

Detection of *Pneumocystis jirovecii* by Grocott-Gomori Methenamine Silver and Indirect Immunofluorescence Assay in Chronic Obstructive Pulmonary Disease (COPD) Patients

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Abstract

Background: *Pneumocystis jirovecii* has emerged as an opportunistic fungal pathogen in immunocompromised patients. The organism has a tropism to colonized lung in patients with chronic pulmonary obstructive disease (COPD) even with no obvious immunosuppressive state.

Aims: This study aimed to investigate the colonization of *P. jirovecii* in patients with COPD and the association of different demographic characteristics with this colonization.

Patients and Methods: Sputum samples were collected from 100 patients with COPD. Samples were processed according to the standard protocols and were examined by two laboratory techniques: Grocott-Gomori methenamine silver (GMS) and indirect immunofluorescence assay (IFA). Data including age, gender, smoking status and hospital stay were extracted from patients through direct interview.

Results: The overall detection rate of *P. jirovecii* from COPD patients using GMS and IFA was 8% and 9% respectively. Older ages and smoking were associated significantly with increased risk of *P. jirovecii* colonization in COPD patients.

Conclusions: These data indicate the importance of *P. jirovecii* as opportunistic pathogen in colonization of the respiratory tract of patients with COPD. Further studies are required to illustrate the clinical implication of this colonization.

Keywords: *Pneumocystis jirovecii*, chronic obstructive pulmonary disease (COPD).

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Introductions

COPD is a progressive lung diseases associated with a chronic inflammatory response involving airways and lung parenchyma [1]. Recent reports considered COPD as the fourth leading cause of mortality worldwide. With less severe cases, there are heavy health burden and high medical cost[2]. Both genetic and environmental factors overlap to introduce COPD. Beside smoking, recent evidence supports the role of infectious agents in the triggering and perpetuating of COPD [3]. *P.jirovecii* is an atypical opportunistic organism belonging to the fungi according to the phylogenetic studies based on whole genome sequencing [4,5]. This organism has a tropism to pulmonary tissues and causes pneumonia especially in immunocompromised individuals [6]. Several evidence has linked *P. jirovecii* with COPD. These include COPD-like feature that take place after pneumocystis pneumonia (PCP), the inflammatory response in COPD and *Pneumocystis* colonization is alike, and the occurrence of airway obstruction in animal models subjected to pneumocystis colonization [3]. It seem that the colonization of *P. jirovecii* is not

associated with impaired function of lung; rather the organism especially colonizes the lung with COPD. Probst *et al.* reported 7% and 40% colonization of *P. jirovecii* in cystic fibrosis and COPD respectively [7]. Furthermore, Helweg-Larsen *et al.* found 4% of patients with suspected bacterial pneumonia were positive for *P. jirovecii*, 63% of whom had COPD, while only 20% of COPD free patients had such colonization [8]. Regarding the source of infection, recent evidence suggested a continuous acquisition of the micro-organism rather than the presence of latent infection. Supporting this assumption is the frequent reported outbreaks in different settings like liver transplant units [9], pediatric oncology wards [10] and hematology ward [11] where *P. jirovecii* DNA was detected in the air surrounding patients with or without active PCP.

There is paucity in the studies regarding the colonization rate of *P. jirovecii* in COPD patients in Iraq. Therefore, this study aimed to investigate the colonization of this fungus in human immunodeficiency virus (HIV)-negative COPD patients and its association with some demographic factors.

Subjects and Methods:

The Study Population and Sampling

Sputum samples were collected from 100 moderate to severe COPD patients who were admitted to respiratory ward in AL- Imamian AL- Kadhumain Medical City during the period from May-2017 to March-2018. Inclusion criteria were all patients with moderate to severe COPD with sera-negative for HIV infection by ELISA and western blot test. Moderate to severe COPD was defined according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria (a ratio of forced expiratory volume in 1 second [FEV1] to forced vital capacity [FVC] of <70% after bronchodilator use and an FEV1 of <80% of the predicted value after bronchodilator use). Clinical manifestations were determined by consultation of Respiratory and Chest diseases specialist. Exclusion criteria were patients diagnosed with asthma or bronchiectasis at the sampling, patients with immunosuppressive drugs, and those with life-threatening disorders. The ethical aspects of this study have been approved by the ethical council in Medical College, Al- Nahrain University.

Samples Collection and Processing:

Sputum samples containing mucous material were added to a 2-fold volume of 0.9% NaCl and mixed by vortexing for 5 minutes. The mixture was then centrifuged at 3000 rpm for 5 minutes, and the supernatants were discarded. Two laboratory methods were used for *P. jirovecii* detection in this study. The first method is staining with GMS stain (abcam®, UK). A portion of precipitate pellets (100µl) was used to prepare smears which were fixed with ethanol and stained with GMS stain according to the standard protocol [12]. The second method was the fluorescent monoclonal antibody test using a commercially ready kit (Axis-Shield Diagnostics, and Dundee, UK) according to the manufacturer's instructions. In this method, the pellets were resuspended with sterile distilled water and washed twice by centrifugation. Several slides were prepared on which smears from the resuspended pellets were prepared and fixed with high quality acetone. After drying at room temperature, the slides were rinsed with distilled water to remove the salts from the specimens. The specimens were then treated with enzyme solution containing trypsin. Monoclonal murine anti-*P. jirovecii* antibody and fluorescently labelled anti-mouse antibody were added and the preparation was incubated at 37°C for 10 min. in a humidified chamber. After incubation, the slides were rinsed and air-dried and examined at ×100 magnification using fluorescent microscope. A positive finding was based on the detection of the fluorescent cysts which have round or elliptical shape [13].

Statistical Analysis

Continuous variables were expressed as mean ± standard deviation (SD) and analyzed with student t-test, while binomial variables were expressed as frequency and percentages, and analyzed with Chi-square. All analyses were conducted with SPSS software. A p-value of 0.05 or less was considered significant.

Results

Demographic and Clinical Characteristics of Patients

The demographic and clinical characteristic of COPD patients are shown in table 1. Most patients were elderly, and relatively more females (57%) were affected than males (43%). This high percentage of female was reflected by lower percentage of smokers (38%) than non-smokers (62%) because most women are non-smokers. Most patients

have 3 days or less hospital staying Cough was predominant and reported in 83% of patients, while about two-third of patients were suffering from shortness of breath.

Table 1: Demographic and clinical characteristics of the study population

Variables	Value, Frequency(%)
Age, year (mean±SD)	66.5 ± 11
Gender	
Male	43(43%)
Female	57(57%)
Hospital stay	
≤3 days	72(72%)
4 days or more	28(28%)
Smoking	
No	62(62%)
Yes	38(38%)
Cough	83(83%)
Shortness of breath	69(69%)
Fever	26(26%)

Detection of *Pneumocystis jiroveci*

Grocott-GomoriMethenamine Silver Stain

Cysts of *P. jiroveci* is the diagnostic form, and under light microscope, it had a characteristic appearance as a spherical, or cup-shaped objects when stained by GMS (Figure 1). Out of 100sputum samples of COPD patients, the organism was detected in 8(8%) with this technique.

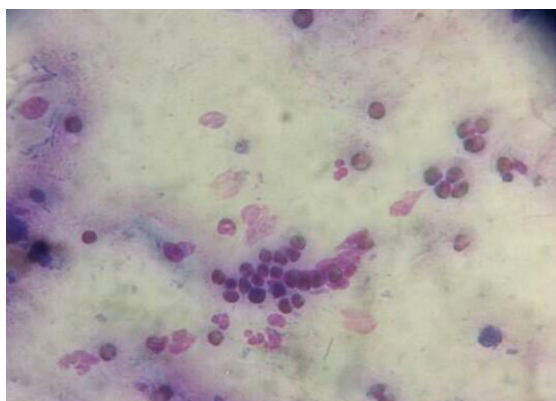


Figure 1: *P. jirovecii* cysts detected by GMS stain (×100).

Immunofluorescence Detection of *Pneumocystis jirovecii*

Under fluorescent microscope, *P. jirovecii* cysts appear as round or elliptical structure with green color (Figure 2). Out of 100sputum samples of COPD patients, the organism was detected in 9(9%) samples by IFA test. Of note, all specimens gave positive results for GMS Stain were positive for fluorescent test, which implies the superiority of IFA monoclonal antibody over GMS stain in detection of *P. jirovecii* cyst.

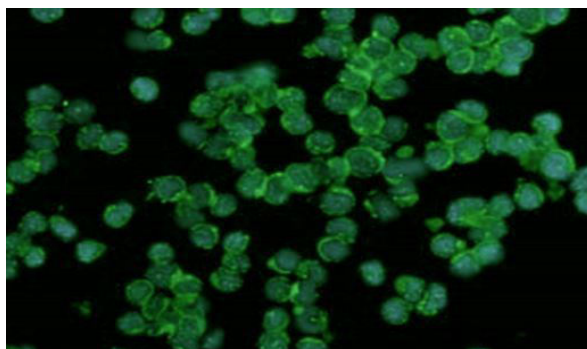


Figure (2): Immunofluorescent staining using monoclonal antibodies against *P.jirovecii*. the cyst appears bright to bright apple green($\times 100$).

Effect of Demographic Characteristics on *P. jirovecii* Colonization

The association of different demographic characteristic with the colonization of *P. jirovecii* was further investigated (Table 2). Both age and smoking status were found to be significantly influence such colonization. Mean age of infected group was 82.44 ± 18.2 years versus 59.92 ± 9.3 years ($P=0.016$). More than three-quarters of infected patients were smokers compared to 34.07% among non-infected ($P=0.011$).

Table 2: Association of *P. jirovecii* infection with some demographic characteristics

Variables	Infected (9)	Non-infected (91)	P-value
Age, year (mean \pm SD)	82.44 \pm 18.2	59.92 \pm 9.3	0.016
Gender			
Male	5(55.56%)	38(43.95%)	0.428
Female	4(44.44%)	53(56.05%)	
Hospital stay			
≤ 3 days	4(44.44%)	68(74.73%)	0.068
4 days or more	5(55.56%)	23(25.27%)	
Smoking			
No	2(22.22%)	60(65.93%)	0.011
Yes	7(77.78%)	31(34.07%)	

Discussion

Pneumocystis jirovecii is an opportunistic pathogen which colonizes lung and associates with COPD. Detection of this colonization is very important for two main reasons. Firstly for better understanding of the organism epidemiology and its correlation with lung disease. Secondly, most cases of colonization represent starting points for PCP especially when there is an immune suppression [14]. The current study reported 9% incidence *P. jirovecii* in patients with COPD. These results are completely in accordance with that obtained in a local study conducted Yehia *et al.* on patients with lower respiratory tract infections [15]. In contrast, Touhali *et al.* reported 14% of immunocompromised patients were positive for Pneumocystosis [16]. This high rate is reasonable because the immune status of the study population. Of note, in these two studies, conventional (not molecular) methods were used for detection of the fungus. Globally, Sheikholeslami *et al.* in Iran reported very close rate (7%) of infection in patients with COPD [17]. More recently, in Iran also, Aboualigalehdari *et al.* subjected 115 BAL specimens from patients with different chronic pulmonary disorders for nested-polymerase chain reaction with specific primers for *P. jirovecii*. They found that 27% of their patients were positive for this opportunistic pathogen [18]. It is obvious from these studies and others that there are several factors which influence the detection rate. These include age, complain and immune status of the patients, specimens type, and, may be the most important, the method of

detection. In this regard, Ng *et al.* reported that samples were considered positive to *P. jirovecii* (i.e., a truly positive specimen) if this organism was detected by two or more of the staining methods [19]. Indeed, this was true before the era of PCR which dramatically changed the standard of diagnosis. In a recent study, Moodly *et al.* compared the efficiency of quantitative PCR (qPCR) with IFA in detection of *P. jirovecii* in sputum samples from patients suspected to have PCP. As expected, qPCR had higher detection rate than IFA (67% vs 51%) [20]. However, this preponderance of molecular methods is not predominant. In an Egyptian study including 50 children with hematological malignancies, three diagnostic methods (Giemsa staining, PCR and direct IFA) were used for *P. jirovecii* detection. Interestingly, IFA was found to have superiority over PCR or Giemsa staining in this study [21]. In fact, even within molecular methods there is some variation in detection rate, and there is a general agreement that real-time PCR is more sensitive than conventional PCR. The current study revealed a significant positive association of age and smoking with the colonization of *P. jirovecii*. For age association, a previous Japanese report confirmed such influence [22]. Another study involving 110 elderly HIV-negative subjects attending a preventive health care program detected *P. jirovecii* in 12.8% which is a relatively high rate [23]. Two factors have been proposed to explain the high incidence of *P. jirovecii* in elderly, both of them related to the immune status. These are the decline in cellular immune response accompanying the aging process (known as immunosenescence), and the medications taken by elderly which may have negative impact on the immune system [22, 24]. Although it was not significant in the current study, hospital stay appears to have positive impact on *P. jirovecii* colonization in the lung. A prolonged hospital stay increases the risk of getting nosocomial infections. In one study, it was found that healthcare workers are able to carry and transmit this pathogen for up to 10 weeks [25]. Smoking is a well-known risk factor for many respiratory infections. The current study showed a significant association between smoking and *P. jirovecii* colonization. This result agrees with the recent study by Tokemoto *et al.* on healthy smokers and non-smokers. The authors reported a significant impact of smoking on the colonization rate of *P. jirovecii* (20% vs 47%) [26]. The exact mechanism by which smoking has such influence is not exactly clarified. However, smoking induces a mechanical damage accompanied with an increase of inflammatory cytokines from alveolar tissues [27]. These changes and may be others could create a favorable microenvironment for *P. jirovecii* colonization. Collectively, these data indicate the significance of *P. jirovecii* in COPD patients. A cohort study investigating the effect of this colonization on lung function and the possible development to PCP is required.

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Conflict of interest

The authors declare that they have no competing interests.

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