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Study on the Presence of Protozoal Coinfections among Patients with *Pneumocystis jirovecii* Pneumonia in Bulgaria

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***Abstract*—**The *Pneumocystis jirovecii* (*P. jirovecii*) and protozoan of the genera *Acanthamoeba, Cryptosporidium*, and *Toxoplasma gondii* are opportunistic pathogens that can cause life-threatening infections in immunocompromised patients. Aim of the study was to evaluate the coinfection rate with opportunistic protozoal agents among Bulgarian patients diagnosed with *P. jirovecii* pneumonia. 38 pulmonary samples were collected from 38 patients (28 HIV-infected) with *P. jirovecii* infection. *P. jirovecii* DNA was detected by real-time PCR targeting the large mitochondrial subunit *ribosomal RNA* gene. Acanthamoeba was determined by genus-specific conventional PCR assay. Real-time PCR for the detection of a *Toxoplasma gondii* and *Cryptosporidium* DNA fragment was used. Pneumocystis DNA was detected in all 38 specimens; 28 (73.7%) were from HIV-infected patients. Three (10,7%) of them were coinfected with *T. gondii* and 1 (3.6%) with Cryptosporidium. In the group of non-HIV-infected (n = 10), *Cryptosporidium* DNA was detected in an infant (10%). *Acanthamoeba* DNA was not found in the tested samples. The current study showed a relatively low rate of coinfections of *Cryptosporidium* spp./*T. gondii* and *P. jirovecii* in the Bulgarian patients studied.

***Keywords*—**Coinfection, opportunistic protozoal agents, *Pneumocystis jirovecii*, pulmonary infections.

I. Introduction

OPPORTUNISTIC infections are diseases that occur in people with compromised immune system, mainly in those living with Human Immunodeficiency Virus (HIV) [1]. Notwithstanding progress in diagnosis and treatment of HIV-infection, opportunistic infections remain a predominant cause of significant morbidity and mortality among HIV/AIDS patients mostly in the low and middle income countries [2].

Opportunistic parasitoses and especially those caused by protozoa *Acanthamoeba* spp., *Toxoplasma gondii (T. gondii)*, *Leishmania donovani (L. donovani)*, *Cryptosporidium* spp., *Cystoisospora belli*, and *Encephalitozoon* spp. were very common in HIV-positive individuals before the introduction of antiretroviral therapy (ART), but they still continue to occur in a large number of cases [3].

The fungus *Pneumocystis jirovecii* (*P. jirovecii*), formerly known as *P. carinii*, causes *Pneumocystis* pneumonia (PCP) [4]. In HIV-infected persons [5], immunosuppressed patients, and premature or malnourished infants [6], [7] the infection can lead to severe complications and increased mortality.

The aim of this study was to evaluate the coinfection rate with other opportunistic protozoal agents among the Bulgarian patients diagnosed with *P. jirovecii* pneumonia.

II. Patients and Methods

A. Study Design

This is a retrospective study of co-morbidity in patients with proven PCP, conducted over a five-year period (2017-2021) in the National Reference Laboratory for Diagnosis of Parasitic Diseases at the National Center of Infectious and Parasitic Diseases, Sofia, Bulgaria.

B. Patients and Samples

38 respiratory samples (32 induced sputum and three bronchoalveolar lavage fluid (adults), and three nasopharyngeal aspirate (infants) specimens) were collected from 38 patients suspected of having *P. jirovecii* infection. Of those examined, 28 were HIV-infected. Of the remaining ten persons, three had other immunosuppressive conditions, and seven had no evidence of compromised immunity (Table I).

Table I presents demographic characteristics and clinical conditions of the polymerase chain reaction (PCR) *P. jirovecii* positive patients involved in the study.

C. DNA Extraction

Genomic DNA was extracted from collected specimens using the PureLink™ Genomic DNA kit (invitrogen, Thermo Fisher Scientific, North America) following the manufacturer's instructions.

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TABLE I
P. JIROVECII POSITIVE PATIENTS (N = 38) WITH PULMONARY SYMPTOMS, STUDIED FOR COINFECTIONS WITH PROTOZOAL AGENTS

	Variables	N	Coinfection with protozoal agent			
			T. gondii positive cases	*Cryptosporidium* spp. positive cases	*Acanthamoeba* spp. positive cases	% coinfected cases
		n = 38	n = 3 /7.9%	n = 2 /5.3%	n = 0	13.2
Sex	Male	34	3 / 8.8%	2/ 5.9%	-	14.7
	Female	4	-	-	-	
Age	< 1 year	3	-	1/ 33.3%	-	33.3
	1-18 years	2	-	-	-	
	> 18 years	33	3 / 9.1%	1/ 3%	-	12.1
Clinical symptom/disease/infection						
with compromised immunity	HIV	28	3 /10.7%	1/ 3.6%	-	14.3
	hematological malignancy	1	-	-	-	
	interstitial pulmonary fibrosis	2	-	-	-	
No data for immuno-suppression	interstitial pneumonia	6	-	1/ 14.3%	-	16.7
	post-COVID 19 disease	1	-	-	-	

D.Real-time PCR for P. jirovecii

For detection of *P. jirovecii* DNA, real-time PCR assay (RIDA®GENE *Pneumocystis jirovecii* real-time PCR Kit, R-biopharm AG) targeting mitochondrial large subunit rRNA (mtLSU rRNA) gene of *P. jirovecii* was used. The amplification of the *P. jirovecii*-specific gene fragment is based on the TaqMan probe technology. An internal control is included in the kit for detection of PCR inhibitors, for reagent integrity monitoring, and for confirmation of successful nucleic acid extraction process.

E. Acanthamoeba spp.-Specific PCR

PCR targeting a 423- to 551-bp *Acanthamoeba*-specific ASA.S1 segment of 18S rDNA, highly specific for the genus *Acanthamoeba*, was applied according to Schroeder et al. using primer set JDP1-JDP2 [8]. Amplification reactions were performed in a 25 µl final reaction volume and cycling conditions were as described in [8]. PCR amplifications were performed in a GeneExplorer Thermal Cycler instrument (Hangzhou Bioer Technology Co., Ltd.).

F. Real-time PCR for Toxoplasma gondii

Qualitative real-time PCR based on the TaqMan probe technology for amplification of the DNA fragment of a nonstructural 200- to 300-fold repeated gene (529 bp long) encoding *T. gondii* protein (AmpliSens® Toxoplasma gondii-FRT PCR kit, InterLabService), was used.

G. Real-time PCR for Cryptosporidium spp.

Real-time PCR procedure based on SYBR Green I fluorescence was applied. A nested PCR assay according to the published data was used [9], [10]. The primer set consisted of genus-specific primers targeting the 18S rRNA gene of *Cryptosporidium* spp. The outer primer pair, N-DIAGF2/R2, had homology with all *Cryptosporidium* species, and the resulting amplicons (expected lengths from 655 to 667 bp) vary depending on the species of *Cryptosporidium* or *C. parvum* genotype. The inner primer pair, CPB-DIAGF/R, targeted a 435 bp long fragment of *Cryptosporidium* spp. [9], [10]. PCR amplifications were performed in a 50 µl final reaction volume. The primary PCR was performed with 2 µl of DNA, and 5 µl of the primary PCR products were used for re-amplification in the secondary PCR. The thermal cycling protocols were as described in [9]. Identification of PCR amplification products was done by melting curve analysis during which the temperature raised from 55 to 95 °C at a rate of 0.02 °C/s.

The different real-time PCR assays were performed in a Gentier Real-Time PCR System 96E instrument (Xi'an Tianlong Science and Technology Co., Ltd.).

Both negative and positive controls were included in every PCR experiment to determine if false positives and negatives were being detected during the amplification process.

H. Agarose Gel Electrophoresis

Amplification products from conventional PCR were analyzed by agarose gel electrophoresis in a 2% agarose gel stained with the peqGREEN DNA/RNA dye (VWR International GmbH, Germany), products were sized using the GeneRuler 100 bp DNA Ladder (Thermo Scientific, Lithuania), and fluorescence detection was performed with SYNGENE gel documentation system (GelVue Model No. GVM20, Synoptics Ltd, UK).

I. Ethical Considerations

Ethical clearance was obtained from NCIPD ethical review board.

III. RESULTS

All patients included in the study were suspected of having PCP by the clinicians in the differential diagnosis as they were presented with signs of fever, cough, dyspnea, and respiratory failure. Respiratory specimens were collected and immediately sent to our laboratory for the analysis.

Pneumocystis DNA was detected in all patient specimens (n = 38). PCP was confirmed by detection of *P. jirovecii*-specific gene fragment using a TaqMan real-time PCR assay. Differentiation of PCP from colonization was done based on the fungal burden in respiratory specimens from HIV-infected and non-HIV-infected patients included in the study. The detection limit of the RIDA®GENE *Pneumocystis jirovecii* real-time PCR was ≥ 10 copies per reaction. Patients were defined with PCP if

their fungal burden was > 100 DNA copies/µl. In our study the lowest fungal burden detected was 9,112 DNA copies/µl and the heist fungal burden was 1,672 x 10^8 DNA copies/µl.

28 (73.68%) of the respiratory specimens examined were from HIV-infected patients. The other ten specimens were obtained from three patients undergoing immunosuppressive therapy due to a) haematological malignancy (n = 1), and b) interstitial pulmonary fibrosis (n = 2); one with post COVID-19 disease; and six with interstitial pneumonia (three infants, one adolescent and two adults).

The results from PCR assays for detection of coinfections with other opportunistic protozoal agents among the Bulgarian patients diagnosed with *P. jirovecii* pneumonia showed the following (see Table I):

a. In the group of patients with HIV (n = 28) three (10.71%) were coinfected with *P. jirovecii* and *T. gondii* and one (3.57%) with *P. jirovecii* and *Cryptosporidium* spp.
b. In the group of non-HIV-infected persons positive result for the presence of *Cryptosporidium* spp., DNA was detected in a 6-month-old baby with acute interstitial pneumonia (10%, 1 of 10 tested).
c. DNA of free-living amoebae of the genus *Acanthamoeba* was not found in the tested samples.

IV. Discussion

Significant changes in the epidemiology of lung infections in HIV and other immunosuppressive conditions have been observed last decades. There are different opinions about the reasons for this. Widespread use of primary prophylaxis for PCP is considered one of them, and the introduction of ART since 1996 is respectively the other main cause [11]. PCP prophylaxis has resulted in a significant reduction in the incidence of *Pneumocystis* infections in the United States and Europe.

In HIV-infected patients, especially those with a high level of immunosuppression, lung infections caused by more than one agent can occur frequently. According to some authors, the percentage of lung infections with polyetiology is about 9% [12]. Other authors have reported that none of the subsequently identified microorganisms were initially suspected as the causative agent of the pulmonary infection [12], [13]. This emphasizes the importance of achieving an accurate etiological diagnosis.

According to a study by Benito et al. (2012) about 80% of pulmonary pathology in HIV-infected persons is mainly due to bacterial infections and those with *Pneumocystis*, and more than one etiological agent is found in about 7% of pneumonia cases [11].

The emergence of HIV/AIDS has led to increased interest in many parasitic infections that can affect the respiratory tract [14]. In immunodeficient individuals and patients suffering from malabsorption, the respiratory infection caused by a parasitic agent can result in severe respiratory tract complications and can be associated with significant mortality. Furthermore, it is now clear that parasitic infections, particularly with protozoa, throughout the course of HIV disease, can increase its progression [15], [16]. Cases of lung infections caused by some parasite species affecting sites other than their predilection sites are described in the literature [14]. The list of "uncommon" protozoa affecting the respiratory tract in HIV infection includes: *Acanthamoeba* spp., *Encephalitozoon* spp., *Leishmania* sp., while pulmonary forms of cryptosporidiosis and toxoplasmosis have been registered in AIDS [11], [17].

Free-living amoebae belonging to the genus *Acanthamoeba* can infect lungs, nasal mucosa, and sinus causing pulmonary nodular infiltration, and pulmonary oedema [18], [19]. In immunodeficient and AIDS patients, infection caused by these amoebae can lead often to a fatal outcome - amoebic meningoencephalitis [18], due to a delayed etiological diagnosis and treatment difficulties.

In immunocompromised individuals, the apicomplexan protozoan organism *T. gondii* can cause localized infection of the central nervous system (in most cases is severe), as well as disseminated infection with lung, heart, and liver involvement [20]. Reactivation of latent toxoplasmosis in the presence of immunosuppression can lead to serious complications in HIV-infected persons, solid organ transplant recipients [21], patients who underwent allogeneic hematopoietic stem cell transplant (HSCT) [20], or in pregnant women (with compromised immunity) who were infected with *Toxoplasma* before their pregnancy (e.g. congenital infection in the fetus) [22], [23].

In humans, the protozoan pathogens of the genus *Cryptosporidium* mainly colonize intestines and infection is often self-limiting in immunocompetent individuals [24]. However, the infection can be life-threatening for HIV-infected/AIDS patients, patients with malignancy [25], [26], bone marrow transplant patients [27], and in patients with hematologic malignancies [28], whose immune system is suppressed due to the underlying disease, as well as for young children (aged under 5 years) [29]. There are a limited number of reported cases of disseminated *Cryptosporidium* spp. infection to the respiratory tract [26], [28], [30]-[33]. In infants (suffering from hyper-immunoglobulin M syndrome) [34], [35] the respiratory infection was associated with chronic diarrhea and subsequent spread to the respiratory tract (by a fecal-oral route or through hematogenous dissemination). Thus, the hypothesis for the inhalation of oocysts of *Cryptosporidium* spp. can serve as an explanation for the presence of the pathogen in pulmonary secretion and its potential transmission to other individuals [17].

In connection with all of the above, we attempted to determine if individuals with PCP were coinfected with protozoan parasite species using the PCR technique. Of the total of 38 patients examined, two - one HIV-infected and one 6-month-old baby with severe interstitial pneumonia (5.26%) were detected with coinfection with *Cryptosporidium* spp. In three HIV-infected individuals (7.89%) *T. gondii* DNA were found. The data show that except one (a 6-month-old infant), all cases of coinfection were found in immunosuppressed individuals (HIV-infected). Despite the limited number of subjects included in our study, its data regarding pneumonias with more than one causative agent in immunocompromised individuals are similar to those reported in the literature [11], [36], [37].

In Bulgaria, studies on the infection caused by *P. jirovecii*

started in the last century with a detection of the pathogen in patients with AIDS (the first case of AIDS-associated pneumocystosis) [38]-[40] and in young children with pneumonia [41], [42]. The current study shows the need of applying molecular methods for improving diagnoses of pulmonary infections, especially in patients with compromised immune system (including those with HIV/AIDS, cancer and transplant patients who are taking immunosuppressive drugs).

V. Conclusion

The increasingly widespread use of various PCR techniques in the diagnosis of infectious pathology, including the pulmonary one, makes it possible to expand the diagnostic range in severe diseases, particularly in people with compromised immunity and children in infancy, and respectively to optimize the therapeutic behavior. To our knowledge, this study is the first for the country with a similar focus, and its data give reason to consider that there is most likely a "hidden" incidence of protozoan lung pathology in patients with a similar profile, which is not detected because it is rarely thought about. In this aspect, it is necessary for clinicians to expand their differential-diagnostic plan to include diseases that, although relatively rare, could cause lung inflammations, or complicate the course and severity of them with another leading cause in the form of coinfection.

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