Pneumocystis jirovecii Colonization and Its Association with Pulmonary Diseases Based on a Multicenter Study by an Improved Loop-Mediated Isothermal Amplification Assay

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Abstract

Background Pneumocystis jirovecii (P. jirovecii) is an opportunistic fungal pathogen and the role of its colonization in pulmonary diseases has become a popular focus in recent years. The aim of this study is to develop an improved loop-mediated isothermal amplification (LAMP) assay for detection of Pneumocystis jirovecii (P. jirovecii) DNA and use it to examine the prevalence and association of P. jirovecii colonization among non-HIV patients with various pulmonary diseases. Methods We modified the previously reported LAMP assay for P. jirovecii by adding real-time detection. This method was used to detect P. jirovecii colonization in pulmonary samples collected from 403 non-HIV patients with various pulmonary diseases enrolled from 5 hospitals in China. We determined the prevalence of P. jirovecii colonization in 7 types of pulmonary diseases and assessed the association of P. jirovecii colonization with clinical characteristics of these diseases. Results The new LAMP assay showed no cross-reactivity with other common pulmonary microbes and was 1,000 times more sensitive than that of conventional PCR. Using the new LAMP assay, we detected P. jirovecii colonization in 281 (69.7%) of the 403 patients enrolled. P. jirovecii colonization was more common in interstitial lung diseases than in chronic obstructive pulmonary disease (COPD) (84.6% vs 64.5%, P < 0.05). Patients with acute exacerbation of COPD had a higher prevalence of P. jirovecii colonization compared to patients with stabilized COPD (67.4% vs 43.3%, P < 0.05). P. jirovecii colonization was associated with decreased pulmonary function, increased levels of 1,3-β-D-glucan and C-reactive protein, and decreased levels of CD4+ T-cell counts (P < 0.05 for each). Approximately 70% of P. jirovecii colonized patients had confections with other fungi or bacteria. Conclusions We developed an improved LAMP assay for detecting P. jirovecii. Our multi-center study of 403 patients supports that P. jirovecii colonization is a risk factor for the development of pulmonary diseases and
highlights the need to further study the pathogenesis and transmission of P. jirovecii colonization in pulmonary diseases.

Introduction

Pneumocystis jirovecii (P. jirovecii) is a unicellular parasitic fungus and an opportunistic pathogen which almost exclusively infects the lungs [1, 2]. P. jirovecii infection may lead to a fatal P. jirovecii pneumonia (PJP) in immunocompromised patients, particularly in those infected with human immunodeficiency virus (HIV). While, the incidence of PJP in HIV-infected patients has decreased dramatically, mainly due to the widespread use of combination antiretroviral therapy, there are growing reports of PJP cases among HIV-negative individuals [3-7]. In addition, asymptomatic P. jirovecii colonization is being increasingly identified in immunocompetent individuals [2, 8]. In recent years, the role of P. jirovecii colonization in pulmonary diseases has become a popular focus, especially in those with chronic obstructive pulmonary disease (COPD) and sudden infant death syndrome [5, 8-11]. These asymptomatic PJP individuals, carrying P. jirovecii, may act as an important reservoirs of nosocomial infections. Therefore, it is necessary to clarify the role of P. jirovecii colonization in the pathogenesis of pulmonary diseases.

Various molecular methods for detection of P. jirovecii have been described previously, including conventional polymerase chain reaction (PCR) and real-time quantitative PCR [12-15]. Loop-mediated isothermal amplification (LAMP), originally developed by Notomi T. et al. [16], is a novel, highly sensitive, and specific method to diagnose infectious diseases. There is one report related to the application of LAMP in the detection of P. jirovecii in patients with chronic pulmonary diseases [5]. However, this method suffers from several drawbacks, including subjective end-point visual detection of the LAMP amplification products, and potential inhibition of the LAMP amplification due to the presence of SYBR Green I [17]. Thus, the aims of this study were to develop an improved
real-time fluorescence LAMP assay for *P. jirovecii* and then, use it to examine the prevalence and association of *P. jirovecii* colonization among non-HIV patients with various pulmonary diseases.

**Materials And Methods**

**Patient enrollment**

This retrospective study was approved by the Institutional Ethics Review Board, China Medical University (2013070). Written informed consent was obtained from all the patients involved in this study. We enrolled a total of 403 patients with various pulmonary diseases hospitalized between October 2016 and January 2018, from 5 hospitals in China, including the First Affiliated Hospital of China Medical University, the Fourth Affiliated Hospital of China Medical University, the Fifth Affiliated Hospital of China Medical University, the Eleventh Affiliated Hospital of Shanxi Medical University, and the General Hospital of Shenyang Military Command. Clinical background information of patients was collected through the Case File System in the participating hospitals.

The patients with different symptoms and signs of pulmonary diseases, with non-HIV infections, without the clinical evidence or history of PJP symptoms, and without the definite diagnosis of PJP were included in the study [14, 18]. Besides considering the predisposing factors for the diseases, the pulmonary diseases were diagnosed not only on the basis of the clinical manifestations of respiratory tract diseases (such as cough, fever, expectoration, wheezing, chest pain, chest tightness, hemoptysis, and dyspnea), but also chest imaging tests, bronchoscopic examination, and laboratory test, primarily including COPD [including the stable stage and acute exacerbations (AECOPD)], acute exacerbations of chronic bronchitis (AECB), interstitial lung diseases (ILDs), bronchiectasis, bronchial asthma, invasive pulmonary aspergillosis (IPA), and type I respiratory failure. Various diseases, included in the study, were diagnosed as follows: i) COPD was diagnosed mainly
by confirming the presence of persistent airflow limitation according to a post-
bronchodilator FEV1/FVC < 70%, history of risk factors such as exposure to cigarette
smoke, and the Global Strategy for the Diagnosis, Management, and Prevention of Chronic
Obstructive Lung Disease 2017 Report: GOLD Executive Summary [19]. In addition, the
assessment of AECOPD met the GOLD guidelines, the diagnostic criteria of AECOPD given
by American College of Chest Physicians (2011), and the European Respiratory Society
guidelines [20]; ii) ILDs were primarily diagnosed by high-resolution computed tomography
(HRCT) depicting the radiological manifestations of fibrosis in ILDs and sometimes, by
bronchoalveolar lavage for the diagnosis of some particular types of ILDs [21, 22]; iii)
AECB was diagnosed by considering the patients with mild to moderate syndromes of
COPD (the diagnosis of COPD as mentioned above), patients who developed the
exacerbated clinical presentations with increased dyspnea, increased sputum volume, and
increased sputum purulence, and meanwhile, excluding pneumonia and other lung
diseases by chest radiography [23]; iv) bronchiectasis was primarily diagnosed by HRCT of
the chest [24], the gold standard for confirming the bronchiectasis, and the considering
the underlying causes such as post-infectious [25]; v) bronchial asthma was diagnosed
through a detailed clinical history, physical examination, lung function, and detection of
allergens [26]; vi) IPA was diagnosed by demonstrating Aspergillus in the respiratory
samples and other contributions by 1,3-β-D-glucan assay, clinical manifestation of lower
respiratory tract infection, and CT clinical characteristic with the air-crescent sign, halo
sign, cavitation lesion, or nodules infarction; vii) type I respiratory failure was diagnosed
by laboratory studies such as evaluating the level of hypoxemia and hypercapnia via
arterial blood gas and oxygen saturation, imaging examination, and pulmonary function
test [27].
Respiratory tract specimens

Before patients received diagnosis, antibiotics, and other treatments, specimens including blood, bronchoalveolar lavage fluid (BALF), and sputum samples were collected. Venous blood was collected in vacutainer tubes with different anticoagulants or coagulants and used immediately for the following tests: \( \text{CD}_4^+ \) T-cell counts (BD FACS Canto, USA), erythrocyte sedimentation rate (ESR) (Vital Monitor-20, Italy), and serum biochemical parameters including lactate dehydrogenase (LDH) (ADVIA 2400, SIEMENS Healthineers, Germany), pro-calcitonin (PCT) (Cobas 8000, Roche Diagnostics GmbH, Germany), C-reactive protein (CRP) (BN ProSpec System, SIEMENS Healthineers, Germany), and 1,3-\( \beta \)-D-glucan (BDG) (Goldstream MB-80, Era Biology Technology Co. Ltd. Tianjin, China).

Pulmonary function (Medisoft S.A. Hyp’ Air, Belgium) and high-resolution computed tomography (HRCT) (BN ProSpec System, SIEMENS Healthineers, Germany) were measured by the following standard protocols in clinical practice. Each patient underwent HIV-1 and 2 antibody testing using the anti-HIV-1 and 2 antibody enzyme-linked immunosorbent assay (ELISA) diagnostic kit (Intec Products, Inc. Xiamen, China) to determine HIV infection status.

Among 403 patients, 324 patients provided sputum samples and 79 patients provided BALF samples. As the nature of the study is retrospectively and severity status of the pulmonary disease was not available in the Case File System, we can only assume that patients who provided BALF sample had more severe disease status, as compared to patients who provide sputum samples. Moreover, patients providing sputum samples were not eligible for the BAL procedure. To collect the sputum samples, patients gargled with saline solution approximately three times to prevent oral microbial contamination before coughing up their morning sputa from the deep respiratory tract. Sputa were then
collected in sterile containers and sent to clinical laboratories for bacterial and/or fungal
culture and identification, respectively using the VITEC-2 system (ATB system, BioMérieux,
Marcy-l’Étoile, France) and the Sensititre™, Aris 2X system (Thermo Fisher Scientific,
USA). The remaining of the sputum samples were used to exact DNA and make slide smear
to identify \( P. \ jiurovecii \) microscopically following GMS and Giemsa staining.
Bronchoscopic examination was performed in 79 patients. BALF was collected using strict
aseptic technique and filtered through a 2-layered nylon gauze to remove the mucus. The
filtered BALF was divided for DNA extraction (described below) and slide smear staining by
GMS and Giemsa staining.

**DNA extraction**

BALF samples were centrifuged at 1,500 rpm for 15 minutes and cell pellets were
collected. The sputa were pretreated with 4% NaOH (w/v %), washed with saline solution
approximately three times, and then centrifuged at 12,000 rpm for 10 minutes. After
centrifugation the supernatant was discarded. All cell pellets were washed three times
with PBS and then subjected to DNA extraction following the traditional protocol involving
proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation. The DNA
extracts were quantitated using the NanoDrop UV-Vis spectrophotometer (Thermo Fisher
Scientific, USA) and stored at -80°C before use.

**Real-time fluorescence LAMP assay**

All primers used in this study, including the external primer set (F3-B3), internal primer
set (FIP-BIP), and loop primer set (LF-LB) have been described previously [5]. The LAMP
reaction mixture contained 1.6 \( \mu \)M FIP-BIP, 0.2 \( \mu \)M F3-B3, 0.4 \( \mu \)M Loop F-B, and 2.5 \( \mu \)l 10×
Isothermal Amplification Buffer including 0.1% Triton X-100 or 100 \( \mu \)g/ml BSA. The
preferred buffer was 50 mM KCl, 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 1 mM DTT, 0.1% Triton X-100, and 50% glycerol (New England Biolabs, USA), 3.5 µl 10 mM dNTPs (Takara Biotechnology Co., LTD., Dalian, China), 1 µl 8 U/µl Bst DNA Polymerase, Large Fragment (New England Biolabs, USA), 1 µL100 mM MgSO₄ (New England Biolabs, USA), 0.5 µl 10µM SYTO 9.0 (Thermo Fisher Scientific, USA), 2 µl 5.0 M Betaine (Sigma-Aldrich, USA), 2 µl DNA template solution, and 6.5 µl RNase-free water (Takara Biotechnology Co., LTD., Dalian, China) up to a final volume of 25 µl. Each experiment included a no template control (with distilled water). The LAMP reaction was performed in the ABI 7500 system (ThermoFisher Scientific, USA) at 63℃ for 60 min, and terminated at 80℃ for 10 min. The entire LAMP amplification process was monitored in real time and the fluorescent signals recorded automatically by the ABI 7500 system. Compared with the conventional LAMP, the real-time LAMP was analyzed by the amplification curves. At the end of the reaction, white precipitates formed at the bottom of the test tube. In the analysis of conventional LAMP, visual detection was observed by adding 2 µl 1000× SYBR Green I (Thermo Fisher Scientific, USA) and the detection of LAMP amplicons were evaluated by 2% agarose gel electrophoresis and stained with GoodView (SBS Genetech Co., Ltd.) for visualization under UV radiation.

**Conventional PCR**

PCR was performed using the external primers targeting the 18S rRNA gene of *P. jirovecii* described elsewhere.[5] PCR mixture contained 12.5 µl of DNA polymerase Premix Taq™ (Takara Biotechnology Co., LTD., Dalian, China), 1-3 µl of DNA samples, 1 µl of 10 µM F3-B3 primer pair, and RNase-free water up to a final volume of 25 µl. Ultrapure distilled water was used as a negative control. PCR was performed under the following conditions: 94℃ for 5 min; 30 cycles of 94℃ for 30 s, 55 ℃ for 30 s, and 72℃ for 30 s, and 72℃ for 8
min. PCR products were separated by electrophoresis on 1.5% agarose gels, stained with GoodView (SBS Genetech Co., LTD. Beijing, China), and visualized by UV radiation.

**Sensitivity and specificity of conventional PCR and the real-time fluorescence LAMP assay**

To prepare a plasmid DNA standard, the PCR product, amplified using the external primers of the *P. jirovecii* 18S rRNA gene described above, was excised from the gel and purified using the agarose gel DNA extraction kit (Tiangen Biotech Co., LTD., Beijing, China). The recovered fragment was cloned into the TA cloning vector pMD18-T (Takara, Biotechnology Co., LTD., Dalian, China) with a standard TA cloning technique. DNA was extracted from one recombinant plasmid clone containing the correct sequence. DNA concentration was measured using the NanoDrop UV-Vis spectrophotometer (Thermo Fisher Scientific, USA). The sensitivities of the LAMP and PCR methods were compared using 10-fold serial dilutions of recombinant plasmid DNA (from $1 \times 10^0$ copies/µl to $1 \times 10^8$ copies/µl) in triplicate.

The specificities of the real-time fluorescence LAMP assay and conventional PCR were determined using genomic DNA from 10 other common respiratory microorganisms, including *Candida albicans, C. tropicalis, C. krusei, C. glabrata, C. parapsilosis, Aspergillus niger, Streptococcus pneumoniae, Escherichia coli, Acinetobacter baumannii, and Methicillin resistant Staphylococcus aureus* (MRSA). The genomic DNA of each microorganism was extracted using the Plant Genomic DNA Kit (Tiangen Biotech Co., LTD., Beijing, China) or the TIANamp Bacteria DNA Kit (Tiangen Biotech Co., LTD., Beijing, China). An aliquot of 10-100 ng of genomic DNA from each microorganism was used in each reaction.
Statistical analysis

All data were analyzed using the statistical analysis software SPSS (version 20.0, IBM Corporation, Chicago, IL, USA). Counting variables are represented as frequencies or percentages. Continuous variables are represented as the mean ± SD. Data related to clinical findings, imaging examinations, serologic parameters, and other indicators were compared using the \( c^2 \) test. Fisher’s exact test was used for the clinical data analysis when the number of samples was less than 40 or the expected value was less than 1. \( P \) values less than 0.05 were considered as statistically significant.

Results

Development of an improved real-time fluorescence LAMP assay for *P. jirovecii* detection

We developed a novel real-time fluorescence LAMP assay of *P. jirovecii*. This assay showed no cross-reactivity with 10 other common respiratory pathogenic microorganisms tested, suggesting its high specificity (Fig 1). The sensitivity of this assay was compared with that of conventional PCR using serial dilutions of recombinant plasmid DNA containing *P. jirovecii* 18S rRNA gene. As shown in Fig 2, the lowest detection threshold was \( 1 \times 10^4 \) copies/\( \mu l \) for the LAMP assay, which was 1,000 times more sensitive than that of conventional PCR (\( 1 \times 10^4 \) copies/\( \mu l \)).

We also compared the sensitivity of the new LAMP assay with conventional PCR using clinical specimens of patients with various respiratory diseases. The positive rate of *P. jirovecii* was 69.7% (281/403) by the LAMP assay and 40.5% (163/403) by conventional PCR. In only one case, positive by both assays, cyst forms of *P. jirovecii* were detected by GMS and Giemsa staining. There was a good coincidence of the *P. jirovecii* detection rate between the LAMP and conventional PCR. All the positive specimens detected by
conventional PCR were also positive in LAMP, however, the all positive specimens detected by LAMP were not positive in conventional PCR. All these data indicate that our modified real-time fluorescence LAMP method is reliable for the detection of *P. jirovecii*.

**Prevalence of *P. jirovecii* colonization in sputa and BALF from patients with various pulmonary diseases**

Of the 403 patients enrolled, the mean age was 66.7±12.5 years, including 248 (61.5%) males and 155 (38.5%) females. The overall prevalence of *P. jirovecii* colonization among all enrolled patients was 69.7% (281/403) (Table 1). The prevalence of *P. jirovecii* colonization in BALF of patients was higher compared with it in sputa, but the comparative differences between them were not significant (75.9% vs 68.2%, \( P > 0.05 \)) (Table 2). The prevalence of *P. jirovecii* colonization in ILDs patients was significantly higher compared with COPD and AECOPD patients (85% vs 67% and 43.3%, \( P < 0.01 \)). Furthermore, the prevalence of *P. jirovecii* colonization in AECOPD patients was higher than those with stabilized COPD (67.4% vs 43.3%, \( P < 0.01 \)) (Table 1).

**Presence of various comorbidities in patients with *P. jirovecii* colonization and pulmonary diseases**

More than half (230 or 57%) of all the enrolled patients presented with coexisting diseases including autoimmune diseases, connective tissue diseases, and other diseases (Table 3). Majority of these patients with comorbidities were positive for *P. jirovecii* (167 vs 63).

There were 24 patients with autoimmune diseases including rheumatoid arthritis (RA), autoimmune thyroid disease (AITD), chronic glomerulonephritis (CGN), Sjogren's syndrome, connective tissue disease (CTD), and mixed connective tissue diseases (MCTD). Notably, the frequency of *P. jirovecii* detection in patients with autoimmune diseases was
higher than those with the hypertension, diabetes, and coronary heart disease. Moreover, particular finding worth mentioning is the presence of a statistically significant difference in the frequency of *P. jirovecii* gene detection between patients with autoimmune diseases and those with the cardiac insufficiency (*P* < 0.05).

**Concurrent infection with other fungi or bacteria in patients with *P. jirovecii* colonization**

Most of the patients with *P. jirovecii* colonization (71/102 or 69.6%) had concurrent infection with other fungi and bacteria (Table 4). There were no significant differences in pair-wise comparison of the *P. jirovecii* colonization rate among patients co-infected with bacteria, those with other fungi, and those with both other fungi and bacteria (66.2% vs 69.6% vs 85.7%, *P* > 0.05).

**Association of *P. jirovecii* colonization with clinical characteristics of patients with various pulmonary diseases**

As illustrated in Table 5, no significant differences in PCT, LDH, and ESR measurements were observed between patients with *P. jirovecii* colonization and patients without (*P* > 0.05). However, patients with *P. jirovecii* colonization showed increased levels of BDG and CRP, and decreased levels of pulmonary function and CD4+ T-cell counts compared to patients without *P. jirovecii* colonization (*P* < 0.05). In addition, compared to patients without *P. jirovecii* colonization, patients with *P. jirovecii* colonization had significantly more abnormal findings on thoracic HRCT imaging, including reticulation opacities, cystic lesions, septal thickening, nodules, irregular linear opacities, and consolidation (*P* < 0.05).

**Discussion**

*P. jirovecii* is a life-threatening opportunistic pathogen in immunocompromised patients. As
extensively reviewed by Morris and Norris, *Pneumocystis* colonization, in contrast to *Pneumocystis* infection, is defined as the presence of *Pneumocystis* organisms (usually identified by PCR-based molecular biological techniques) without signs or symptoms of acute pneumonia.[2, 8] Colonization with *P. jirovecii* can even occur in individuals with normal immunity due to the development of highly efficient strategies to escape both innate and acquired immune defenses in host [1, 2]. These colonized individuals may serve as reservoirs of nosocomial infections. A high-level prevalence of *P. jirovecii* colonization in immunocompetent individuals has been reported in numerous studies throughout the world [2, 28]. The role of *P. jirovecii* colonization in certain pulmonary diseases, particularly among patients with COPD, has gained increasing attention [5, 8, 9, 29]. In the present multicenter study involving 403 non-HIV patients with various pulmonary diseases, we found a high prevalence of *P. jirovecii* colonization and this was associated with some clinical and laboratory characteristics of patients with pulmonary diseases, supporting the involvement of *P. jirovecii* colonization in pulmonary diseases. To better assess the prevalence of *P. jirovecii* colonization, we developed an improved real-time fluorescence LAMP assay for the detection of *P. jirovecii*, which showed high sensitivity and specificity. To avoid the inhibitory effect of SYBR Green I on LAMP amplification [17, 30], we chose to use SYTO-9 fluorescent dye (Thermo Fisher Scientific), which can be added into the reaction mixture without detectable inhibitory effect. This advantage allowed us to add real-time detection capacity to the LAMP assay, which demonstrated higher amplification efficiency and sensitivity compared to the previously described LAMP assay with end-point detection [5]. (Fig 3).

The overall frequency of colonization by *P. jirovecii* observed in this study (69.7%) appears to be higher than that reported in previous studies (16%-63.3%) [5, 31]. Moreover, the prevalence of *P. jirovecii* colonization in BALF of patients in this study (75.9%) is higher
than that previously reported (21/30 or 70%) [29]. In addition, in the this study, Nested PCR (mt LSU rRNA as Pneumocystis target gene) was compared with real-time fluorescence LAMP, and the positive rate was 58.56% (236/403), which was lower than the positive rate of real-time fluorescence LAMP. The reasons for these differences are unclear, but may be related to different target gene, different detection methods, or different patient populations tested. Another possibility is the high-dose, long-term steroid treatment in our patient population. P. jirovecii cysts were detected by GMS and Giemsa in BALF from only one of the four IPA patients. This patient had received prolonged steroid treatment (~ 4 months) due to multiple recurrent infections with other fungi and bacteria. Although, the steroid treatment is helpful in some pulmonary diseases, especially COPD, IPA, and bronchial asthma, high-dose steroids for a longer time lead to compromised lung function and immunity in patients, which might promote the colonization of P. jirovecii or the occurrence of PJP. The high prevalence of P. jirovecii colonization observed in this study is consistent with elevated serum beta-glucan levels in patients with P. jirovecii colonization compared to patients without P. jirovecii colonization (Table 5), as has been reported in many previous studies [32-34]. In addition to the high prevalence of P. jirovecii colonization in various pulmonary diseases, this study provides several other lines of evidence supporting P. jirovecii colonization as a risk factor for the development of pulmonary diseases. In this study, HRCT imaging findings suggested that the ground-glass opacity (GGO) could be a result of P. jirovecii colonization in patients with pulmonary diseases. Compared to patients without P. jirovecii colonization, patients with P. jirovecii colonization showed a decreased pulmonary function as assessed by the FEV1/FVC values (Table 5), which may suggest a possibility of damage to the respiratory tract due to long-term colonization. This possibility is further supported by the elevated serum C-reactive protein (CRP) levels in patients with P. jirovecii colonization, as CRP is a sensitive marker
of inflammation and tissue damage. Finally, consistent with previous studies [35-37], we also observed decreased CD4+ T-cell counts in patients with *P. jirovecii* colonization compared to patients without *P. jirovecii* colonization, which suggests an impaired T cell-mediated immunity.

Patients with chronic pulmonary diseases frequently suffer from various comorbidities (Table 3), which may also contribute to the severity of diseases. In this study, we found a high rate of underlying autoimmune diseases in patients with *P. jirovecii* colonization (Table 3). This finding can be explained by the widespread administration of systemic high-dose steroids in these patients [38, 39].

Despite increasing reports of *P. jirovecii* colonization, there are only a few studies of coinfection with other respiratory pathogens in patients with *P. jirovecii* colonization [40-42]. This study found that the majority of individuals with *P. jirovecii* colonization had concurrent infections with other fungal or bacterial pathogens (Table 4). Further studies are needed to assess the differential contribution of these pathogens to the pulmonary diseases.

This study did have certain limitations. First, there were certain heterogeneities in the sample types (sputum and BALF samples) and underlying clinical conditions, which might have caused biases in data interpretation. Second, we did not subtract coinfection with other fungi or bacteria when assessing the association of *P. jirovecii* colonization with clinical characteristics due to the small sample size after subtraction. Third, sputum specimens are not the best specimens to examine the prevalence of coinfection with other fungi or bacteria in the lungs due to unavoidable contamination with normal microbial flora in the oral cavity and nasopharynx. In this study, we used a large number of sputum specimens, due to the limited availability of BALF samples. Lastly, we represented the data on the basis of individual pulmonary diseases; however, due to retrospective nature
of this study, we lacked the data on severity of individual pulmonary disease and thus, could not represent the data on the basis of severity, such as the GOLD staging for COPD and the severity classification of asthma.

In summary, we have developed an improved LAMP assay for detecting *P. jirovecii*. This multi-center study involving 403 patients, using this assay, has provided additional new evidence for the involvement of *P. jirovecii* colonization in the development of pulmonary diseases in non-HIV patients, and highlights the need to further study the pathogenesis and transmission of *P. jirovecii* in pulmonary diseases.

**Abbreviations**

PJP: *Pneumocystis jirovecii* pneumonia; COPD: Chronic obstructive pulmonary disease; HIV: Human immunodeficiency virus; LAMP: Loop-mediated isothermal amplification; AECOPD: acute exacerbations of chronic obstructive pulmonary disease; AECB: acute exacerbations of chronic bronchitis; ILDs: interstitial lung diseases; IPA: invasive pulmonary aspergillosis; BALF: bronchoalveolar lavage fluid; ESR: erythrocyte sedimentation rate; LDH: lactate dehydrogenase; PCT pro-calcitonin; CRP: C-reactive protein; BDG: 1,3-β-D-glucan; HRCT: high-resolution computed tomography; GGO: the ground-glass opacity.

**Declarations**

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Clinical Laboratory of the Eleventh Affiliated Hospital of Shanxi Medical University and the Department of Respiratory Internal Medicine of the General Hospital of Shenyang Military Command.

Contributions
This study was designed and conceived by Ting Xue and Chun-Li An, and performed and analyzed by Ting Xue. Data acquisition, statistical analysis and manuscript preparation were performed by Ting Xue. Zhuang Ma, Fan Liu, Wei-Qin Du, and Li He participated in the specimen collection and patients’ management. Jin-Yan Wang and Chun-Li An reviewed and edited the manuscript. All authors contributed to drafting and revising the paper, as well as analyzing data. All authors have read and approved the final manuscript and are responsible for all aspects of this study.

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Availability of data and materials
The data used in this study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
This retrospective study was approved by the Institutional Ethics Review Board, China Medical University (2013070). Written informed consent was obtained from all the patients involved in this study.

Consent for publication
Not applicable.

Conflict of interest statement
The authors declared no conflict of interests.

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Tables

Table 1. *P. jirovecii* colonization rate in patients with pulmonary diseases
|                              | Patients with colonization (n = 281) | Patients without colonization (n = 122) | All patients (n = 403) | Positive rate (%) |
|------------------------------|-------------------------------------|----------------------------------------|------------------------|-------------------|
| Age (years)                  | 66.21 ± 13.23                       | 67.98 ± 10.63                          | 66.74 ± 12.51          |                   |
| Male (%)                     | 66.5                               | 33.5                                   | 61.5                   |                   |
| COPD                         | 160                                | 88                                      | 248                    | 64.52*            |
| AECOPD                       | 147                                | 71                                      | 218                    | 67.43**           |
| Stable stage                 | 13                                 | 18                                      | 30                     | 43.33             |
| Interstitial lung diseases   | 55                                 | 10                                      | 65                     | 84.62             |
| IIP                          | 47                                 | 8                                       | 55                     | 84.45             |
| Sarcoidosis                  | 8                                  | 0                                       | 8                      | 100               |
| PAP                          | 0                                  | 2                                       | 2                      | 0                 |
| AECB                         | 27                                 | 13                                      | 40                     | 67.50             |
| Bronchiectasis               | 30                                 | 8                                       | 38                     | 78.95             |
| Bronchial asthma             | 2                                  | 3                                       | 5                      | 40.0              |
| IPA                          | 4                                  | 0                                       | 4                      | 100               |
| Type 1 respiratory failure   | 3                                  | 0                                       | 3                      | 100               |

Data are expressed as numbers (%). *P < 0.01 compared with ILDs; P > 0.05 compared with AECB, bronchiectasis and bronchial asthma; ** P < 0.01 compared with the stable stage.

Abbreviations: COPD, chronic obstructive pulmonary disease; AECOPD, acute exacerbation of COPD; AECB, acute exacerbations of chronic bronchitis; IPA, invasive pulmonary aspergillosis; PAP, pulmonary alveolar proteinosis; IIP, idiopathic interstitial pneumonia.

Table 2. *P. jirovecii* colonization rate in BALF and sputa of the patients
Patients with colonization (n=281) | Patients without colonization (n=122) | Total (n=403) | Positive rate (%) |
---|---|---|---|
*P. jirovecii* colonization in BALF | 60 | 19 | 79 | 75.94* |
*P. jirovecii* colonization in sputa | 221 | 103 | 324 | 68.20 |

* P > 0.05 compared with *P. jirovecii* colonization in sputa.

Table 3. Comorbidities in patients with *P. jirovecii* colonization

| Comorbidities                          | Patients with colonization | Patients without colonization | Total | Positive rate (%) |
|----------------------------------------|---------------------------|------------------------------|-------|------------------|
| Hypertension                           | 45                        | 12                           | 57    | 78.95            |
| Cardiac insufficiency                  | 31                        | 20                           | 51    | 60.78            |
| Diabetes mellitus                      | 25                        | 8                            | 33    | 75.76            |
| Coronary heart disease                 | 24                        | 9                            | 33    | 72.73            |
| Autoimmune diseases                    | 24                        | 5                            | 29    | 82.75#           |
| Systemic inflammatory response syndrome| 9                         | 2                            | 11    | 81.82            |
| Others                                 | 9                         | 7                            | 16    | 56.25            |
| **Total**                              | **167**                   | **63**                       | **230** | **72.61**        |

# P > 0.05 compared with hypertension, diabetes, coronary heart disease and systemic inflammatory response syndrome; P < 0.05, > 0.01, compared with the cardiac insufficiency group.

Table 4. Concurrent infection with other fungi and bacteria in patients with *P. jirovecii* colonization
| Microorganism                        | Positive | Negative | Total | Positive rate (%) |
|-------------------------------------|----------|----------|-------|-------------------|
| **Bacteria**                        |          |          |       |                   |
| *Streptococcus pneumoniae*          | 10       | 5        | 15    | 66.67             |
| *Pseudomonas aeruginosa*            | 11       | 3        | 14    | 78.57             |
| *Klebsiella pneumoniae*             | 5        | 4        | 9     | 55.56             |
| *Haemophilus influenzae*            | 4        | 3        | 7     | 57.14             |
| *Acinetobacter baumannii*           | 4        | 1        | 5     | 80.00             |
| **Others**                          | 9        | 6        | 15    | 60.00             |
| **Other fungi**                     | 16       | 7        | 23    | 69.57             |
| *Candida albicans*                  | 11       | 6        | 17    | 64.71             |
| *Aspergillus fumigatus*             | 4        | 0        | 4     | 100               |
| **Others**                          | 1        | 1        | 2     | 50.00             |
| **Other fungi and bacteria**        | 12       | 2        | 14    | 85.71             |
| *P. aeruginosa and C. albicans*     | 3        | 0        | 3     | 100               |
| **Others**                          | 9        | 2        | 11    | 81.82             |
| **Total**                           | 71       | 31       | 102   | 69.61             |

* P > 0.05 compared with fungal infection alone and infection with both fungi bacteria.

**Table 5.** Association of *P. jirovecii* colonization with laboratory findings of the patients
| Test                        | Positive  | Negative | Total | Positive rate (%) |
|-----------------------------|-----------|----------|-------|--------------------|
| Serum BDG levels            |           |          |       |                    |
| Positive (>10 pg/ml)        | 34        | 5        | 39    | 81.18\(^a\)        |
| Negative (<10 pg/ml)        | 128       | 50       | 178   | 71.91              |
| Serum PCT levels            |           |          |       |                    |
| Positive (>0.5 ng/mL)       | 59        | 22       | 81    | 72.84\(^b\)        |
| Negative (<0.5 ng/mL)       | 103       | 33       | 136   | 75.74              |
| Serum LDH levels            |           |          |       |                    |
| Positive (>225 U/L)         | 84        | 33       | 117   | 71.79\(^c\)        |
| Negative (<225 U/L)         | 197       | 89       | 286   | 68.88              |
| Serum CRP levels            |           |          |       |                    |
| Positive (>5 mg/L)          | 171       | 59       | 230   | 74.35\(^d\)        |
| Negative (<5 mg/L)          | 110       | 63       | 173   | 63.58              |
| ESR test                    |           |          |       |                    |
| Abnormal                    | 78        | 33       | 111   | 70.27\(^e\)        |
| Normal                      | 109       | 51       | 160   | 68.13              |
| Thoracic HRCT findings      |           |          |       |                    |
| GGO positive                | 67        | 14       | 81    | 82.72\(^f\)        |
| GGO negative                | 95        | 41       | 136   | 69.85              |
| Pulmonary function          |           |          |       |                    |
| FEV1/FVC positive (<70%)    | 25        | 6        | 31    | 80.65\(^g\)        |
| FEV1/FVC negative (>70%)    | 14        | 11       | 25    | 56.00              |
| CD\(_4^{+}\) T-cell count   |           |          |       |                    |
| < 410/mm\(^3\)             | 23        | 2        | 25    | 92.00\(^h\)        |
| > 410/mm\(^3\)             | 53        | 24       | 77    | 68.83              |

\(^a\)P < 0.05, >0.01, Compared with serum BDG < 10 pg/mL.\(^b\)P > 0.05 Compared with serum PCT < 0.5 ng/mL.

\(^c\)P > 0.05 compared with LDH levels < 225 U/L.\(^d\)P < 0.05, > 0.01, compared with CRP negative (< 5mg/L).

\(^e\)P > 0.05 compared with ESR normal. ESR abnormal (> 15 mm/h for men and > 20 mm/h for women). ESR normal (≤ 15 mm/h for men and ≤ 20 mm/h for women).

\(^f\)P < 0.05, >0.01, compared with GGO negative.\(^g\)P < 0.05, > 0.01, compared with FEV1/FVC negative (>70%).
hP < 0.05, > 0.01, compared with CD4+ T-cell count > 410/mm³.

Abbreviations: BDG, 1,3-β-D-glucan; PCT, pro-calcitonin; LDH, lactate dehydrogenase; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; GGO, ground-glass opacity; HRCT, high-resolution computed tomography; FEV1, forced expiratory volume in one second; FVC, forced vital capacity.

Figures

Figure 1

The specificity of the real-time fluorescence LAMP assay for P. jirovecii detection.

A) A representative result of the LAMP assay. The green curve (labeled as 1) represents the LAMP amplification plot of one positive specimen of P. jirovecii.
Other flat curves near the bottom represent amplification plots of control samples, including the negative control (with water) and other common respiratory microbes such as Candida albicans, C. tropicalis, C. krusei, C. glabrata, C. parapsilosis, Aspergillus niger, Streptococcus pneumoniae, Escherichia coli, Acinetobacter baumannii, and Methicillin resistant Staphylococcus aureus (MRSA). B) A representative result of 2% agarose gel electrophoresis analysis of LAMP products. M, DNA size marker; lane 1, no template control; lane 2, P. jirovecii-positive specimen. Lanes 3-12 are C. albicans, C. tropicalis, C. krusei, C. glabrata, C. parapsilosis, A. nige, S. pneumoniae, E. coli, A. baumannii, and Methicillin resistant S. aureus (MRSA), respectively.
Comparison of the sensitivity of real-time fluorescence LAMP assay and conventional PCR using 10-fold serial dilutions of recombinant plasmid DNA
containing P. jirovecii 18S rRNA gene. A) Amplification plots of the real-time fluorescence LAMP assay (from 1×1010 copies/μl for curve 1 to for 1×100 copies/μl curve 11, and no template control for curve 12). B) 2% agarose gel electrophoresis of LAMP products from serial dilutions of plasmid from 1×1010 copies/μl for lane 1 to for 1×100 copies/μl lane 11. M, DNA size marker. C) 2% gel electrophoresis of conversional PCR products from serial dilutions of plasmid from 1×1010 copies/μl for lane 1 to for 1×100 copies/μl lane 11. M, DNA size marker.
Comparison of different methods for detecting LAMP products. A) Amplification plots of real-time fluorescence LAMP. Curve 1, no template control; curve 2, P. jirovecii-negative patient specimen; curves 3-5, P. jirovecii-positive specimens. B) Direct visual detection of end-point products as white precipitate at the bottom of reaction tubes: Tube 1, no template control (no precipitate); tube 2, P. jirovecii-negative patient specimen (no precipitate); tubes 3-5, P. jirovecii-positive specimens (precipitate at the bottom). C) Visual detection of end-point products mixed with SYBR Green I fluorescent dye (Thermo Fisher Scientific). Tube 1, No template control (orange color); tube 2, P. jirovecii-negative patient specimen (orange); tubes 3-5, P. jirovecii-positive patient specimens (emerald green).
