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Investigation of *Pneumocystis jirovecii* in Lung Cancer Patients with the Nested PCR Method

Akciğer Kanserli Hastalarda Pneumocystis jirovecii'nin Nested PZR Yöntemi ile Araştırılması

Ahmed Galip Halidi[1], Merhamet Ölçen[2], Esra Gürbüz[3], Abdurrahman Ekici[4], Selahattin Aydemir[4], Hasan Yılmaz[4]

[1]Muş Alparslan University, Bulanık Vocational School, Muş, Turkey
[2]Şanlıurfa Training and Research Hospital, Clinic of Chest Diseases, Şanlıurfa, Turkey
[3]University of Health Sciences Turkey, Van Training and Research Hospital, Clinic of Infectious Diseases and Clinical Microbiology, Van, Turkey
[4]Van Yüzüncü Yıl University Faculty of Medicine, Department of Parasitology, Van, Turkey

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ABSTRACT

Objective: *Pneumocystis jirovecii* (*P. jirovecii*) is an opportunistic pathogen in humans. Early diagnosis and optimal treatment of patients with *P. jirovecii* pneumonia (PJP) remains a key priority. This study investigated *P. jirovecii* in patients with lung cancer using the nested-polymerase chain reaction (PCR) method and examined the relationship between *P. jirovecii* and clinical findings.
Methods: The study included 60 patients with lung cancer and 30 patients without lung cancer. The bronchoalveolar lavage (BAL) fluid samples of these 90 individuals were taken for diagnostic purposes in the University of Health Sciences Turkey, Van Training and Research Hospital, Clinic of Chest Diseases. Patient information was recorded. After DNA isolation from the BAL fluid samples taken from patients, the nested-PCR protocol for amplification of mtLSUrRNA in *P. jirovecii* was performed.
Results: *P. jirovecii* DNA was detected in 40 (66.67%) of the lung cancer patients included in the study and in six (20%) patients without lung cancer, that is, in 46 (51.11%) patients. The rate of nested-PCR positivity in the lung cancer group was significantly higher than that in the non-lung cancer group (p=0.0001). Additionally, a statistically significant correlation was found between anorexia and weight loss, fever and sputum *P. jirovecii* positivity in patients with lung cancer (p<0.005).
Conclusion: These findings suggest that lung cancer patients should be evaluated for PJP.
Keywords: *Pneumocystis jirovecii*, bronchoalveolar lavage, nested PCR, Turkey

ÖZ

Amaç: *Pneumocystis jirovecii* (*P. jirovecii*) insanlar için fırsatçı bir patojendir. *P. jirovecii* pnömonisi (PJP) olan hastaların erken teşhisi ve optimal tedavisi önemli bir öncelik olmaya devam etmektedir. Bu çalışmada, akciğer kanserli hastalarda *P. jirovecii*'nin nested-polimeraz zincir reaksiyon (PZR) yöntemi ile araştırılması ve *P. jirovecii* ile klinik bulgular arasındaki ilişkinin değerlendirilmesi amaçlanmıştır.
Yöntemler: Çalışmaya Sağlık Bilimleri Üniversitesi, Van Eğitim ve Araştırma Hastanesi, Göğüs Hastalıkları Kliniği'nde tanısal amaçla bronkoalveolar lavaj (BAL) sıvı örnekleri alınan 60 akciğer kanserli hasta ve 30 akciğer kanseri olmayan hasta dahil edildi. Hastalara ait bilgiler kaydedildi. Hastalardan alınan BAL sıvı örneklerinden DNA izolasyonu yapıldıktan sonra *P. jirovecii* mtLSUrRNA gen bölgesinin amplifikasyonu için nested-PZR protokolü uygulandı.
Bulgular: Çalışmaya dahil edilen akciğer kanserli hastaların 40'ında (%66,67), akciğer kanseri olmayan hastaların 6'sında (%20) olmak üzere toplam 46 (%51,11) hastada *P. jirovecii* DNA'sı saptandı. Akciğer kanseri grubunda nested-PZR pozitiflik oranı, akciğer kanseri olmayan gruba göre istatistiksel olarak anlamlı derecede yüksek yüksekti (p=0,0001). Ayrıca akciğer kanserli hastalarda iştahsızlık ve kilo kaybı, ateş ve balgam ile *P. jirovecii* pozitifliği arasında istatistiksel olarak anlamlı bir ilişki bulundu (p<0,005).
Sonuç: Sonuç olarak akciğer kanseri hastalarının, PJP yönünden mutlaka değerlendirilmesi gerektiği kanaatindeyiz.
Anahtar Kelimeler: *Pneumocystis jirovecii*, bronkoalveolar lavaj, nested PZR, Türkiye

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Address for Correspondence/Yazar Adresi: Selahattin Aydemir, Van Yüzüncü Yıl University Faculty of Medicine Department of Parasitology, Van, Turkey
Phone/Tel: +90 530 777 87 08 **E-mail/E-Posta:** saydmr23@gmail.com **ORCID ID:** orcid.org/0000-0002-0941-2779

Türkiye PARAZİTOLOJİ Dergisi

INTRODUCTION

Pneumocystis jirovecii (*P. jirovecii*) is a single-celled eukaryotic microorganism and an opportunistic pathogen for humans (1,2). *P. jirovecii* shows tropism to the lung alveoli. There are trophozoite, precyst, and cyst forms in its life cycle (3).

The mode of transmission of *P. jirovecii* is not fully understood, and its environmental source is not known. It is thought that the infection is mostly transmitted by air. *P. jirovecii* infections are asymptomatic in immunocompetent individuals (3,4). The clinical course may be severe in patients with AIDS or those whose immune system is suppressed for other reasons (5). The most common clinical symptoms of patients are dyspnea, tachypnea, non-productive cough, mild fever, and cyanosis. Rarely, chest pain may also occur (6,7).

Early diagnosis and optimal treatment of patients with *P. jirovecii* pneumonia (PJP) remains a key priority (8,9). Definitive diagnosis of infection is made by microscopic detection of cysts and/or trophozoites of the agent in respiratory system samples. Although bronchoalveolar lavage (BAL) fluid samples are the first choice for diagnosis, presence of this parasitic agent is also tested in patient samples such as induced sputum, bronchial lavage, nasopharyngeal aspiration, lung biopsy, and oral washing fluid (1,10). These samples are stained with various staining methods such as methenamine silver, toluidine blue O, Wright-Giemsa, and Gram-Weigert (10). Direct and indirect immunofluorescence staining methods are also frequently used (1). However, the low number of parasitic agents in non-AIDS immunosuppressed patients or individuals with intact immune system reduces the sensitivity of microscopic diagnosis. This makes molecular methods, which are more sensitive in diagnosis and based on the detection of *P. jirovecii* DNA, more attractive (11).

Detection of *P. jirovecii* DNA in clinical samples does not mean that the patient has definitive PJP. In clinically asymptomatic individuals, detection of the organism or its DNA is defined as colonization. Clinical evaluation is very important to distinguish between infection and colonization (12,13).

This study investigated *P. jirovecii* in patients with lung cancer using the nested-polymerase chain reaction (PCR) method and examined the relationship between *P. jirovecii* and clinical findings.

METHODS

The study was approved by the Scientific Research and Publication Ethics Committee of Muş Alparslan University (27/09/2019-29). Patients who did not receive anti-PJP treatment and whose BAL fluid samples were taken for diagnostic purposes in the Chest Diseases Department of the University of Health Sciences Turkey, Van Training and Research Hospital between January 2020 and November 2021 were included in this study. BAL fluid samples taken from 60 patients with lung cancer and 30 without lung cancer (19 pneumonia, 9 idiopathic pulmonary fibrosis, 2 pulmonary vasculitis) were brought to Van Yüzüncü Yıl University Faculty of Medicine Department of Parasitology Research Laboratory. Patient information was recorded.

Sample Preparation

On average, 2 mL of BAL liquid samples were placed in centrifuge tubes. Then, distilled water was added to make a total volume of 10 mL. These tubes were centrifuged at 1.500 rpm for 10 minutes. After centrifugation, the supernatant was taken, and the bottom precipitate was removed and stored at -20 °C.

DNA Extraction

DNA was extracted from stored BAL samples using an GeneJET Genomic DNA Purification Kit (Thermo Scientific, Lithuania) according to the manufacturer's recommendations.

Nested-PCR

The nested-PCR protocol for amplification of mtLSUrRNA in *P. jirovecii* was performed as previously described by Özkoç et al. (1). The primers pAZ102-E (5'-GATGGCTGTTTCCAAGCCCA-3') and pAZ102-H (5'-GTGTACGTTGCAAAGTACTC-3') were used in the first step of the nested-PCR. The reaction was adjusted to a total volume of 50 µL containing 25 µL of Tag 2x Master Mix (12.5 mM $MgCl_2$), 0.5 mM $MgCl_2$, 0.2 µM from each primer, and 1 µL of sample DNA. 1 µL of the obtained amplicon was used for the second step of the nested-PCR. In the second step, the primers pAZ102-X (5'-GTGAAATACAAATCGGACTAGG-3') and pAZ102-Y (5'-TCACTTAATAATTAATTGGGGAGC-3) were used, which amplify the 267 bp long region. The reaction was carried out under the conditions specified in the previous step.

Reactions were run on the Applied Biosystems SimpliAmp Thermal Cycler PCR instrument. The same amplification protocol was used for both PCRs. The PCR was programmed for a total of 35 cycles of 30 seconds each at 94 °C, 30 seconds at 50 °C (49 °C for the second step), and 90 seconds at 72 °C. In both PCR processes, an additional 15-minute denaturation step at 95 °C was applied before the first cycle, and a 10-minute extension step at 72 °C was applied after the last cycle.

Agarose-gel Electrophoresis

In order to display the results of the nested-PCR process, 15 µL agarose gel (1.5%) from the reaction products was subjected to electrophoresis and displayed in the UVP gel documentation system.

Statistical Analysis

Comparisons of the *P. jirovecii* prevalence for research groups, gender, and clinical signs and symptoms were analyzed by independent two proportions Z (t) test. The statistical significance level was considered as 5% ($p<0.05$) (MINITAP; ver: 14).

RESULTS

P. jirovecii DNA was detected in the BAL samples by the nested-PCR method (Figure 1) in 40 (66.67%) of patients with lung

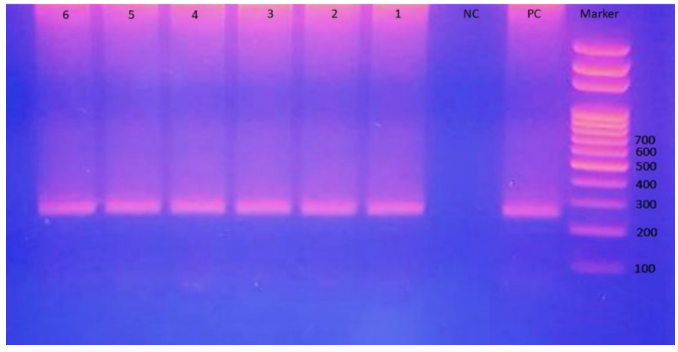

Figure 1. Agarose gel image of positive samples of *P. jirovecii*

cancer diagnosis and 6 (20%) of the patients without lung cancer. That is, *P. jirovecii* DNA was detected in the BAL samples in a total of 46 (51.11%) patients. Twenty-seven (58.7%) of these patients were male; 19 (41.3%) were female (Table 1). Their mean age was 52.1 years.

In the study, the rate of nested-PCR positivity in the lung cancer group was significantly higher than in the non-lung disease group (p=0.0001). However, no significant correlation was found between the incidence of *P. jirovecii* and age and gender.

The clinical signs and symptoms of the patients with lung cancer diagnosis and *P. jirovecii* positivity are compared in Table 2. Of the 40 *P. jirovecii* nested-PCR positive patients, 35 (87.5%) had cough, 21 (52.5%) had dyspnea, 24 (60%) had sputum, 9 (22.5%) had anorexia and weight loss, and 15 (37.5%) had fever. Of the 20 *P. jirovecii* nested-PCR negative patients, 15 (75%) had cough, 9 (45%) had dyspnea, 5 (25%) had sputum, none had anorexia and weight loss, and none had fever. Although a statistically significant relationship was found between sputum, anorexia and weight loss, fever and *P. jirovecii* positivity, no statistically significant relationship was found between other clinical signs and symptoms and *P. jirovecii* positivity (Table 2).

DISCUSSION

P. jirovecii is an important pathogen in immunocompromised patients (14). It is an important risk factor especially for patients with organ transplantation, solid tumors, and hematological malignancies and in individuals with inflammatory and rheumatic diseases (15). The use of chemotherapy regimens and immunomodulatory drugs has increased the number of patients at high risk for *P. jirovecii* (16). Especially in patients with primary brain cancer or metastatic cancer, the use of high-dose systemic corticosteroids increases the risk (17). In addition to these, there is a high rate of *P. jirovecii* colonization in patients with chronic lung complaints (18). Detection of *P. jirovecii* DNA in individuals without pneumonia signs and symptoms has been defined as colonization (12). Colonization of *P. jirovecii* does not indicate that the person has PJP, but it may affect the pathophysiology of respiratory diseases and may suggest that the person is at risk for *P. jirovecii* disease (13).

In other studies conducted with BAL samples, it was reported that the sensitivity of the PCR method was 100% and the specificity was between 84% and 93%. Although positive results in conventional PCR cannot distinguish colonization from infection, it is a reliable method for detecting individuals at risk (4). In this study also, it was not determined whether there was colonization or infection in *P. jirovecii* positive patients determined by PCR method.

The data obtained in some studies show that chronic lung diseases pave the way for the colonization of *P. jirovecii*, and the data obtained in some other studies show that *P. jirovecii* causes inflammatory changes in the lungs and these changes play a role in the pathophysiology of chronic lung diseases (1,18-20). Both

Table 1. The distribution of *P. jirovecii* positivity according to research groups and gender

Group	*P. jirovecii*		p
	Positive n (%)	Negative n (%)	
Patients with lung cancer diagnosis (n=60)	40 (66.7)	20 (33.3)	**0.001**
Patients without lung cancer (n=30)	6 (20)	24 (80)	
Pneumonia (n=19)	4 (21.1)	15 (78.9)	0.763
Idiopathic pulmonary fibrosis (n=9)	2 (22.2)	7 (77.8)	
Pulmonary vasculitis (n=2)	0 (0)	2 (100)	
Male (n=49)	27 (55.1)	22 (44.9)	0.406
Female (n=41)	19 (46.4)	22 (53.6)	
Total (n=90)	46 (51.1)	44 (48.9)	-

Table 2. Relationship between *P. jirovecii* prevalence and some clinical symptoms in patients with lung cancer diagnosis

Clinical signs and symptoms		Patients with lung cancer diagnosis (n=60)		
		P. jirovecii positive n (%)	*P. jirovecii* negative n (%)	p
Cough	Positive (n=50)	35 (70)	15 (30)	0.221
	Negative (n=10)	5 (50)	5 (50)	
Dyspnea	Positive (n=30)	21 (70)	9 (30)	0.584
	Negative (n=30)	19 (63.3)	11 (36.7)	
Sputum	Positive (n=29)	24 (82.8)	5 (17.2)	**0.011**
	Negative (n=31)	16 (51.6)	15 (48.4)	
Anorexia and weight loss	Positive (n=9)	9 (100)	0 (0)	**0.021**
	Negative (n=51)	31 (60.8)	20 (39.2)	
Fever	Positive (n=15)	15 (100)	0 (0)	**0.002**
	Negative (n=45)	25 (55.6)	20 (44.4)	

possibilities have prompted researchers to investigate *P. jirovecii* colonization in people with lung disease. One study showed that 31.8% of the patients with positive PCR results had a history of PJP or will develop PJP (21). Therefore, although colonization-infection distinction was not made, the positivity rate determined in patients with lung cancer diagnosis in this study shows that patients with lung cancer are at risk for PJP.

In studies conducted with patients with lung disease symptoms, *P. jirovecii* colonization has been reported to be in the range of 2.6-55% (10,11,18,20). In an autopsy study evaluating lung cancer cases, *P. jirovecii* colonization was detected in all 10 patients who died of small cell lung cancer (22). Özkoç et al. (1) detected *P. jirovecii* colonization in eight (57.1%) of 14 patients with lung cancer. The data of these two studies (22) and the data obtained in our study may be consistent or convergent.

HIV-infected patients with PJP typically present with subacute symptoms beginning with cough, dyspnea, and fever (14,23). Studies have reported that some symptoms may also occur in patients who are not infected with HIV but are found to have *P. jirovecii*. Özmen et al. (10) reported that the clinical findings of patients with *P. jirovecii* colonization included cough, sputum, dyspnea, hemoptysis, chest pain, and fever.

In a study by Roux et al. (24), in which they compared the clinical signs and symptoms of patients with and without AIDS diagnosed with PJP, they reported that patients in both groups had fever and dyspnea, and cough was more common in AIDS patients with a statistically significant difference. Bienvenu et al. (25) also compared the clinical signs and symptoms of patients with and without HIV who were diagnosed with PJP. In their study, it was reported that cough and dyspnea were observed less frequently in non-HIV patients compared to HIV patients, and fever was a symptom seen in both groups. In our study, the patients did not have HIV infection, and some clinical signs and symptoms such as fever and weight loss were detected in the patients. Considering the data obtained from the studies conducted by Roux et al. (24) and Bienvenu et al. (25) and the data in our study, we suggest that fever and sputum may be a clinical symptom in lung cancer patients with *P. jirovecii* colonization, and cough, dyspnea may develop independently of lung colonization.

In this study, anorexia and weight loss were seen more frequently in lung cancer patients with *P. jirovecii* colonization compared to patients without colonization. No information could be found in the literature on the relationship between anorexia and weight loss and *P. jirovecii* colonization. Therefore, further studies are needed to confirm this finding.

CONCLUSION

The colonization rate of *P. jirovecii* in patients with lung cancer is high and should not be ignored. Colonization may turn into an infection following the use of immunosuppressive drugs in patients with cancer. For this reason, lung cancer patients should be evaluated for PJP during the treatment process.

*Ethics

Ethics Committee Approval: The study was approved by the Scientific Research and Publication Ethics Committee of Muş Alparslan University (27/09/2019-29).

Informed Consent: Informed consent was taken from all present patients in this study.

Peer-review: Internally and externally peer-reviewed.

*Authorship Contributions

Concept: A.E., A.G.H., E.G., Design: A.E., S.A., H.Y., Data Collection or Processing: E.G., M.Ö., Analysis or Interpretation: S.A, A.E, A.G.H., Literature Search: S.A., A.E, E.G., A.G.H., Writing: S.A., A.E, E.G., A.G.H.

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