

# STUDY OF THE DISTRIBUTION OF PNEUMOCYSTOSIS IN BULGARIA BETWEEN SEPTEMBER 2017 AND APRIL 2019 BY USING REAL-TIME PCR

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## ABSTRACT

*Pneumocystis jirovecii* is an opportunistic organism that inhabits predominantly the human pulmonary alveoli. The fully sequenced genome of *P. jirovecii* was first reported in 2012. According to some authors, *P. jirovecii* is an obligate pathogen because genes encoding virulence factors and most enzymes for amino acid biosynthesis were not found in the genome. This suggests that the microorganism normally colonises the human lungs but causes disease only in immunocompromised persons. Pneumocystis pneumonia (PCP) is one of the most common opportunistic infections in patients developing acquired immune deficiency syndrome (AIDS). The use of conventional microscopic methods in diagnosis is associated with considerable limitations. Therefore, detection of *Pneumocystis* DNA in clinical samples by PCR techniques leads to significant advances in the diagnosis of PCP.

The aim of this study is to determine the importance of PCR-based methods in the diagnosis of human pneumocystosis and to evaluate their diagnostic

value in comparison with conventional microscopy methods. For a period of 20 months in the National Reference Laboratory "Diagnosis of Parasitic Diseases" at the National Centre of Infectious and Parasitic Diseases 33 patients were tested by real-time PCR and 11 of them were found positive for the presence of *P. jirovecii* DNA. Eight of the patients (72.7%) were HIV-infected. Although limited in extent, this is the first real-time PCR study on the distribution of human pneumocystosis in Bulgaria. Our data shows that PCR techniques have higher sensitivity and specificity than microscopic methods and provide new opportunities for the diagnosis of *Pneumocystis* pneumonia.

## KEYWORDS:

*Pneumocystis jirovecii*, real-time PCR, compromised immunity

## INTRODUCTION

In the 1940s of the 20th century, *Pneumocystis* was first recognised as a pathogen causing pneumonia in malnourished or prematurely born children. Before the 1980s, *Pneumocystis jirovecii* pneumonia (PCP) was diagnosed mainly in persons with malignant haematological diseases (1). Following the outbreak of the global human immunodeficiency virus (HIV) epidemic, the incidence of PCP significantly increased. The introduction of antiretroviral therapy and prophylaxis led to a decrease in PCP morbidity in HIV-positive individuals, but still pneumocystis pneumonia is one of the most common opportunistic infections in patients developing acquired immune deficiency syndrome (AIDS) (2, 3).

*Pneumocystis* spp. are single-celled organisms that can complete their life cycle in the lungs of many mammals (4). *Pneumocystis jirovecii* lives predominantly in the human pulmonary alveoli. Morphological studies have revealed three distinct forms: trophozoite (trophic form), often forming clusters; sporozoite (precystic form) and the cyst which contains several intracystic bodies (spores) (5). The trophic form, adhering tightly to the alveolar epithelial cells type I, is with diameter of 1-4 µm, and the mature cyst is 8-10 µm in diameter. During lung infection in humans, the ratio between trophic and cystic forms is approximately 10:1 (6, 7).

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Initially *Pneumocystis* was classified as a protozoan organism because of the morphological characteristics of the two identified forms in the life cycle – the small trophozoite and a larger cyst form, and also because of the response of *Pneumocystis jirovecii* infection to treatment with antiprotozoal drugs (5). Based on DNA sequence analysis, *Pneumocystis* is currently classified as a fungus, although it is difficult to cultivate in a standardised system. The fully sequenced genome of *P. jirovecii* was first reported in 2012 (8). Genes encoding virulence factors and most enzymes for the biosynthesis of amino acids were not found in the genome. This suggests that the microorganism normally colonises the human lungs but causes disease only in immunocompromised persons. Although the whole genome analysis is completed, explanation of the life cycle and sensitivity to drugs is hampered by the difficulty in isolating *Pneumocystis* in pure culture (3, 5). A major problem in the evaluation of diagnostic tests for PCP is the lack of a golden standard, mainly because there is no system for culturing *P. jirovecii* (9). There are serious limitations in the sensitivity of microscopy diagnosis, the low-level invasion particularly in non-HIV-infected patients, and also in using non-invasive methods for collection of pulmonary specimens (10). Stained smears for microscopy diagnosis are most often prepared from Gomori's methenamine silver (GMS), Calcofluor white, Giemsa, toluidine blue and commercially available specific *Pneumocystis* immunofluorescent diagnostic kits (11, 12).

Detection of *Pneumocystis* DNA in clinical specimens by using polymerase chain reaction (PCR) analysis led to a significant advance in the diagnosis of PCP (9). This is the most sensitive method for detection of *Pneumocystis* and should be considered as the first choice among diagnostic tests. In fact, real-time PCR is currently regarded as the main tool for diagnosing PCP (10, 11).

Bronchoalveolar lavage fluid is the optimal clinical specimen type for PCR analysis but induced sputum is also acceptable, especially in HIV-infected patients. PCR assays have showed that *Pneumocystis* DNA can be detected in oropharyngeal washes and nasopharyngeal aspirates (3, 6).

The aim of this research is to determine the importance of PCR-based methods in the diagnosis of human pneumocystosis and to evaluate their diagnostic value in comparison with conventional microscopy methods.

## MATERIAL AND METHODS

### Patients

For a period of 20 months, in the National Reference Laboratory "Diagnosis of Parasitic Diseases" at the National Centre of Infectious and Parasitic Diseases, were tested 33 patients suspected for *Pneumocystis* pneumonia. Eleven were with HIV infection. From all 33 tested patients, 14 were children and adolescents between 0-19 years and 19 were adults. Gender distribution showed that 8 of the patients (24.2%) were female and 25 (75.8%) – male.

### Clinical samples

During the study period the following samples were tested: bronchoalveolar lavage fluid (n = 2), tracheal aspirate (n = 2), induced sputum (n = 28), post-mortem material from lungs (n = 1).

### Microscopic diagnosis

From 15 of the obtained samples were prepared smears stained by the Romanovsky-Giemsa method and toluidine blue. Microscopy was performed with magnification of 10x100.

### DNA isolation and real-time PCR

All 33 samples were subjected to DNA analysis. DNA was extracted according to the manufacturer's protocol using the commercial kit PureLink® (Genomic DNA Kits, Invitrogen, Life technologies) based on the selective binding to silica membranes in the presence of chaotropic salts. Real-time PCR analysis was performed by using the commercial kit RIDA®GENE *Pneumocystis jirovecii* (R-Biofarm AG) with amplification of a DNA fragment of the mitochondrial large subunit gene, specific for *P. jirovecii*. The kit contains internal control that monitors PCR inhibition and confirms whether nucleic acid extraction is successful. The reaction was carried out in LightCycler®480 II, Roche.

## RESULTS

For a period of 20 months a total of 33 samples were tested by real-time PCR for the presence of

*P. jirovecii* DNA. Twenty-two samples were from patients without established HIV infection or AIDS, from immunocompetent or patients with other form of immunosuppression, and 11 were from HIV-infected persons (Table 1).

In the group of HIV-positive patients we found 8 positive samples (72.7%). Among HIV-negative patients there was a significantly fewer number of positive results – 3 (13.6%). *P. jirovecii* DNA was found in clinical materials from induced sputum

(n = 10) and tracheal aspirate (n = 1). We prepared stained smears from 15 samples for light microscopy diagnosis and results were negative. The same 15 samples were tested by real-time PCR and 12 of them were negative, but in 3 there was amplification of the target sequence.

The affected persons were aged from 0 to 49 years. The largest number of cases was in the age group 30-34 years, followed by 0-4 years and 45-49 years (Table 2).

**Table 1.** Distribution of pneumocystosis cases by primary diagnosis.

Primary diagnosis	Number of cases	Positive for <i>P. jirovecii</i> DNA	Positive cases in %
HIV	11	8	72.7
Following liver transplantation	2	0	0
Patients with severe pneumonia	17	3	17.6
Other (respiratory distress syndrome, death of a newborn, nephrotic syndrome with acute respiratory failure)	3	0	0

**Table 2.** Distribution of cases by age group.

Age group	0-4	5-9	10-14	15-19	20-24	25-29	30-34	35-39	40-44	45-49	50-54	55-59	60-64	75-79
Number of tested persons	8	0	0	6	1	3	4	4	1	3	1	0	1	1
<i>P. jirovecii</i> DNA-positive persons	2	0	0	1	0	1	3	1	1	2	0	0	0	0

## DISCUSSION

Transmission of *P. jirovecii* in the human population is airborne. There is an assumption that about 95% of people are infected during childhood but healthy adults are asymptomatic carriers. The disease usually develops in cases of compromised immunity. The prevalence of HIV-associated *P. jirovecii* pneumonia varies worldwide: in Europe it is about 16%, in Africa reaches 39% and in Southeast Asia (Malaysia) – up to 63% (2, 13). Data on the prevalence of *P. jirovecii* in Bulgaria are limited. Kurdova et al. (2004) reported results of an 11-year study (1993-2003) on opportunistic parasites causing morbidity in

HIV-infected persons. Among the 165 patients examined with microscopy 10 (6.06%) were with positive results for pneumocystosis (14). Our study showed significantly larger percentage of pneumocystosis cases among HIV-infected people (72.7%). We believe that this is due to the higher sensitivity of PCR techniques in general, as well as the small number of examined patients and the shorter study period. Our data suggest that besides in HIV-infected, the disease is registered also in other cases of compromised immunity: 4- and 6-months-old infants (n = 2) and a boy at the age of 15 years who developed PCP in association with congenital

agranulocytosis and massive immunosuppressive therapy. In this respect, our data correlate with data in the literature. Ten of the patients with *P. jirovecii* infection were male (90.9%) and only 1 person was an HIV-positive female.

According to the Centres for Disease Control and Prevention (CDC, USA), *P. jirovecii* infection mortality in untreated immunocompromised patients is 100% and is reduced to 5-40% with aetiological treatment (15). Therefore, it is essential to determine the aetiological diagnosis promptly and initiate treatment. According to literature data, the type of clinical specimen and choice of diagnostic method are important factors for a better diagnosis.

Laboratory diagnosis of PCP using bronchoalveolar lavage fluid exhibits sensitivity of 98% or higher and this is the preferred material, although sensitivity with induced sputum (50-90%) is also acceptable (3, 16). According to some authors, PCR methods have the highest detection limits, whereas for microscopic methods they are significantly lower. According to Doyle et al. (2017), sensitivity of toluidine blue staining is between 71.4% and 85.7% depending on the type of clinical specimen, for histology – 71.4%-75% and surprisingly low for cytology – 43%. As for PCR, data shows 100% sensitivity. The same team established that PCR-based methods have negative predictive value of 100% and positive predictive value of 93.1% (11). Therefore, it is not uncommon to detect the presence of *P. jirovecii* DNA with real-time PCR, even though microscopic examination shows negative result (16). Data obtained from our survey, despite being limited, confirms this fact.

## CONCLUSIONS

Although limited in extent, this is the first real-time PCR study on the distribution of human pneumocystosis in our country. PCR methods provide new opportunities for the diagnosis of *Pneumocystis pneumonia* with higher sensitivity

and specificity than microscopic methods. PCR techniques also enable detection of *P. jirovecii* even at low pathogenic load compared to conventional microscopic methods. The small amount of pathogens in the clinical material may be due both to the level of immunosuppression and the initial stage of disease. Early diagnosis and initiation of treatment improves the chances of a favourable patient outcome.

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