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Investigation of Pneumocystis Jiroveci Carriage in Patients with Various Lung Diseases

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Summary

Pneumocyctis jiroveci is one of the major reasons of morbidity especially in immunosuppressive individuals. Recently, a large number of asymptomatic colonization has been noted. The aim is to investigate the frequency of P. jiroveci colonization in patients (Group I) with lung cancer who do not take oral corticosteroid and the patients (Group II) with other lung problems by using immunofluorescence (IF) method and polymerase chain reaction.

Incidence of P. jiroveci has been investigated in the bronchoalveolar lavage samples taken by bronchoscopy for diagnose and treatment by using IF method and P. jiroveci DNA has been defined with PCR method.

In Group I, 1 (1.8%) IF and 3 (5.5%) PCR positivism (one of them is the case which is found positive by using IF method) have been acquired. In Group II, both of the methods have produced no positivism.

Colonization of P. jiroveci is noted in patients with HIV negative. The fact that the cases only with lung cancer have positivity supports the idea that lung cancer is important risk factor.

The patients with lung cancer should be monitored closely for prophylaxis and treatment and the treatment should not be applied to each PCR positive case.

Key words: Pneumocyctis jiroveci, PCR, immunofluorescence, lung carcinoma

Introduction

Pneumocyctis pneumonia (PCP) is one of the major reasons for diseases and mortalities in the immunodeficiency patients. The disease has been defined in immunosuppressed patients over the years but after the epidemic of AIDS its incidence has increased seriously [1, 2]. More than 3000 PCP cases have been reported by Centers for Disease Control and Prevention. One third of this figure is children under the age of 15 with AIDS [3, 4]. The agent has been reported in the situations which the immunity system is suppressed such as malign diseases, organ transplantations, and chemotherapy and recently it has been reported as well in moderate level immunosuppressed patients with lung problem such as pneumonia, fibrosis and Chronic Obstructive Pulmonary Disease (COPD) [5-8].

The agent of the disease has been known as *Pneumocyctis carinii* f.sp *hominis* before and it has been classified as protozoa. After the application of molecular methods, this action has been classified as a separate kind of fungus by Frenkel in 1999 and named as Pneumocyctis *jiroveci* [9].

Seroconversion generally emerges in the early childhood and increases in correlation with the age. Recently, *P. jiroveci* has been found related with the disease in healthy infants [10]. *P. jiroveci* is considered as being acquired in childhood and it is the reactivation of latent infection in a later period. However, *P. jiroveci* is thought to be a "de novo" infection agent which stems from another resource as well [2, 5, 8, 10, 11].

Insufficiency of T lymphocyte is important for the pathogenesis of the infection and when the number of these cells decreases or their function reduces, the infection risk increases [1, 3, 6]. In clinical practices, when the number of CD_4 T lymphocytes drops under a certain level, antibiotic prophylaxis is given for PCP, but this application is led to side effects and high cost in the cases when there is no colonization.

PCP diagnose requires the investigation of the samples of the lower respiratory tract. Among these samples, bronchoalveolar lavage (BAL) is a gold standard for diagnose [1, 12, 13]. Different stain methods are applied in these secretions to stain the pathogens (Methenamine Silver Nitrate, Toluidine Blue O, Acridine Orange, Gram-Weigert, etc.). Although some of them are quite cheap and easily applicable, they have less sensitivity [13-15]. However, commercial immunofluorescence (IF) kits which contain monoclonal antibodies have higher sensitivity rates [1, 16-18]. *P. jiroveci* PCR is being used as a potential diagnostic test with the selection of appropriate primary, probe and target gene and the specificity ratios of this test has almost reached %100 [12, 19-23]. Demonstration of PCR and a limited number of *P. jiroveci* organisms in the BAL samples of patients with no acute PCP symptoms is evaluated as pulmonary colonization [6, 24, 25].

The aim of this prospective study is to investigate the formation of *P. jiroveci* colonization in the BAL samples, respiratory tracts of the patients with lung cancer or other lung pathologies by using IF and PCR methods and to evaluate its relation to the underlying risk factors.

Material and Methods

Patients and clinical samples: This prospective study was undertaken with patients who were under inpatient

treatment in Atatürk Chest Disease and Surgery Center, Department of Respiratory Medicine, on 15th September 2002-15th March 2003. Of the patients who were admitted to the hospital for diagnose and treatment, those with lung cancer were classified as Group I and those with other lung diseases were classified as Group II. Upon the consent of all patients for diagnostic procedures and treatment monitoring, BAL samples were collected and the study which will be conducted with these samples was approved by Atatürk Chest Disease and Surgery Center, the Board of Ethic. The presence of *P. jiroveci* was investigated in all BAL samples by the methods of IF and PCR. All patients were over 18 and their anti-HIV test results were negative.

Bronchoscopy and BAL were carried out according to a standardised protocol. The bronchoscope was wedged into a segment of the right middle lobe, and three 50-mL aliquots of sterile saline solution, warmed at 37°C, were instilled into the subsegmental bronchus. Fluid was gently aspirated immediately after each aliquot was introduced, and collected in a sterile container. During the bronchoscopy, oxygen saturation and ECG tracings were continuously monitored.

Immunofluorescence staining (MerIFluor Pneumocyctis, Meridian Diagnostics, Ohio, USA): IF study was carried out in Kirikkale University, Laboratory of Infectious Diseases and Clinical Microbiology. BAL samples were centrifuged in 1800g for 10 minutes and thrown away supernatant; they were transferred to a microscope slide and dried in the air. Microscope slides which were dried in the air were fixed with acetone. Of 50 ml Detection Reagent (monoclonal antibody labeled with fluorescein isothiocyanate) were dropped to the slides (samples and control). The samples were incubated for 30 minutes in a moist environment. The slides were washed with distilled water and put into wash-basin. Then, the preparations were dried with the aid of blotting paper. 1-2 drops of mounting medium was poured on each slide and the slides were covered with the cover-glass of the microscope and they were inspected in a fluorescent microscope in 200-300x magnification. Preparations were protected from the light. The cysts died in light-green fluorescent color were evaluated as positive. As stated in the guide, preparations with 5 or more cysts are positive, those with 4 or less cysts are suspicious and those with no cysts are negative.

DNA extraction and PCR: Amplification of *P. jiroveci* DNA by PCR has been made in Gülhane Military Medical Academy, Laboratory of Microbiology and Clinical Microbiology.

DNA extraction. DNA was extracted from BAL specimens by a modification of the procedure described by Nelson et al. (22). Two hundred and fifty microliters of K buffer and 10 microliters pronase E was added to micro-centrifuge tubes containing 100 microliters BAL specimens. The tubes were vortexed, and then incubated at 42°C for 60 min. Following incubation, 250 µl of alkali phenol and 250 µl chloroform + isoamyl alcohol were added the tubes, the tubes were vortexed and centrifuged at approximately 12000 rpm, at 4^0C for 15 min. The supernatant was decanted, and 500 ml of 70% ethanol was added to the tube and vortexed. After 5 min of centrifugation at 12000 rpm, at 4^0C the ethanol was decanted. Air-dried DNA pellets at 37 ^{0}C were suspended in 100 ml of sterile distilled H_2O. Extracted DNA from specimens and controls was added to PCR mixtures immediately or stored at -20°C.

Amplification of P. jiroveci DNA. One microliter of extracted DNA, equivalent to 1/100 of the original BAL specimen, was added to 0.5-ml thin-walled PCR tubes containing 50 µl of PCR mix and was overlaid with mineral oil. Final PCR mixtures consisted of 5 µl 10x PCR buffer; 2.5 mM $MgCl_2$; 10 mM dNTP (Sigma, Germany); 1.5 U of Taq DNA polymerase (xxxxx.); and 100 pmol/µl primers BP1 (5'-TCGGACTAGGATATAGCTGG-3') and BP2 (5'-CCCTTTCGACTATCTACC-3') (Metabion, Planegg-Martinsried, Germany). PCR tubes were placed in a model xxx thermal cycler (AmpliTaq; PE Applied Biosystems, Weiterstadt, Germany), denatured at 94°C for 5 min, then subjected to 40 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 35 s, and 72°C for 5 min. Positive and negative controls were included in all runs.

Detection of PCR products. Products of PCR were investigated by agarose gel electrophoresis, stained with ethidium bromide, and analyzed under UV light. PCR analysis was performed without prior knowledge of the conventional-staining diagnosis. Contamination precautions included use of aerosol barrier pipette tips and the performance of master mix preparation, DNA extraction, PCR procedure, and specimen detection in separate rooms. Several positive (from BAL specimens of PCP patients) and negative (autoclaved water and the PCR mixture minus the DNA template) controls were tested simultaneously. For PCR-positive samples with negative conventional-staining results (PCR-positive samples were reexamined microscopically by two different persons) DNA isolation was repeated and an additional PCR was performed. A positive result was accepted when this PCR determination was also positive.

Statistical analyses: Statistical evaluation of the results acquired in the study has been made in SPSS (version 10.0) program (SPSS Inc, Chicago, USA). Positive ratios of the groups were compared with chi-square (x^2) and/or Fisher's exact test. A p value of <0.05 was considered significant.

Table 1: Distribution of the groups according to age and sex

	Group I		Group II		Total	
	man	woman	man	woman	man	woman
Number (n)	48	7	55	10	103	17
Percent (%)*	87.3	12.7	84.6	15.4	85.8	14.2
Percent (%)**	45.8		54.2		100	
Age range (yrs)	33-80		20-85		20-85	
Mean age (yrs)	59.036 ± 1.556		54.753 ± 1.855		56.716 ± 1.245	

* column percentage ** among the all patients

Table 2: Diagnostic Distribution of Group II patients

Diagnose	Number (n)	Percent (%)
Pneumonia	20	30.8
COPD	16	24.6
Post-tuberculosis sequelae	12	18.5
Other	17	26.2
Total	65	100

Table 3: Distribution of the positive results obtained by PCR and IF methods

	Group I		Group II	
	PCR	IF	PCR	IF
Number (n)	3*	1	0	0
Percent (%)	5.5	1.8	0	0
p	>0.05	>0.05		

* The ages of the patients were 55, 57 and 33 respectively and all were male. The diagnosis was primary lung cancer diagnose was made out for each of them.

Results

No meaningful difference could be statistically observed between the average age of Group I and Group II. (p>0.05) (Table 1). Group I covered the patients diagnosed with lung cancer (90% primary lung cancer, 10% metastatic lung cancer) and none of them had gone under the treatment of chemotherapy or glucocorticoid. 30.8 % of Group II was the patients with pneumonia and 24.6 % were those with COPD. None of patients with COPD and with some other lung problems used oral glucocorticoid (Table 2). By using IF method, positivism was detected in only one of the Group I patients and in 3 of them positivism and PCR were obtained. Of these 3 patients, only one was IF positive. After using both methods, positivism could not be obtained in Group II (Table 3).

Discussion

P. jiroveci colonization is seen mostly in patients (8-40%) with immunodeficiency, in patients (10%) with COPD without immunodeficiency and lastly in patients (6%) whose respiration system has been damaged [5, 6, 20, 24]. Excluding AIDS, corticosteroid treatment, chemotherapy and other immunosuppressive agents demonstrate the clinical risks of the disease. PCP is rarely reported without predisposing factors. In this study, *P. jiroveci* positivism with PCR was obtained in 3 patients who were diagnosed with lung cancer. None of the cases used glucocorticoid. Positivism was not obtained in Group II.For a long time, it was thought that *P. jiroveci* caused to reactivation considering the high level of antibody positivism at early ages. However, the fact that no carriage was observed in animal hosts and immunocompetant individuals after primary infection supports the view that the disease develops in the form of a new infection [10, 11, 26, 27]. Absence of latent *P. jiroveci* in healthy persons isn't demonstrated there is no agent. Presence of the organism in any part of the body is enough to continue latent infection. It can be considered as a temporary colonization rather than a permanent one [2]. The result that 3 cases with cancer are positive leads us to think underlying disease increases predisposition and causes reactivation. In the same way, that there is no PCP tables in positive cases again suggests reactivation.

It is known that the specificity and sensitivities of the kits containing monoclonal antibody against *P. jiroveci* are quite higher than the histological paints [14, 16, 17, 19]. 92 % *P. jiroveci* positivism was observed in the BAL and endotracheal aspiration samples of 31 patients with immunodeficiency by using IF method [18]. The number of studies which prove the eligibility of PCR technology for PCP diagnose has increased for the last years and this method has turned out to be

more sensitive than the standard diagnose methods [12, 19, 22, 23]. Its ability to define both living and dead organisms is a great advantage. Wakefield has developed all primers necessary for DNA amplification of all *Pneumocyctis spp* [21]. When these primers used in human-oriented samples are ability to detect only *P. jiroveci* DNA but they can not detect microorganisms in the lung tissues of other mammals. Little amount of organism which can not be detected by microscopy could be observed by PCR in the BAL fluids of patients who do not have

Acute PCP manifestations and are both immunocompetant and with impaired immunity [5, 6]. Only one of the three positive cases was detected by the method of IF. As in the earlier studies, PCR gave better results than the stain methods.

PCR positivism in an asymptomatic individual may demonstrate a temporary carriage with contamination, colonization or low number of *P. jiroveci*. In a study, 3 different PCR methods were used and the same result was obtained [12]. In 20% of 169 HIV negative patients with moderate or serious immunosuppression, *P. jiroveci* positivism were obtained by nested-PCR but none of the patients developed PCP [28]. In different studies, 17-28 % of immunosuppressive individuals with HIV negative pulmonary disease but not with PCP were found PCR positive [6, 16, 23, 24, 29, 30]. With the aid of "real time quantitative PCR" which has been recently developed, it became possible to detect the mRNA of *P. jiroveci* and measure its viability with a molecular method [22]. On the contrary of these ratios, Helweg-Larsen and et al obtained 4% PCR positivism in the community-acquired pneumonia cases [12]. While Visconti and et al obtained 2.5 % positivism in 78 immunosuppressive patients, Oz and et al could not obtain any positive cases in 258 upper respiratory tract samples of 86 immunosuppressive patients [20, 24]. Clinical importance of a positive *P. jiroveci* PCR result is not clear. In the studies, when compared with the control group (18%), high level of mortality (54%) was observed. Since the autopsy was not rendered, real reasons of death could not be determined [31]. Wakefield could not detect *P. jiroveci* in the BAL samples of 10 healthy persons (HIV negative) by using PCR [32]. Peter obtained the same result in the postmortem lung tissue of 15 immunocompetant patients (HIV negative) [27]. It has been demonstrated that PCR does not provide an additional advantage to the traditional diagnose methods for the immunocompetant patients [23]. During our study, in 3 lung cancer cases with *P. jiroveci* DNA positive, no PCP findings were observed. The evaluation of positive results obtained by PCR still remains uncertain.

Since the *P. jiroveci* DNA can be defined in immunocompetant individuals who do not go under pneumocyctis treatment, subclinical infection or colonization may be observed in these adults. Quantitative *P. jiroveci* PCR may be helpful for pneumocyctis risk patients to differentiate the colonization and clinical disease. *P. jiroveci* should be investigated with PCR in patients who are not HIV positive and especially with underlying pulmonary disease. The fact that the cases only with lung cancer have positivity supports the idea that lung cancer is important risk factor. According to the acquired results, the patients should be monitored closely for prophylaxis and treatment and the treatment should not be applied to each PCR positive case. Also, the possible pathogenic role of *P. jiroveci* should be supported by other studies.

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