficiency (n=1) (Table 1). According to the results, this infection was not significantly related to gender, age, acceptation or underlying diseases.
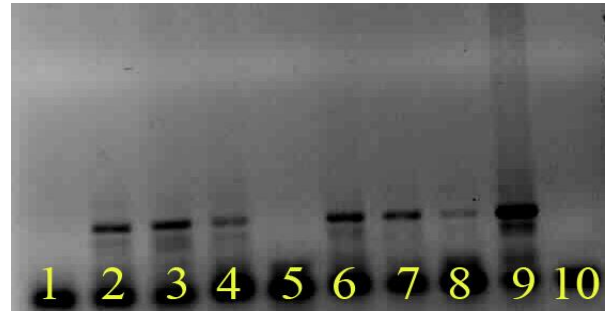
Figure 1. Gel electrophoresis of PCR products in clinical pneumocystis; line 1: negative control; lines 2, 3, 4, 6, 7, 8 and 9: positive samples; line 5 and 10: negative samples; last line: the ladder

Discussion

By the end of the Second World War, the first case of PCP was reported in an orphanage in Europe [31]. The most frequent cases of PCP have been observed among patients with AIDS, although other individuals with immunosuppression are also at risk of this infection. Different types of malignancies, congenital immune deficiency [9], diabetes [7], organ transplantation; malnutrition [3, 32-23] and immunosuppressive drugs [5, 20, 34] are the main predisposing factors for PCP.

The majority of studies have been performed on patients with AIDS and pulmonary disorders, whereas a limited number of studies have evaluated individuals, suffering from other types of immunodeficiencies. The present study was performed on patients with compromised immunity and pulmonary disorders. Considering the inclusion and exclusion criteria, 60 patients (24 females and 36 males) were included in the present research.

In the present study, the results of PCR and sequencing tests demonstrated that 10 BAL samples were colonized by *P. jirovecii* infection. Similarly, a study performed by Azoulay in 2004 in Paris showed the rate of positive samples to be 8.7%. In this study, it seems that prophylactic treatment was effective for decreasing the rate of infection [38]. In

Table 1. Clinical information of the study population and PCR-positive samples

Risk factors	PCR-positive	Total
Diabetes	3	19
Chemotherapy	2	8
Malignancy	2	9
Extensive surgery	1	8
Congenital immunodeficiency	1	1
Immunosuppressive drugs	1	1
An unknown form of immunodeficiency		1
Long-term antibiotic therapy		12
Hypereosinophilia syndrome		1
Total	10	60

another study on a similar population in Iran, use of co-trimoxazole was an exclusion criterion, and *P. jirovecii* infection was reported in 12.60% of cases [35].

Among 60 examined samples for *P. jirovecii*, one sample belonged to a four-year-old child with congenital immunodeficiency; however, the condition of this child was different from other patients. Considering the results reported in previous studies, the rate of this infection seems to be rather high in children. In this regard, in one study on hospitalized children in Denmark, the incidence of *P. jirovecii* infection was estimated at 16% [36].

Among different immunocompromised patients, diabetic patients are not very susceptible to this infection. However, disorders resulting from uncontrolled diabetes considerably decrease the level of immunity [37]. The reason is the higher sensitivity of diabetic patients to viral and bacterial infections.

Diabetes mellitus is a condition which decreases the activity level of body cells, responsible for immunity [7]. These activities include infiltration, chemotaxis, phagocytosis and natural killer cell activities; however, impairment is reversible if the disease is controlled [37]. On the other hand, the high level of blood glucose may lead to weakened immune system, increased adherence of cell surface, impairment of humoral immunity and repression of superficial cell receptors [37].

According to epidemiological studies, the prevalence of PCP has been estimated at 10% in different populations. This rate is insignificantly higher than the incidence rate of AIDS; therefore, it is necessary to follow-up patients with pulmonary disorders. In the present study, the rate of infection among diabetic patients was estimated at 18.8%. *P. jirovecii* infection was also reported in type II diabetic patients [7].

In the present study, molecular examination of BAL samples, obtained from patients with malignancy, showed that only two samples (22.2%) suffered from PCP; this finding was highly similar to the results reported by Khodadadi and colleagues [35]. Based on the findings of similar studies in different countries, the incidence of *P. jirovecii* infection

was estimated at 16-22% [38, 39]. Also, the main clinical signs and symptoms among patients in this study were chest pain, rales, fever and dry cough, which are common among patients with pneumocystis, as reported in similar studies.

Among 60 BAL samples examined in this study, only one direct Geimsa-stained smear was positive for *P. jirovecii*, while nested PCR confirmed the presence of *P. jirovecii* in 10 samples. This indicates that the sensitivity of nested PCR is 10 times higher than that of conventional methods. The sensitivity of molecular method has been confirmed by many researchers [30, 36, 40-44].

The present study was in fact the first report on *P. jirovecii* infection in Mashhad, Iran. However, this does not prove the absence of this infection before this study, since conventional methods are not sensitive enough to provide accurate results.

Conclusion

P. jirovecii infection is one of the causes of pulmonary disorders. Use of molecular methods is highly useful for detecting misdiagnosed infections. Infected patients can act as a source of infection for other susceptible individuals present in the same room or section. Therefore, identification of *P. jirovecii* infection is indicated for patients with pulmonary disorders.

Acknowledgements

Authors' contributions

M.P. obtained the specimens, performed all the tests and wrote the draft of the manuscript. A. F. designed and managed the research and edited the draft. MJ. N. supervised the research and F. R. referred the patients to the section.

All authors reviewed and approved the final manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

Financial disclosure

Financial interests related to the materials of this study have been declared.

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