The mycological and molecular study of *Pneumocystis jiroveci* pneumonia among HIV and non-HIV immunocompromised patients hospitalized in pulmonary units in Guilan, northern Iran

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**ABSTRACT**

**Background and Objectives:** *Pneumocystis jirovecii* pneumonia (PJP) is a serious infection that usually affects those with a weak immune system. Since the prevalence of this infection in Iran and in the world is not clearly defined, the present study aimed to evaluate the incidence, clinical spectrum, and demographic characteristics of PJP among HIV and non-HIV immunocompromised patients.

**Materials and Methods:** Bronchoalveolar Lavage (BAL) specimens were obtained from 3 groups of immunocompromised patients, including acquired immunodeficiency syndrome (AIDS) patients, diabetic patients, and patients receiving immunosuppressive therapies. All were hospitalized in pulmonary units. The specimens were examined using microscopic methods (Giemsa and calcofluor white staining) and the nested-PCR technique based on mtLSU-rRNA gene.

**Results:** A total of 120 BAL samples were collected. From 12.5% (5 from 40) of HIV-infected patients, 5% (2 from 40) of patients receiving immunosuppressive therapies, and 2.5% (1 from 40) of diabetic patients *Pneumocystis jirovecii* was isolated. There was not any association between the prevalence of PJP and the patient's gender ($p = 0.557$) and age ($p = 0.681$). Fever and dyspnea ($n=7, 87.5\%$), nonproductive cough and abnormal auscultation sound ($n=5, 62.5\%$), and also chills and weight loss ($n=2, 25\%$) were the documented clinical symptoms of PJP. Also, the results showed that none of the samples had positive results for *P. jiroveci* with microscopic tests while using the nested-PCR method 8 samples had positive results.

**Conclusion:** Since PJP often causes symptoms that are similar to other illnesses, such as the flu or tuberculosis, clinical and laboratory findings should be used simultaneously for making the final decision on drug administration.

**Keywords:** Pneumonia; Pneumocystis; Human immunodeficiency virus; Diabetes mellitus; Immunosuppression; Epidemiology; Iran
INTRODUCTION

Pneumocystis jiroveci, formerly known as P. carinii, is an unusual opportunistic fungus that was considered a protozoon. In the 1980s it emerged as a major pathogen with the onset of the acquired immunodeficiency syndrome (AIDS) epidemic (1). The disease caused by the fungus is used to be called Pneumocystis jiroveci pneumonia (PJP) (2). Patients at particular risk of infection include those with AIDS, underlying diseases such as hematologic malignancies and solid tumors, diabetics, those receiving immunosuppressive therapies, and organ transplant recipients (3). P. jiroveci is thought to be transmitted from person to person through an airborne route. Asymptomatic lung colonization can occur in people with normal immune systems, and they may unknowingly become reservoirs (asymptomatic carriers) for the spread of the fungus to immunocompromised individuals. Occult pneumocystosis may lead to chronic lung inflammation (4).

PJP can cause death is 27%, increasing to 50% in severely immunocompromised patients (5). Pneumocystis selectively attaches to squamous (type I) alveolar epithelial cells, interferes with the pulmonary surfactant, causing marked thickening of the alveolar septa and profound hypoxia, which can be fatal if not treated aggressively (6). PJP may be difficult to diagnose owing to its nonspecific symptoms. Radiological presentation is also nonspecific or even normal in the case of mild disease. Therefore, the single most important diagnostic tool for PJP is a high clinical suspicion (7). Because PJP is one of the most frequent opportunistic infections, physicians should consider it when an HIV-infected patient or a patient who is immunosuppressed for reasons other than HIV complains of fever, shortness of breath, and/or cough (8, 9). In more than 95% of cases, P. jiroveci infection is restricted to the lung, but in severely immunocompromised individuals, the rare disseminated infection (thyroid, kidney, bone marrow, liver, spleen, retina, ear, and skin) may occur (10-14). The major problem in Pneumocystis research is the absence of an in vitro culture system (15). Although the incidence of PJP in developed countries has reduced as a result of prophylaxis, in developing countries like Iran, it is a significant concern because of limited care resources and the absence of enough data about the epidemiology of the infection (16). Besides, the epidemiology of Pneumocystis infections has still remained unde-
PJP according to a pulmonary diseases specialist opinion (dyspnea, nonproductive cough, fever, chest pain, weight loss, and abnormal auscultation sound).

(2) Patients with diffuse pulmonary involvement (e.g., bilateral infiltrates and/or ground-glass opacities and/or nodules and/or alveolar condensation on high-resolution computed tomography scan).

Patients who had taken co-trimoxazole before enrollment were excluded from the present study in order to prevention of false-negative results.

Demographic features including age and gender were recorded. Bronchoalveolar Lavage (BAL) specimen from each patient were obtained. Samples were homogenized, centrifuged (at 1500 g for 5 minutes) and sediments were collected in sterile conical tubes.

**Microscopic examination.** The BAL pellets were smeared, dried, and fixed with methanol and then stained with calcofluor white (Fungi-Fluor, Polysciences, Inc., Pennsylvania, USA) in accord with the manufacturer’s instructions. Giemsa staining was performed (with 2 mL of Giemsa stain in 40 mL of 6.7 mM phosphate buffer at pH 7.2) on methanol-fixed BAL smears, as described previously (17). The experiments were examined twice in parasitology and mycology laboratories, and positive or negative findings were confirmed by a professional parasitologist and mycologist. We considered the specimen microscopically positive if *P. jirovecii* was detected by either Giemsa or calcofluor white.

**DNA extraction.** *P. jirovecii* DNA was extracted by using commercially available DNA extraction kits (GeneAll Bldg, 303-7 105 Dongnam-ro, Songpa-gu, Seoul, South Korea) according to manufacturer’s instructions.

**Primers.** PCR was performed for mitochondrial large subunit (mtLSU) ribosomal ribonucleic acid (rRNA) gene, using previously published primers (18). External primers namely pAZ102-E (5'-GAT GGC TGT TTC CAA GCC CA-3') and pAZ102-H (5'-GTC TAC GTC CCA AG TAC TC-3') were used in the first round, which produced a 346 bp amplicon, and the internal primers namely pAZ102-X (5'-GTC AAC TAC AAA TCG GAC TAG G-3') and pAZ102-Y (5'-TCA CTT ATT ATT AAT TGG GGA GC-3') were used in the second round to amplify a 260 bp fragment (18).

**PCR and sequencing.** Each PCR reaction contained 2.5 µL of 10x reaction buffer, 2 mM MgCl₂, 500 µM dNTPs mixture, 1.25 µM Taq polymerase (Prime Taq, Genet Bio, South Korea), 25 pmol of each primer, 2 µL of DNA template solution, and enough distilled water to a final volume of 25 µL. First-round was fulfilled under the following condition: 95°C for 4 min, 30 cycles of 94°C for 45 s, 60°C for 1 min and 72°C for 1 min; and a final extension for 10 min in 72°C. Two microliters of the first PCR product was used as DNA template in the second PCR reaction having the following conditions: 95°C for 4 min, 30 cycles of 94°C for 30 s, 60°C for 45 s and 72°C for 45 s; and a final extension of 72°C for 10 min. PCR products were analyzed by electrophoresis on 1.5% agarose gels containing 0.5 µg/mL ethidium bromide and the bands were observed under UV light of the transilluminator. To rule out any false-positive results, aerosol barrier pipette tips were used and different steps of the preparations including DNA extraction, master mix preparation, PCR reactions and product detection were done in different rooms. 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 adjusted and exported to Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI) database for species recognition.

**Statistical tests.** The data analysis was performed by SPSS software (IBM SPSS Statistics for Windows, Version 21.0, IBM Corp, Armonk, NY, USA). The study was assessed using Fisher’s exact test and 95% Confidence intervals (CI). A p-value ≤ 0.05 was considered statistically significant.

**RESULTS**

A total of 120 BAL samples were obtained from 3 groups of immunocompromised patients hospitalized in pulmonary units. In the current study, the prevalence of PJP among HIV and non-HIV immunocompromised patients hospitalized in pulmonary units was 6.66%. Of this population, in 12.5% (5
from 40) of HIV-infected patients, 5% (2 from 40) of patients receiving immunosuppressive therapies, and 2.5% (1 from 40) of diabetic patients *P. jiroveci* was isolated. In the present study, according to the host factors (clinical symptoms, radiology findings, and predisposing factors which were defined as inclusion criteria), and the positive results in nested-PCR indicating the presence of *P. jiroveci* DNA in the BAL specimen in patients who were screened and tested negative for other respiratory pathogens the infection was confirmed. The results showed that none of the samples had positive results with microscopic tests (Giemsa staining and calcifluor white) while using the nested-PCR method based on mtLSU-rRNA gene, 8 samples had positive results for *P. jiroveci* (Fig. 1). Among the 8 positive cases in this research, most isolates were from male patients (n=6, 75%) with a mean ± SD age of 58.3 ± 16.3 years (Table 1). Statistical analysis could not find any association between the prevalence of PJP in HIV and non-HIV immunocompromised patients hospitalized in pulmonary units and the patient’s gender (p= 0.557) and age (p= 0.681).

Based on clinical symptoms, 7 positive patients (87.5%) had fever and dyspnea, 5 cases (62.5%) presented with nonproductive cough and abnormal auscultation sound, and 2 positive patients (25%) had chills and weight loss. Statistical analysis showed that there was not a significant relationship between the prevalence of PJP in HIV and non-HIV immunocompromised patients hospitalized in pulmonary units and clinical symptoms (p = 0.21). The detailed information related to 8 patients with PJP is shown in Table 1.

The GenBank accession numbers for the sequences obtained in this work are SRR6399953-SRR6399960.

**DISCUSSION**

*P. jiroveci* causes PJP with a high rate of morbidity and mortality in immunocompromised patients especially in HIV infected persons and other immunosuppressed groups such as diabetic patients, and patients with autoimmune disorders (who treated with steroid or monoclonal antibodies directed against cell-mediated immune system mediators). In this study, a total of 120 BAL specimens related to HIV and non-HIV immunocompromised patients hospitalized in pulmonary units were examined and in 8 (6.66%) cases PJP was diagnosed. Of this population, in 12.5% (5 from 40) of HIV-infected patients, 5% (2 from 40) of patients receiving immunosuppressive therapies, and 2.5% (1 from 40) of diabetic patients *P. jiroveci* was isolated. In a study in Tehran, Iran the rate of *P. jiroveci* among non-HIV-infected patients

![Fig. 1](http://ijm.tums.ac.ir)  
Fig. 1. Agarose gel electrophoresis of nested-PCR products of *P. jiroveci* partial mtLSU-rRNA gene. From left: Lane 1: DNA ladder (100 bp), Lanes 2,3: Negative control, Lane 3: positive control, Case 4 and Case 9: positive clinical samples having specific 260 bp bands representative of *P. jiroveci*, Cases 1-3, 5-8, and 10,11: no representative band indicating negative clinical samples.
was 12.5%. They have shown that *P. jirovecii* colonization rate in patients with malignancy, transplant recipients, immunosuppressive therapy recipients and patients with other various lung diseases was 21.7%, 20.3%, 12.7%, and 7.3%, respectively (17). On the other hand, the frequency of *P. jirovecii* was reported 11.9% among HIV-infected patients with a CD4 count < 200 cells/µL in Tehran (19). In a study conducted by Aboualigaledhari et al. in Ahvaz, Iran the prevalence of *P. jirovecii* was totally 27% including, 32.1% in tuberculosis patients, 25.3% in other chronic pulmonary disorders, and 25.3% in HIV patient (20). In another study by Parian et al. the results of PCR and sequencing tests demonstrated that 10 BAL samples were colonized by *P. jirovecii* (21). Also, a study performed by Azoulay in 2004 in Paris showed the rate of positive samples to be 8.7% (22).

Furthermore, the results of the present study showed that among different immunocompromised patients, HIV-infected patients are more susceptible PIP. Different studies have demonstrated that in HIV-infected patients, the risk of PIP is rapidly increased when the number of CD4⁺ lymphocytes decrease to 200 × 10⁶ per mL (23, 24).

Diabetes mellitus was observed in 12.5% of patients with PIP in the current study. Diabetes mellitus is a condition that decreases the activity level of body cells, responsible for immunity. These activities include infiltration, chemotaxis, phagocytosis, and natural killer cell activities (25, 26). On the other hand, the high level of blood glucose may lead to a weakened immune system, increased adherence of cell surface, impairment of humoral immunity, and repression of superficial cell receptors. These are reasons for the higher sensitivity of diabetic patients to fungal, viral, and bacterial infections (25, 26, 37).

Also, receiving immunosuppressive therapy was observed in 25% of patients with PIP in the present study. The results of a study conducted by Calero-Bernal et al. demonstrated that corticosteroids were in the medication lists of 98 (76%) of non-HIV patients with PIP (27). In a study conducted by Bryan et al., most patients who had PIP had received a corticosteroid dose equivalent to 16 mg or greater administered for at least 8 weeks (28).

Furthermore, the main clinical signs and symptoms among patients in this study were dyspnea, nonproductive cough, fever, and abnormal auscultation sound, which are common among patients with pneumocystis, as reported in similar studies (19-23).

The sex distribution of *P. jirovecii* colonization varies in several reports. The female: male ratio of *P. jirovecii* was 3:3:1 in Togashi et al. (29) report. Also, in a study conducted by Izadi et al. the frequency of *P. jirovecii* colonization among males was 2.5 rather than females (30). Our results showed that the frequency of PIP among males was 3 (M: F; 6:2) greater than females.

PCR-based diagnosis of *Pneumocystis* in respiratory specimens is a valuable tool for screening this organism. Single-step PCR cannot precisely detect the infectious agent in some cases, especially in low template DNA conditions. Therefore, in this study, the more sensitive and reliable nested PCR was used due to its ability to amplify very small quantities of DNA (31). Among 120 BAL samples examined in this study, none of the samples had positive results for *P. jirovecii* with microscopic tests while using the nested-PCR method 8 samples had positive results. This indicates that the sensitivity of nested PCR is

| Variables                        | Patients                  | Number | Percentage |
|----------------------------------|---------------------------|--------|------------|
| Age groups (years)               | ≥ 50                      | 6      | 75%        |
|                                  | < 50                      | 2      | 25%        |
| Gender                           | Female                    | 2      | 25%        |
|                                  | Male                      | 6      | 75%        |
| The type of underlying condition | Human immunodeficiency virus infection | 5 | 62.5% |
|                                  | Receiving immunosuppressive therapies | 2 | 25% |
|                                  | Diabetes Mellitus         | 1      | 12.5%      |
| Clinical symptoms                | Fever and dyspnea         | 7      | 87.5%      |
|                                  | Nonproductive cough and abnormal auscultation sound | 5 | 62.5% |
|                                  | Chills and weight loss    | 2      | 25%        |
higher than that of conventional methods. The sensitivity of the molecular method has been confirmed by many researchers (32-36).

CONCLUSION

The current study is the first report of the epidemiology of PJP from Guilan, northern Iran. The results showed a frequency of 6.66% for PJP among 3 groups of immunocompromised patients, including AIDS patients, diabetic patients, and patients receiving immunosuppressive therapies. Also, our results showed that the nested-PCR might be a reliable technique for the diagnosis of *P. jirovecii*. Here we found that the Giemsa and calcofluor white staining were not effective staining methods for the detection of *Pneumocystis*. Special attention to these patients is essential for preventing nosocomial infections or emerging drug resistance isolates.

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