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Rate of *Pneumocystis pneumonia* in Iranian HIV+ Patients with Pulmonary Infiltrates

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A B S T R A C T

Background: *Pneumocystis jirovecii* (formerly known as *P. carinii*) is one of the most opportunistic agents, which frequently leads to hospitalization and death in immunocompromised patients, especially in HIV-infected individuals.
Objectives: The current study is the first report on the rate of the infection in Iranian HIV-infected individuals.
Patients and Methods: We used two nested PCR and PCR-RFLP assays to amplify mt LSU rRNA and DHPS genes in 126 samples obtained from the respiratory systems of Iranian HIV-patients with CD4 count < 200 cells/μL, who were referred to two Research Centers from August 2010 to March 2011.
Results: In the group of studied patients, 112 were male (88.9%). The mean age of the patients was 35.12 ± 9.75 years. Median CD4 T cell count of the patients was 93 cells/μL. Thirty nine patients (31%) were hospitalized, and 24 patients (18.9%) tested positive for *Mycobacterium tuberculosis*. In spite of receiving *Pneumocystis pneumonia* prophylaxis and highly active anti-retroviral therapy, *P. jirovecii* was detected in 15 samples (11.9%). Just one patient (0.8%) was co-infected with *Mycobaterium tuberculosis*. The mortality rate due to *Pneumocystis pneumonia* (PCP) was 26.6%. None of the isolates showed any mutation in codons 55 and 57, which are associated with resistance to sulfa/sulfon drugs. The sequencing results showed that the genetic patterns of Iranian isolates were similar to Indian and Spanish isolates (99% identity).
Conclusions: The study indicated that the rate of *P. jirovecii* infection is similar to those reported from Asian, Indian and African countries. The similarity of the genetic pattern between Iranian and Indian isolates is probably due to their close geographical proximity.

Keywords: Pneumocystis pneumonia ; Nested PCR

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Implication for health policy/practice/research/medical education:
Based on the relatively frequent occurrence of the parasite *Pneumocystis jirovecii* in HIV positive patients in Iran, we have to consider it to be a major risk factor for the transmission of *P. pneumonia* in these patients.

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1. Background

Pneumocystis jirovecii (formerly *Pneumocystis carinii*) is an opportunistic microorganism, causing severe pulmonary infection in immunocompromised patients, especially those infected with HIV (1). Patients at risk for *P. jirovecii* pneumonia can be divided into two categories; HIV positive and HIV negative (2). Several risk factors have been identified in the HIV-negative group including immunosuppressive therapy, or an inherited or acquired immunodeficiency (3, 4). Due to the cellular immunity condition of HIV-infected persons, it is an important cause for hospitalization and/or death of these patients (3, 5, 6). The mortality rate of HIV-infected individuals with *P. pneumonia* (PCP) is much higher than those without the disease (1).

Although the incidence of PCP has declined significantly in recent years, particularly in HIV-positive patients, which is due to the introduction of highly active antiretroviral therapy (HAART), PCP chemoprophylaxis, and an increase in the percentage of patients receiving chemotherapy, *P. jirovecii* remains one of the most common opportunistic infection in HIV/AIDS patients (3, 7). The incidence of PCP in developing countries has continued to increase (7, 8).

Untreated PCP is associated with high morbidity and mortality, particularly in HIV-positive patients, thus, developing a rapid and reliable diagnostic method is vital (3, 8). The current diagnosis of PCP relies on Giemsa and/or immunofluorescent staining of induced sputum or bronchoalveolar lavage (BAL) fluid samples (9). The main drawbacks of the method are its complexity and the need for trained microscopists (10). Therefore, a rapid diagnostic technique that can identify the presence of a low number of cysts is needed. Nucleic acid amplification tests, such as polymerase chain reaction (PCR), play an important role in the detection of *P. jirovecii* (11).

PCP has been described in immunocompromised patients for many years, including outbreaks in malnourished young children in the 1950s, in Iranian orphanages (3). Owing to technical difficulties in the diagnosis of this disease, the number of studies on the disease has declined in recent years. Consequently, the prevalence rates of the disease remain unknown in HIV-infected Iranian patients.

2. Objectives

It is very important to detect the *Pneumocystis* organism in respiratory samples of immunocompromised patients, especially HIV positive persons. In the current study, we tried to detect the rate of *P. jirovecii* pneumonia in HIV-infected patients with infiltrate pneumonia using molecular assays. Moreover, evaluation of whether or not there are probable mutations in the dihydropteroate synthase (DHPS) gene associated with drug resistance to sulfa/sulfon drugs was also conducted. Finally, we tried to find the identity of Iranian isolates by comparing them with isolates from other regions of the world.

3. Patients and Methods

3.1. Patients

Respiratory samples were collected from 120 HIV-infected patients with infiltrated pneumonia, who were referred to the Research Center of HIV/AIDS and National Research Institute of Tuberculosis and Lung Diseases in Tehran, Iran, from August 2010 to February 2011. The samples included; 30 sputum (23.8%), 8 bronchoalveolar lavage fluid (6.4%), 3 tracheal aspirate (2.3%), 1 nasopharyngeal swab (0.8%) and 84 induced sputum (66.7%). The samples were investigated simultaneously for *Mycobacterium tuberculosis* (MTB) and *P. jirovecii*.

3.2. Inclusion Criteria

The samples were collected from HIV infected patients with a CD4 count < 200 cells/μl and suspected of PCP because they had at least one sign of pneumonia, for example; cough, fever, dyspnea, pneumothorax, and/or abnormal chest X-ray *(Table)*. Patients with severe disease and who needed mechanical ventilation were hospitalized. However, the other patients were able to stay in their homes with clinical support. All Iranian HIV-infected patients, especially those with a CD4 count < 200 cells/μl, received HAART and PCP prophylaxis.

3.3. DNA Extraction

Two separate respiratory samples were taken from each patient. One of these was sent for routine bacterial examination and the other was given to the Mycobacteriology Research Center in order to detect MTB or *P. jirovecii*. These samples were treated with NaOH to decontaminate them and then they were neutralized with HCl. Samples were divided into three parts: One part for Ziehl-Nelseen staining to detect MTB, the second for inoculation on Lowenstein-Jensen medium which is a special medium for the culture of MTB, and the third for DNA extraction. The DNA was extracted using QIAamp DNA Mini and Blood Mini (QIAGEN, Germany) according to the instructions of the manufacturer.

Polymerase Chain Reaction (PCR): Two nested PCR and PCR-RFLP (restriction fragment length polymorphism) methods were performed to amplify mitochondrial large subunits of rRNA (mt LSU rRNA) and DHPS genes of *P. jirovecii*, respectively, as described by Wakefield *et al.* Montes-Cano *et al.* and Totet *et al* (3, 12, 13). A negative control (distilled water without any DNA) and a positive control (sputum from an HIV/AIDS patient with *P. jirovecii*) were included in each experiment.

Table. Baseline Characteristics of Patients

	Data [b]
Age, y (range)	35.12 (25-45)
Male, No. (%)	112 (89)
Patients receiving HAART [a], No. (%)	126 (100)
CD4 count, mean	93cells/μl
Patients receiving PCP prophylaxis, No. (%)	126 (100)
Respiratory symptoms at hospital admission, No. (%)	
Fever	105 (83.3)
Cough	86 (68.2)
Dyspnea	92 (73)
Lung auscultation, No. (%)	
Bilateral crackles	73(58)
Unilateral crackles	12 (9.5)
Ronchi or wheezes	5 (4)
Normal	36 (28.5)
Oxygenation at hospital admission, No. (%)	
Arterial oxygen saturation on room air	90 (84-93)
PaO2 on room air	68 (50-80)
Chest radiograph findings at admission	
Diffuse interstitial infiltrates	76 (60.4)
Diffuse alveolar-interstitial infiltrates	12 (9.5)
Unilateral interstitial infiltrates	8 (6.3)
Normal	30 (23.8)
Hospitalization	
Yes	39 (31)
No	87(69)
Causes of *pneumonia*	
P. jirovecii	15 (11.9)
M. tuberculosis	24 (19.05)
Other bacterial agents	53 (42.1)
Viral agents	34 (26.95)

[a] Abbreviation: HAART, Highly active anti-retroviral therapy ADDIN
[b] Data are presented as No. (%) or median (IQR)

3.4. Sequencing

To confirm that the 260-bp band belongs to the mt LSU rRNA gene of *P. jirovecii*, eight positive samples were randomly selected and sequenced. The sequences were submitted to the *National Center for Biotechnology Information* (NCBI) and were released with the accession number JF733748-JF733758. These sequences were aligned by BioEdit software (V7.0.5) and compared with sequences previously submitted to GenBank (*Figure*).

The work was approved by the ethical committees of Tarbiat Modares University, Iranian HIV/AIDs Research Center and Mycobacteriology Research Center. Written informed consent was obtained from all subjects.

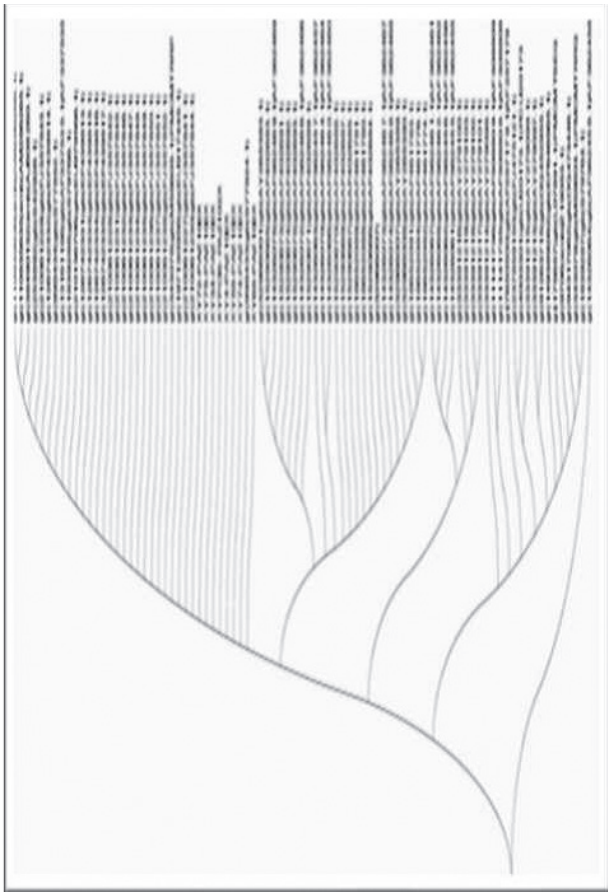

Figure. Phylogenetic Tree of P. jirovecii Isolated from Iranian HIV Infected Patients Based on Maximum Likelihood Analysis.

4. Results

In a retrospective study, 126 respiratory samples were collected from HIV-infected patients with CD4 count < 200 cells/μL. All patients were Iranian except two, who were originally from Uzbekistan and Afghanistan, but they had lived in Iran for many years (more than 10 years). The patients included 112 males (~89 %), with a mean age of 35.12 ± 9.75 years and the median age was 33 years. The patients' median number of CD4 T cells was 93 cells/μL. All of the Iranian HIV infected patients, especially those whose CD4 count < 200 cells/μL, received HAART and co-trimoxazole as prophylaxis against *P. jirovecii*. The outpatients were examined by a physician if they had clinical signs of pneumonia.

All of the patients introduced to this project, either had a; cough, dyspnea, and/or fever. Most of the patients had

an abnormal chest X-ray (76.2%). Diffuse interstitial infiltrate and diffuse alveolar interstitial infiltrate was the most frequent radiological pattern (70%), and bilateral interstitial pneumonia and pneumothorax was confirmed by a physician. The patients with severe pneumonia and hypoxemia (hypoxemia was defined as arterial PaO2 < 70 mm Hg in room air or a requirement for supplemental oxygen) were hospitalized (31%). Whereas, the remaining patients (69%) had more stable conditions and were able to stay in their homes with clinical support *(Table)*.

Twenty four samples (19.05%) were positive for *M. tuberculosis*. In spite of receiving PCP prophylaxis and HAART treatment, the DNA of *P. jirovecii* was detected in 15 samples (11.9%). In more detail the 15 cases included; 9/84 induced sputum (10.7%), 1/3 tracheal aspirate (33.3%), 3/30 sputum (10%) and 2/8 bronchoalveolar lavage samples (25%). All of the positive cases were detected among the hospitalized patients. It means that 38.5% (15/39) of the hospitalized HIV-infected patients with respiratory disease in two Medical Research Centers in Iran were due to PCP. In these patients, no other microorganisms were detected by culture. However, Giemsa staining was positive for *P. jirovecii* in 12 cases. The causes of pneumonia in the remaining 87 patients were due to other bacterial agents (42%) and/or viral agents (27%). Just one patient (0.8%) was co-infected with both *P. jirovecii* and *M. tuberculosis*. The mortality rate of the PCP infection was 26.6% in these patients (*Table*).

All samples were tested for probable mutations in the DHPS gene using a PCR-RFLP assay. All positive samples showed positive results with DHPS primers. None of these showed any mutation in codons 55 and 57, which are associated with resistance to sulfa/sulfon drugs.

In the current study, eight positive samples were randomly selected, and then sequenced to confirm the results of PCR amplification of mt LSU rRNA. The sequenced samples were aligned and compared with GenBank data. The results of alignment in the eight samples showed that all isolates belong to *P. jirovecii*. However, because of the low polymorphic changes, this gene is not suitable for phylogenetic studies, the phylogeny analysis showed that seven of these isolates *(XY-58pAZ102X, XY-Pos pAZ102X, XY-4pAZ102X, XY-34pAZ102X, XY-75pAZ102X, XY-74pAZ102X, and XY-72pAZ102X)* are similar to isolates from Indian patients. Just one isolate (XY-68pAZ102X) had close similarity to a Spanish isolate. None of the isolates were similar to; *Pneumocystis carinii forma specialis (f.sp.) ratti, P. carinii f.sp. carinii*, or *P. carinii f.sp. macaca* (*Figure*). The similarity of isolates from Iranian patients with those from Indian and Spanish was determined to be 99%.

5. Discussion

PCP is one of the most opportunistic diseases found in HIV-infected patients, and it can cause hospitalization and/or mortality in these patients (1, 9). The disease was first described in malnourished children in World War II, as well as in malnourished children in Iranian orphanages, in the 1950s (3). Since that time, there have been no studies detailing this threatening infection in Iranian individuals. Studies from Asia have also been infrequent. Fisk *et al.* in a comprehensive review of PCP found a few Asian studies from Thailand, South Korea, Taiwan and the Philippines. There was no evidence concerning the rate of PCP in Iran (8). For this reason, a retrospective review of Iranian HIV-infected patients was conducted. This study showed that 11.9% of Iranian HIV positive patients with infiltrated pneumonia are affected by *P. jirovecii*.

Despite the decline in the incidence of the disease in developed countries, this is due to the administration of PCP prophylaxis, the disease frequently occurs in the majority of HIV-infected patients; especially in those with CD4 count < 200 cells/μl (3, 4). The current incidence of PCP in Western Europe and the United States among individuals with AIDS is 2-3 cases per 100 person-years, or a 2-3% annual incidence rate (14).

Based on the estimate of the World Health Organization (WHO), 42 million people will be infected with the HIV virus by the end of 2020, 95% of whom will live in developing countries (3, 4). Most HIV-infected individuals live in Southeast Asian countries and sub-Saharan regions of Africa (3). Based on a report by Fallahzadeh *et al.* the rate of HIV/AIDS infection in Iranian individuals has increased over the period 1997-2004 from 1.38 to 4.6 cases per 100 000 population per year. The cumulative number of reported cases of HIV/AIDS among Iranians, up to the end of September 2006, was 13 702 (15). Infection with the HIV virus has also increased in Iran as in other regions of the world such as; Latin America, Eastern Europe, and Asian countries (3).

In most developing countries, PCP is still one of the most opportunistic diseases (3-5, 14). One study showed that 27-40% of HIV-infected patients were treated for PCP in Taiwan (3, 4, 8). Another study carried out in Brazil reported that 55% of HIV positive patients who had respiratory distress were hospitalized due to PCP. The general rate of the disease was 24-29% in Brazil (3, 4). Our study showed that 38.5% (15/39) of hospitalized HIV-infected patients with respiratory disease in two Medical Research Centers in Iran were due to PCP. The general rate of PCP is lower than in Brazil, too. The probable reason for this significant difference between our country and Brazil is most likely owing to the administration of prophylaxis against PCP in Iranian HIV-positive patients.

In Africa, PCP is rare in adults. The prevalence of the disease varies from 0-11% in HIV-infected individuals in different regions of Africa (3, 4). The results of the study indicated that the prevalence of PCP in Iranian HIV-infected patients is 11.9%, which is similar to the prevalence of the disease reported in other Asian and developing countries, particularly from some parts of Africa (3, 4).

The rate of PCP in Iranian HIV/AIDS patients is similar to that reported for Indian patients by Gupta *et al.* (16) and Udwadi et al (17). The low rate of the disease reported for Africa may be due to; poor diagnosis, high prevalence of bacterial pneumonia and tuberculosis (which causes the quick death of patients), environmental factors such as seasonal changes, and the existence of resistant strains or strains with low virulence (3).

Up to the present time, there have been no reports in Iran on the rates of PCP in immunocompromised patients especially in HIV infected patients, except for some case reports (18, 19). Since the symptoms of the disease are very similar to those of other respiratory infections such as tuberculosis, it seems that the disease is not detected in most cases. There is no reliable method for detecting *P. jirovecii*. Most laboratories in our country use the Giemsa staining assay to detect the cysts of the organism, however, this method has major drawbacks as it needs a well-trained microscopist (10).

Considering the technical problems in the detection of the organism in body fluids or respiratory samples of patients, molecular methods such as PCR, which can amplify low copy numbers of the organisms DNA, have recently been used in developed countries. Using single or nested PCR increases the sensitivity of detection, and it also increases the risk of detecting subclinical colonization of *P. jirovecii* (20-25). *Pneumocystis* colonization, is defined as the detection of the organism or its DNA without the signs or symptoms of pneumonia. Furthermore, PCR-positive patients with pulmonary infiltrates diagnosed as pneumonia other than PCP were also considered to have a colonization of *P. jirovecii*. Differentiation between PCP and colonization can only be done by clinical diagnosis (26).

The risk of colonization is decreased in HIV-infected patients with progressively decreasing immune function. In such patients the positive nested PCR may therefore indicate clinical PCP or a risk for later manifested PCP (24, 27). In HIV patients, 90% of PCP cases occurred with a CD4+ count < 200 cells/μL (14). Other risk factors include; CD4+ cell percentage <14%, history of prior PCP, oral candidiasis, recurrent bacterial pneumonia, unintentional weight loss, and higher rates of plasma HIV RNA (14). Considering what has been mentioned above, we used the nested PCR method to amplify the mt LSU rRNA gene of *P. jirovecii* in samples obtained from HIV infected patients with CD4 count < 200 cells/μl. All of the patients introduced to this project were suspected of having PCP due to their clinical signs or symptoms. In this study the clinical signs and symptoms were confirmed by the laboratory data. On the other hand, no other microorganisms were detected by culture in the positive cases. While, the *P. jirovecii* organisms were detected by Giemsa staining in 80% of positive cases (*Table*).

Just one patient was detected with a positive culture result for *M. tuberculosis*. This patient was co-infected with either M. tuberculosis or *P. jirovecii* (0.8 %). The rate of co-infection in Iran is significantly lower than in African countries such as Ethiopia (28). It is likely to be due to; firstly, the administration of PCP prophylaxis to HIV-infected patients in Iran especially those patients with a CD4 count of less than 200 cells/μL, and secondly, to a lower prevalence of tuberculosis in Iran in comparison with African countries.

Our study also showed that the rate of tuberculosis disease is higher than PCP in patients. One cause of the low rate of PCP in African countries is related to the death of patients from more dangerous and opportunistic diseases such as tuberculosis (3, 4, 14). In HIV-infected patients, the most dangerous opportunistic disease, tuberculosis, is often the cause of fatal infections. Since tuberculosis is endemic in Iran and can cause disease sooner than PCP (with CD4 count above 200 cells/μL), logically more patients will die from tuberculosis before they became infected with PCP. Furthermore, all Iranian HIV positive patients received PCP prophylaxis and HAART treatment, which in turn has an effect on the rate of the disease.

PCP predominantly occurs in individuals with previously undiagnosed HIV disease or those not currently receiving HIV care (14). Prophylaxis for PCP is recommended for patients with a CD4+ count < 200 cells/μL or when the history or symptoms suggest oropharyngeal candidiasis. Individuals with a history of an AIDS-defining illness are potential candidates for prophylaxis (14). We believe that the rate of PCP would have been higher in the HIV-infected Iranian patients if they had not received any prophylaxis or HAART treatment.

However, an important question for us is, why had the disease progressed in 11.9% of the patients in spite of receiving PCP prophylaxis. Could it be due to resistance against sulfa/sulfon drugs such as co-trimoxazole? Therefore, we investigated the probable mutations in DHPS gene that are associated with resistance to sulfa/sulfon drugs. Two point mutations in codons 55 and 57 are associated with probable drug resistance to the organism (29, 30). Nevertheless, we did not find any mutations in these two points. Therefore, development of the disease in spite of receiving PCP prophylaxis might be due to unresponsiveness to HAART treatment in HIV-positive patients. It is also possible that some patients did not use the prophylaxis drug correctly.

In the current study, seven samples and also the positive control sample were sequenced based on the mt LSU rRNA gene to confirm the results obtained from PCR and to show that these isolates were definitely *P. jirovecii*. Comparing the results obtained with those recorded in the NCBI showed that most Iranian isolates are similar to Indian isolates (99% identity) *(Figure)*. There are two possible explanations; firstly, the geographical closeness of the two regions, and secondly, the scarcity of reports on

evaluation and sequencing of this gene from other regions of the world such as Southeast Asia, sub-Saharan Africa, North Europe, Australia, South America, and Canada.

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Ethical Approval

The work has been approved by the ethical committees of Tarbiat Modares University, Iranian HIV/AIDs Research Center and the Mycobacteriology Research Center. Written informed consent was obtained from all subjects.

Authors' Contribution

None declared.

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