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Role of *Pneumocystis jirovecii* in patients with different pulmonary underlying condition using a nested-PCR

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ABSTRACT

Introduction. The prevalence of *Pneumocystis jirovecii* colonization and its role in pulmonary disease remains unclear. PCR methods have shown an improved sensitivity in the detection of this fungus. It has been suggested that the PCR results be combined with another test such as IFA to create a diagnostic algorithm.

Material and methods. A multiplex nested-PCR procedure with a 16S rRNA gene as the internal amplification control was evaluated to determine the role of *P. jirovecii* in pulmonary disease.

Results. A 20% of the 199 bronchoalveolar lavage samples were PCR-positive, 13.5% samples were PCR-inhibited, and the rate of Pneumocystis-colonisation was 6.4%. The sensitivity, specificity, positive predictive value and negative predictive value of the nested-PCR were 100%, 93%, 70% and 100%, respectively. The sensitivity of the nested-PCR was higher than the current "gold standard" immunofluorescence assay (IFA) (p< 0.0001). PCR-negative and PCR-positive patients did not show any clinical or radiological differences in the medical variables studied.

Conclusion. PCR could help the diagnosis of *Pneumocystis* pulmonary disease given the high negative predictive value of the technique. *P. jirovecii* DNA can frequently be detected in healthy population, so the analysis of the patient medical history is critical to make the correct clinical decision.

Keywords: *Pneumocystis jirovecii*; nested-PCR; internal control; colonisation; pulmonary disease.

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Papel de *Pneumocystis jirovecii* en pacientes con diferente patología pulmonar de base usando una PCR anidada

RESUMEN

Introducción. La prevalencia de la colonización por *Pneumocystis jirovecii* y su papel en la enfermedad pulmonar sigue sin estar clara. Los métodos de PCR han demostrado una sensibilidad mejorada en la detección de este hongo. . Se ha sugerido que los resultados de PCR se combinen con otra prueba como IFA para crear un algoritmo de diagnóstico.

Material y métodos. Se evaluó una PCR múltiple anidado con el gen 16S rRNA como control interno de amplificación para determinar el papel de *P. jirovecii* en la enfermedad pulmonar. **Resultados.** Un 20% de las 199 muestras de lavado broncoalveolar fueron positivas para PCR, 13,5% muestras fueron inhibidas por PCR, y la tasa de colonización por *Pneumocystis* fue de 6,4%. La sensibilidad, especificidad, valor predictivo positivo y valor predictivo negativo de la PCR fueron del 100%, 93%, 70% y 100%, respectivamente. La sensibilidad de la PCR fue mayor que el ensayo de inmunofluorescencia "gold-standard" (IFA) actual (p <0,0001). Los pacientes PCR-negativos y PCR-positivos no mostraron diferencias clínicas o radiológicas en las variables médicas estudiadas.

Conclusión. La PCR podría ayudar al diagnóstico de la enfermedad pulmonar por *Pneumocystis* dado el alto valor predictivo negativo de la técnica. El ADN de *P. jirovecii* se puede detectar con frecuencia en poblaciones sanas, por lo que el análisis del historial médico del paciente es fundamental para tomar la decisión clínica correcta.

Palabras clave: *Pneumocystis jirovecii*; PCR anidada; control interno; colonización; enfermedad pulmonar.

INTRODUCTION

Pneumocystis jirovecii is an opportunistic fungal pathogen that causes pneumonia (PJP) in immunocompromised hosts. Over the past decade, despite the decreased number of PJP cases among HIV-infected patients, PJP has become a serious problem in immunodeficient patients with other immunosuppressive conditions [1, 2]. *P. jirovecii* has a global distribution; most people have serologic evidence of infection during early childhood [3, 4], and normal healthy individuals can carry this fungus. The colonisation may develop into PJP if there is a worsening of the underlying disease and the patients does not receive appropriate prophylaxis [2, 5, 6]. However, the prevalence of *P. jirovecii* infection without disease remains unclear and complicates the interpretation of positive results becoming the clinical diagnosis of PJP into a challenging question.

The detection of the fungus in the laboratory by direct microscopic immunofluorescent staining of respiratory smears and tissue specimens has long been considered the major diagnostic tool and the current "gold standard". The development of PCR technology with increased sensitivity allowed the detection of subclinical infections and colonisations [7-9]. These new approaches are especially important for non-HIV immunocompromised patients where the diagnosis is more difficult [10]. Currently, molecular detection is superior to microscopic evaluation [11, 12].

PCR assays vary significantly in their detection technology, turnaround time, type of clinical sample and capacity to yield a quantitative versus qualitative result [8, 13-20]. Simple DNA extraction and nested PCR in bronchoalveolar lavage specimens has been shown to be a sensitive test [6], which may be performed in clinical laboratories [21], with similar or even better sensitivity than real-time PCR [22]. Nevertheless, bronchoalveolar lavage (BAL) samples may contain inhibitors of the PCR reaction. The use of an internal control (IC) can identify inhibitory substances and monitor the PCR reaction [23].

The objectives of this work were to determinate the role of *Pneumocystis jirovecii* in the pulmonary disease and to evaluate a simple multiplex nested-PCR in bronchoalveolar samples to detect *P. jirovecii*.

MATERIAL AND METHODS

From April 2013 to April 2014, 199 BAL samples sent to the laboratory for the detection of any infectious agents from 197 patients at the 1250-tertiary bed University Hospital (CHUVI) were collected.

Sample preparation for staining. BAL samples were mixed V/V with Sputasol (Oxoid) and mixed vigorously for 5 min. Samples were centrifuged at 3000 g for 10 min, and the pellets were resuspended 1/10 in 0.9%NaCl. The resuspended pellet was used to prepare smears. An immunofluorescence assay (IFA) was performed with a flouresceinisothiocyanate-conjugated monoclonal antibody to *P. jirovecii* following the manufacturer's instructions (MONOFLUO™ *Pneumocystis jirovecii* IFA Test Kit, Bio-Rad, Laboratories, USA).

Sample preparation for DNA extraction. One ml of BAL samples were aliquoted in 200 µl each and were stored at -20°C until they were studied. DNA was extracted from stored BAL samples using an InstaGene Matrix kit (Bio-Rad Laboratories, USA) according to the manufacturer's recommendations.

The nested-PCR protocol for amplification of mtLSUr-RNA in *P. jirovecii* was performed as previously described

Table 1	Primers sequences and thermocycling nested-PCR conditions.				
Reaction	Target	Primer name	Primer sequences	Cycling conditions	
Primary amplification					
	mtLSUrRNA	pAZ102-E	5´-GATGGCTGTTTCCAAGCCCA-3´	94°C 10 min	
		pAZ102-H	5´-GTGTACGTTGCAAAGTACTC-3´	40 cycles	94°C/1 min
					50°C/1 min
		U1	5´-CCAGCAGCCGCGGTAATATCG-3´		72°C/2 min
	16sRNA	U2	5´- ATCGG(C/T)TACCTTGTTACGACTTC-3´		
				72°C 10 min	
Secondary amplification					
	mtLSUrRNA	pAZ102-X	5´-GTGAAATACAAATCGGACTAGG-3´	94°C 5min	
		pAZ102-Y	5´-TCACTTAATATTAATTGGGGAGC-3´	45 cycles	94°C/20sec
					50°C/20sec
					72°C/20sec
				72°C 10 min	

by Wakefield et al.[13]. The external primers pAZ102-E and pAZ102-H to mtLSUrRNA *P. jirovecii* gene amplification were used in the first amplification round which produced a 346 bp amplicon, being included in the same reaction as an internal control 16s rRNA gene amplification primers (U1 and U2), which produced a 996 bp product [24]. The internal primers pAZ102-X and pAZ102-Y were used in a second round to amplify a 260 bp fragment.

Each PCR reaction contained 5 μL of the extraction product, 12.5 μL QIAGEN Multiplex PCR MasterMix and 0.2 μM concentration of each primer in a total volume of 25 μL (QIAGEN Multiplex PCR kit,Qiagen, Hilden, Germany);5 μL of the first PCR product was used as the DNA template for the second PCR reaction. The PCR products were analysed by electrophoresis on 1% agarose gel stained with RedSafe TM Nucleic Acid Staining Solution (iNtRON Biotechnology Inc., Sungnam, Kyungki-Do, Republic of Korea) (table 1).

External controls, contamination prevention and validation of the nested-PCR product. During each PCR, a positive control (a BAL fluid sample from a patient with PJP, *P. jirovecii* positive immunofluorescence assay) and ultra-pure water as the negative control were used. Amplification of the 16s RNA was performed to confirm successful DNA extraction and the absence of PCR inhibitions. To avoid contamination, all steps (master mix preparation, DNA extraction, amplification, and addition of the PCR product) were performed in separate areas. To validate the usefulness of the primers U1/U2 to identify oropharyngeal bacteria by PCR that usually contaminate the BAL samples, the following bacteria were studied: *Streptococcus mitis* ATCC 49456, *Streptococcus oralis* ATCC 35037, *Streptococcus dysgalactiae subps equsimilis* ATCC 10009, *Corynebacterium striatum* ATCC 7094, *Hemophilus parainfluenzae* ATCC 9796 and *Neisseria cinerea* ATCC 14685, getting a 996 bp product in all of them. The nested-PCR product was purified using a QIAquik PCR purification Kit (Qiagen, Hilden, Germany) and sequenced with the forward primer pAZ102-X. A BLAST search was performed to verify the PCR-amplicon result.

Clinical and microbiological data collection. Demographic and clinical data including: age, sex, haematological malignancies, solid tumours, transplant recipients (bone marrow or solid organ), immunosuppressive therapy, tobacco smoke exposure, inflammatory lung disease, HIV infection, anti-PJP prophylaxis or treatment, clinical symptoms, season of the year and other organisms identified in the specimen. All samples were studied according to the procedures, serological studies and molecular studies for difficult to culture bacteria and virus were performed on request by the clinician [25].

Colonisation or Pneumocystis infection. Patients with a negative staining result who were PCR-positive were classified as colonised or infected based on a medical chart review. Colonisation was defined in cases involving a patient without symptoms, no previous infection or treatment for PJP, no radiological abnormalities and favourable clinical outcome without specific treatment or symptoms attributed to another basic pathology. *Pneumocystis* infection (true-PJP) was defined in the setting of symptoms (fever or low grade fever, cough, dyspnea, weight loss) and compatible radiography or CT scan [26], provided that no other infectious agent or immune-allergic aetiology were identified.

Statistical analysis. Analysis was performed using SPSS version 20.0 (Chicago, IL, USA). All clinical variables were compared between the PCR-positive and PCR-negative groups. Continuous variables were compared using Student`s *t*-test, and categorical data were compared using the Chi-squared test. A two-tailed *p* value <0.05 was considered statistically significant.

Sensitivity, specificity and positive predictive value (PPV) and negative predictive value (NPV) of IFA and PCR were calculated. Positive-PCR results in patients with pneumonia were considered true-positive. False positive was considered the positive-PCR results in colonized patients. To determine the predictive values, we assumed a PJP prevalence of 20%, which is considered the median rate of colonisation in immunocompetent adults [27].

RESULTS

Nested-PCR results. For the 199 BAL specimens tested by nested-PCR, 35 (20.3%) were positive, 137 (68.8%) were negative and 27 (13.5%) were PCR-inhibited.

There was an initial inhibition of the PCR reaction in 47 samples, but the inhibition problems were resolved in 20 of them, with a second DNA extraction of the original specimens. This produced a total of 27 samples that were definitely considered as PCR-inhibited.

Comparison of classic staining/nested-PCR. Only 6 of the PCR-positive samples were positive based on IFA, and no negative PCR results with positive microscopy results were found.

Based on the criteria established in the materials and methods section, we estimated the sensitivity and specificity of each technique: IFA sensitivity and specificity was 25% (IC 95%: 0.08-0.42) and 100% (IC 95%: 0.97-1), respectively, and the nested-PCR sensitivity and specificity was 100% (IC95%: 0.85-1) and 93% (IC 95%: 0.88-0.97), respectively. There was a significant difference in sensitivity between PCR and IFA (P<0.0001). The PPV of the technique was 70% (IC95%: 0.5-0.9), and the NPV was 100%.

A good correlation between the IFA and PCR results was found in HIV-positive patients (2/2), but PCR detected more possible PJP patients among the non-HIV immunocompromised groups.

Characteristics of the patients. The mean age of the 170 patients was 61 +- 14 years, 105 (62%) were male. The most common underlying diseases were haematologic malignancies or solid tumours (35%); only 11 patients were HIV positive (6.5%), 88 (51.8%) had chronic lung disease (CPOD, asthma, pneumoconiosis, or interstitial lung disease). Active or previous exposure to tobacco was identified in 50% of the patients and

Table 2		Clinical data of positive–PCR patients							

Age	Sex	Underlying disease	Clinical presentation	Immunosuppressive therapy	Tobacco[a] exposure	Pneumocystis prophylaxis	Radiological signs	Sampling season	Colonisation/PJP
62	Male	Crohn´s disease	Asymptomatic	Yes	Ex	No	Infiltrate	Spring	Colonisation
71	Female	Chronic lymphocytic leukaemia B	Dyspnoea, cough, fever	Yes	No	No	Infiltrate	Spring	Pneumonia
44	Female	Bronchial asthma	Asthma exacerbation	Yes	No	No	Infiltrate	Spring	Pneumonia
64	Female	Microscopic polyangiitis	Cough, fever	No	Ex	Yes	Normal	Spring	Pneumonia
39	Female	Breast cancer	Cough, dyspnea	Yes	No	No	Infiltrate	Spring	Pneumonia
68	Male	Hypersensitivity pneumonitis	Asymptomatic	No	Yes	No	Normal	Spring	Colonisation
46	Male	Silicosis	Asymptomatic	No	No	No	Infiltrate	Spring	Colonisation
70	Male	Colon cancer	Dyspnoea, cough, fever	Yes	Ex	No	Consolidation	Summer	Pneumonia
69	Female	COPD	Cough, fever	Yes	Yes	No	Infiltrate	Summer	Pneumonia
61	Male	Silicosis	Dyspnoea, cough	No	Ex	No	Infiltrate	Summer	Pneumonia
33	Male	HIV	Dyspnoea, cough	No	No	No	Infiltrate	Summer	Pneumonia
53	Male	Chest chondrosarcoma	Dyspnoea, cough, fever	Yes	No	No	Infiltrate	Summer	Pneumonia
64	Female	Peritoneal pseudomyxoma	Dyspnoea, fever	No	No	No	Infiltrate	Autumn	Pneumonia
55	Male	Bronchial asthma	Asymptomatic	Yes	No	No	Normal	Autumn	Pneumonia
73	Male	Pulmonary fibrosis	Asymptomatic	No	Ex	No	Infiltrate	Autumn	Colonisation
64	Male	ILD	Asymptomatic	Yes	No	No	Infiltrate	Autumn	Colonisation
56	Female	ILD	Dyspnoea	Yes	Ex	No	Infiltrate	Autumn	Pneumonia
60	Male	Silicosis	Asymptomatic	Yes	No	No	Nodule	Autumn	Colonisation
63	Female	Chronic bronchitis	Asymptomatic	No	Yes	No	Infiltrate	Autumn	Colonisation
52	Female	Hypersensitivity pneumonitis	Asymptomatic	No	Yes	No	Infiltrate	Autumn	Colonisation
75	Female	Common variable immunodeficiency	Dyspnoea, weight loss	Yes	No	No	Infiltrate	Autumn	Pneumonia
53	Male	Lung cancer	Cough, fever, chest pain	Yes	Yes	No	Infiltrate	Autumn	Pneumonia
63	Male	Myelofibrosis	Fever	Yes	Yes	Yes	Infiltrate	Winter	Pneumonia
79	Female	Bronchiectasis	Dyspnoea, cough, fever	No	No	No	Infiltrate	Winter	Pneumonia
66	Female	Breast cancer	Fever, cough	No	No	No	Infiltrate	Winter	Pneumonia
53	Male	Silicosis	Asymptomatic	No	Ex	No	Consolidation	Winter	Colonisation
64	Female	Bronchiectasis	Cough, low grade fever	No	Yes	No	Nodule	Winter	Pneumonia
73	Male	Acute pneumonia	Dyspsnoea, cough, fever	No	Ex	No	Nodule	Winter	Pneumonia
81	Male	Lung abscess	Cough, weight loss	No	No	No	Infiltrate	Winter	Colonisation
84	Male	No disease	Dyspnoea	No	Yes	No	Infiltrate	Winter	Pneumocystis pneumonia
74	Male	Ureter cancer and leukaemia	Cough, fever	Yes	No	No	Infiltrate	Winter	Pneumonia
73	Female	Pulmonary fibrosis	Cough, fever	Yes	Yes	No	Infiltrate	Winter	Colonisation
37	Female	No disease	Cough, fever	No	Yes	No	Infiltrate	Winter	Pneumonia
57	Female	Common variable immunodeficiency	Cough, fever	No	No	No	Nodule	Winter	Pneumonia
49	Male	HIV	Dypsnoea, chest pain	No	No	No	Infiltrate	Spring	Pneumonia

[a]Ex: previous exposure to tobacco. IDL: Interstitial lung disease. COPD: chronic obstructive pulmonary disease. PJP: *Pneumocystis jirovecii* pneumonia

43.5% of the patients received immunosuppressive therapy including corticosteroids or chemotherapeutic agents. Seventeen percent of patients were treated or received prophylactic therapy for PJP.

PJP was diagnosed in 24 patients, and *P. jirovecii*-colonisation was diagnosed in 11 patients on basis of the criteria described in the material and methods. Table 2 summarises the clinical data from *P. jirovecii* PCR-positive patients. Direct flu-

Table 3	Comparisons of the clinical features PCR+ and PCR– patients.		
	PCR-positive (n=35)	PCR-negative (n=137)	p-value
Age, years	62 ± 13	61 ± 14	0.954
Sex, Female	15 (42.8%)	49 (35.7%)	0.279
Underlying disease			
Solid tumor	6 (17.1%)	21 (15.3%)	0.42
Haematological malignancy	4 (11.4%)	29 (21.1%)	
Immunosuppressive disease	5 (14.3%)	19 (13.9%)	0.949
Inflammatory lung disease (COPD/Asthma)	11 (31.4%)	77 (56.2%)	0.0015
HIV infection	2 (5.7%)	9 (6.5%)	0.726
Organ transplantation	3 (8.5%)	16 (11.7%)	0.601
Corticosteroids	14 (40%)	60 (4.4%)	0.68
Tobacco exposure			
Active	10 (28.6%)	34 (24.8%)	0.89
Previous exposure to tobacco	10 (28.6%)	32 (23.4%)	
Anti- PcP prophylaxis	3 (8.5%)	27 (19.7%)	0.12
Positive microbiology culture results	8 (22.8%)	35(25.5%)	0.74
X-ray findings			
Nodules	4 (11.4%)	25 (18.2%)	
Infiltrates	25 (71.4%)	70 (51.1%)	0.142
Consolidations	3 (8.6%)	30 (21.9%)	
Normal	3 (8.6%)	12 (8.8%)	

orescence microscopy examination was ordered by clinicians in 15 (42.8%) of the PCR-positive samples. Furthermore, all these patients had some immunosuppressive condition that increased the risk of PJP. The seasonality of the infection was related with the cold months of the year, 12 patients in winter and 10 patients in autumn.

Comparisons of clinical and radiological results between PCR-positive and PCR-negative patients. The clinical features of the PCR-positive and PCR-negative patients are shown in table 3. The PCR-positive results were more likely in females and in patients with prophylaxis, although there was no statistical significance. Nodules, consolidations and normal X-ray findings were also more common than infiltrates in PCR-negative patients. A diagnosis of an underlying lung disease was significantly associated with a negative PCR result. Other clinical conditions were not associated with a positive result for *P. jirovecii*.

Microbiological results. Eight (22.8%) of the 35 PCR-positive samples and 35 (25.5%) of the PCR-negative samples were positive for other pathogens. In the PCR-positive samples, the most commonly isolated microorganisms were *Staphylococcus aureus* (n=2), *Aspergillus fumigatus* (n=2) and *Haemophilus* spp. (n=2). In the PCR-negative samples, non-fermenting

gram-negative bacilli (n=7) were the most common isolated microorganisms, followed by *Enterobacteriaceae* spp. (n=5), *Haemophilus* spp. (n=5) and *A. fumigatus* (n=5). Microbiological results of the culture positive samples are summarised in Table 4.

Clinical outcome. IFA and PCR positive patients were treated for PJP. Three patients of the 35 PCR-positive patients died, the cause of death could not be attributable to PJP.

DISCUSSION

PJP is an opportunistic infection of increasing importance in non-HIV patients. In this study, only 2/35 (5.3%) of the PCR-positive patients were HIV-infected. It is well acknowledged that the diagnosis of PJP is particularly difficult in non-HIV patients, who can develop rapidly progressive PJP even with low fungus loads. In addition, in non-HIV patients, false negative results from tinctorial methods are more common [8, 9].

In most studies that compared microscopy with PCR methods for PJP diagnosis, PCR exhibited superior sensitivity for organism detection in patients with chronic lung disease and/or those on steroid treatment [3, 28, 29]. A recent me-

Table 4	Microbiological isolates obtained from *P. jirovecii* PCR positive and negative samples.

P. jirovecii PCR-positive	*P. jirovecii* PCR-negative
8/37 (23%)[a]	31/137 (23%)[a]
Staphylococcus aureus (n=2)	*Aspergillus fumigatus* (n=5)
Aspergillus fumigatus (n=2)	*Haemophilus* spp.[b] (n=5)
Haemophilus spp. (n=2)	*Pseudomonas* spp.[c] (n=4)
Pseudomonas aeruginosa (n=1)	*Escherichia coli* (n=3)
Mycobacterium lentiflavum (n=1)	*Candida* spp.[d] (n=3)
	Mycobacterium spp.[e] (n=3)
	Streptococcus pneumoniae (n=3)
	Other non-fermenting gram-negative[f] (n=3)
	Staphylococcus aureus (n=2)
	Serratia marcescens (n=2)
	Metapneumovirus (n=1)

[a]Number of isolated microorganisms/total samples studied

[b]*H. influenzae* (n=6); *H. parainfluenzae* (n=1)

[c]*P. aeruginosa* (n=3); *P. fluorescens* (n=1)

[d]*C. albicans* (n=3); *C. glabrata* (n=1)

[e]*M. avium* (n=1); *M. lentiflavum* (n=1); *M. tuberculosis* (n=1)

[f]*Burkholderia cepacia* (n=1); *Brevundimonas diminuta* (n=1); *Stenotrophomonas maltophilia* (n=1).

ta-analysis showed a very high accuracy of PCR in BAL samples for the diagnosis of PJP in patients who are at risk and a pooled sensitivity of 98.3% and a specificity of 91.0% [9]. To select the targets and primers, Roberts et al. compared 9 PCR assays with different primers/targets and found that the most sensitive PCR technique should consider a mtLSUrRNA nested reaction with the potential of producing false positive results [30]. Consistent with published data [11, 29, 31] the mtLSUrRNA PCR method used in the present study detected 18 more *P. jirovecii*-infected and 11 more *P. jirovecii*-colonised patients than IFA, improving the sensitivity from 25% to 100% (p<0.01). IFA exhibited excellent specificity but lacked sensitivity, whereas PCR was much more sensitive and also detected colonised patients. In particular, all confirmed-PJP cases in the HIV patient group were detected by both methods, perhaps in relation to the higher fungus loads in the HIV-infected patients [32, 33]. However, nested PCR exhibited a higher sensitivity in the non HIV group.

The high NPV allowed excluding PJP. Nevertheless, clinical and radiological criteria are essential to interpret a PCR-positive result because the low PPV of this technique. Clinical diagnosis in conjunction with IFA and PCR are considered the cornerstones for PJP patient management [10]. Although *P. jirovecii* was not recognised as the main cause of disease, it might play an important role as a comorbidity cofactor in patients with a severe underlying disease [33].Recent studies

showed that 31.8% of the patients with positive PCR results have a history of PJP or will develop PJP [29]. Surprisingly, in this study, the laboratory test to detect *P. jirovecii* was requested in less than 50% of patients, and half of the unrequested PCR-positive samples were from patients who were at risk of developing PJP.

The actual prevalence of *P. jirovecii* colonisation or subclinical infection in immunocompetent patients remains unclear. The results of the current study show a low rate of colonisation of 6.4%. Nevertheless, similar studies reported differences in the rate of colonisation. Recent studies have shown a *P. jirovecii* colonisation prevalence from 2.6% to 55% [27, 28]. The differences in the prevalence rate could be due to the respiratory sample used (BAL vs. sputum), differences in the studied patients, such as underlying disease and comorbidities or differences in the recognition of a positive case given the absence of universally accepted criteria to establish the diagnosis for PJP. On the other hand, recent studies have shown that colonised-patients with low loads could be candidates for *P. jirovecii* prophylaxis [34]. Obviously, the detection of a *P. jirovecii* infection has a direct therapeutic impact on the choice of appropriate antimicrobial therapy. Understanding the role of *P. jirovecii* colonisation in patients with underlying pulmonary or systemic disease may help identify patients at risk of developing PJP [35].

Calderón et al. showed that *P. jirovecii* carriage could be involved in the progression of COPD by means of the capacity of *P. jirovecii* during very early stages of the infection to induce, in animal models, alveolar macrophage activation, pro-inflammatory interleukin elevation, and changes in pulmonary surfactant [36]. In the present study, 10% of the CPOD patients were PCR positive for *P. jirovecii*. Recent studies in Europe found rates of carriage in patients with chronic diseases between 6 and 40% [37, 38]. However, the association between high rates of *P. jirovecii* colonisation and chronic lung diseases is debatable. In the present study, there were more PCR-positive *P. jirovecii* patients in the group without any lung disease. This result could be related to the proportion of patients with malignancies that were higher in the group of patients without any pulmonary disease than in the group of patients with lung disease (48% versus 21%).

In the present study, it was not clear whether the presence of *P. jirovecii* with other pathogens contributed to the exacerbation of pneumonia, because of the low number of PJP in which another potential respiratory pathogen was isolated.

As far as we know, this is the first conventional-PCR method to detect *P. jirovecii* that uses a non-competitive bacterial internal amplification control (IAC) to reveal reaction failure due to the presence of inhibitory substances in the sample [39]. A current issue that limits the reliability and sensitivity of PCR is the degree of inhibition caused by inhibitory substances, especially in respiratory samples [40]. Many other PCR designed to detect *P. jirovecii* do not use IAC [13, 22], use nonbacterial IAC as exogenous internal commercial control or complex recombinant plasmids [7, 8, 15, 19]. Another strategy used was

a second round of amplification with the addition of a DNA template to exclude the presence of inhibitors [18, 41]. In this work, 13.5% of samples were inhibited, demonstrating the clinical utility of IAC. Therefore, reporting false-negative results is avoided. Previous studies found inhibition rates of 23.7% [40]. As Döskaya et al. observed in retesting diluted samples [40] in 20 (11.6%) samples, inhibition problems were resolved with a second extraction round. Maybe the use of more efficient extraction systems would improve the results.

In conclusion, PJP could be a serious problem for non-HIV patients, where the diagnosis by PCR has produced better results than traditional staining methods. The use of an internal control is necessary to ensure the reliability of the results, especially in samples with a high presence of PCR inhibitors such as respiratory samples. The PCR strategy used in this work has proven to be useful for routine clinical laboratories without access to more specialized diagnostic procedures, which are more expensive for the detection of *P. jirovecii* in respiratory samples. Our results could help in the understanding of the clinical features that are associated with colonisation or infection with this microorganism. However, more studies are needed to clarify these findings.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest

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