

Colonization of *Pneumocystis jirovecii* in Chronic Obstructive Pulmonary Disease (COPD) patients and the rate of *Pneumocystis pneumonia* in Iranian non-HIV⁺ immunocompromised patients

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ABSTRACT

Background and Objectives: With increasing rate of immunodeficiency diseases in the world, opportunistic micro-organism such as *Pneumocystis jirovecii* (*P. jirovecii*) become more important. Little information is available on prevalence of this life-threatening microorganism in Iran. This study was designed to determine the colonization and the rate of active disease caused by *P. jirovecii* in two groups of Iranian immunosuppressed patients.

Materials and Methods: Two hundred and fifty five pulmonary samples were collected from two groups of immunosuppressed patients to detect a 260bp fragment of mt LSU rRNA gene of *P. jirovecii* by nested PCR. The first group was COPD patients consumed oral, inhaled or injectable corticosteroid and the second group was patients with malignancies under chemotherapy. Both groups were referred to National Research Institute of Tuberculosis and Lung Disease and Imam Khomeini hospital because of pulmonary symptoms. All patients introduced to this project were confirmed HIV sera-negative by ELISA and western blot test.

Results: The mean age of COPD patients was 66.5 ± 11 (41-88) years and all of them were men. The mean age of patients with malignancy (PMs) was 43 ± 11 (23-65) years and 51.6% were men. The *P. jirovecii* was colonized in 7 of 89 COPD patients (7.9%) and its DNA was isolated from 11 of 153 PMs (7.2%). The microorganism could cause active disease in 7 of 67 (10.5%) PMs who suffered from pneumonia.

Conclusion: The study showed that *P. jirovecii* was one of colonizing agents in the COPD patients, but it could cause active disease in PMs. Generally, the microorganism can exist in the lung of non-HIV⁺ immunosuppressed patients. Therefore, it should be considered as a potential infective agent in non-HIV⁺ immunocompromised patients.

Keyword: Colonization, *P. jirovecii*, COPD, Non-HIV⁺ immunocompromised patients

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INTRODUCTION

Today, human faces to conditions that suppress the immune system directly or indirectly, such as malignant disease or many kinds of chronic diseases like Chronic Obstructive Pulmonary Disease (COPD). Increasing of immunosuppressive diseases requires more attention to human opportunistic microorganisms. One of the most dangerous of these microorganisms is a yeast-like fungus of the genus *Pneumocystis*. *P. jirovecii* is one of the most important human pathogens; particularly among immunocompromised hosts. It can cause severe pneumonia in human beings. In 1940's, the first epidemic of *Pneumocystis pneumonia* (PCP) was occurred in malnourished children in the European countries and it was reported in the premature infants in Iranian orphanages during the Second World War (1). For the first time, etiologic association between *P. jirovecii* and plasma cell interstitial pneumonia was found in malnourished children (2). The study showed the disease was cured when the life returned to normal condition and patient's diet was improved. After 1954, the sporadic infection by *pneumocystis* was reported in all of the countries throughout the world (3). The relationship between PCP and immunosuppressive disease was recognized in 1964 (4). A great list of related PCP diseases were published including premature infants, marasmus and malnourished children, congenital immune deficiency or acquired immune deficiency, patients with malignancies (PMs) under chemotherapy, recipients of organs and finally HIV positive patients (3). Thereafter, *P. jirovecii* was detected in 10-51% of HIV positive patients and 5.8-32% of non HIV positive immunosuppressed patients without any clinical symptoms using molecular assays (5-12). Today, a direct relationship between mortality and PCP in immunosuppressed patients has been proven (13). Therefore, patients who take suppressive drugs, PMs under chemotherapy, patients with transplanted organs or bone marrow, patients with vascular collagen and HIV positive patients are at a high risk of getting *pneumocystis pneumonia* (14, 15). Generally, two types of patients are at risk of *P.jirovecii*: 1) HIV positive patients and 2) non-HIV positive immune compromised patients (16). *Pneumocystis pneumonia* occurs in two distinct individuals: the first main victims are premature and malnourished children in overcrowded orphanages or hospital situation and the other is the patients with

chronic diseases such as PMs who are being under chemo or radio therapy (17). Although *P. jirovecii* is one of the potential opportunistic agents in HIV positive patients, it can also be able to infect most of non HIV positive immunodeficiency patients (1, 18). After application of highly active antiretroviral therapy and prophylactic treatment against pneumocystis in developed countries, the incidence rate of PCP was decreased in HIV positive patients. However, the rate remains high in developing countries and in the immunocompromised patients (19). Though PCP had been reported in Iranian orphanages in 1950's (1), there is no any report about the rate of disease in Iranian immunosuppressed patients. Therefore, we tried to detect DNA of *P. jirovecii* in clinical specimens of HIV positive and HIV negative patients to find the rate of disease in Iranian immunosuppressed patients. In this study we report the rate of colonization and active disease of *P. jirovecii* in two groups of immunosuppressive patients.

MATERIALS AND METHODS

Samples and patients. Two groups of immunosuppressive patients were studied. One hundred and fifty five bronchoalveolar lavage samples (BAL) were collected from 153 PMs under chemotherapy treatment. In addition, 62 sputum and 38 nasopharyngeal washing samples were collected from 89 COPD patients who referred to National Research Institute of Tuberculosis and Lung Disease (NRITLD) during the period of June 2010 to December 2011. Both groups of patients were seronegative for HIV as confirmed by ELISA and western blot test.

Extraction of DNA. To reduce the mucosa of samples, they were treated with 4% NaOH and then were neutralized with normal HCl. DNA was extracted by QIAamp Mini Kit according to manufacturer instruction. Briefly, 200 µl PBS was added to pellet of samples after centrifuging at 3000 rpm for 10 minutes. Then 20 µl proteinase K and 200 µl of AL buffer were added and after brief shaking they incubated at 56°C for 10 minutes. After treating with proteinase K, 200 µl absolute alcohol was added and suspension was transferred to column prepared by the manufacturer. The columns were washed twice with washing buffer prepared by kit and 100 µl of elution buffer was added. Then DNA was collected in a new 1.5 ml microtube after 1 minute incubation at

room temperature. The quality and concentration of extracted DNA was calculated by spectrophotometry. The extracted DNA was used for PCR directly or incubated at -20 degree centigrade for a long time.

Nested PCR. Two nested PCR was performed to amplify mt LSU rRNA and DHPS genes of *P. jirovecii* (20-22). To prevent of contamination, a negative control (distilled water without any DNA) was added in each step of procedure. To confirm the accuracy of PCR, a positive control (sputum of HIV/AIDS patient with *P. jirovecii*) was also included in each experiment.

Electrophoresis. The PCR product was run on 1.5% agarose gel besides a 100 bp ladder to see the amplified 260 bp fragment of mt LSU rRNA gene. The gel was stained with ethidium bromide and visualized using UV trans-illuminator. The work has been approved by the ethical committees of Tarbiat Modares University, Iranian HIV/AIDS Research center and Mycobacteriology Research Center. A written informed consent has been obtained from all subjects.

RESULTS

Totally, 255 pulmonary samples were collected from 153 PMs and 89 COPD patients to detect DNA of *P. jirovecii* by a nested PCR assay. Thirty eight nasopharyngeal washing and 62 sputum samples were collected from the COPD patients hospitalized in the NRITLD because of pulmonary infection. The mean age of these patients was 66.5 years and all of them were men. 35% of patients suffered from high level stage of COPD disease (stage III or IV). Pulmonary infection was occurred in 75% of admitted patients more than one time. All of patients were confirmed to be HIV seronegative by ELISA and western blot test. Cough was the most common symptoms in the studied patients and 86% of them were suffered from breath shortness, 59% had sputum with cough. In lung auscultation, wheezing was heard in 73% of patients. The result of spirometry was accessed for seventy four patients. It showed that 53 patients had FEV1 less than 70% that means about 60% of patients suffered from exacerbate COPD (Table 1).

One hundred and fifty five Bronchoalveolar lavage (BAL) samples were collected from 153 malignant

patients under chemotherapy as the second group in this study. All of them were also HIV seronegative as confirmed by ELISA and Western blot test. The mean age of patients was 43 years and 51.6% were men. These patients were hospitalized because of pulmonary disease. The cause of their hospitalization in 44% of patients was pneumonia, and 20% of these patients showed mono-lateral or bi-lateral infiltration in the CT scan. Others were hospitalized due to pulmonary edema, hemorrhage, pain chest, fever and chilling and trauma. Legionellosis, infeluenza and aspergillosis were diagnosed in 15 patients. Pulmonary symptoms were the cause of hospitalization in 7 other patients because of background disease (malignancy). Six patients were hospitalized because of unclear reason (Table 2). The result of CT scan was normal in 8% of patients. There was not any CT scan report for 72% of patients (Table 2). We could isolate DNA of *P. jirovecii* from 11 malignant patients (7.2%) and 7 COPD patients (7.9%). Totally, the rate of *P. jirovecii* infection in these two groups of Iranian patients was 7.4% (Table 3). In this survey, DNA of *P. jirovecii* was isolated from 7/67 malignant patients who suffered from pneumonia. No other fungus or bacterial growth were observed in the routine laboratory culture media and it is determined that the virus agents was not cause of pneumonia in these patients. Generally, this study showed that the cause of pneumonia in 10.5% of PMs was *P. jirovecii*. However, this organism was mainly colonized in COPD patients.

DISCUSSION

P. jirovecii is a eukaryotic microorganism which can infect most of mammalian in addition human beings. One of the most important characteristics of these organisms is their pathogenesis in immunosuppressive patients. This characteristic of microorganism causes that it is known as one of the most dangerous microorganism in the immunodeficiency patients particularly HIV positive ones (23, 24). Throughout history, people have been faced several times with the outbreaks of the disease. The first epidemic of the disease was reported during the Second World War in the European countries (2). Although, it was detected as a dangerous disease in the Iranian orphanages in 1950's (25), there is no any systematic study about the disease and its prevalence in Iranian patients. One of probably reason is

Table 1. Demographic characteristic of AECOPD patients.

Characters	Data	
	No.	%
Age (years)	66.5 ± 11 (41-88)	
Gender (Men)	89	100
HIV sero positive	0	0
Pulmonary signs & symptoms		
Cough	85	95.5
Shortness of breath	77	86.5
Sputum	53	59.5
Fever	17	19.1
Lung auscultation		
Wheezing	65	73
Normal	24	27
Spirometry result		
FEV1 < 70%	53	59.5
80% < FEV1 < 120%	14	15.7
Unknown	22	24.8
GOLD result		
Stage I	11	16.4
Stage II	25	37.4
Stage III	27	40.3
Stage IV	4	5.9
Frequency of recurrence		
One time	22	24.7
Two times	26	29.2
Three to five times	17	19.1
Frequent	24	26.9

the problem of detection. In most laboratories the detection of organism is based on Giemsa staining, a method depending on the experience of microscopist with low sensitivity. Nowadays, in most developed countries molecular assays such as nested PCR are used to detect the colonized or active cases of *P. jirovecii*. For this reason we tried to diagnosis the *P. jirovecii* in immunosuppressive patients by nested PCR and find the rate of active disease and colonized cases in these categories of patients in comparing with clinical symptoms. *P. jirovecii* is known as one of the most important colonization agents in COPD patients and can be dangerous for them. Asymptomatic carriers serve as reservoirs and sources of infection (13). Therefore, it is very important to detect the active and colonized cases in patients whose immune system is defected. Based on the result, we could isolate DNA of *P. jirovecii* from 7.9% of admitted COPD patients

without any signs and symptoms of pneumonia. *Pneumocystis* can be recognized occasionally in the lungs of healthy human beings or animals. It is also possible that the person has subclinical infection with *P. jirovecii* until his immune system become deficient (26). Immune system of COPD patients was suppressed because of consumption of corticosteroids. The susceptibility of these patients to infectious disease is high. Some studies in European countries reported a prevalence of 6-40% for *P. jirovecii* among patients with COPD (7, 27-29). A positive result for PCR and pneumonia symptoms are indicators of an active disease in the patient. But none of our studied COPD patients hospitalized in the ICU ward showed pneumonia symptoms. They were admitted because of influenza, sore throat, wheezing, purulent sputum, fever, body pain and hoarseness. Although, they were referred to our hospital for more than one time, the clinical and radiological symptoms did not show any

Table 2. Demographic characteristic of malignant patients.

Characters	Data	
	No.	%
Age (years)	43 ± 11 (23-65)	
Gender (Men)	79	51.6
HIV sero positive	0	0
Prophylaxis against PCP	21	14
Cause of hospitalization		
Pneumonia	67	43.8
Legionellosis	6	4
Pulmonary edema	18	11.75
Pleurisies	29	19
Influenza	8	5
Heamorahgia	7	4.6
Malignancy	7	4.6
Aspirogellosis	1	0.65
Fever and Chilling	2	1.3
Chest pain	1	0.65
Trauma	1	0.65
unknown	6	4
Radiologic findings		
Diffuse interstitial secretions	31	20.3
Normal	12	7.8
Unknown	110	71.9

evidence of pneumonia. Therefore, our deduction was the positive result of PCR is due to colonization of microorganism in the lung and not the disease in these patients. The reported rate of colonization of *P. jirovecii* in lung of COPD patients is inconsistent with ours (30). It is possible due to the different kind of specimens. In our study, the nasopharyngeal washing or sputum was tested because of no cooperation of patients, while in similar study conducted by Morris *et al.*, bronchoalveolar lavage or induced sputum specimens were tested. It is noted that the sensitivity of PCR assay to detect DNA of *P. jirovecii*, in the pulmonary specimens' specially induced sputum and bronchoalveolar lavage is more than nasopharyngeal washing. *P. jirovecii* is one of the most important infective agents that trigger the severity of COPD (31). The effect of microorganism on intensifying of the COPD is still unknown.

Based on Global Health Initiative on Obstructive Lung Disease (GOLD) the studied COPD patients were divided in 4 groups (stages I, II, III, IV). Our study showed that *P. jirovecii* was colonized in the patients at stage II and III. In this study just five percent of patients were in stage IV, while in Morris *et al.* study more patients were classified in stage IV. They want to detect the relationship between colonization and the severity of COPD (30). Our study in comparing with theirs had two important differences: 1- The kind of specimens and 2- The stage of disease. Therefore, our results were conflict with theirs. Although our results were not exactly showed the association of severity of disease with colonization of *P. jirovecii*, the percent of FEV1 was under 70% in six from seven positive cases (85.7%). Therefore, it could be concluded that colonization of *P. jirovecii* is one of causes for intensifying the COPD.

Table 3. Frequency of *P. jirovecii* DNA in different kinds of disease

Background disease	<i>P. jirovecii</i> DNA positive	Total patients
Malignant patients	11 (7.2%)	153 (63.2%)
COPD patients	7 (7.9%)	89 (36.8%)
Total patients	18 (7.4%)	242 (100%)

The second group of patients in this survey was PMs under chemotherapy. Recently, sporadic cases or outbreaks of *Pneumocystis pneumonia* were reported in patients receiving immunosuppressive therapy for transplantation or malignancy (32, 33). The chemotherapy with cytotoxic drugs suppresses the immune system and the patients under chemotherapy were susceptible to infectious diseases especially opportunistic agents such as *P. jirovecii*. We could find DNA of *P. jirovecii* from 7.2% of malignant patients. Physicians confirmed that 43.8% of these patients were hospitalized because of pneumonia; the cause of pneumonia was *P. jirovecii* in 10.5% of them (7/67). In these cases, no other viral or bacterial microorganisms were isolated. For this reason we dedicated these patients suffer from *Pneumocystis pneumonia* (PCP). Although *P. jirovecii* was one of colonization agents in COPD patients, it could cause active disease in about 91% of PMs (10/11). Only one patient suffered from Legionellosis in our study. So the rate of colonization in PMs is about 0.6% (1/153). One of the possible reasons of these dramatic differences of colonization in two categories of studied patients is the type of immunodeficiency. Most of PMs consumed cytotoxic drugs such as methotrexate which hurt their cellular immunity especially T cells, however in COPD patients humoral immunity was attenuated because of corticosteroid. Logically, the microorganism can cause active disease in patients whom cellular immunity defected, i.e. malignant patients in our study. It should be considered that the colonization of *P. jirovecii* can exacerbate the COPD.

CONCLUSION

Despite the lack of any report on the prevalence of PCP among the Iranian immunocompromised patients, it should be considered as a potential dangerous and infectious disease. Our study showed that *P. jirovecii* was one of the opportunistic agents which could be colonized in lung of COPD patients, while it could cause pneumonia in PMs under chemotherapy. Since most of the times it remains unknown in laboratory routine test, we suggest that it is to be diagnosed with more sensitive methods such as PCR. Our results suggest that *P. jirovecii* is an infectious agent that may play a role in the pathophysiology of COPD. However, future studies are needed to further define the role of *Pneumocystis* infection in COPD.

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