Review

Pneumocystis jirovecii Pneumonia: Current Advances in Laboratory Diagnosis

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Abstract:

Pneumocystis jirovecii pneumonia (PcP) remains a major cause of respiratory illness among immunocompromised patients. PcP is difficult to diagnose, in particular in non-HIV-infected patients, due to the lack of associated specific clinical data. Since *P. jirovecii* could not be cultivated for many years, microscopic visualization of cystic or trophic forms in respiratory specimens based on cytochemical or immunofluorescence staining are the standard procedure to identify this fungus. Polymerase chain reaction (PCR)-based methodologies have been developed to overcome the low sensitivity of microscopy in respiratory specimens, especially those with low fungal load and in non-HIV-infected patients. Real-time quantitative PCR is the only format suitable for a quantitative diagnosis, and these results have been used to differentiate PcP active disease (high fungal load) from carriage/colonization (low fungal load). However, its use is inconclusive with limited results in intermediate fungal loads. New strategies based on measurement of blood biomarkers

may be a viable alternative to perform PcP diagnosis non-invasively. Several studies explored the usefulness of candidate serum biomarkers, such as (1-3)-β-D-Glucan, Krebs von den Lungen-6 antigen, lactate dehydrogenase, and S-adenosylmethionine, with the former presenting the most promising results. More recently, approaches based on the detection of specific anti-*P. jirovecii* antibodies in patients' sera are showing encouraging results that could enable a faster and inexpensive screening and diagnosis of this opportunistic infectious disease, helping to improve therapeutic interventions, disease control, and provide retrenchment to healthcare systems.

Keywords
Pneumocystis jirovecii; pneumonia; laboratory diagnosis; current methods; new alternatives

1. *Pneumocystis* Pneumonia Infection

Pneumocystis jirovecii (previously called *Pneumocystis carinii f. sp. hominis*) is an opportunistic fungus of ubiquitous distribution with specificity restricted to humans. This pathogen is capable of causing fatal interstitial pneumonia (PcP) in immunocompromised hosts, especially in those infected by the human immunodeficiency virus (HIV), but also in patients who are undergoing immunosuppressive treatments related to other pathologies. This disease is also emerging as a co-morbidity factor associated with chronic diseases, such as chronic obstructive pulmonary disorder (COPD), and can cause asymptomatic infection in immunocompetent persons [1, 2, 3]. *Pneumocystis* infections are, as a rule, confined to the lungs. However, infections in other organs or tissues have been reported [4].

During the last 30 years, PcP proved to be an extremely important disease associated with HIV infection and the acquired immune deficiency syndrome (AIDS) epidemic. During the first years of the HIV/AIDS epidemic, PcP functioned as the main indicator associated with HIV infection; in recent years, it still plays a relevant role in the clinical picture of HIV-infected patients, since recent reports point to PcP as the most common AIDS-defining illness in Europe in 2016. This disease was recorded in 20.0% of the cases, followed by pulmonary and/or extra-pulmonary tuberculosis (15%), oesophageal candidiasis (11%), and wasting syndrome due to HIV (10%). This demonstrates that even in the era of combination antiretroviral therapy (cART), PcP remains a significant cause of morbidity and mortality in patients with HIV/AIDS in developed countries [5]. In developing countries, the scenario is even worse because in addition to the large number of HIV-infected patients, access to cART, PcP diagnosis, and prophylaxis is limited due to lack of resources and expertise [6-9].

Therefore, the control and prevention of this disease is still an area that requires much attention from a public health point of view in all countries. One way to meet this need is by improving the tools for an early and accurate diagnosis of *Pneumocystis* pneumonia.

2. Clinical and Laboratory Assessment of PcP

PcP does not present pathognomonic clinical, radiologic or gasometric findings. Therefore, the diagnosis of this disease depends on a clinical assessment based on non-specific clinical

manifestations, pulmonary function, arterial blood gas and radiological testing, plus non-specific and specific laboratory tests. Nevertheless, PcP presentation depends on the underlying disease and usually the clinical and laboratory findings are less severe in patients immunocompromised by other pathologies rather than HIV infection [10].

The non-specific clinical manifestations of a patient with PcP include fever, non-productive cough and dyspnea. The most common radiological presentation of this pathology is a pattern of bilateral interstitial pneumonia, but may vary depending on the degree of immune deficiency, the presence of other concomitant infections, or the use of pentamidine in the prophylaxis of PcP. Blood gas analysis shows hypoxemia that worsens with exercise and a partial pressure of oxygen (PaO_2) in peripheral blood $\leqslant$ 9.3 kPa (70 mmHg) is indicative of PcP [1, 11]. In terms of non-specific laboratory tests, the measurement of increased levels of lactate dehydrogenase (LDH) can be used as a prognostic tool and to assess response to therapy [12]. Since the risk of developing the disease increases with a $CD4^+$ T cell count $\leqslant$ 200/mm^3 in all patients [1, 13, 14], this analysis is crucial not only for the diagnosis, but also for the prevention and control of PcP. In spite of the usefulness of all these clinical and laboratory data, a definitive diagnosis still depends on the detection of *P. jirovecii* in the affected tissues, which is only possible with specific laboratory methodologies.

In contrast to most pathogenic microorganisms, the absence of a sustained, stable, and reproducible *P. jirovecii* culture medium has been a significant limitation for the disease diagnosis [15]. Although a promising culture system to propagate *P. jirovecii in vitro* was developed in 2014 [16], it still needs to be validated since some authors were unable to reproduce the results obtained in 2014 [17]. Thus, the isolation, cultivation, and propagation of this fungus remains a challenge for diagnostic purposes.

Meanwhile, the evolution and improvement of classical diagnostic methods (such as cytochemical or immunofluorescent staining and detection of DNA of the organism by molecular techniques) have enabled an improvement in the detection of *P. jirovecii*, mainly in respiratory specimens. However, the successful diagnosis of PcP based on these methods is dependent on the resources and expertise of the laboratory team, as well as the type of biological specimen analysed. Therefore, finding simple methods and minimally invasive specimens, such as blood for rapid and effective detection of the causative agent, has become an urgent need. This need is especially critical in low-to-middle income countries, where it is difficult to implement classical laboratory diagnostic techniques and the appropriate methods for respiratory specimen collection, since both require specialized personnel, expensive equipment, and structures that are not readily available, in most cases [9, 18]. This urgency leads to developing new strategies and new approaches for PcP diagnosis.

2.1. Classical Methods for PcP Laboratorial Diagnosis

The classical diagnosis of PcP is based on the identification or detection of *P. jirovecii* in the affected tissues by cytochemical staining, immunofluorescent staining with monoclonal antibodies (IFI-MAb), and/or the detection of *Pneumocystis* DNA by molecular techniques. The most important characteristics of the commonly applied and studied laboratory diagnostic methods for PcP classical diagnosis are summarized in Table 1.

Table 1 Characteristics of the classical laboratory methods for PcP diagnosis (adapted from [26]).

Method	Technique	Sensitivity	Specificity	Specimens Recommended	Advantages	Disadvantages	References
Microscopy	GMS	79% (BALF)	99% (BALF)	BALF or Biopsy	Allows semi-quantification methods; only needs an optical microscope.	Needs experienced microscopist; needs invasive and costly samples; time-consuming protocol; only identify cystic forms; recommended combination with Giemsa or Giemsa-like stains.	[83]
	TBO	68% (BALF)	100% (BALF)	BALF or Biopsy			[84]
	Giemsa or DQ	68% (BALF)	88% (BALF)	BALF or Biopsy	Rapid/easy protocol; allows identification of trophic forms and spores; allows semi-quantification methods; only needs an optical microscope.	Needs experienced microscopist (difficult to read); needs invasive and expensive samples; recommended combination with GMS or TBO; allows semi-quantification methods.	[85]
	IF	97% (BALF)	100% (BALF)	BALF, IS or Biopsy	Excellent sensitivity/ specificity (robustness); most accurate/ robust microscopic method; easy to read; allows identification of cystic and/or trophic forms; allows semi-quantification methods.	Needs invasive and expensive samples; needs expensive/ specific equipment (fluorescence microscope).	[86]
Molecular	nPCR	76-100% (BALF)	53-86% (BALF)	BALF, IS or Biopsy (OW, SS and NA also possible)	Alternative non-invasive samples may be used; detection of low fungal burdens; allows genotyping.	Needs experienced/qualified staff; high rate detection of colonised patients (false positives); needs expensive/ specific equipment (thermocycler).	[47]

	RT-qPCR	94-99% (BALF)	89-96% (BALF)	BAL, IS or Biopsy (OW, SS and NA also possible)	Alternative non-invasive samples may be used; detection of low fungal burdens; allows quantification.	Needs experienced/qualified staff; high rate detection of colonised patients (false positives); needs expensive/ specific equipment (real-time apparatus).	[31]

GMS, Grocott's Methenamine Silver stain; TBO, Toluidine Blue O; DQ, Diff-Quick; IF, immunofluorescence staining; nPCR, nested-PCR; RT-qPCR, real-time quantitative PCR; BALF, bronchoalveolar lavage fluid; IS, induced sputum; OW, oropharyngeal washing; SS, spontaneous sputum; NA, nasopharyngeal aspirate.

Biological specimens. Specimens of the lower respiratory tract, such as bronchoalveolar lavage fluid (BALF) or induced sputum (IS), are the most commonly utilized for the diagnosis of PcP. These specimens and others, such as open-lung biopsy (LB), transbronchial biopsy (TBB), and bronchial secretions (BS), are obtained by invasive techniques that, in addition to being onerous, are difficult to perform in patients with respiratory failure, children, and especially difficult to implement in low-to-middle income countries.

Lung biopsy (LB) is the gold standard procedure for the assessment of inflammatory lung conditions in immunocompromised patients [19], allowing the observation of the microorganism in more than 95% of the infection cases [20]. BALF, which is collected through fiber optic bronchoscopy, allows diagnosis in more than 80% of all patients with PcP, and in more than 95% of patients concurrently infected with HIV [21, 22]. Sputum induction, which is obtained in a less invasive way through inhalation of 1.8% saline with the aid of an ultrasonic nebulizer, can be applied with good diagnostic yield in AIDS patients. However, in patients with other forms of immunodeficiency who generally have a lower burden of *P. jirovecii*, IS may have less diagnostic utility [23]. The non-specific staining of IS specimens detects *Pneumocystis* in 30-55% of cases of infection and the results are sometimes difficult to interpret [19, 24]. Sensitivity can be improved to 60-97% with IS liquefaction using dithiothreitol, followed by cell sedimentation and analysis by immunofluorescence staining with anti-*P. jirovecii* monoclonal antibodies (MAb-IF) or PCR techniques [23-25]. As an alternative, other specimens like spontaneous sputum (SS), nasopharyngeal aspirate (NA), or oropharyngeal washing (OW), which are obtained less invasively, can also be used for diagnosis of PcP but have even lower diagnostic yields than BALF or IS [26]. Molecular techniques can be used as an alternative to improve the sensitivity of detection when using these less invasive respiratory specimens (SS, NA, OW) for PcP diagnosis [26-31], due to their lower fungal burden.

The diagnosis of extrapulmonary *P. jirovecii* infection is performed by the demonstration of developmental forms of the organism in the specific infected tissues. In these cases, non-specific as well as specific staining methods or a PCR method to detect *Pneumocystis* DNA can be applied, depending on the biological specimen available.

Staining methodologies. Several cytochemical staining methods exist for PcP diagnosis: Gomori methenamine silver (GMS); Gram-Weigert (GW); Giemsa; rapid Giemsa-like stains such as Diff-Quik (DQ); toluidine blue O (TBO); cresyl echt violet; and calcofluor white. These methods, despite being able to reveal the characteristic morphology of the cystic and/or the trophic forms of *Pneumocystis*, are non-specific and can stain other microorganisms.

The GMS stain technique was first described by Gomori and then modified by Grocott (1955) and Musto (1982) [32, 33] and was considered the gold standard for the diagnosis of PcP for many years. The reagent selectively stains the wall of the cystic forms of *Pneumocystis*, which appears dark brown (Figure 1A) [32]. Cystic forms of *P. jirovecii* can also be identified by GW and TBO staining, which have a good affinity for the cystic form wall components, selectively staining them in purple/blue or reddish violet (Figure 1B), respectively [34]. The cresyl echt violet technique (which is similar to TBO) and the chemifluorescent reagent calcofluor white (which binds non-specifically to β-linked polysaccharide polymers of the cell wall of *Pneumocystis* [35]) can also be used to identify *Pneumocystis* cystic forms, but not the other forms. Conversely, Giemsa and Diff-Quik do not stain sporocytic or cystic walls, but stain the nuclei of all *Pneumocystis* life cycle stages (Figure 1C) [36]. All of these methods can be applied in any kind of clinical specimen, but they are

non-specific, presenting affinity to other lung pathogens. Therefore, the reading of the microscopic slides requires training and expertise, especially when dealing with patients with low fungal burden. When only non-specific staining methods are available, a good strategy for a more accurate PcP diagnosis is the concomitant use of a method that stains the nuclei of the developing forms (Giemsa or Diff-Quik) and another one that stains the wall of the cystic form (GMS, GW, TBO, calcofluor, or cresyl echt violet) in different smears from the same specimen [14, 27].
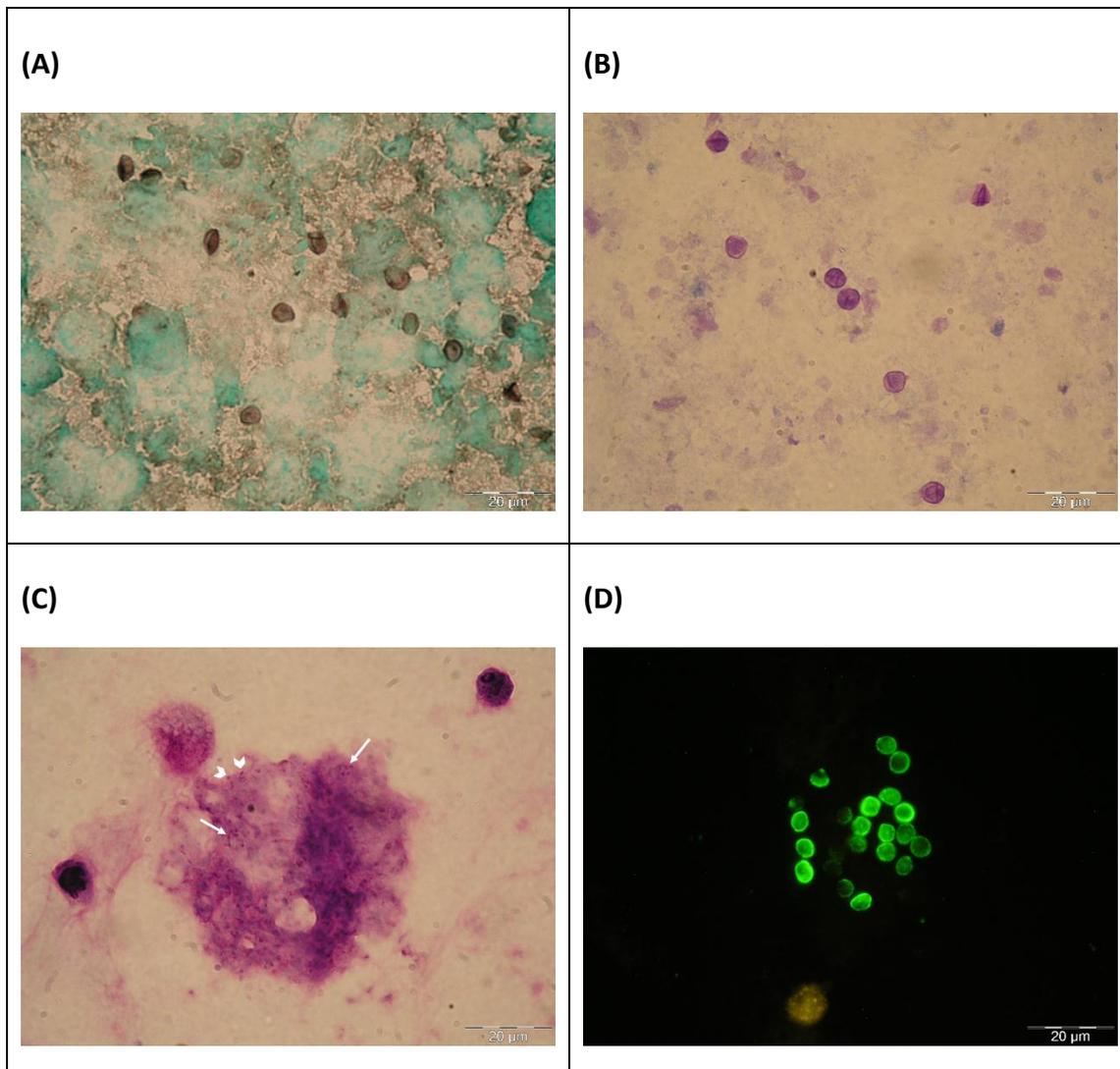

Figure 1 Rat and human respiratory specimens with *Pneumocystis* after proper staining (magnification X 1,000). **(A)** Rat-derived *Pneumocystis* cystic forms stained with Gomori methenamine silver (GMS). **(B)** Rat-derived *Pneumocystis* cystic forms stained with toluidine blue O (TBO). **(C)** Cluster of *Pneumocystis jirovecii* mature cystic forms (arrows) and trophic forms (arrowhead) in BALF stained with Giemsa. **(D)** Clustered cystic forms of *Pneumocystis* in BALF stained with IF-MAb anti-*Pneumocystis jirovecii.*

Specific staining techniques appeared with the development of monoclonal antibodies (MAbs) specific for *P. jirovecii* in 1986, which allowed the appearance of immunofluorescent techniques for the diagnosis of PcP [37, 38]. These staining methods are considered more sensitive and specific than the cytochemical ones, and have begun to be applied as the preferred method in less

than optimal specimens (IS, BS, NA, and OW) [24, 25, 38-40]. Currently, it is also possible to find some direct and indirect immunofluorescent assays (DFA and IFA, respectively) in the market for clinical use. The IFA identifies only the cystic forms while the DFA identifies both cystic and trophic forms. In these commercial kits, the anti-*P. jirovecii* MAbs are conjugated with fluorescein isothiocyanate (FITC) and, when they bind to the specific antigen of *P. jirovecii*, the cystic and/or trophic forms appear with a characteristic apple-green fluorescence when exposed to a given wavelength (Figure 1D). Nowadays, because of its reliability, these immunofluorescent stains are the most commonly used technique in the diagnosis of PcP [26, 41-43].

Molecular biology methodologies. The ability to detect *P. jirovecii* DNA in clinical specimens by applying molecular tools has brought important advances in the diagnosis, epidemiology, and management of PcP in the last few decades. PCR methods allow the early detection of *P. jirovecii* DNA in respiratory specimens from patients that tested negative via a microscopic examination [29, 44, 45]. Thus, reducing the time from the onset of symptoms to the diagnosis and treatment of the disease, improving the prognosis, and avoiding the progression from the initial case of PcP to a serious illness with associated respiratory failure and significant mortality [12, 26].

The efficiency of the molecular methods depends on the type of specimen analysed. Biopsies (which are highly invasive) are rarely used, while BALF is the standard specimen. Less invasive specimens like IS, NA, and OW can also be used but compromise the final overall sensitivity of the molecular PcP diagnostic protocol [26, 44, 46-49]. However, a positive PCR test associated with a negative microscopy causes ambiguity in the diagnosis of PcP, since it may reflect cases of either PcP or *P. jirovecii* colonization. This doubt can only be resolved by combining the laboratory results with the patient's clinical picture [14]. In clinical practice, a case of *P. jirovecii* colonization is considered when *P. jirovecii* DNA is detected by PCR in a biological specimen of an immunocompromised or immunocompetent individual without clinical manifestations of PcP, and is also accompanied by a negative staining result [1, 2, 50]. In these cases, the false negative results of the microscopic examinations are due to the very low fungal burdens that are difficult to detect [51].

Different PCR techniques and gene targets have been reported for PcP molecular diagnosis [26, 52-69]. The mitochondrial large subunit rRNA (mtLSUrRNA) nested-PCR procedure, with a detection threshold that can reach values of 0.5-1 organism/µL of sample [14, 26, 29], is the most used. Reports have shown that it is the most sensitive method among nine PCR assays evaluated for detection of *P. jirovecii* DNA [70]. This technique produced less false negative results and presented higher concordance with microscopic data than mtLSUrRNA single-PCR, internal transcribed spacers (ITS) nested-PCR, dihydropteroate synthase (DHPS) single- and nested-PCR, dihydrofolate reductase (DHFR) nested-PCR, major surface glycoprotein (MSG) heminested-PCR, 18S ribosomal RNA (18S rRNA) 1-tube nested-PCR, and 5S ribosomal RNA (5S rRNA) real-time quantitative PCR (RT-qPCR) [70]. In addition, the DHPS and DHFR PCR assays showed low diagnostic specificity in several studies [71-73]. The fact that mitochondrial mtLSU rRNA is a multicopy gene in the *P. jirovecii* genome, compared with other target genes that are single nuclear encoded genes (e.g. DHPS, DHFR, ITS), contributes largely to the higher successful amplification rates of the PCR procedures that target this gene, especially with the nested-PCR technique [26, 56, 74, 75]. A bivariate meta-analysis and systematic review of 16 studies of PCR-based assays of a total of 1857 BALF from 1793 patients recorded a sensitivity of 98.3% (95% CI, 91.3%-99.7%) and specificity of 91.0% (95% CI, 82.7%-95.5%), which suggests that the application

of these methodologies in BALF is a very accurate method for the diagnosis of PcP [47]. In particular, nested-PCR-based *P. jirovecii* detection techniques demonstrated a high sensitivity of 98% (95% CI, 76-100%) and a relatively medium specificity of 73% (95% CI, 53-86%) [47]. Once again, this could be explained by colonized/asymptomatic patients that test as false positive by nested-PCR although the symptomatic infection is not established. Therefore, this indicates that although nested-PCR protocols present high sensitivity in the detection of *P. jirovecii* DNA in respiratory specimens, a positive result with a negative microscopic examination always needs to be clinically investigated because it can correspond to a case of colonization, a symptomatic case, or even to a case of contamination [47, 76].

P. jirovecii burden quantification is another theme of interest that prompted the development of several molecular strategies. The application of RT-qPCR protocols targeting the MSG multigene family, β-TUB, KEX1 genes, mtLSUrRNA, and cdc2 genes of *P. jirovecii* have been reported [45, 74, 77-80]. A meta-analysis study assessing the use of RT-qPCR protocols for the diagnosis of PcP in immunocompromised patients from 10 individual studies from 1990 to 2010 showed an overall sensitivity of 97% (95% CI, 93%-99%) and specificity of 94% (95% CI, 90%-96%) [31]. In the subgroup of HIV-infected patients, *P. jirovecii* DNA was detected with a sensitivity of 97% (95% CI, 93%-99%) and a specificity of 93% (95% CI, 89%-96%). The DNA detection in BALF demonstrated a sensitivity of 98% (95% CI, 94%-99%) and specificity of 93% (95% CI, 89%-96%) [31]. Despite good diagnostic accuracy results, RT-qPCR protocols still need to be investigated in new studies to identify any differences in the diagnostic performance of this method in HIV-infected versus other immunocompromised patients as well as in differentiating colonization from active disease [26]. Also, in order to improve accuracy in the management of PcP, thresholds should be assessed according to underlying diseases and other clinical and radiological parameters [80]. To guarantee clinical value of the results obtained, it is essential that both specificity and sensitivity are ⩾95%. In addition, when interpreting the significance of the fungal burden, the quality of the biological specimen under study and the underlying condition should also be taken into consideration.

In conclusion, although nested-PCR and RT-qPCR assays (especially the ones targeting the mtLSUrRNA gene) are consistently indicated as the most sensitive and specific molecular tools for *P. jirovecii* DNA detection [46, 51, 57, 73, 77, 81, 82], it will be most helpful for diagnostic laboratories to choose standardized commercial tests that have developed a criterion for results interpretation.

2.2. Alternatives to the Classical Laboratory Diagnosis of PcP

The success of classical methods for PcP diagnosis depends on: resources and technology of the laboratory, the team's experience, and the type of biological specimen to be analysed. The desire to use biological specimens obtained by less invasive techniques and less restricted technologies attracted the attention to and interest in the blood and serum. Blood specimens started to be tested as an alternative to respiratory specimens in the 1990s. At the beginning, once a bloodborne phase of the infection was suggested but never demonstrated, the attention was focused on the detection of *P. jirovecii* in blood by molecular methods such as PCR [87-93]. However, five in seven studies that applied this method showed low to very low sensitivity (0–30%), depending on the locus analysed [87-93]. Recently, other alternative strategies for a less-invasive PcP diagnosis emerged, based on the measurement of blood biomarkers that reflect the

host–pathogen interaction [18, 94, 95]. Currently, the focus is on the detection of anti-*P. jirovecii* sera antibodies, because several reports using recombinant antigens of *P. jirovecii* and antibody immunodetection techniques have shown potential application in the diagnosis and epidemiological studies of PcP [96-107].

Blood biomarkers for non-invasive diagnosis of PcP. In the past few decades, many biomolecules that could be detected in the serum of patients were studied for use in the diagnosis of PcP. Molecules of the microorganism, such as (1-3)-β-d-glucan (BG), and also host molecules such as lactate dehydrogenase (LDH), Krebs von den Lungen-6 antigen (KL-6), as well as S-adenosylmethionine (SAM), have been considered as potential candidates for use as biomarkers in the serological diagnosis of PcP [18, 94, 95, 108-113]. However, serum levels of all these metabolites are not strictly specific for *P. jirovecii* infection.

Although it is a structural molecule of the cell wall of *P. jirovecii*, the polysaccharide BG presents a panfungal character, which compromises the specificity rates for the diagnosis of PcP [94, 112, 114]. Additionally, it was suggested that false positive results can also be induced by the administration of certain agents that are filtered through cellulose membranes [115]. Otherwise, the fungal burden is another parameter that could compromise the serum concentrations of BG and, consequently, the reliability of the BG test for the diagnosis of PcP in patients without HIV infection, as the number of *P. jirovecii* organisms in the lungs of non-HIV-infected patients is usually lower than that observed in the lungs of HIV-infected patients [116, 117]. Another major problem in the application of BG serum levels for PcP diagnosis is the absence of a definitive optimal cut-off limit, which would enable distinguishing between colonization and disease [18, 118, 119].

A variety of studies suggest that increased levels of the mucin-like glycoprotein KL-6 are present in patients with PcP, which is compatible with the fact that high serum levels of this glycoprotein are known to be an indicator of interstitial lung disease and acute lung damage [18, 117]. However, since KL-6 is not a *P. jirovecii*-related molecule and is strongly expressed on type II alveolar pneumocytes and bronchiolar epithelial cells, increased levels may be more specific to underlying injury to the lung parenchyma rather than a specific marker of *P. jirovecii* infection. This could lead to false positive cases and consequently a lack of specificity to diagnose PcP [117].

The LDH enzyme is another biomarker that is widely expressed in human tissues, which is released into the blood stream after cell membrane damage. Therefore, although high serum levels of this biomarker are known to be elevated in patients with PcP, false positives can appear due to underlying lung injury and inflammation caused by *P. jirovecii* and/or other pathogen presence in the lungs, and even due to a variety of extrapulmonary disorders [18, 120].

Finally, the applicability of SAM serum levels causes disagreement among several authors since conflicting data exist about the need for exogenous SAM and the presence of a functional SAM synthetase gene in *P. jirovecii* [110, 116, 121, 122]. While some studies show SAM as an accurate biomarker for PcP [110, 121], others show that this biomarker is unable to differentiate between patients with and without the disease [113, 116], revealing very low diagnostic strength. Regardless, this biomarker is the one with the lowest potential to be used in the diagnosis of PcP, since the relationship between its serum levels and the presence of *P. jirovecii* infection is not well-defined.

Despite all of this, several studies have shown a good correlation between serum BG levels and disease severity, as well as demonstrating the ability to distinguish between *P. jirovecii* infection

and other fungal infections, which is common in immunocompromised patients [18, 113]. Two meta-analyses estimated that the measurement of BG serum levels for PcP diagnosis presents high sensitivity (95-96%), medium specificity (84-86%), and negative predictive value varying between 98.5% and 98.9% for a PcP prevalence of 20% in a population with a majority of HIV-infected patients [111, 123]. However, recent studies reveal a lower sensitivity (85%) when testing non-HIV-infected patients [124]. As serum BG detection presents high sensitivity to diagnose PcP, a negative result allows exclusion of PcP in a patient at risk of the disease. However, the lack of specificity and the absence of a consensus threshold for its application in PcP diagnosis makes it impossible to consider a BG positive result by itself as diagnostic of PcP [112, 125, 126]. Even so, the serum measurement of this biomarker has shown to be able to contribute to PcP diagnosis as well as to exclude the disease when associated with PCR of upper respiratory tract specimens [126] and with other clinical diagnostic conditions indicative of PcP, especially in immunocompromised non-HIV-infected patients. Furthermore, the combination of BG and KL-6 tests showed great accuracy and can be an alternative for a minimally invasive and less costly diagnosis of PcP, compared to the classic diagnostic approach [112, 113]. Table 2 briefly shows variables such as sensitivity, specificity, advantages, and disadvantages of the application of these blood biomarkers in the laboratory diagnosis of PcP [26, 112, 113].

Immunodiagnosis for less invasive detection of *P. jirovecii* infection. Reports of PcP in patients with genetic mutations affecting immunoglobulin production [127], along with reports of high incidence of positive serology for *P. jirovecii* in healthy adults [128], highlights the important role of humoral immunity during PcP and supports the idea that a serological test is viable.

Currently, several studies have shown that human serum antibodies are able to recognize recombinant antigens of *P. jirovecii* proteins [96-107]. Until now, the major surface glycoprotein (MSG) has received the most attention. This is a protein highly specific to this pathogen that plays a central role in the interaction of *Pneumocystis* with its host, eliciting humoral and cellular protective immune responses [107, 129]. The three individual recombinant fragments that cover the entire length of MSG (MsgA, MsgB, and MsgC) were analysed in human serum specimens for *P. jirovecii* antibody detection and this analysis revealed that the carboxyl-terminal domain (MsgC) is the most conserved and reactive region of this glycoprotein [96, 98, 103-105]. This data suggests that epitopes that stimulate at least part of the human antibodies against *P. jirovecii* may be located in this region. Taking this into consideration, a recombinant synthetic antigen with three antigenic regions of the Msg protein, specifically, one from the terminal portion of MsgB and two from MsgC (Figure 2), was recently designed and produced [107]. The antigen was purified and applied as an antigenic tool in an ELISA assay (Figure 3) for anti-*P. jirovecii* antibody detection. The results showed increased IgM anti-*P. jirovecii* levels in PcP patients compared with patients without PcP. This ELISA assay presented a sensitivity of 100% and a specificity of 80.8% when associated with the clinical diagnosis of PcP of each patient [107]. These results suggest that, based on the immunogenic behaviour of *P. jirovecii* proteins, newly recombinant synthetic multi-epitope antigenic peptides (RSAs) are one of the most promising approaches to developing diagnostic platforms for routine screening of PcP [130-132]. This technology is based on synthetic amino acid sequences designed to contain more than one reactive region of each selected antigen, increasing the sensitivity and specificity of the serological test as well as making it cheaper and easier to standardize [130, 131].
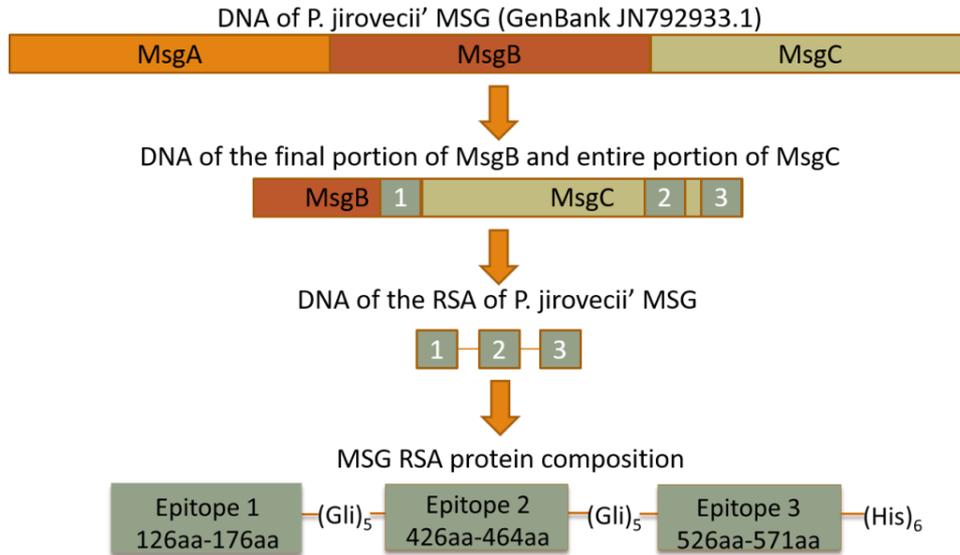
Figure 2 Scheme illustrating the design and composition of the three selected epitopes of the MSG RSA applied in an ELISA platform for PcP diagnosis.
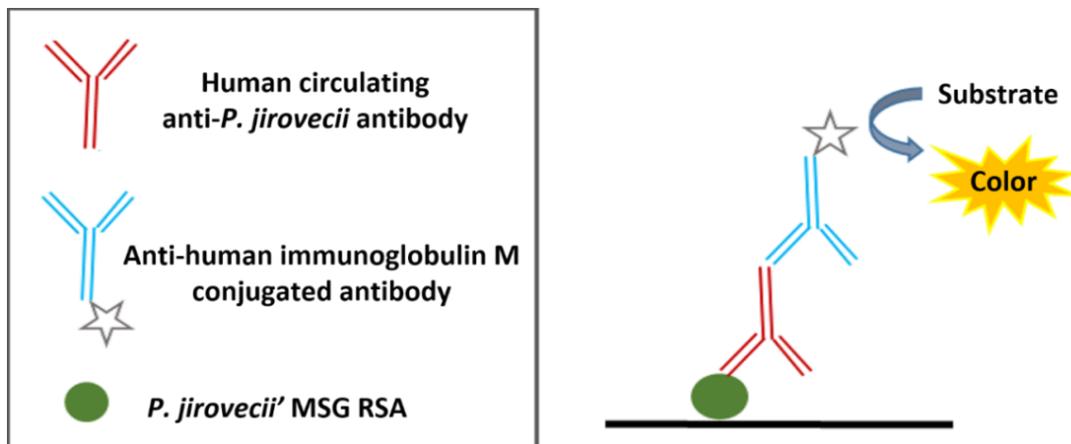

Figure 3 Indirect ELISA assay developed for serological diagnosis of PcP.

More recently, the attention of researchers began to focus on other proteins of *P. jirovecii* such as kexin-like serine protease (KEX1) and the GPI-anchored cell surface protein MEU10. KEX1 is a nuclear single-copy gene involved in the processing of proteins that maintain *P. jirovecii* cell surface integrity such as the proteolytic processing of MSG [104,133]. Recombinant KEX1 segments have been developed to study humoral responses to *P. jirovecii* and some variants were found useful in identifying acute cases of PcP, or even as indicators of subsequent risk of PcP [98, 103-105]. Also, a new MEU10 antigenic epitope, a GPI-anchored protein that appears to be in the surface of both the trophic and the cystic forms and is also conserved in *P. jirovecii*, was recently described to be capable of inducing a humoral response during *Pneumocystis* infection [134]. Thus, there is a need to better characterize the serologic responses to epitopes from these proteins and use standardized antigen preparations to assess the host immune response to *P. jirovecii* infection.

Moreover, a new tool based on innovative nanotechnology approaches is being developed. Biosensors become faster, more sensitive, and flexible when gold nanoparticles (AuNPs) are used as tags or labels [135-138], because AuNPs have high surface areas and unique physicochemical

properties, e.g., tunable bright color, that make them ideal candidates for developing biomarker platforms [137]. AuNPs can be used in the development of methods suitable for clinical diagnosis, where AuNPs serve as signal transducers and as scaffolds for bio-recognition [138]. Thus, the possibility of combining the powerful technologies of recombinant synthetic multi-epitope antigens (RSAs) with the extraordinary properties and high sensitivity of AuNPs may have a major impact on the point-of-care diagnosis of PcP. The innovative nature of this approach is the use of RSAs in association with AuNPs to design new platforms for PcP diagnosis at point-of-care to detect specific anti-*P. jirovecii* antibodies using a simple, fast, sensitive, specific, and inexpensive solid-phase (e.g., strip-based) test and a readily available, less expensive, and minimally invasive sample, such as blood. Preliminary results [139] not yet published have shown that this approach is possible to achieve, and that it could result in a point-of-care platform that will enable a faster and cheaper screening and diagnosis of this opportunistic infectious disease, helping to refine therapeutic interventions, improve disease control, and provide retrenchment of healthcare systems.

Therefore, it is expected that in the near future this and other approaches will emerge, based on these or other measurable serum biomarkers in the search for new tools for definitive, non-invasive PcP diagnosis.

Table 2 Characteristics of the tests based on measurement of blood biomarkers for PcP diagnosis (adapted from [26]).

Blood biomarker	Sensitivity	Specificity	Advantages	Disadvantages
BG	91%	77%	Minimally invasive or inexpensive samples; good for screening; can allow indirect quantification; rapid technique.	Positive results in other fungal infections (false positives); recommended confirmation of results with GMS and Giemsa or TBO and Giemsa or IF; needs expensive and specific equipment (microplate reader).
KL-6	72%	79%	Minimally invasive or inexpensive samples; rapid technique.	Positive results in other interstitial lung diseases (false positives); needs combination with GMS and Giemsa or TBO and Giemsa or IF; not quantitative; needs expensive and specific equipment (microplate reader).
LDH	80%	52%	Minimally invasive or inexpensive samples; rapid and inexpensive technique.	Positive results in organ damage cases (false positives); low specificity; needs combination with GMS and Giemsa or TBO and Giemsa or IF; not quantitative; low cost, however needs expensive and specific equipment (microplate reader).

SAM	68%	52%	Minimally invasive or inexpensive samples; rapid technique.	Low robustness and accuracy; needs combination with GMS and Giemsa or TBO and Giemsa or IF; not quantitative; needs expensive/specific equipment (microplate reader).
BG/LDH	97%	72%	Minimally invasive or expensive samples.	Low specificity; needs combination with GMS and Giemsa or TBO and Giemsa or IF; not quantitative; needs expensive and specific equipment (microplate reader); the combined tests is time consuming.
LDH/KL-6	89%	74%	Minimally invasive or expensive samples.	Low specificity; needs combination with GMS and Giemsa or TBO and Giemsa or IF; not quantitative; needs expensive and specific equipment (microplate reader); the combined tests is time consuming.
BG/KL-6	94%	90%	Minimally invasive or expensive samples; most accurate serologic method; suitable for screening;	Not quantitative; needs combination with GMS and Giemsa or TBO and Giemsa or IF; needs expensive and specific equipment (microplate reader); the combined tests is time consuming.

BG, (1-3)-β-d-Glucan quantification assay; KL-6, Krebs von den Lungen-6 antigen quantification assay; LDH, lactate dehydrogenase quantification assay; SAM, S-adenosylmethionine quantification assay; BG/LDH, combination test using BG and LDH quantification assays; LDH/KL-6 combination test using LDH and KL-6 quantification assays; BG/KL-6, combination test using BG and KL-6 quantification assays.

GMS, Grocott's Methenamine Silver stain; TBO, Toluidine Blue O; IF, immunofluorescence staining.

Serologic combination tests (BG/KL-6, BG/LDH, LDH/KL-6) are considered positive when both biomarkers levels are indicative of PcP, negative when both biomarkers levels are below the cut-off level for PcP, and undetermined when either one of the two biomarker assays yields contradictory results.

3. Conclusions

Despite the availability of cART and prophylaxis against PcP, this disease remains a significant cause of mortality and morbidity in HIV-infected and non-HIV-infected patients.

Early diagnosis of PcP is crucial for a timely implementation of treatment and for a better prognosis. Lately, new approaches for the diagnosis of PcP emerged. Several clinical support tools, such as clinical history, physical examination, and nonspecific radiological and laboratory tests may suggest the disease, but none of them is decisive. The definitive diagnosis of PcP still depends on specific laboratory methodologies, which have improved dramatically in the last 30 years. The classical methods, based on cytochemical or immunofluorescent staining and molecular biology assays, could be applied to a variety of more or less invasive respiratory specimens, which influence the sensitivity, specificity, cost, and complexity of the diagnosis. New alternatives based on the measurement of blood biomarkers of infection, such as the detection of anti-*P.jirovecii* antibodies, continue to be investigated. When selecting which laboratorial method to utilize, the

local incidence of PcP, the type of biological specimen available, as well as the level of local resources and expertise should be taken into consideration. Improving the accuracy of *P. jirovecii* detection, enabling PcP diagnosis through less invasive biological specimens, and developing a way to discriminate between patients with PcP and those who are carriers still need to be further researched. New diagnostic platforms based upon nanotechnology using non-invasive biological specimens are promising tools for an easier and cheaper detection of PcP biomarkers, enabling an early implementation of therapeutic and prophylactic measures and facilitating disease control, especially in resource-limited settings. However, these innovative approaches still need optimization and validation for their application in PcP diagnosis and implementation in clinical practice.

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Author Contributions

Ana Luísa Tomás and Olga Matos contributed equally to this work.

Competing Interests

The authors have declared that no competing interests exist.

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