Iranian Journal of Microbiology
Volume 7 Number 6 (December 2015) 333-337

ORIGINAL ARTICLE

The prevalence of *Pneumocystis jirovecii* among patients with different chronic pulmonary disorders in Ahvaz, Iran

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Received: June 2015**, Accepted:** October 2015

ABSTRACT

Background and Objectives: *Pneumocystis jirovecii* pneumonia (PJP) is a chronic fungal infection that caused by *P. jirovecii*. Disease is more prevalent among the HIV-infected patients. The colonization of pneumocystis in human respiratory system is associated with the airway inflammation and obstruction. The current study was conducted to identify the prevalence of *P. jirovecii* among the patients with chronic pulmonary disorders in Ahvaz, Iran.
Materials and Methods: In the present study, 115 bronchoalveolar lavage (BAL) specimens were collected from patients. Samples were subjected to Nested-PCR with specific primers. The second PCR products were used for sequencing analysis.
Results: Our findings demonstrated that 31(27.0%) of samples were positive for *P. jirovecii*. Nine patients (29%) have tuberculosis (TB) followed by, 1(3.2%) HIV positive and 21(67.7%) miscellaneous pulmonary disorders. Our results show that there was no significant differences between sex (male:female ratio, 17:14), TB, HIV and *P. jirovecii* in BAL samples (P>0.05).
Conclusion: The current study is the first report from Ahvaz and it showed a relatively high frequency (27%) of *P. jirovecii* among patients with different pulmonary disorders. In addition Nested-PCR might be reliable technique for diagnosis of *P. jirovecii*, while the Grocott's methenamine silver (GMS) have a low sensitivity, which only two positive patients were identified.

Keywords: *Pneumocystis jirovecii* pneumonia; HIV-infected patients; Tuberculosis, Ahvaz-Iran

INTRODUCTION

Pneumocystis jirovecii pneumonia (PJP) formerly known as *Pneumocystis carinii* pneumonia (PCP) is an opportunistic fungal infection of the lungs with a significant morbidity and mortality among immunocompromised patients (1-3). The different species of pneumocystis are obligate fungal pathogens and unculturable *in vitro* (4). Disease is often cause of pneumonia in immunocompromised hosts such as hematologic malignancies, organ transplants, immunosuppressive drugs users, pulmonary tuberculosis (TB) and patients infected with human immunodeficiency virus (HIV) (3, 5). In addition, in some cases,

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Pneumocystis can survive for a long time without causing clinical symptoms in the human body. Sheikholeslami et al. have shown that there are a high rate of co-infection of *Mycobacterium tuberculosis* and *P. jirovecii* in patients with HIV (6).

When the immune system is suppressed, the *P. jirovecii* could bind to the alveolar epithelium and proliferate (7). Although suppression of immune system is associated with infection, sometimes immunosuppression could unmask an previous occult infection (8). In addition to causing active disease, *P. jirovecii* has been identified in respiratory tract of healthy individual (9). However, an autopsy study reported *Pneumocystis* colonization rate 65% (10), other relevant studies involving immunocompetent patients did not find any evidence of *Pneumocystis* colonization (11). Additionally, investigations on animal models demonstrate that *Pneumocystis* may cause chronic inflammation (12) and a case of subclinical *Pneumocystis* pneumonitis in a patient without apparent immune-compromised conditions has been reported (13).

Because of *P. jirovecii* is more associated with inflammatory response and symptomatic disease in patients with impaired cell-mediated immunity, we postulate that co-infection with this fungus is possible in patients whose cell-mediated immunity has failed to control *M. tuberculosis*, consequently, leads to more severe pulmonary disease. Therefore, the current study was conducted to identify the frequency of *P. jirovecii* in the patients with chronic pulmonary disorders in Ahvaz, Iran.

PATIENTS AND METHODS

Sampling specimens. One hundred and fifteen bronchoalveolar lavage (BAL) specimens were collected from patients with chronic pulmonary disorders during February-September 2014 in Ahvaz, Iran. Samples were homogenized, centrifuged (at 1500 g for 5 minutes) and sediments were collected in sterile conical Falcon tubes. The sediments were aliquoted into 2 equal volumes, one for DNA extraction (kept frozen at -20 °C) and another for direct examination. This project approved in the ethical committee of Ahvaz Jundishapur University of Medical Sciences (Ajums. Rec. 1392.234). All patients or parents were signed consent form before sampling.

Smear preparation and staining with Grocott's methenamine silver. The BAL pellets were smeared, dried and fixed with methanol and then stained with Grocott's methenamine silver (GMS) (Pajouhesh Asia, Iran) according to manufacturer instruction (14). The smears were carefully evaluated and circular to oval black or gray cysts considered as *P. jirovecii.*

DNA extraction. DNA was extracted by phenol chloroform method (15). Briefly, the specimens centrifuged for 5 minutes and then lysis buffer (50 mM KCl, 15 mM Tris-HCl (pH = 8.3)) and 20 µL proteinase K (Cinnagen, Iran) were added and incubated for 30 minutes in 56 °C. Then phenol chloroform iso-amyl alcohol (Sigma. USA) added and centrifuged at 12000 rpm for 10 minutes. The supernatant was collected and mixed equality with phenol chloroform iso-amyl alcohol (Sigma, USA) and centrifuged at 12000 rpm for 10 minutes. Then, supernatant mixed equally with 2-propanol (Merck, Germany) following diluted 1:10 with sodium acetate (Merck, Germany) and were kept at -20 °C for 20 minutes. Then, it centrifuged at 12000 rpm for 10 minutes. 300 µL of ethanol 70% (Kimia Alcohol, Iran) added to sediment and centrifuged at 12000 rpm for 10 minutes. Finally 50 µL of deionized distilled water added to dried precipitate and stored at -20 °C for further experiment.

Molecular investigation. The extracted DNA from BAL specimens was subjected to Nested PCR (16). The first round of PCR was performed with primers pAZ102-H and pAZ102-E, designed for mitochondrial large subunit ribosomal RNA (mt LSU rRNA) target gene of *P. jirovecii* and second round was done with primers PAZ102-X as forward primer and PAZ102-Y as reverse primer. The primers were designed by Wakefield et al. (17). Five microliters of extracted DNA, 12.5 µL of master mix (Amplicon, Denmark) 5.5 µL of deionized distilled water and 2 µL of primers were used for the first round. Polymerase chain reaction in first step was performed by an initial denaturation at 94 °C for 5 minutes followed by 35 cycles of 94 °C for 30 seconds (denaturation), 55 °C for 30 seconds (annealing) and 72 °C for one minute (extension), and final extension at 72 °C for 5 minutes in a thermocycler (Analytik Jena, Germany). Then, 2 µL of first round PCR product was utilized for the second round PCR reaction as the

template.

The nested round was performed by primary denaturation at 94 °C for five minutes followed by 35 cycles of 94 °C for 30 seconds (denaturation), 58.6 °C for 30 seconds (annealing) and 72 °C for 45 seconds (extension), and final extension at 72 °C for 5 minutes. PCR products were separated by electrophoresis on a 1.5% agarose gel (Cinnagen, Iran) containing ethidium bromide and the amplicons were visualized under ultraviolet transillumination device (G. Box, UK). The positive and negative controls were a confirmed sample of *P. jirovecii* (Courtesy Dr. Fatemeh Maryam Sheikholeslami) and deionized distilled water, respectively. Thirty one (27%) positive samples for *P. jirovecii* were produced a 260 bp band in the amplification, as indicated in Fig. 1.

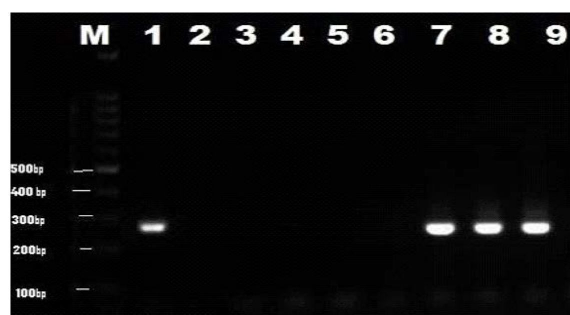

Fig. 1. *Pneumocystis jirovecii* results after second round PCR. M, marker (100bp); lane 1, positive control (260 bp); lane 2, negative control; lanes 3 – 6, specimens with negative results; lanes 7 – 9, samples with positive reaction for *P. jiroveci*

Sequence analysis. The PCR products sent to Bioneer Company (Bioneer, Daejeon, South Korea) for sequencing and the results of DNA sequencing were run in Chromas Lite program (Technelysium, Version 2.4) to analyze the similarity to the sequenced gene in GenBank library.

Statistical analysis. Statistical analysis was done using the SPSS statistical package software for the determination of significant difference via Chi-square between *P. jirovecii* with TB and sex (Version 16.0; SPSS, Chicago, IL).

RESULTS

A total of 115 BAL specimens were collected, 66 (57.4%) male and 49 (42.6%) female in Western health center in Ahvaz. According to patient's history, 28 (24.3%) patients were confirmed to have pulmonary tuberculosis, 4 (3.5%) patients had HIV and the rest of them (72.2%) have miscellaneous pulmonary disorders. The findings demonstrated that out of 115 suspected patients, 31 (27%) were positive for *P. jirovecii,* which 17 (54.8%) and 14 (45.2%) were male and female, respectively (male: female ratio, 1.2:1).

Our results shows that out 28 patients with TB, 9 (32.1%) samples were positive for *P. jirovecii* and the other 21(25.3%) *P. jirovecii* positive samples obtained from patients who have showed negative results for TB and had other chronic pulmonary disorders. In addition, in HIV patients only 1 of 4 (25%) sample was positive for *P. jirovecii.* Generally, 31(27.0%) of samples were positive for *P. jirovecii* using nested-PCR while only two positive results obtained by GMS method. Statistically there was no significant difference between sex and *P. jirovecii* also *P. jirovecii* and TB ($P \geq 0.05$). The sequence analysis demonstrated 100% identity with *P. jirovecii* (Genbank accession numbers: LC107420.1; LC107278.1; LC106980.1; LC102362.1)

DISCUSSION

Pneumocystis jirovecii pneumonia or *Pneumocystis* pneumonia is a chronic, severe and relatively life-threatening infection of lung airways. Disease has remained as one of the most important opportunistic fungal infections among patients infected with HIV (6), lung transplant recipients (16), renal transplant recipients (18), chronic pulmonary disorders (19), hematologic malignancies (3), chemotherapy (20) and specific immunosuppressive drugs users such as high dose corticosteroids and tumor-necrosis factor inhibitors (3, 21). In addition some reports shows that there are a co-infection between *P. jirovecii* with TB (6) and *P. jirovecii* with *Aspergillus fumigatus* infection (22). In addition, some researchers were recognized *P. jirovecii* in the lungs of healthy human or animals (19, 23), and the prevalence of *Pneumocystis* colonization among healthy individuals ranged from 0 to 20% (11).

There are a few available data about the prevalence of PJP and *P. jirovecii* in Iran. As a results, the epidemiological pattern of PJP infection is unknown.

In a study in Tehran, the rate of *P. jirovecii* among Non-HIV-infected patients was 12.5%. They have shown that *P. jirovecii* colonization rate in patients with malignant patients, transplant recipients, immunosuppressive therapy recipients and patients with other various lung diseases was 21.7%, 20.3%, 12.7% and 7.3%, respectively (15). 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 (24). Lung transplant recipients were also considered as one of the most important patients group that colonize to *P. jiroveci.* Izadi et al. (16) has shown that 21.9% of lung transplant recipients in Tehran were colonized by *P. jiroveci*. The prevalence of *P. jirovecii* among sampled patients in our study was totally 27% including, 32.1% in TB patients, 25.3% other chronic pulmonary disorders and 25.3% in HIV patient. Sheikholeslami et al. report shows that co-infection TB and PJP was only detected at 10% of patients with HIV (6), whereas in our study 32.1% of TB patients contaminated to *P. jirovecii.* In contrast, Suk et al. have believed that coinfections of *P. jirovecii* and TB in non-HIV-infected patients are rare (25). On the other hand PCP and TB are more common among HIV infected patients (26).

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. (27) report. Although, Izadi et al. studied few cases of *P. jirovecii* colonization, the frequency of colonization among male was 2.5 rather than female (16). Our results showed that the frequency of *P. jirovecii* colonization among male was 1.2 (M:F, 17:14) greater than female. In a study conducted by Vidal et al. a higher percentage of males smokers was found among *P. jirovecii* carriers (28).

In conclusion, the current study is the first report from Ahvaz and it showed a relatively high frequency (27%) of *P. jirovecii* among patients with different pulmonary disorders. As a result, it is recommended more study for screening *P. jirovecii* among patients with respiratory tract infection. Also, our results showed that the Nested-PCR might be a reliable technique for diagnosis of *P. jirovecii,* while the GMS had a low sensitivity.

ACKNOWLEDGEMENT

We are thankful Health Research Institute, Infectious and Tropical Diseases Research Centre, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran for supporting this study. This study was an MSc thesis (Elham Aboualigalehdari) supported by a grant (No: 92140) from the Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.

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