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Babić-Erceg, Andrea; Vilibić-Čavlek, Tatjana; Erceg, Marijan; Mlinarić-Missoni, Emilija; Begovac, Josip

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Prevalence of *Pneumocystis jirovecii* pneumonia (2010-2013):

The first Croatian report

Short title: *Pneumocystis jirovecii* in Croatia

Andrea Babic-Erceg[1,2], Tatjana Vilibic-Cavlek[1,2] M. Erceg[1],

Emilija Mlinaric-Missoni[1,2], J. Begovac[2,3]

[1] Croatian National Institute of Public Health, Zagreb, Croatia

[2] School of Medicine University of Zagreb, Zagreb, Croatia

[3] University Hospital for Infectious Diseases "Dr Fran Mihaljevic", Zagreb, Croatia

Corresponding author: Andrea Babić-Erceg, Croatian National Institute of Public Health, Rockefellerova 7, 10000 Zagreb, Croatia

Tel. ++385/1/4863 260

Fax:++385/1/4863017

Mob.++385/98/276 006

e-mail: andrea.babic.erceg@hzjz.hr

Abstract

Pneumocystis jirovecii is an important cause of interstitial pneumonia particularly among immunocompromised hosts. We analyzed the prevalence of *P. jirovecii* pneumonia (PCP) among HIV-infected and HIV-uninfected patients presented with interstitial pneumonia or acute respiratory syndrome hospitalized in six Croatian tertiary care hospitals. Over four-year period (2010-2013), a total of 328 lower respiratory tract samples: 253 (77.1%) bronchoalveolar lavage fluid, 43 (13.1%) tracheal aspirates and 32 (9.8%) bronchial aspirates from 290 patients were examined by real-time polymerase chain reaction (PCR). PCP was detected in 23 (7.9%) patients. The prevalence of PCP differed significantly among tested groups (χ^2=95.03; d.f. =3; p<0.001). HIV-infected patients were more often positive (56.6%, 95%CI=37.3-72.4) compared to other groups (patients with malignant disease 7.7%, 95%CI=2.6-20.3; transplant patients 7.7%, 95%CI=2.2-24.1; patients with other diagnosis 1.5%, 95%CI=0.5-4.4). Majority of HIV-positive patients (80%) were newly diagnosed cases. Our results indicate that HIV-infected patients still represents the main risk group for *P. jirovecii* infection. PCP is responsible for pneumonia in 56.6% HIV-positive patients in Croatia, primarily those who do not know that they are HIV infected.

Key words: *Pneumocystis jirovecii*, prevalence, Croatia, real-time PCR

Introduction

Pneumocystis jirovecii is an opportunistic fungal pathogen causing human pneumocystis pneumonia (PCP) [1]. PCP remains a major cause of morbidity and mortality in patients with compromised immune system caused by HIV, chemotherapeutic regimens for malignancies and immunosuppressive therapy after organ transplantation. However, any patient with an impaired immunity such as those receiving corticosteroids for autoimune diseases or other immunosupressive medications are also at significant risk [2]. PCP is still a frequent AIDS-defining opportunistic infection [3, 4]. Without specific prophylaxis, 60-80% of HIV-infected individuals will develop PCP during the course of their illness [5].

Pneumocystis cannot be cultured. The diagnosis is based on direct microscopic examination using staining techniques (methenamine silver and modified Giemsa stain). Immunofluorescent staining with monoclonal antibodies and molecular methods (conventional polymerase-chain reaction-PCR or real-time PCR) are more sensitive [6, 7].

There has been lack of epidemiological studies on the occurrence of PCP in Croatia so far. The aim of this study was to analyze the prevalence of PCP among HIV- infected and HIV-uninfected patients hospitalized with clinical and radiologic findings of interstitial pneumonia.

Patients and methods

Patients

Over a four-year period (2010-2013), a total of 328 samples: 253 (77.1%) broncho-alveolar lavage fluids (BAL), 43 (13.1%) tracheal aspirates (TA) and 32 (9.8%) bronchial aspirates (BA) were tested for *P.jirovecii* using real-time PCR. Samples were collected from 290 hospitalized patients presented with interstitial pneumonia or acute respiratory syndrome in six tertiary care hospitals in Croatia. Two hundred and seventy-seven samples were additionally tested using IFA. There were 190 (65.5%) males and 100 (34.5%) females, aged from 6 months to 73 years. Twenty-seven were

HIV-infected and 263 HIV- uninfected. Among HIV-uninfected patients, 39 (14.8%) had malignant disease and 26 (9.9%) were transplant patients. The majority of the remaining 198 (75.3%) patients had no clear evidence of predisposing immunodeficiency.

Methods

Real-time PCR was performed using an ABI 7500 sequence detector system (Applied Biosystems, Foster City, CA). DNA was extracted using an automatic vacuum extraction method QIA extractor (Qiagen, Germany) according to the manufacturer's protocol. Primers and probe for amplification were designed with the Primer Express software (Applied Biosystems, Foster City, CA) in order to specifically amplify the *P. jiroveci* beta-tubulin gene (Genbank accession no. AF170964) - the forward primer (1186F-52GATCCGAGACATGGTCGCTATT), reverse primer (1257R-52TTCAACCTCCTTCATGGAAACAG) and TaqMan probe (1212T-52TGTTGCAGCGATTTTCCGCGGTA). The specimen was considered positive if the results exceeded the threshold with the Ct values < 40 cycles. To detect samples containing PCR inhibitors, an internal positive control (IPC) (TaqMan Exogenous Internal Positive Control, Applied Biosystems, Foster City, CA) was used. A negative result for the target and a positive result for the IPC DNA indicate that no target sequence is present. A negative result for each suggests PCR inhibition [8]. Indirect immunofluorescence assay (IFA) was performed using a commercial test (MONOFLUO Pneumocystis jirovecii IFA test kit, Bio-Rad, Marnes la Coquette, France).

Statistical analysis: Prevalence of *Pneumocystis jirovecii* pneumonia in Croatian patients by underlying diseases is expressed with associated confidence intervals and the chi-squared test was used to compare differences between groups. Statistical analyses were performed using MedCalc for WINDOWS version 7.0. $P<0.05$ was considered to be statistically significant.

Results

P. jirovecii was detected in 23 (7.9%) patients. The prevalence of PCP differed significantly among tested groups (χ^2=95.03; d.f. = 3; p<0.001). The highest prevalence (15/27; 56.6%; 95%CI=37.3-72.4) was documented in HIV-positive patients. PCP occurred with equal frequency in patients with malignant disease (3/39; 7.7%; 95%CI=2.6-20.3) and transplant patients (2/26; 7.7%, 95%CI=2.2-24.1). The lowest prevalence (3/1.5%, 95%CI=0.5-4.4) was reported in the group of 198 patients without clearly defined immunodeficiency (Table 1).

Demographic, clinical and laboratory data of 23 *P.jirovecii* positive patients are presented in the table 2. Majority of HIV-positive patients (15/19; 80%) were newly diagnosed patients. Of seven HIV-uninfected patients, there were three patients with hematological malignancy and three patients with transplant kidney. Six of *P.jirovecii* positive patients died (Table 2).

Thirty of 328 (9.1%) of samples tested positive for *P. jirovecii* using PCR: 26/253 (10.3%) BAL 3/32 (9.4%) BA and 1/43 (2.3%) TA. IFA was positive in 21/217 (9.7%) BAL and 1/30 (3.3%) BA samples.

Disscusion

Despite a decline in the incidence of PCP in the era of highly active antiretroviral therapy (HAART), it remains a frequent and serious opportunistic disease in HIV-infected patients [4]. In 2008, World Health Organization reported that PCP was the leading AIDS-indicator disease accounting for 16.4% of the AIDS cases diagnosed in adults and adolescents that year [9]. The incidence of PCP in low- and middle- income countries differed from that in the developed world [10]. In developed countries, the incidence has declined owing to the routine use of HAART and cotrimoxazole prophylaxis [11]. People who live in developing countries continue to be devastated by HIV and PCP occurs frequently. Data from Central and South America showed

PCP prevalence in HIV-infected patients between 24% and 55% [10]. Clinical studies from Africa in HIV-infected patients with pneumonia report that PCP accounted for 0.8 to 38.6% of cases [12, 13]. The apparently lower prevalence of PCP in Africa may be explained in part by early mortality from other infections such as tuberculosis or bacterial pneumonia and possibly also due to the lack of sensitive diagnostic methods [14]. In contrast, in some other resource-limited countries such as India, PCP appears to occur more frequent (45.2%) [15].

The prevalence of PCP pneumonia in Croatian HIV-infected patients (57.7%) was comparable to that recorded in some developing countries such as Thailand (57%) [16], Malaysia (62.7%) [17] and Vietnam (55%) [18].Some developed countries such as China, reported even higher prevalence of PCP (70.22%) [19].In this study, the prevalence of HIV-infected patients was significantly higher prevalence of PCP compared to other groups. PCP pneumonia occurs primarily among HIV-positive persons who were not receiving antiretroviral therapy or PCP prophylaxis, and many were unaware of their HIV infection at the time of presentation [20]. Majority of patients in this study (80%) were newly diagnosed cases.

In the last decade, *P.jirovecii* infections are becoming increasingly recognized in non-HIV immunosuppressed patients [21-24]. Our results showed that 7.7% transplant patients were positive for *P.jirovecii*. A Spanish study detected *P.jirovecii* in 15% solid organ transplants [25] while in two French studies, solid organ transplant recipients accounted for 17.4% [26] and 7.0% [27] of PCP among HIV-negative patients. Lower prevalence rate (2.2%) was reported in Slovenian kidney transplant recipients [28].

Patients with cancer are another group with substantial risk for *P.jirovecii* infection. In patients with hematological malignancies, PCP prevalence rates ranging from 8.4% to 10% [29, 30] were reported. A similar rate of PCP in patients with malignant disease (7.7%) was found in this study. The lowest prevalence rate of PCP (1.5%) was detected in patients without clear predisposing

immunodeficiency. Comparing PCR to IFA results, the real-time PCR assay was more sensitive for the detection of *P. jirovecii* from all tested clinical samples like in other similar sudies [6, 7] .

Our study has some limitations. In addition to a limited number of patients in each risk group, some of data on the possible underlying disease were incomplete.

Despite these limitations, the results of this first Croatian prevalence study showed that HIV-infected persons still represents the main risk group for *P. jirovecii* infection. PCP was detected in 56.6% HIV-positive patients, primarily those who do not know that they are HIV infected.

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Addresses of the authors:

Andrea Babic-Erceg, MD, PhD, specialist in microbiology, Croatian National Institute of Public Health, Rockefellerova 7, Zagreb, Croatia andrea.babic.erceg@hzjz.hr

Tatjana Vilibic-Cavlek MD, PhD, specialist in microbiology, Croatian National Institute of Public Health, Rockefellerova 7, Zagreb, Croatia tatjana.vilibic-cavlek@hzjz.hr

Marijan Erceg MD, PhD, specialist in epidemiology, Croatian National Institute of Public Health, Rockefellerova 7, Zagreb, Croatia marijan.erceg@hzjz.hr

Emilija Mlinarić-Missoni, prof. MD, PhD, specialist in microbiology, Croatian National Institute of Public Health, Rockefellerova 7, Zagreb, Croatia emilija.mlinaric-missoni@hzjz.hr

Josip Begovac, prof. MD, PhD, specialist of infectious diseases, University Hospital for Infectious Diseases, Mirogojska 8, Zagreb, Croatia josip.begovac@bfm.hr

Table 1. Prevalence of *Pneumocystis jirovecii* pneumonia in Croatian patients (2010-2013)

Underlying disease	Tested N (%)	PCR positive N (%)	95%CI
HIV-infected	27 (9.3%)	15 (56.6%)	37.3 - 72.4
Malignant disease	39 (13,5%)	3 (7.7%)	2.6 - 20.3
Transplant organ	26 (9.0%)	2 (7.7%)	2.2 - 24.1
Pneumonia, ARDS	198 (68,2%)	3 (1.5%)	0.5 - 4.4
TOTAL	290 (100%)	23 (7.9%)	

χ^2=95.03; d.f.=3; p=<0.001

Table 2. Demographic, clinical and laboratory data of 23 patients with *Pneumocystis jirovecii* pneumonia

Case	Age	Gender	Sample	PCR	IFA	Underlying disease	Outcome
1	39	M	BAL	Positive	Positive	HIV	Died
2	50	M	BAL	Positive	Positive	HIV	Died
3	53	M	BA	Positive	Positive	HIV	Died
4	64	F	BAL	Positive	Negative	HIV	
5	1	M	BAL	Positive	Positive	[a]SCID	
6	2	M	BAL	Positive	Positive	Malignant disease	Died
7	47	M	BAL	Positive	Positive	Kidney transplant	
8	63	M	BAL	Positive	Positive	Kidney transplant	
9	34	M	BAL	Positive	Positive	HIV	
10	0.6	M	BAL	Positive	Positive	HIV	
11	23	M	BA	Positive	Negative	HIV	
12	58	M	BAL	Positive	Positive	HIV	Died
13	23	M	BA	Positive	Negative	HIV	
14	30	M	BAL	Positive	Positive	HIV	
15	53	M	TA	Positive	Negative	HIV	
16	73	F	BAL	Positive	Positive	Tuberculosis	
17	49	M	BAL	Positive	Positive	HIV	
18	55	M	BAL	Positive	Positive	Brain abscess	Died
19	4	M	BAL	Positive	Positive	Malignant disease	
20	41	F	BAL	Positive	[b]NT	HIV	
21	47	F	BAL	Positive	[b]NT	HIV	
22	38	M	BAL	Positive	[b]NT	HIV	
23	68	M	BAL	Positive	[b]NT	[c]NHL	

[a]SCID = severe combined immunodeficiency

[b]NT = not tested

[c]NHL = non-Hodgkin lymphoma