The mycological and molecular study of *Pneumocystis jiroveci* pneumonia among HIV and non-HIV immunocompromised patients hospitalized in pulmonary units in Guilan, northern Iran

Zahra Rafat[1,2], Keyhan Ashrafi[1*], Seyed Jamal Hashemi[2,3*], Elahe Sasani[4], Alireza Naserani[1], Hasti Kamali Sarvestani[2], Farshid Hashemi[5]

[1]Department of Medical Microbiology, School of Medicine, Guilan University of Medical Sciences, Rasht, Iran
[2]Department of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
[3]Food Microbiology Research Center, Tehran University of Medical Sciences, Tehran, Iran
[4]Department of Mycology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran
[5]Department of Pharmacognosy, School of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran

ABSTRACT

Background and Objectives: *Pneumocystis jirovecii* pneumonia (PJP) is a serious infection that usually affects those with a weak immune system. Since the prevalence of this infection in Iran and in the world is not clearly defined, the present study aimed to evaluate the incidence, clinical spectrum, and demographic characteristics of PJP among HIV and non-HIV immunocompromised patients.

Materials and Methods: Bronchoalveolar Lavage (BAL) specimens were obtained from 3 groups of immunocompromised patients, including acquired immunodeficiency syndrome (AIDS) patients, diabetic patients, and patients receiving immunosuppressive therapies. All were hospitalized in pulmonary units. The specimens were examined using microscopic methods (Giemsa and calcofluor white staining) and the nested-PCR technique based on mtLSU-rRNA gene.

Results: A total of 120 BAL samples were collected. From 12.5% (5 from 40) of HIV-infected patients, 5% (2 from 40) of patients receiving immunosuppressive therapies, and 2.5% (1 from 40) of diabetic patients *Pneumocystis jiroveci* was isolated. There was not any association between the prevalence of PJP and the patient's gender ($p= 0.557$) and age ($p= 0.681$). Fever and dyspnea (n=7, 87.5%), nonproductive cough and abnormal auscultation sound (n=5, 62.5%), and also chills and weight loss (n=2, 25%) were the documented clinical symptoms of PJP. Also, the results showed that none of the samples had positive results for *P. jiroveci* with microscopic tests while using the nested-PCR method 8 samples had positive results.

Conclusion: Since PJP often causes symptoms that are similar to other illnesses, such as the flu or tuberculosis, clinical and laboratory findings should be used simultaneously for making the final decision on drug administration.

Keywords: Pneumonia; Pneumocystis; Human immunodeficiency virus; Diabetes mellitus; Immunosuppression; Epidemiology; Iran

*Corresponding author: Keyhan Ashrafi, Ph.D, Department of Medical Microbiology, School of Medicine, Guilan University of Medical Sciences, Rasht, Iran.
Tel: +98-13-33690921
Fax: +98-1333690036
Email: k_fashi@yahoo.com; k_ashrafi@gums.ac.ir

*Corresponding author: Seyed Jamal Hashemi, Ph.D, Department of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran; Food Microbiology Research Center, Tehran University of Medical Sciences, Tehran, Iran.
Tel: +98-21-42933150
Fax: +98-21-88951392
Email: sjhashemi@tums.ac.ir

INTRODUCTION

Pneumocystis jiroveci, formerly known as *P. carinii*, is an unusual opportunistic fungus that was considered a protozoon. In the 1980s it emerged as a major pathogen with the onset of the acquired immunodeficiency syndrome (AIDS) epidemic (1). The disease caused by the fungus is used to be called *Pneumocystis jiroveci* pneumonia (PJP) (2). Patients at particular risk of infection include those with AIDS, underlying diseases such as hematologic malignancies and solid tumors, diabetics, those receiving immunosuppressive therapies, and organ transplant recipients (3). *P. jiroveci* is thought to be transmitted from person to person through an airborne route. Asymptomatic lung colonization can occur in people with normal immune systems, and they may unknowingly become reservoirs (asymptomatic carriers) for the spread of the fungus to immunocompromised individuals. Occult pneumocystosis may lead to chronic lung inflammation (4).

PJP can cause death is 27%, increasing to 50% in severely immunocompromised patients (5). *Pneumocystis* selectively attaches to squamous (type I) alveolar epithelial cells, interferes with the pulmonary surfactant, causing marked thickening of the alveolar septa and profound hypoxia, which can be fatal if not treated aggressively (6). PJP may be difficult to diagnose owing to its nonspecific symptoms. Radiological presentation is also nonspecific or even normal in the case of mild disease. Therefore, the single most important diagnostic tool for PJP is a high clinical suspicion (7). Because PJP is one of the most frequent opportunistic infections, physicians should consider it when an HIV-infected patient or a patient who is immunosuppressed for reasons other than HIV complains of fever, shortness of breath, and/or cough (8, 9). In more than 95% of cases, *P. jirovecii* infection is restricted to the lung, but in severely immunocompromised individuals, the rare disseminated infection (thyroid, kidney, bone marrow, liver, spleen, retina, ear, and skin) may occur (10-14). The major problem in *Pneumocystis* research is the absence of an *in vitro* culture system (15). Although the incidence of PJP in developed countries has reduced as a result of prophylaxis, in developing countries like Iran, it is a significant concern because of limited care resources and the absence of enough data about the epidemiology of the infection (16). Besides, the epidemiology of *Pneumocystis* infections has still remained unde-

termined because the detection of the fungus in clinical specimens by microbiological methods such as direct microscopy does not have enough sensitivity. Furthermore, the organism cannot grow on microbiological media. It should be noted since 1979, no studies have evaluated *P. jirovecii* isolates in Guilan province, Northern Iran.

The present study evaluated the incidence of PJP among HIV and non-HIV immunocompromised patients hospitalized in pulmonary units, based on microscopic methods (Giemsa and calcofluor white staining) and the Nested-PCR technique in Guilan province. Determining the clinical spectrum, and demographic characteristics associated with PJP were the other purposes of the current study.

MATERIALS AND METHODS

Ethical considerations. This study was approved by the ethical committee of Tehran University of Medical Sciences (the number of Ethics Committee protocol: IR.TUMS.SPH.REC.1397.002). Written informed consent was obtained from all subjects prior to sample collection. All data were de-identified.

Patients and sampling. This descriptive cross-sectional study was carried out over a period of two years, from March 2017 to March 2019 in Guilan province, located in the northern region of Iran.

A total of 120 patients hospitalized in pulmonary units who were suspected of PJP (including 57 males and 63 females) divided by the type of underlying condition into 3 groups as follows:

(1) 40 HIV-infected patients

(2) 40 diabetic patients

(3) 40 patients receiving immunosuppressive therapy (patients receiving chemotherapy within the last 3 months before admission, steroid use: at least 4 mg methylprednisolone (or equivalent) per day for at least 7 days in the past 3 weeks before admission or a cumulative dose of at least 250 mg of methylprednisolone (or equivalent) in the past 3 months before enrollment, and recipient of any other immunosuppressive treatments (tacrolimus, cyclosporine, methotrexate, cyclophosphamide, and sirolimus).

In addition to the underlying condition, the following criteria were used for patient selection in this study:

(1) Patients with clinical symptoms indicative of

PJP according to a pulmonary diseases specialist opinion (dyspnea, nonproductive cough, fever, chest pain, weight loss, and abnormal auscultation sound).

(2) Patients with diffuse pulmonary involvement (e.g., bilateral infiltrates and/or ground-glass opacities and/or nodules and/or alveolar condensation on high-resolution computed tomography scan).

Patients who had taken co-trimoxazole before enrollment were excluded from the present study in order to prevention of false-negative results.

Demographic features including age and gender were recorded. Bronchoalveolar Lavage (BAL) specimen from each patient were obtained. Samples were homogenized, centrifuged (at 1500 g for 5 minutes) and sediments were collected in sterile conical tubes.

Microscopic examination. The BAL pellets were smeared, dried, and fixed with methanol and then stained with calcofluor white (Fungi-Fluor, Polysciences, Inc., Pennsylvania, USA) in accord with the manufacturer's instructions. Giemsa staining was performed (with 2 mL of Giemsa stain in 40 mL of 6.7 mM phosphate buffer at pH 7.2) on methanol-fixed BAL smears, as described previously (17). The experiments were examined twice in parasitology and mycology laboratories, and positive or negative findings were confirmed by a professional parasitologist and mycologist. We considered the specimen microscopically positive if *P. jirovecii* was detected by either Giemsa or calcofluor white.

DNA extraction. *P. jirovecii* DNA was extracted by using commercially available DNA extraction kits (GeneAll Bldg, 303-7 105 Dongnam-ro, Songpa-gu, Seoul, South Korea) according to manufacturer's instructions.

Primers. PCR was performed for mitochondrial large subunit (mtLSU) ribosomal ribonucleic acid (rRNA) gene, using previously published primers (18). External primers namely pAZ102-E (5'-GAT GGC TGT TTC CAA GCC CA-3') and pAZ102-H (5'-GTG TAC GTT GCA AAG TAC TC-3') were used in the first round, which produced a 346 bp amplicon, and the internal primers namely pAZ102-X (5'-GTG AAA TAC AAA TCG GAC TAG G-3') and pAZ102-Y (5'-TCA CTT AAT ATT AAT TGG GGA GC-3') were used in the second round to amplify a 260 bp fragment (18).

PCR and sequencing. Each PCR reaction contained 2.5 μL of 10× reaction buffer, 2 mM $MgCl_2$, 500 μM dNTPs mixture, 1.25 μM Taq polymerase (Prime Taq, Genet Bio, South Korea), 25 pmol of each primer, 2 μL of DNA template solution, and enough distilled water to a final volume of 25 μL. First-round was fulfilled under the following condition: 95ºC for 4 min, 30 cycles of 94ºC for 45 s, 60ºC for 1 min and 72ºC for 1 min; and a final extension for 10 min in 72ºC. Two microliters of the first PCR product was used as DNA template in the second PCR reaction having the following conditions: 95ºC for 4 min, 30 cycles of 94ºC for 30 s, 60ºC for 45 s and 72ºC for 45 s; and a final extension of 72ºC for 10 min. PCR products were analyzed by electrophoresis on 1.5% agarose gels containing 0.5 μg/mL ethidium bromide and the bands were observed under UV light of the transilluminator. To rule out any false-positive results, aerosol barrier pipette tips were used and different steps of the preparations including DNA extraction, master mix preparation, PCR reactions and product detection were done in different rooms. Positive and negative controls were included in each experiment. The positive control was the DNA extracted from BAL smears, which were positive, based on Giemsa staining and PCR experiments. On the other hand, sterile distilled water was used as the negative control in each run of the test. All positive PCR products of mtLSU-rRNA gene were sequenced. Sequences in this study were adjusted and exported to Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI) database for species recognition.

Statistical tests. The data analysis was performed by SPSS software (IBM SPSS Statistics for Windows, Version 21.0, IBM Corp, Armonk, NY, USA). The study was assessed using Fisher's exact test and 95% Confidence intervals (CI). A p-value ≤ 0.05 was considered statistically significant.

RESULTS

A total of 120 BAL samples were obtained from 3 groups of immunocompromised patients hospitalized in pulmonary units. In the current study, the prevalence of PJP among HIV and non-HIV immunocompromised patients hospitalized in pulmonary units was 6.66%. Of this population, in 12.5% (5

from 40) of HIV-infected patients, 5% (2 from 40) of patients receiving immunosuppressive therapies, and 2.5% (1 from 40) of diabetic patients *P. jiroveci* was isolated. In the present study, according to the host factors (clinical symptoms, radiology findings, and predisposing factors which were defined as inclusion criteria), and the positive results in nested-PCR indicating the presence of *P. jirovecii* DNA in the BAL specimen in patients who were screened and tested negative for other respiratory pathogens the infection was confirmed. The results showed that none of the samples had positive results with microscopic tests (Giemsa staining and calcofluor white) while using the nested-PCR method based on mtLSU-rRNA gene, 8 samples had positive results for *P. jiroveci* (Fig. 1). Among the 8 positive cases in this research, most isolates were from male patients (n=6, 75%) with a mean ± SD age of 58.3 ± 16.3 years (Table 1). Statistical analysis could not find any association between the prevalence of PJP in HIV and non-HIV immunocompromised patients hospitalized in pulmonary units and the patient's gender (*p*= 0.557) and age (*p*= 0.681).

Based on clinical symptoms, 7 positive patients (87.5%) had fever and dyspnea, 5 cases (62.5%) presented with nonproductive cough and abnormal auscultation sound, and 2 positive patients (25%) had chills and weight loss. Statistical analysis showed that there was not a significant relationship between the prevalence of PJP in HIV and non-HIV immunocompromised patients hospitalized in pulmonary units and clinical symptoms (*p* =0.21). The detailed information related to 8 patients with PJP is shown in Table 1.

The GenBank accession numbers for the sequences obtained in this work are SRR6399953–SRR6399960.

DISCUSSION

P. jirovecii causes PJP with a high rate of morbidity and mortality in immunocompromised patients especially in HIV infected persons and other immunosuppressed groups such as diabetic patients, and patients with autoimmune disorders (who treated with steroid or monoclonal antibodies directed against cell-mediated immune system mediators). In this study, a total of 120 BAL specimens related to HIV and non-HIV immunocompromised patients hospitalized in pulmonary units were examined and in 8 (6.66%) cases PJP was diagnosed. Of this population, in 12.5% (5 from 40) of HIV-infected patients, 5% (2 from 40) of patients receiving immunosuppressive therapies, and 2.5% (1 from 40) of diabetic patients *P. jiroveci* was isolated. In a study in Tehran, Iran the rate of *P. jirovecii* among non-HIV-infected patients
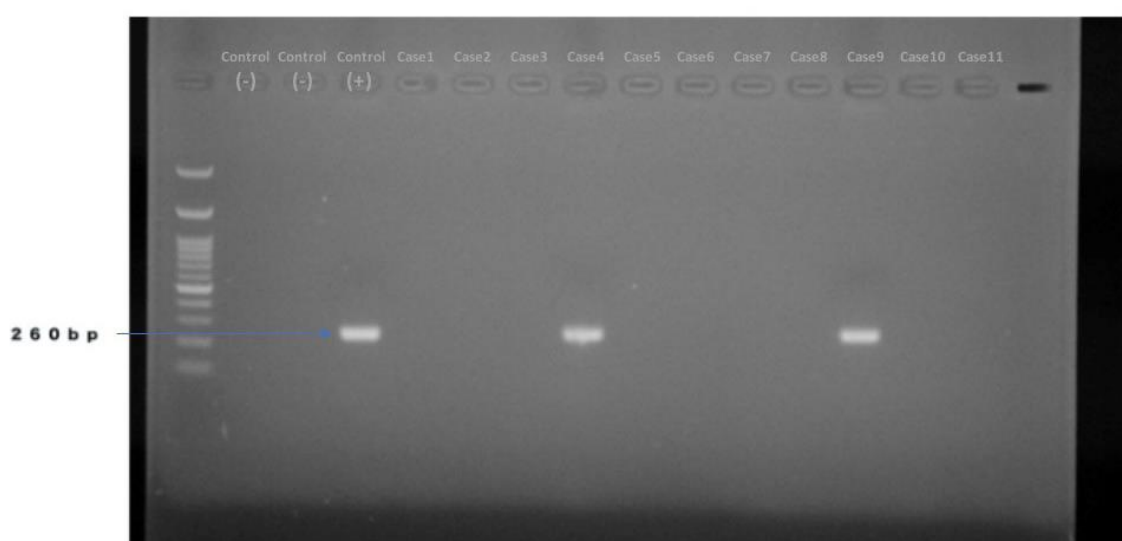
Fig. 1. Agarose gel electrophoresis of nested-PCR products of *P. jirovecii* partial mtLSU-rRNA gene. From left: Lane 1: DNA ladder (100 bp), Lanes 2,3: Negative control, Lane 3: positive control, Case 4 and Case 9: positive clinical samples having specific 260 bp bands representative of *P. jirovecii*, Cases 1-3, 5-8, and 10,11: no representative band indicating negative clinical samples.

Table 1. The detailed information related to 8 patients with *Pneumocystis jiroveci* pneumonia

Variables		Patients	
		Number	Percentage
Age groups (years)	≥ 50	6	75%
	< 50	2	25%
Gender	Female	2	25%
	Male	6	75%
The type of underlying condition	Human immunodeficiency virus infection	5	62.5%
	Receiving immunosuppressive therapies	2	25%
	Diabetes Mellitus	1	12.5%
Clinical symptoms	Fever and dyspnea	7	87.5%
	Nonproductive cough and abnormal auscultation sound	5	62.5%
	Chills and weight loss	2	25%

was 12.5%. They have shown that *P. jirovecii* colonization rate in patients with malignancy, transplant recipients, immunosuppressive therapy recipients and patients with other various lung diseases was 21.7%, 20.3%, 12.7%, and 7.3%, respectively (17). On the other hand, the frequency of *P. jirovecii* was reported 11.9% among HIV-infected patients with a CD4 count < 200 cells/ μL in Tehran (19). In a study conducted by Aboualigalehdari et al. in Ahvaz, Iran the prevalence of *P. jirovecii* was totally 27% including, 32.1% in tuberclosis patients, 25.3% in other chronic pulmonary disorders, and 25.3% in HIV patient (20). In another study by Parian et al. the results of PCR and sequencing tests demonstrated that 10 BAL samples were colonized by *P. jirovecii* (21). Also, a study performed by Azoulay in 2004 in Paris showed the rate of positive samples to be 8.7% (22).

Furthermore, the results of the present study showed that among different immunocompromised patients, HIV-infected patients are more susceptible PJP. Different studies have demonstrated that in HIV-infected patients, the risk of PJP is rapidly increased when the number of CD4 $^+$ lymphocytes decrease to 200×10^6 per mL (23, 24).

Diabetes mellitus was observed in 12.5% of patients with PJP in the current study. Diabetes mellitus is a condition that decreases the activity level of body cells, responsible for immunity. These activities include infiltration, chemotaxis, phagocytosis, and natural killer cell activities (25, 26). On the other hand, the high level of blood glucose may lead to a weakened immune system, increased adherence of cell surface, impairment of humoral immunity, and repression of superficial cell receptors. These are reasons for the higher sensitivity of diabetic patients

to fungal, viral, and bacterial infections (25, 26, 37).

Also, receiving immunosuppressive therapy was observed in 25% of patients with PJP in the present study. The results of a study conducted by Calero-Bernal et al. demonstrated that corticosteroids were in the medication lists of 98 (76%) of non-HIV patients with PJP (27). In a study conducted by Bryan et al., most patients who had PJP had received a corticosteroid dose equivalent to 16 mg or greater administered for at least 8 weeks (28).

Furthermore, the main clinical signs and symptoms among patients in this study were dyspnea, nonproductive cough, fever, and abnormal auscultation sound, which are common among patients with pneumocystis, as reported in similar studies (19-23).

The sex distribution of *P. jirovecii* colonization varies in several reports. The female: male ratio of *P. jirovecii* was 3.3:1 in Togashi et al. (29) report. Also, in a study conducted by Izadi et al. the frequency of *P. jirovecii* colonization among males was 2.5 rather than females (30). Our results showed that the frequency of PJP among males was 3 (M: F, 6:2) greater than females.

PCR-based diagnosis of *Pneumocystis* in respiratory specimens is a valuable tool for screening this organism. Single-step PCR cannot precisely detect the infectious agent in some cases, especially in low template DNA conditions. Therefore, in this study, the more sensitive and reliable nested PCR was used due to its ability to amplify very small quantities of DNA (31). Among 120 BAL samples examined in this study, none of the samples had positive results for *P. jiroveci* with microscopic tests while using the nested-PCR method 8 samples had positive results. This indicates that the sensitivity of nested PCR is

higher than that of conventional methods. The sensitivity of the molecular method has been confirmed by many researchers (32-36).

CONCLUSION

The current study is the first report of the epidemiology of PJP from Guilan, northern Iran. The results showed a frequency of 6.66% for PJP among 3 groups of immunocompromised patients, including AIDS patients, diabetic patients, and patients receiving immunosuppressive therapies. Also, our results showed that the nested-PCR might be a reliable technique for the diagnosis of *P. jirovecii*. Here we found that the Giemsa and calcofluor white staining were not effective staining methods for the detection of *Pneumocystis*. Special attention to these patients is essential for preventing nosocomial infections or emerging drug resistance isolates.

REFERENCES

1. Wang RJ, Miller RF, Huang L. Approach to fungal infections in human immunodeficiency virus–infected individuals: pneumocystis and beyond. *Clin Chest Med* 2017;38: 465-477.

2. Salzer HJF, Schäfer G, Hoenigl M, Günther G, Hoffmann C, Kalsdorf B, et al. Clinical, diagnostic, and treatment disparities between HIV-infected and non-HIV-infected immunocompromised patients with *Pneumocystis jirovecii* pneumonia. *Respiration* 2018;96: 52-65.

3. Guo F, Chen Y, Yang SL, Xia H, Li XW, Tong ZH. *Pneumocystis pneumonia* in HIV-infected and immunocompromised non-HIV infected patients: a retrospective study of two centers in China. *PLoS One* 2014;9(7): e101943.

4. Truong J, Ashurst JV. *Pneumocystis (Carinii) Jiroveci* Pneumonia. In: StatPearls. StatPearls Publishing, Treasure Island (FL); 2019.

5. Gaborit BJ, Tessoulin B, Lavergne RA, Morio F, Sagan C, Canet E, et al. Outcome and prognostic factors of *Pneumocystis jirovecii* pneumonia in immunocompromised adults: a prospective observational study. *Ann Intensive Care* 2019;9: 131.

6. Gigliotti F, Limper AH, Wright T. Pneumocystis. *Cold Spring Harb Perspect Med* 2014;4: a019828.

7. Arsić Arsenijevic V, Vyzantiadis TA, Mares M, Otasevic S, Tragiannidis A, Janic D. Diagnosis of *Pneumocystis jirovecii* pneumonia in pediatric patients in Serbia,

8. Bienvenu AL, Traore K, Plekhanova I, Bouchrik M, Bossard C, Picot S. *Pneumocystis pneumonia* suspected cases in 604 non-HIV and HIV patients. *Int J Infect Dis* 2016;46: 11-17.

9. Tandukar S, Singh N, Naseer MS, Chand R, Brunet H, Shokouh-Amiri HM. Role of serum (1, 3)-B-D-glucan to screen for *Pneumocystis pneumonia* in kidney transplant recipients. *Transplant Proc* 2021;53: 1075-1079.

10. Karam MB, Mosadegh L. Extra-pulmonary *Pneumocystis jiroveci* infection: a case report. *Braz J Infect Dis* 2014;18: 681-685.

11. Anuradha, Sinha A. Extrapulmonary *Pneumocystis carinii* infection in an AIDS patient: a case report. *Acta Cytol* 2007; 51:599-601.

12. O'Neal CB, Ball SC. Splenic pneumocystosis: an atypical presentation of extrapulmonary pneumocystis infection. *AIDS Read* 2008;18: 503-508.

13. Panos GZ, Karydis I, Velakoulis SE, Falagas ME. Multi-skeletal *Pneumocystis jiroveci (carinii)* in an HIV-seropositive patient. *Int J STD AIDS* 2007;18: 134-137.

14. Siddiqi AE, Liu AY, Charville GW, Kunder CA, Uzel G, Sadighi Akha AA, et al. Disseminated *Pneumocystis jirovecii* infection with osteomyelitis in a patient with CTLA-4 haploinsufficiency. *J Clin Immunol* 2020; 40:412-414.

15. Wakefield AE. *Pneumocystis carinii. Br Med Bull* 2002;61: 175-188.

16. Cillóniz C, Dominedò C, Álvarez-Martínez MJ, Moreno A, García F, Torres A, et al. *Pneumocystis pneumonia* in the twenty-first century: HIV-infected versus HIV-uninfected patients. *Expert Rev Anti Infect Ther* 2019; 17:787-801.

17. Khodadadi H, Mirhendi H, Mohebali M, Kordbacheh P, Zarrinfar H, Makimura K. *Pneumocystis jirovecii* colonization in non-HIV-infected patients based on nested-PCR detection in bronchoalveolar lavage samples. *Iran J Public Health* 2013;42: 298-305.

18. Chabe M, Herbreteau V, Hugot JP, Bouzard N, Deruyter L, Morand S, et al. *Pneumocystis carinii* and *Pneumocystis wakefieldiae* in wild Rattus norvegicus trapped in Thailand. *J Eukaryot Microbiol* 2010;57: 213-217.

19. Sheikholeslami FM, Sadraei J, Farnia P, Forozandeh M, Kochak HE. Rate of *Pneumocystis pneumonia* in Iranian HIV+ patients with pulmonaryn infiltrates. *Jundishapur J Microbiol* 2013;6: 295-300.

20. Aboualigalehdari E, Mahmoudabadi AZ, Fatahinia M, Idani E. The prevalence of *Pneumocystis jirovecii* among patients with different chronic pulmonary disorders in Ahvaz, Iran. *Iran J Microbiol* 2015;7: 333-337.

21. Parian M, Fata A, Najafzadeh MJ, Rezaeitalab F.

Molecular detection of *Pneumocystis jirovecii* using polymerase chain reaction in immunocompromised patients with pulmonary disorders in northeast of Iran. *Curr Med Mycol* 2015;1: 13-18.

22. Azoulay E, Bergeron A, Chevret S, Bele N, Schlemmer B, Menotti J. Polymerase chain reaction for diagnosing *Pneumocystis pneumonia* in non-HIV immunocompromised patients with pulmonary infiltrates. *Chest* 2009;135: 655-661.

23. Morris A, Lundgren JD, Masur H, Walzer PD, Hanson DL, Frederick T, et al. Current epidemiology of *Pneumocystis pneumonia*. *Emerg Infect Dis* 2004;10: 1713-1720.

24. Kaplan JE, Hanson D, Dworkin MS, Frederick T, Bertolli J, Lindegren ML, et al. Epidemiology of human immunodeficiency virus associated opportunistic infections in the United States in the era of highly active antiretroviral therapy. *Clin Infect Dis* 2000; 30 Suppl 1: S5-14.

25. Rafat Z, Hashemi SJ, Ashrafi K, Nikokar I, Jafari A, Foroushani AR, et al. Fungal isolates of the respiratory tract in symptomatic patients hospitalized in pulmonary units: a mycological and molecular epidemiologic study. *J Multidiscip Healthc* 2020;13: 661-669.

26. Rafat Z, Hashemi SJ, Ashrafi K, Nikokar I, Jafari A, Foroushani AR, et al. Epidemiology, laboratory diagnosis and clinical aspects of fungal pulmonary infections in 384 patients hospitalized in pulmonary units in Guilan province, Iran. *Iran J Microbiol* 2020;12: 353-363.

27. Calero-Bernal ML, Martin-Garrido I, Donazar-Ezcurra M, Limper AH, Carmona EM. Intermittent courses of corticosteroids also present a risk for *Pneumocystis pneumonia* in non-HIV patients. *Can Respir J* 2016;2016: 2464791.

28. Krajicek BJ, Thomas CF, Limper AH. *Pneumocystis pneumonia*: current concepts in pathogenesis, diagnosis, and treatment. *Clin Chest Med* 2009;30: 265-278.

29. Togashi Y, Masago K, Ito Y, Sakamori Y, Okuda C, Fukuhara A, et al. *Pneumocystis jiroveci* pneumonia and colonization in patients with advanced lung cancer. *Oncol Lett* 2013;5: 601-604.

30. Izadi M, Jafari NJ, Sadraei J, Poornaki AM, Rezavand B, Zarrinfar H, et al. The prevalence of *Pneumocystis jiroveci* in bronchoalveolar lavage specimens of lung transplant recipients examined by the Nested PCR. *Jundishapur J Microbiol* 2014;7(12): e13518.

31. Sheikhi N, Rafat Z, Zaker Boostan Abad S, Ahmadi SM, Azizian R, Hamidi M. Evaluating the clinical application of nested PCR in the diagnosis of fungal infections. *New Zealand J Med Lab Sci* 2020;74: 115-118.

32. Summah H, Zhu YG, Falagas ME, Vouloumanou EK, Qu JM. Use of real-time polymerase chain reaction for the diagnosis of *Pneumocystis pneumonia* in immunocompromised patients: a meta-analysis. *Chin Med J (Engl)* 2013;126: 1965-1973.

33. Wilson JW, Limper AH, Grys TE, Karre T, Wengenack NL, Binnicker MJ. *Pneumocystis jirovecii* testing by real-time polymerase chain reaction and direct examination among immunocompetent and immunosuppressed patient groups and correlation to disease specificity. *Diagn Microbiol Infect Dis* 2011;69: 145-152.

34. Mahmoodzadeh A, Hajia M, Rezaiemanesh MR. Comparison of Gomori's methenamine silver method with PCR technique on oral swab, bronchoalveolar lavage and lung Ho-mogenate specimens in detection of pneumocystis. *Iran J Parasitol* 2008;3 :21-25.

35. Tang YW. Molecular diagnostics of atypical pneumonia. *Acta pharmacol Sin* 2003;24:1308-1313.

36. Procop GW, Haddad S, Quinn J, Wilson ML, Henshaw NG, Reller LB, et al. Detection of *Pneumocystis jiroveci* in respiratory specimens by four staining methods. *J Clin Microbiol* 2004;42: 3333-3335.

37. Abdollahi A, Kamali Sarvestani H, Rafat Z, Ghaderkhani S, Mahmoudi-Aliabadi M, Jafarzadeh B, et al. The Association between the level of serum 25 (OH) vitamin D, obesity, and underlying diseases with the risk of developing COVID-19 Infection: a case-control study of hospitalized patients in Tehran, Iran. *J Med Virol* 2021;93: 2359-2364.