Article

Title: Study on the prevalence of Pneumocystis jirovecii as a causative agent of lung pathology in people with different immune status

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Abstract: Background: *Pneumocystis* pneumonia (PCP) commonly affects immunocompromised individuals, whereas in immunocompetent persons, it occurs relatively rarely, and in most cases the *Pneumocystis* infection is detected as an asymptomatic colonization. The present study aimed to establish the prevalence of *Pneumocystis jirovecii* infection in human hosts with different immune status (immunocompromised and immunocompetent), using molecular diagnostic methods, and to compare their diagnostic value with that of classical staining methods. Methods: We used the collected to this moment data from a prospective study on the prevalence of pneumocystosis among the Bulgarian population. Clinical specimens (including throat secretion, induced sputum, tracheal aspirate, and bronchoalveolar lavage collected from 220 patients suspected of PCP (153 immunocompetent and 67 immunocompromised patients) were examined with staining microscopic methods and real-time PCR for detection of *P. jirovecii.* Results: DNA of the pathogen was detected in 38 (17%) specimens (32 immunocompromised patients and 6 immunocompetent subjects). From all 220 clinical samples examined by staining methods, only in five (2%) *P. jirovecii* cysts were detected by the Gomori's stain. All patients with PCP were treated with trimethoprim-sulfamethoxazole, but in ten of them (HIV- positive patients) the disease was with fatal outcome. Conclusions: This study is the first for the country including the main available laboratory methods for diagnosis of human pneumocystosis in Bulgaria. Regarding the etiological diagnosis of PCP, in our study the sensitivity of real-time PCR was higher compared to the staining methods. The choice of a method for sample collection and examination has an important role in the efficiency of the laboratory diagnostics.

Keywords: *Pneumocystis* pneumonia; immune status; real-time PCR; staining methods

1. Introduction

The first reports of the involvement of *Pneumocystis* in human pathology date back to the 1940s as a pathogen causing pneumonia in malnourished or premature infants. Until the 1980s, *Pneumocystis* pneumonia (PCP) in the elderly was considered as a rare but fatal infection mainly among patients with acute leukemia and other haematological malignancies [1]. In the 1980s, after the onset of

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the global epidemic of human immunodeficiency virus (HIV) infection, interest in PCP increased dramatically as it was one of the leading co-infections being the cause of death in patients with Acquired Immune Deficiency Syndrome (AIDS). Due to advances in access to antiretroviral therapy (ART) and routine prophylaxis against PCP, its incidence in the HIV-infected population has decreased in most industrialized countries. However, PCP still remains the most common opportunistic infection among AIDS patients in many countries [2–4].

Pneumocystis can be present in the respiratory system without leading to the clinical presentation of severe pneumonia. Detection of *Pneumocystis* in individuals without clinical symptoms is defined as colonization [5]. Pulmonary colonization with *P. jirovecii* is common in immunocompromised patients, but is less frequent among immunocompetent individuals with lung disease [6–8].

In Bulgaria, pneumocystosis has been the subject of scientific interest for years. *P. jirovecii* was found in young children with pneumonia and in patients with AIDS [9 – 11]. The first case of pneumocystosis associated with AIDS in our country was diagnosed at the National Center of Infectious and Parasitic Diseases [12–14]. In a study (covering the period 1986-2000) of the prevalence and features of different types of opportunistic infections in HIV-infected and AIDS patients in Bulgaria, *Pneumocystis* was detected in 26 (16.25%) of 160 patients by direct microscopic examination (staining methods and immunofluorescence assay). The clinical manifestation of pneumocystosis was represented by pneumonia with a disseminated type of infection in one case [15]. In another study conducted between 1993 and 2003, pneumocystosis was diagnosed in 6.06% of the examined (n=165) patients [16].

The present study aimed to establish the prevalence of pneumocystosis in different groups of immunocompromised and immunocompetent individuals using molecular diagnostic methods, and to compare their diagnostic value with that of classical staining methods.

2. Materials and Methods

2.1. Study design

This article is based on data from a prospective study on the prevalence of pneumocystosis among the Bulgarian population, beginning in January 2019.

2.2. Ethical considerations

The study was reviewed and approved by the Institutional review board (IRB) 00006384 and informed consent was obtained from the patients. No information that could reveal the identity of the patients who participated in the study was used.

2.3. Patients and samples

Clinical specimens (including throat secretion, induced sputum, tracheal aspirate, and bronchoalveolar lavage (BAL)) collected from a total of 220 patients suspected of having pneumocystosis (Group 1 - immunocompetent patients and Group 2 - patients with compromised immune system) were examined.

Group 1 included a total of 153 individuals presenting with cough (n = 106), evidence of unspecified pneumonia (n = 23), shortness of breath (n = 4), respiratory failure (n = 3), hemoptysis (n = 3), bronchitis (n = 2), fatigue (n = 2), respiratory distress syndrome (n = 1), pharyngitis (n = 1), lung abscess (n = 1), and 7 with COVID-19 related pneumonia.

Group 2 consisted of 67 individuals - 47 with HIV infection and 20 on immunosuppressive therapy (including haematological disease, n = 7; interstitial pulmonary fibrosis, n = 3; nephrotic syndrome, n = 3; bronchiectasis, n = 3; solid organ transplantation, n = 2; asthma, n = 1, and disseminated lupus, n = 1).

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2.4. Methods for detection of pathogen

2.4.1. Real-time PCR for qualitative and quantitative detection of P. jirovecii:

For extraction and purification of *P. jirovecii* DNA from clinical samples, PureLink™ Genomic DNA Mini Kit (Life Technologies Corporation, Carlsbad, CA 92008 USA) was used. Amplification of the gene encoding the mitochondrial large subunit of ribosomal RNA (mtL SU rRNA) was done by using RIDA®GENE kit (r-biofarm AG, Germany) according to the manufacturer's instructions.

2.4.2. Staining methods for detection of *P. jirovecii:*

Three staining methods for direct detection of *P. jirovecii* were applied. Six smears from each clinical material (throat secretion, induced sputum, tracheal aspirate, or bronchoalveolar lavage) of the PCP-suspected patient using clean glass microscope slides were prepared. After drying, two smears were stained by each of the three methods.

- Romanowski-Giemza staining (for trophozoites and cysts of *P. jirovecii).* Commercial Giemsa stain, modified solution (Sigma-Aldrich) was used. Dried thin smears were fixed with methyl alcohol for 5-10 minutes, dried, stained with a working solution of Giemsa stain for 20-22 minutes (the exposure was determined during the initial testing of the stain), washed with tap water and allowed to dry in a vertical position at room temperature.
- Toluidine blue staining (selective method for cysts of *P. jirovecii*). The thin smears from each clinical material were immersed for 5 minutes in sulfate reagent (prepared by mixing 25 ml diethyl ether and 25 ml concentrated sulfuric acid), rinsed with tap water, and stained with toluidine blue solution for 3 minutes. Differentiation was then performed in 2 shifts of isopropyl alcohol for 15-30 seconds, lightening with xylene and finally drying.
- Staining with methenamine-silver nitrate according to Gomori (for cysts of *P. jirovecii*). The method is considered the "gold standard" for microscopic visualization of *P. jirovecii* cysts. Microscopy Methenamine silver plating kit acc. to Gomori (Cat. No. 1.00820.0001; Merck KGaA, 64271 Darmstadt, Germany, Sigma-Aldrich Canada Co. or Millipore, Canada Ltd.) was used. The dried smears of the relevant clinical material were fixed for 30 minutes in 3.5% formalin and stained according to the manufacturer's protocol. The color of the cyst wall varies from gray to black (their surface membranes are visible).
- The samples were examined under a light microscope (Euromex IS.1153-Pli, The Netherlands) at 400x and 1000x magnification and visualized using color digital camera (Euromex DC.6000s, The Netherlands).

3. Results

We applied real-time PCR targeting the mtL SU rRNA gene of *P. jirovecii* for qualitative and quantitative detection of the pathogen from clinical specimens of the patients included in the study. *P. jirovecii* DNA was detected in the specimens of 38 (17%) out of 220 subjects examined. Positive PCR results were obtained in specimens of 6 patients with pneumonia of the 153 individuals of group 1. In group 2, amplification of a fragment of the target gene was obtained in 26 of the HIV-infected patients and 6 patients receiving suppressive therapy (Table 1).

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Table 1. Demographic, immunological and clinical data for study participants.

Demographic data	Age groups (range)				Gender		Total N (%)
	0-12 months	**1-9 years**	10-18 years	> 18 years	Male sex	Female sex	
No of cases	25	36	31	128	137	83	220
Real-time PCR positive	3	2	2	31	32	6	38 (17.3%)
Real-time PCR negative	22	34	29	97	105	77	182 (82.7%)
Light microscopy (RG[1]/ TB[2]/ GMS[3]), positive	0	0	0	5	5	0	5 (2.3%)
Light microscopy, negative	25	36	31	123	132	83	215 (97.7%)
Groups distributed by immunological status and clinical presentation (primary diagnosis)							
Group 1 - patients without data of immunosuppression	23	34	26	70	86	67	153
pneumonia	7	1	1	14	16	7	23 (15%)
respiratory distress syndrome	0	0	1	0	1	0	1 (0.7%)
pharyngitis	0	0	0	1	1	0	1 (0.7%)
respiratory failure	1	0	0	2	1	2	3 (1.9%)
dyspnea	0	0	1	3	1	3	4 (2.6%)
pulmonary abscess	0	0	0	1	1	0	1 (0.7%)
bronchitis	0	1	0	1	0	0	2 (1.3%)
fatigue	0	0	0	2	2	0	2 (1.3%)
hemoptysis	0	0	0	3	1	2	3 (1.9%)
cough	15	32	23	36	56	50	106 (69.3%)
COVID-19	0	0	0	7	4	3	7 (4.6%)
Real-time PCR positive	3	0	1	2	6	0	6 (3.9%)
Real-time PCR negative	20	34	25	68	80	67	147 (96.1%)
Light microscopy, positive	0	0	0	0	0	0	0
Group 2 - patients with compromised immune system	2	2	5	58	51	16	67
HIV infection	0	1	0	46	42	5	47 (70%)
hematological malignancy	1	0	1	5	4	3	7 (10.5%)
interstitial pulmonary fibrosis	0	0	0	3	2	1	3 (4.5%)
nephrotic syndrome	0	1	2	0	1	2	3 (4.5%)
solid organ transplantation	1	0	1	0	2	0	2 (3%)
long-term use of inhaled corticosteroids due to bronchiectasis and asthma	0	0	0	4	0	4	4 (6%)
disseminated lupus	0	0	1	0	0	1	1 (1.5%)

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Real-time PCR positive	0	2	1	29	26	6	32 (47.8%)
Real-time PCR negative	2	0	4	29	25	10	35 (52.2%)
Light microscopy, positive	0	0	0	5 (**GMS**[3])	5	0	5 (7.5%)
Light microscopy, negative	2	2	5	53	46	16	62 (92.5%)

[1]RG - Romanowski-Gimza staining; [2]TC- toluidine blue staining; [3]GMS - Gomori's methenamine silver stain

Commercial kit RIDA®GENE *Pneumocystis jirovecii* (r-biofarm AG, Germany), containing standards with a certain number of copies of *P. jirovecii* (Standard A: 101 copies/µl, Standard B: 103 copies/µl, Standard B: 105 copies/µl; analytical sensitivity: ≥ 10 DNA copies per reaction) was used for pathogen load assessment in the tested samples. In the post-treatment period for *Pneumocystis* pneumonia, 4 patients of the immunocompromised group and 3 patients without immunosuppression were additionally tested in follow-up studies for assessment of their response to the therapy. The reason for these additional tests was the patient's continuing complaints of shortness of breath, cough, and ongoing fever. Except for one HIV-infected patient, in all others, we found that the control sample taken within one month after the end of therapy did not contain *P. jirovecii* DNA. The complaints of the patient with HIV infection, whose control sample had a positive PCR result one month after the examination of the primary clinical specimen (induced sputum), continued despite the prescribed etiological treatment. On the background of assigned secondary prophylaxis with TMP/SMX, a total of 9 control tests a month apart from each other were performed for monitoring the response of the treatment, and only in the last sample no DNA of the pathogen was detected [17].

All 220 clinical samples of patients suspected of having pneumocystosis from Groups 1 and 2 were examined by staining methods. In patients from Group 1, staining methods showed no evidence of *P. jirovecii* cysts, while in five of Group 2 patients, the presence of *P. jirovecii* cysts was detected only by the Gomori's methenamine silver stain (Table 1, Figure 1).

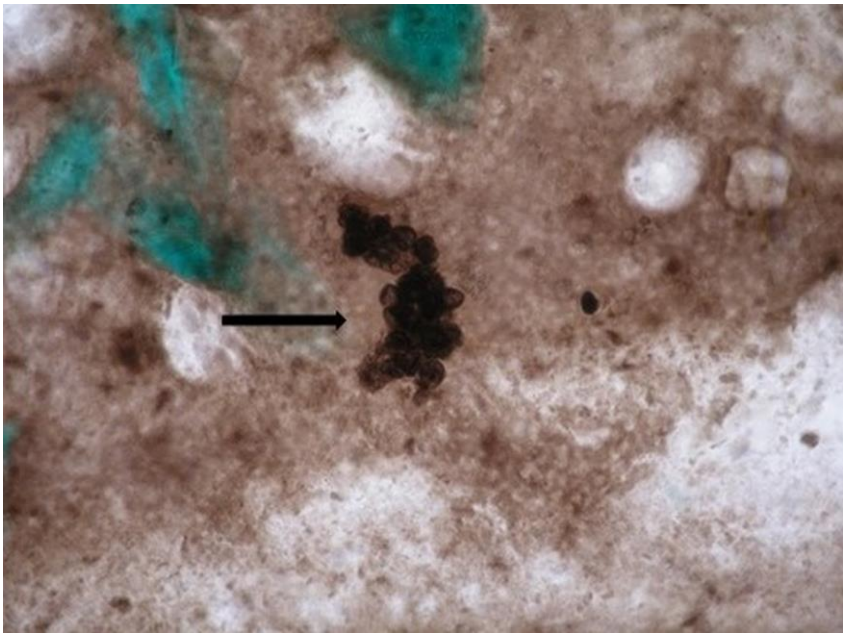

Figure 1. Sputum smear of HIV+ patient, stained with methenamine-silver nitrate (according to Gomori). A cluster of black-stained oval and rounded cysts of *P. jirovecii* (arrow). Light microscopy, magnification 1000x.

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The results of Gomori's staining, quantification of DNA load, and PCR cycle (Ct value), in which a fluorescent signal was reported as a result of multiplication of the target region of DNA molecule in the test sample are presented in Table 2.

Table 2. Comparison of data from studies with the Gomori's methenamine silver stain and the real-time PCR (the specific target gene of *Pneumocystis jirovecii* is mtL SU rRNA) of 5 patients with positive results by both techniques.

Patients	Staining method Specimen type - induced sputum	Real-time quantitative PCR		Ct
	GMS	*P. jirovecii* DNA concentration (copies/µl)		
		In 1µl of the reaction solution	In 200 µl of the initial sample	
P1 HIV+	**Clusters of cysts**	5,035 x 10^5	1,007 x 10^8	18.074
P2 HIV+	Clusters of cysts	4,669 x 10^5	9,338 x 10^7	18.176
P3 HIV+	Single cysts	2,179 x 10^1	4,358 x 10^3	31.566
P4 HIV+	Single cysts	5,790 x 10^1	1,158 x 10^4	30.254
P5 HIV+	Single cysts	4,703 x 10^2	9,406 x 10^4	27.441

In some patients, we also performed a comparative study on the concentration of *P. jirovecii* DNA depending on the type of clinical specimen (Table 3).

Table 3. Concentration of *P. jirovecii* DNA depends on the type of clinical specimen.

Type of clinical specimen	Patients/age group	Real-time quantitative PCR		Ct
		Concentration of *P. jirovecii* DNA (copies/µl)		
		In 1µl of the reaction solution	In 200 µl of the initial sample	
Tracheal aspirate	**A 4-month-old baby with pneumonia**	0,8123 x 10^3	0,162480 x 10^6	35.37
	A 6-month-old baby with severe interstitial pneumonia	359,6 x 10^3	71,92 x 10^6	26.67
Bronchoalveolar lavage	A 60-year-old man with interstitial pulmonary fibrosis	1,265 x 10^3	253 x 10^6	24.87
	A 45-year-old man with bilateral interstitial pneumonia	87,52 x 10^3	17,504 x 10^6	29.69

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4. Discussion

Cases of HIV-associated PCP are reported at fluctuating rates throughout the world. While the clinical manifestation of PCP in HIV-positive patients is well known and consists most often of the triad of dyspnea, fever, and cough, the presentation of PCP in HIV-negative patients is atypical and occurs suddenly with oxygen desaturation and rapid dead if left untreated [19]. This pathology is a serious public health problem not only because of the severity of the disease but also because PCP is a life-threatening condition in HIV-negative immunocompromised patients [18]. Another public health problem is the number of colonized patients in hospital wards, where other patients may be at high risk of infection or colonization [20].

According to the literature, the diagnosis of PCP or colonization depends on a complex algorithm based on the patient's medical history, laboratory and radiological data, treatment, and clinical evolution of the patient's condition. If a positive microscopic examination leads to a high probability of PCP, a negative quantitative PCR (qPCR) result cannot rule out the diagnosis, especially in HIV-negative patients. In most cases, qPCR is sensitive enough to allow the diagnosis of PCP in HIV patients, however, the presence of a gray area of Ct values prevents this analysis from becoming a reference method [21]. In the case of Ct value in the gray area, the physician will have to choose between prophylactic or active treatment according to the clinical parameters of the disease and the patient's condition [18].

With this study, we demonstrate that the use of real-time PCR can significantly improve the differential diagnosis in patients suspected of having *P. jirovecii* infection and to clarify the infection epidemiology in immunocompromised patients and those without immunosuppression.

Our data shows that of the 153 examined individuals without evidence of compromised immunity, 4% (n = 6) tested positive for *P. jirovecii* DNA. All of them were with severe pneumonia and PCP-specific radiological findings. While in 66.7% (n = 4) of them the condition can be explained by their infant (0 - 12 months) and child age (14 years), the remaining 33.3% (n = 2) were adults over 18 years old and the development of PCP was difficult to explain. The most significant risk factors for PCP in HIV-free patients are the use of glucocorticoids and the presence of cell-mediated immune defects, which lead to changes in lung surfactant, thus predisposing the patient to pneumonia [22]. There have been several reports of PCP in patients without underlying immunosuppressive disease. A study by Kano et al. described five patients who developed PCP without any underlying immunosuppressive conditions, and in their literature review, they identified only 11 other reported cases [23]. However, the exact mechanisms that lead to the development of PCP in patients without evidence of immunosuppressive status remain unclear [24].

From included in the study individuals with compromised immunity and lung pathology, 47.8% (n = 32) showed the presence of *P. jirovecii* DNA. Of them, 26 (38.8%) were HIV infected and 6 (8.96%) were with other immunosuppressive conditions (Table 1). The age distribution shows a prevalence of patients over 18 years of age (n = 29, 90.6%), while three of the patients (9.4%) were in the age group from 1 to 9 years. One of the children was HIV-positive, and two were non-HIV-infected. One child developed PCP symptoms on the background of oncohematological disease, and the other was on long-term corticosteroid therapy for nephrotic syndrome. In the group of immunocompromised patients, the distribution by sex showed a predominance of males (n = 26, 81.3%) compared to females (n = 6, 18.7%). In general, our data are similar to those in the literature for people at risk for developing PCP pneumonia [25].

Regarding the etiological diagnosis of *Pneumocystis* pneumonia, staining with methenamine-silver nitrate according to Gomori is considered as the "gold standard" for microscopic visualization of *P. jirovecii* cysts. However, our study gives us reason to consider that the real-time PCR is more useful for diagnostic purposes than the staining methods (Table 1). Patients with compromised immunity were more likely to have detectable cysts of the pathogen in obtained from them clinical specimens than those without immunosuppression. The data from our study prove the existence of a correlation between the levels of the pathogen load and the detectability of *P. jirovecii* cysts with staining methods (Table 2).

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The choice of a method for sample collection and examination has an important role in the efficiency of the laboratory diagnostics. Results showed that various clinical specimens (induced sputum, tracheal aspirate, and BAL) can be tested to diagnose pneumocystosis. Our initial observations revealed that in infants and young children, the tracheal aspirate is a good enough clinical sample for genetic testing.

In Bulgaria, the first-line agent for the treatment of PCP is trimethoprim-sulfamethoxazole, regardless of the patient's immune status, while the use of additional drugs and oxygen therapy depends on the patient's condition and is in accordance with generally accepted international guidelines.

In the post-treatment period for *Pneumocystis* pneumonia, 4 patients in the immunocompromised group and 3 patients without immunosuppression had follow-up studies to assess their response to the therapy. The reason for this was continuing complaints such as shortness of breath, cough, and fever. With the exception of one HIV-infected patient, in all others, we found that the control sample taken within one month after the end of therapy did not contain *P. jirovecii* DNA.

Unfortunately, in ten of the patients (HIV-positive people) the disease was fatal. The mortality rate among people with PCP for the period studied by us was 26.3%, and our data are similar to the literature [26, 27].

5. Conclusions

This study is the first in the country including the main available laboratory methods for the diagnosis of human pneumocystosis in Bulgaria. Regarding the etiological diagnosis of PCP, based on our study, the sensitivity of real-time PCR is higher compared to the staining methods. The choice of a method for sample collection and examination plays an important role in the efficiency of the laboratory diagnostics.

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Institutional Review Board Statement: Written consent was obtained according to the Declaration

of Helsinki. The Institutional review board (IRB) 00006384 of the National Centre of Infectious and Parasitic Diseases (protocol number 2 from 11 May 2020) approved this retrospective study's protocol.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The datasets generated during the current study are available from the corresponding author upon reasonable request.

Conflicts of Interest: The authors declare no conflict of interest.

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