High incidence of *Pneumocystis jirovecii* pneumonia in oncological patients: a 19-year study

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ABSTRACT

Aim: In the past, *Pneumocystis jirovecii* belonged to the Protozoa group, but is currently taxonomically included in the kingdom Fungi. *P. jirovecii* is an opportunistic pathogen, responsible for pneumocystis pneumonia with frequent complications of immunocompromised patients. Delayed initiation of appropriate therapy increases the risk of death in immunocompromised patient. The aim of this work was to determine and evaluate the reliability of methods of laboratory diagnosis of pneumocystosis used in routine laboratories as well as the occurrence of this disease in patients from Slovakia during 19 years.

Material and Methods: The diagnosis is based on microscopic examination (Giemsa- and Gram-Weigert-staining) and detection of parasite DNA by classical or real-time PCR in bronchoalveolar lavage and sputum.

Results: Pneumocysts were detected in 190 persons (5.7%) from the whole group of patients. Cancer patients represented the riskiest group in terms of pneumocystosis, which was confirmed by the highest percentage (57.9%) of individuals infected with *P. jirovecii*. Compared with the PCR, 33.7% sensitivity and 100% specificity of microscopy was calculated by using a binary classification test. Molecular methods are more sensitive in the detection of *P. jirovecii* compared to microscopic evidence and currently represent a reliable detection system in the diagnosis of pneumocystosis.

Conclusion: In view of the increasing number of immunocompromised persons, diagnostics of *P. jirovecii* in patients with pulmonary complications is essential. This was also confirmed in our study, where the number of examinations and detection of this opportunistic pathogen increased over the years.

KEYWORDS

Pneumocystis jirovecii – pneumocystosis – microscopic evidence – polymerase chain reaction (PCR)

SÚHRN

Boldiš V., Ondriska F., Kováč Ľ., Steinhübel J., Bastlová M.: Vysoký výskyt pneumónie spôsobenej *Pneumocystis jirovecii* u onkologických pacientov: 19-ročná štúdia

Cieľ: *Pneumocystis jirovecii* patril v minulosti do skupiny prvokov, ale v súčasnosti je taxonomicky zaradený do ríše húb. *P. jirovecii* je oportúnny patogén, zodpovedný za pneumocystovú pneumóniu s častými komplikáciami u imunokompromitovaných pacientov. Oneskorené začatie vhodnej liečby zvyšuje riziko úmrtia u pacientov s oslabenou imunitou. Cieľom práce bolo zistiť a zhodnotiť spoľahlivosť metód laboratórnej diagnostiky pneumocystózy používaných v rutinných laboratóriách ako aj výskyt tohto ochorenia u pacientov zo Slovenska za 19 rokov.

Materiál a metódy: Diagnostika je založená na mikroskopickom dôkaze (farbenie podľa Giemsa a Gram-Weigerta) a detekcii DNA parazita klasickou alebo real-time PCR v bronchoalveolárnej laváži a spúte.

Výsledky: Pneumocysty boli zistené u 190 osôb (5,7 %) z celého súboru pacientov. Onkologickí pacienti predstavovali najrizikovejšiu skupinu z hľadiska infekcie pneumocystami, čo sme potvrdili ich najvyšším podielom (57,9 %) z jedincov s pneumocystózou. Na základe binárneho klasifikačného testu sme vyhodnotili 33,7 % citlivosť a 100 % špecifickosť mikroskopického dôkazu v porovnaní s PCR. Molekulárne metódy sú v porovnaní s mikroskopickým dôkazom citlivejšie v detekcii *P. jirovecii* a v súčasnosti predstavujú spoľahlivý detekčný systém v diagnostike pneumocystózy.

Záver: Vzhľadom na narastajúci počet imunokompromitovaných osôb je diagnostika *P. jirovecii* u pacientov s pľúcnymi komplikáciami nevyhnutná. To sa potvrdilo aj v našej štúdii, kde v priebehu rokov stúpal počet vyšetrení a záchytov tohto oportúnneho patogénu.

KĽÚČOVÉ SLOVÁ

Pneumocystis jirovecii – pneumocystóza – mikroskopický dôkaz – polymerázová reťazová reakcia (PCR)

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INTRODUCTION

Pneumocystis jirovecii is an opportunistic microorganism formerly known as *Pneumocystis carinii*. This atypical fungus causes a serious infection in humans called pneumocystis pneumonia (PcP) [1]. It most often affects people with a weakened immune system and in some cases can be seriously life-threatening. Patients at risk include people with various conditions such as cancer, HIV infection, organ transplants, or the use of immunosuppressive therapy and drugs. Patients with pneumocystosis show clinical symptoms of fever, cough, dyspnoea, and in severe cases, respiratory failure and death may occur. *Pneumocystis* is primarily transmitted from human to human by air, but other routes of transmission have been described. It can also be spread through asymptomatic carriers, ie people with a normal immune system who are colonized. Despite the status of the most common and serious opportunistic respiratory infection in AIDS patients, the incidence of the disease is currently declining in the population due to the introduction of prophylaxis and effective antiretroviral therapy [2]. However, pneumocystosis is increasingly occurring in HIV-negative patients treated with anticancer chemotherapeutics and immunosuppressive therapy [3, 4].

The standard used in the laboratory diagnosis of pneumocystosis in immunocompromised patients is the detection of *P. jirovecii* in bronchoalveolar lavage (BAL) and induced sputum samples by microscopic evidence [5]. Microscopic detection of cysts or trophic forms of the parasite in the lung material is performed using several staining techniques [6]. However, high-sensitivity molecular methods (PCR) for *P. jirovecii* detection have gradually been developed and conventional diagnostic methods have become less reliable [7]. Serological methods represent a minimally invasive way of diagnosing pneumocystosis.

Due to the high efficacy and availability of oral and parenteral forms, trimethoprim-sulfamethoxazole is the drug of choice in the treatment of mild to severe pneumocystosis in HIV-infected patients as well as in non-HIV-infected patients [8, 9].

MATERIAL AND METHODS

Biological material

During the 19-year period (2001–2019), we examined in our routine diagnostic laboratory 3,330 patients from various clinics and hospitals in Slovakia for suspected *P. jirovecii* infection. Bronchoalveolar lavages and sputum were the majority of the samples. Materials such as serum and cerebrospinal fluid were very rarely examined (the objectivity of the results in these cases is questionable).

Microscopic evidence

Giemsa staining was used to detect trophozoites and Gram and Weigert staining to determine cysts [10, 11].

Molecular methods

A commercially available DNeasy kit (Qiagen, Hilden, Germany) was used to isolate DNA from the samples.

From molecular biology methods, a classical polymerase chain reaction (PCR) using primers pAZ102-E and pAZ102-H was applied, which amplify part of the mitochondrial rRNA large subunit gene and the expected fragment length of 345 bp [12]. The initial denaturation step consisted of heating the reaction to 94 °C for 120 s. Thirty cycles consisting of denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s, and extension at 72 °C for 30 s were performed. Thereafter, a final extension for 5 min. at 72 °C was done. PCR products were investigated by agarose gel electrophoresis (1.5%), stained with ethidium bromide, and illuminated under UV light. The detection limit of this PCR assay was 1 organism/µl of extracted DNA.

Real-time PCR was performed using the commercial LightMix® Kit *Pneumocystis jirovecii* (TIB MOLBIOL, Berlin, Germany). A 244-base pair (bp) fragment from the multicopy MSG (multicopy surface glycoprotein) gene of *P. jirovecii* was amplified through rt-PCR, by a pair of primers labeled with the Red 640 probe that is detected in the 640λ channel. The rt-PCR reaction had an internal control of a 278 bp fragment, consisting of a hybridization probe labeled with the Red 690 probe that is detected on the 660λ channel. The amplification process was performed on the cobas z 480 under the following conditions: denaturation at 95 °C for 10 min, amplification by 50 cycles (95 °C for 5 s, 62 °C for 5 s and 72 °C for 15 s) and a cooling step at 40 °C for 30 s. The detection limit was 10 copies/reaction. The cycle number of the Crossing Point (Cp) of each sample was calculated automatically. For data interpretation, samples with Cp < 38 were considered positive. Due to the variability of different MSG gene copies, there is no accurate correlation between the amount of pathogen and Cp value. In case of low level of fungal DNA detection, the attending physician were recommended to repeat the sample collection from the patient.

Statistical analysis

The results were analyzed by OpenEpi statistical software, version 3.03 (http://www.openepi.com/Menu/OE_Menu.htm) and a chi-square test was used to evaluate statistical differences, where a P value < 0.05 was considered significant [13]. The sensitivity, specificity, positive and negative predictive values were calculated from 2 × 2 tables, and this analysis of was undertaken using the MedCalc diagnostic test evaluations program version 11.6.1, for Windows (MedCalc Software; http://www.medcalc.org/calc/diagnostic test.php).

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RESULTS

During 19 years, the number of examinations and detection of *P. jirovecii* is increasing, which is indicated in figure 1 by trend linear lines. The highest increase in samples is observed in the years 2008 to 2015, where the number of patients multiplied 6-fold during 8 years (387: 61 = 6.35). In the following years, since 2016, the number of examined patients is relatively stable in the range of 323 to 369.

Pneumocysts were identified in 5.7% (190 of 3330) of examined patients by PCR methods, while microorganisms were confirmed by microscopy in 1.9% (64 of 3330) of cases. The infection was detected in 163 and 25 patients in BAL and sputum specimens, respectively. The presence of *P. jirovecii* was confirmed mainly in immunocompromised persons with oncological disease 57.9% (110 of 190; lymphatic leukemia 18.2%; non-follicular lymphoma 15.5%; myeloid leukemia 10%; Hodgkin's lymphoma 9.1%; non-Hodgkin's lymphoma 8.2%; malignant tumor of the breast 5.5%, malignant tumor of the bronchi and lungs 4.6%; other oncological diseases 1–4%), but also in patients with respiratory diseases 23.2% (44 of 190; pneumonia caused by unspecified microorganisms 40.9%; acute respiratory failure 25%; bacterial pneumonia 13.6%; respiratory failure 6.8%; other diseases of the respiratory system 2–4%), patients with diagnoses of infectious and parasitic diseases 9.4% (18 of 190), with other diseases 7.4% (14 of 190; mainly diseases of the digestive system; patients with abnormal clinical and laboratory findings) and with HIV 2.1% (4 of 190). The most sensitive groups of patients for pneumocyst infection were oncology individuals and patients with respiratory diseases.

In the years 2001–2012, 803 patients were examined from the whole group using classical PCR, where *P. jirovecii* DNA was detect in 48 (6%) patients. In 12 (1.5%) of them, pneumocysts were also confirmed by microscopic evidence. In the years 2013–2019, the DNA of *P. jirovecii* was determined using real-time PCR in 142 (5.6%) out of 2527 persons, and pneumocysts were also confirmed by microscopy in 52 (2.1%) of them. Figure 2 shows one of the positive specimens staining by the Gram-Weigert. The morphology of the microorganisms is irregular. Pneumocysts have the shape of such a „defective ball", and their sizes vary from 4 to 8 μm in diameter. The wall of pneumocystis cysts was selectively stained by the Gram-Weigert, it was purple or blue, and other material (eosinophils, alveolar cells, pneumocytes) pink or purple.

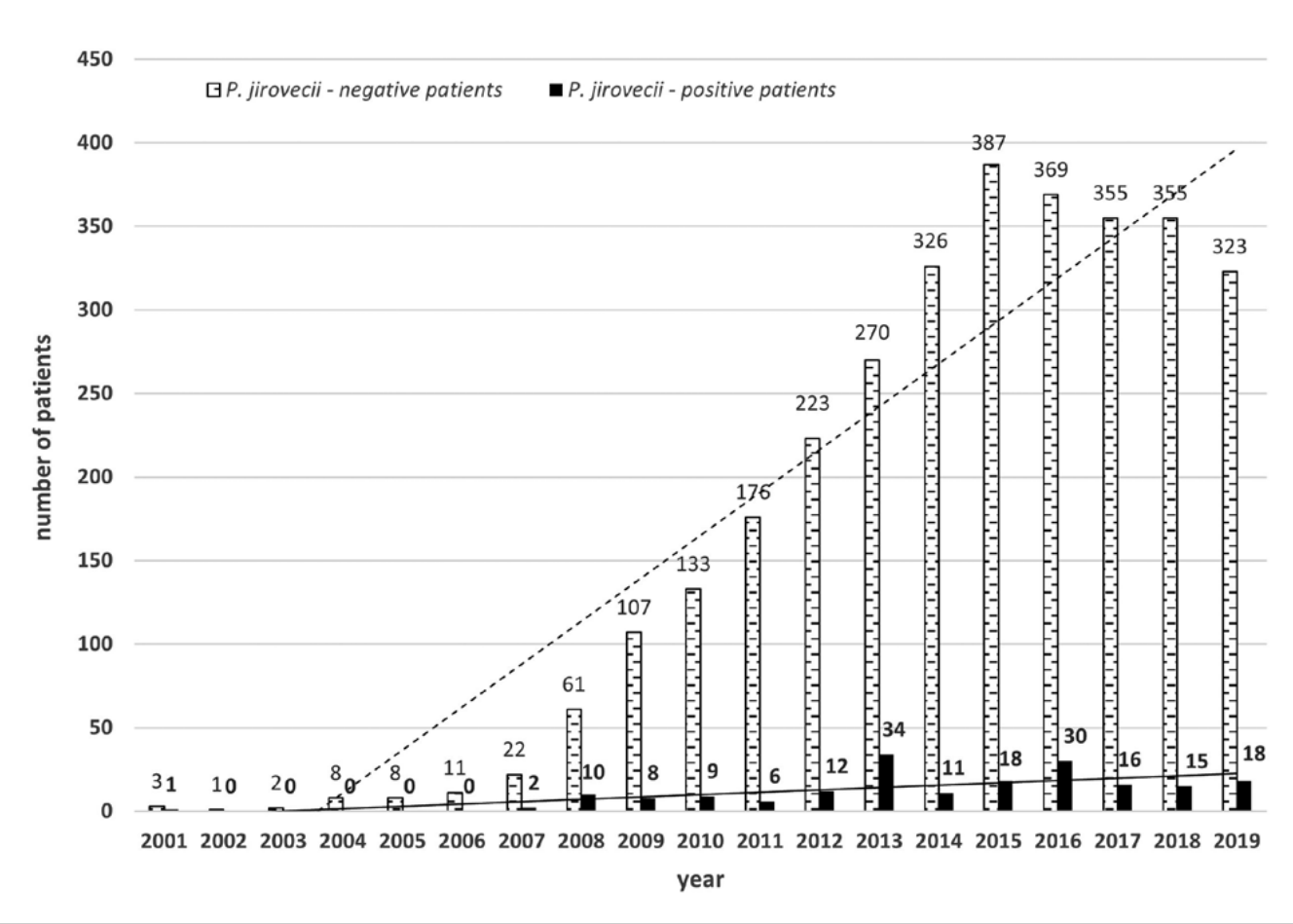

Figure 1. The number of examined samples for *P. jirovecii*

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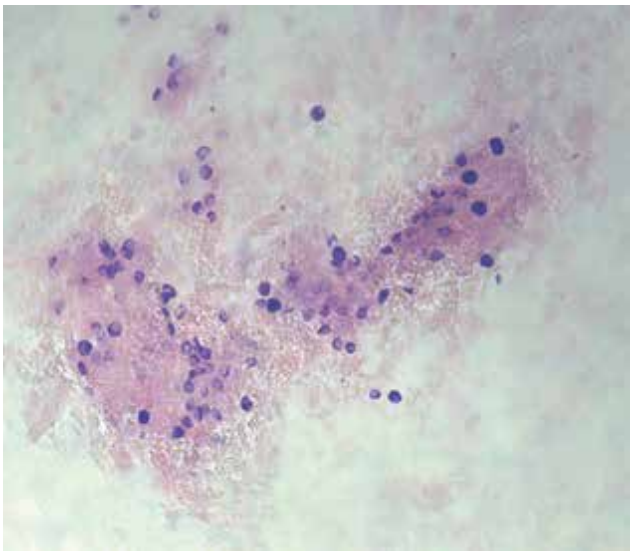

Figure 2. Cysts of *P. jirovecii* after staining according to Gram and Weigert

In order to approximate the clinical manifestation of *P. jirovecii* infection in an oncology patient, where etiological agents were confirmed in our laboratory, an X-ray image with a complicated pneumocystic infection with a marked spotting of both lung wings is attached (Figure 3).

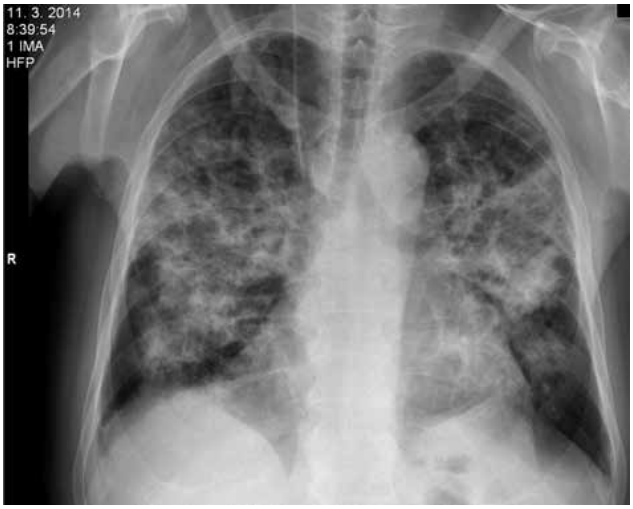

Figure 3. X-ray findings in the lungs of a patient with pneumocystosis

(photo by Dr. M. Franeková)

Statistically significant differences for patients with pneumocystosis were confirmed with respect to age group, collected biological material and established diagnosis. The highest proportion of infected persons was detected in adults aged 21–40 years (9.7%), followed by children 0–10 years (6%), the elderly over 61 years (5.2%), patients between 41 and 60 years of age (4.8%) and at least 10–20-year-olds (1.6%). These differences were statistically significant ($p = 0.009$). A larger proportion of individuals infected with pneumocysts were in the group in which sputum collection was indicated (9.8%) than in BAL collection (5.4%; $p = 0.029$). Significant differences in the number of patients with pneumocystosis were also observed in connection with the predicted diagnosis ($p = 0.02$). The highest percentage (12.5%) of patients with pneumocystosis was in the group diagnosed with infectious and parasitic diseases. Patients with a diagnosis of respiratory diseases (5.8%) were the second most represented group of those infected, followed by diagnoses like others (5.7%), oncological diseases (5.1%), blood and hematopoietic diseases involving immune mechanisms (3.7%) and circulatory system diseases (3.1%). No significant association was found between the presence of pneumocystic infection and gender ($p > 0.05$).

Statistical indicators of the reliability of microscopic evidence of *P. jirovecii* were expressed with respect to PCR, which is considered the gold standard. Compared with the PCR, sensitivity, specificity, positive predicted value and negative predicted value for microscopy were 33.7%, 100%, 100% and 96.1%, respectively.

DISCUSSION

Only 2 papers were published in Slovakia, which documented the occurrence or diagnostic possibilities of laboratory detection of *P. jirovecii* [14, 15]. This work provides a comprehensive overview of the presence of *P. jirovecii* in the examined patients with pulmonary complications in Slovakia for a period of 19 years and describes the possibility of laboratory diagnostics in the conditions of a routine laboratory.

From the whole group of examined individuals, pneumocysts were detected mainly in persons with oncological diseases (especially lymphatic leukemia). Sing et al. [16] diagnosed pneumocysts mainly in people with HIV (6%), followed by cancer (1.8%) and transplant patients (1.5%). In our cohort, there were not as many HIV people (1.1%; 36/3330) as documented in the study by Sing et al. (26.7%; 89/334), so the percentage of HIV patients infected with pneumocysts was much lower. However, most patients infected with *P. jirovecii* were immunosuppressed (60%), similar to the work of Sing et al. (94.3%). Fillatre et al. [17] also studied 293 cases of pneumocystosis in their research. One hundred and fifty-four (52.6%) cases out of the total group of patients were HIV negative. Hematological malignancies (32.5%) were identified as the most common disorders in the development of pneumocystosis, followed by solid tumors (18.2%), inflammatory diseases (14.9%), solid organ transplants (12.3%) and vasculitis (9.7%). The study by Liu et al. [3] confirmed that the most common cause of

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PcP was hematological malignancies (29.1%) similar to the study by Fillatre et al. [17]. Approximately the same results were documented by Kim et al. [5], where malignancies reached up to 68.7%, and Gazaignes et al. [18] who detected PcP in cancer patients (60%). Abastabar et al. [19] similar to us found that the prevalence of *P. jirovecii* varies according to the age of the patients. However, in their study, PcP was more common in patients aged 71–80 years (> 80%) and 81–90 years (100%). They also found no significant difference between men and women in the prevalence of the disease, similar to our work.

In most papers, a significantly higher PCR sensitivity was found compared to standard cytological staining techniques in the examination of BAL, both induced and non-induced sputum [20–24]. Wakefield et al. [25] reported 90% sensitivity of PCR compared to 35% sensitivity of classical microscopic staining. Lipschik et al. [26] and Chouaid et al. [24] documented 93% and even up to 100% sensitivity of PCR in BAL and induced sputum, respectively. As in most works in the literature, a significantly lower sensitivity of microscopy compared to the PCR method was confirmed in our study. In our examinations, 66.3% of the samples were false negative by microscopic evidence and mainly cystic stages and in one case intracystic bodies of pneumocysts were detected. Due to the fact that the examinations were carried out in a routine diagnostic laboratory, where patients suspected of pneumocystosis were examined, it was not possible to include a negative control group (healthy patients) in the examined set. Accordingly, we did not observe any confirmed false positive case during the study period. Similar to the work of Alanio et al. [27], there is little doubt that qPCR has the potential to replace microscopy in the future because of reliability of the results, quantification and low workload when automation becomes possible (nucleic acid extraction and amplification).

Distinguishing colonization from infection may be used to guide therapeutic decisions. The most studies suggest a 'grey zone' between rt-PCR cut-offs providing near 100% positive/negative predictive values for PcP diagnosis [28], some of them found a large and overlapping distribution precluding the assessment of a reliable cut-off [29]. In this work, we did not analyze the performance of quantitative *P. jirovecii*-specific PCR to discriminate PcP from colonization according to the fungal load. The cut-off of the PCR used in this study (the range of Cp values, Ct cycles or copies per ml) was not specified by the manufacturer for distinguishing between colonization and infection. Only values lower than 38 Cp were considered positive for the presence of *P. jirovecii*. The aforementioned differentiation of PcP from colonization for this used rt-PCR may be the subject of further study.

CONCLUSION

Due to the increasing number of immunosuppressed individuals, the diagnosis of *P. jirovecii* is necessary. This was also confirmed in our study, where the number of examinations and detection of this opportunistic pathogen increased over the years. The causal agent responsible for serious pulmonary complications in immunocompromised patients was detected in 190 cases from Slovakia over 19 years.

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