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Diagnosis and management of *Pneumocystis jirovecii* infection

P. Lewis White*, Matthijs Backx* and Rosemary A. Barnes*,*

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**ABSTRACT**

**Introduction:** *Pneumocystis jirovecii* is a ubiquitous fungus, which causes pneumonia in humans. Diagnosis was hampered by the inability to culture the organism, and based on microscopic examination of respiratory samples or clinical presentation. New assays can assist in the diagnosis and even aid with the emergence of resistant infections.

**Areas covered:** This manuscript will provide background information on *Pneumocystis* pneumonia (PcP). Diagnosis, from radiological to non-microbiological (e.g. Lactate dehydrogenase) and microbiological investigations (Microscopy, PCR, β-D-Glucan) will be discussed. Recommendations on prophylactic and therapeutic management will be covered.

**Expert commentary:** PcP diagnosis using microscopy is far from optimal and false negatives will occur. With an incidence of 1% or less, the pre-test probability of not having PcP is 99% and testing is suited to excluding disease. Microscopy provides a high degree of diagnostic confidence but it is not infallible, and its lower sensitivity limits its application. Newer diagnostics (PCR, β-D-Glucan) can aid management and improve performance when testing less invasive specimens, such as upper respiratory samples or blood, alleviating clinical pressure. Combination testing may allow PcP to be both diagnosed and excluded, and molecular testing can assist in the detection of emerging resistant PcP.

1. **Introduction**

*Pneumocystis jirovecii* pneumonia (PcP) was an early indicator of the HIV epidemic and occurred in 70–80% of AIDS patients [1–3]. The incidence of PcP associated with HIV has fallen, a result of earlier HIV diagnoses, better antiretroviral therapy, and the use of prophylaxis. Most HIV-associated cases of PcP now occur in patients undiagnosed HIV [4,5]. There is an increasing population of susceptible non-HIV-infected patients, including those with solid malignancies, solid organ transplant and hematopoietic stem cell transplant recipients, patients receiving immunosuppressive therapies for autoimmune and inflammatory conditions and those with genetic primary immune deficiency disorders [6]. A national study over the decade 2000–2010 showed an increase in incidence of PcP, and the largest cohort associated with PcP were those suffering from underlying hematological malignancy [5]. Cases of PcP have also been diagnosed in less typical scenarios, such as in non-HIV individuals suffering from Dengue fever and those with preexistent lung disease [5,7].

Children are exposed to *Pneumocystis* at early age, between the ages of 2 and 4 years old over 80% of children will have generated antibodies [8]. Reactivation of latent infection was the presumed source of infection in susceptible hosts later in life [8–11]. However, with infection caused by multiple different strains of *Pneumocystis* and an association between infective strain and location at diagnosis, plus several documented PcP outbreaks confirming anthropophilic transmission, likely by airborne dispersal, reactivation of latent infection seems less likely [10,11]. Increased risk for developing PcP is associated with immunosuppression, primarily a reduction in the CD4 lymphocyte count or lymphocyte dysfunction. A summary of risk factors is listed in Table 1.

The primary manifestations are associated with the respiratory tract, with extrapulmonary disease, potentially associated with any organ, a rare manifestation. Symptoms are generally nonspecific, including fever, nonproductive cough, worsening chest pain, shortness of breath (especially on exertion), with the severity of symptoms often greater in non-HIV-infected patients [12]. Microscopic examination and molecular investigation is acute, often fulminant, particularly after corticosteroid administration [15]. The mortality rate in HIV-infected patients ranges from 17 to 30%, whereas in non-HIV-infected patients are higher ranging from 28 to 53% [15].

Given the nonspecific nature of the clinical findings, further investigations specific to *Pneumocystis* are necessary to confirm a diagnosis of PcP even in symptomatic high-risk patients, and diagnosis should not be based on clinical presentation and radiology [16]. Microscopic examination and molecular testing of respiratory samples are available but both have different performance limitations. Alternatively, serum/plasma samples can be tested for the presence of (1–3)-β-D-Glucan (BDG), although this assay cannot differentiate between the
Table 1. A summary of risk factors for Pneumocystis pneumonia.

<table>
<thead>
<tr>
<th>Underlying condition</th>
<th>Risk factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV/AIDS*</td>
<td>CD4 count &lt;200 cells/µl, CD4 cell percentage &lt;14%, Previous PCP, Oral candidiasis, Higher HIV burden, Ongoing bacterial pneumonia.</td>
</tr>
<tr>
<td>Hematological malignancy*</td>
<td>CD4 count &lt;200 cells/µl, Lymphopenia, Immunosuppression to prevent rejection of allogeneic hematopoietic SCT. For autologous SCT patients receiving purine analogs or high-dose corticosteroids. GVHD. ALL patients or those with lymphoproliferative disorders (CML, NHL, and multiple myeloma) as a result of chemotherapy including R-CHOP14, FCR, AVBD, gemcitabine or high-dose methotrexate. Monoclonal antibodies (e.g. rituximab).</td>
</tr>
<tr>
<td>Solid-organ transplantation*</td>
<td>CD4 count &lt;200 cells/µl, Corticosteroids, Antilymphocyte therapy, Mycophenolate mofetil, Calcineurin inhibitors, CMV disease, Graft rejection, Prolonged neutropenia, Exposure to cases of PCP.</td>
</tr>
<tr>
<td>Inflammatory disease</td>
<td>Administration of multiple (≥3) immunomodulatory medications, including: calcineurin inhibitors and/or anti-TNF therapy. Corticosteroids.</td>
</tr>
</tbody>
</table>

*Information collated from CDC, NIH, HIVMA/IDSA guidelines [12].

**Information collated from ECIL guidelines [13].

†Information collated from American Society of Transplantation guidelines [14].

broad range of fungal pathogens it is capable of detecting. Clinical investigations (e.g. radiology) can provide insight in likelihood of PCP, by showing evidence of the disease process or potential host response to infection, but again lack etiological specificity. Recent guidelines for the diagnosis and management of PCP are available but the evidence is lacking or weak in many areas [12–18].

1.1. The incidence of pneumocystis pneumonia

While PCP remains one of the most significant infections during HIV infection, the actual incidence of disease for this cohort in developed countries has fallen over the past decade [5,19]. US data on AIDS defining opportunistic infections showed and approximate fivefold reduction in events per 100,000 person-years between 2000 and 2010 [19]. This was supported by data from England where the number of case of PCP in HIV-infected patients approximately halved over the same period [5]. Contrary to this rate of PCP in England over the same period has actually increased at an annual rate of 9%, particularly in the over 50 years old population, peaking in those aged between 60 and 69 years old [5]. While the authors could not exclude whether this increase in PCP was a result of an increase in laboratory testing it was felt that the correlation with an older cohort was more likely a contributing factor. Over this period, there was an increased diagnosis of hematological malignancy (30% increase from 2000 to 2010) and an increase in the number of patients receiving renal transplantation (25% increase from 2000 to 2010), but this could not fully explain the 388% and 209% increase in PCP infections in renal and hematology patients, respectively [5]. It was concluded that a possible reason for the increase in cases of PCP was a lack of appropriate prophylaxis in potentially novel at risk patients, with risk associated with an increase in the severity of the immunosuppressive drugs used in these populations. Alternatively, it was proposed that there was potentially increase transmission of PCP within these populations due to changing clinical practice [5]. The estimated incidence of PCP in solid organ transplant recipients in the UK was 5.8%, 5.5%, 1.2%, and 0.3% for lung/heart and lung, heart, liver, and kidney transplants, respectively [20]. The global incidence of PCP in hematology patients post allogeneic and autologous stem cell transplantation between 1995 and 2005 was 0.63% and 0.28%, respectively [21]. The incidence of PCP in US HIV-infected patients over the last decade was 0.7% [19].

2. Diagnosis

2.1. Understanding test formats

The incidence of a disease influences utility of diagnostic tests, and can determine the optimal testing strategy in different clinical settings. Focus is often on a purely diagnostic approach to confirm disease, but with a low incidence of disease tests are better suited to exclude a diagnosis, avoiding the need for unnecessary therapy but also indicating that an alternative diagnosis should be sought. Testing can also be used prognostically to monitor disease and assess the duration and response to therapy.

For most cohorts, the incidence of PCP is relatively low and the pretest probability of disease is small compared to the pretest probability of not having disease (see Expert Commentary). Consequently, negative results are better suited to excluding disease through a high sensitivity and negative predictive value (NPV). With high sensitivity comes potential false positivity, but specificity can be improved by intensifying the diagnostic work-up through repeat and combination testing, and multidisciplinary interpretation of results. In respect to PCP testing, different sample types, for example lower and upper respiratory tract specimens, may shift the emphasis of diagnostic testing and can determine the optimal testing strategy in different clinical settings. Focus is often on a purely diagnostic approach to confirm disease, but with a low incidence of disease tests are better suited to exclude a diagnosis, avoiding the need for unnecessary therapy but also indicating that an alternative diagnosis should be sought. Testing can also be used prognostically to monitor disease and assess the duration and response to therapy.

2.2. Radiological investigations

Chest radiography (CXR) may be normal during the early stages of disease, but can worsen rapidly, particularly in the non-HIV-infected population [12,14,15]. Computerized tomography (CT) scans are more sensitive than conventional radiographic techniques, providing evidence of infection even during the early stages of disease in non-HIV-infected patients, and there is a role for CT despite CXR negativity [22,23]. CXR typically presents with bilateral, diffuse interstitial infiltrates that progress to bilateral consolidations [12,22,24].
Differential diagnosis by CT is generally achieved by visualizing predominant ground-glass opacification (GGO) with a diffuse, bilateral and central location, sparing the sub-pleural spaces [23]. Consolidations may be present in mid or late stages of disease [15,22]. In a study investigating the progression of disease, 20% of cases of early PCP presented with diffuse GGO on CT, leading to GGO and patchy consolidation in 47% of mid-stage cases and predominant consolidations in 80% of late-stage disease [22]. Other findings include nodules, cysts, pneumothoraces, upper lobe localization, linear opacities and septal thickening [12,15,24,25]. Cavitation, intrathoracic adenopathy and pleural effusions are less likely [12,13,25]. The radiological presentation of PCP is not specific and can imitate other pathogens (e.g. bacterial or CMV pneumonia) [26]. Radiology cannot provide an etiological diagnosis, but may be used to initiate therapy in high-risk patients. This should trigger efforts to achieve a mycological diagnosis of the organism from the respiratory tract.

Recent developments for the imaging of PCP include the successful application of ultra low dose chest CT, fluorodeoxyglucose positron emission tomography (FDG-PET) and bronchoscopic probe-based confocal laser endomicroscopy [27–29]. CT has also been used to determine the severity and prognosis of PCP infection [22,25].

### 2.3. Non-microbiological laboratory investigations

Overall lymphocyte count should be determined, as values <10% of the norm has been associated with a poor prognosis in PCP infection [30]. While lymphocyte count is obviously important, lymphocyte function may also be significant, with the influence of recent immunosuppressant drugs and other biological response-modifying agents on lymphocyte function requiring consideration.

Hypoxemia will vary depending on the severity of disease and HIV status, and regularly presents as a mild and severe reduction arterial oxygen in HIV-infected and non-HIV-infected patients, respectively [12,15]. Serum lactate dehydrogenase (LDH) elevation is a suggestive marker, with levels >500 ml/dl associated with PCP [12]. Extracellular LDH indicates cell damage or cell death, with elevated levels correlating with lung tissue damage, but it is not specific for PCP and is of little use outside the HIV-infected population. In a study of LDH performance in HIV-infected and non-HIV-infected cohorts the specificity and sensitivity were 100%/47% and 63%/43%, respectively, showing that within HIV-infected cohort a negative result could be used to confidently exclude disease, but positivity required confirmatory testing [31]. The use of procalcitonin serum concentration to differentiate PCP from other respiratory infections and/or colonization is not clear [32–34].

Clinical factors have also been used to predict mortality. In large observational cohort study of 451 HIV-infected patients, five significant predictors (Age, recent intravenous drug use, total bilirubin, serum albumin and alveolar-arterial oxygen gradient) were determined through multivariate analysis and incorporated into model to predict PCP mortality [35].

### 2.4. Conventional techniques – culture

The difficulty in culturing *Pneumocystis* has hindered both diagnosis and research and development. Several methods using various coculture cell lines were described but failed to attain widespread use [36–40]. Most attempts have used rat models and subsequently *P. carinii* not *P. jiroveci*. In 1999, *P. carinii* initially isolated from rat lung was cultured using continuous axenic cultivation [37]. This complex technique has been successfully applied to the recovery of *P. carinii* from lungs and broncholalveolar (BAL) fluid of rats and used to investigate life-cycle, but difficulty in successfully reproducing axenic culture has limited its use, especially in routine diagnostics [38–40].

In 2014, the first successful cultivation and propagation of *P. jiroveci* direct from BAL was achieved using a three-dimensional air-liquid interface culture system formed by CuFi-8 respiratory epithelial cell line [36]. While this represents a major breakthrough and provides the potential to perform antifungal susceptibility testing, it still requires cell culture, limiting its use in routine diagnostics laboratories, being replaced by direct molecular methods and it still requires verification in other laboratories.

### 2.5. Conventional techniques – microscopy

The gold standard for the diagnosis of PCP remains the histological and microscopic identification of ascus (cysts containing ascospores) and trophic forms using Wright’s-Giemsa, toluidine blue O, calcofluor white or Grocott-Gomori stains, in tissue, BAL and induced sputum. While Gomori stains the cell wall of the ascus form, Giemsa will stain the nuclei of all *Pneumocystis* life cycle stages but does not stain the cell wall. Toluidine blue is a generic stain for nucleic acids and polysaccharides, while calcofluor white stains chitin and cellulosic, neither is specific for *Pneumocystis*, and given the inability of *Pneumocystis* to synthesize chitin the suitability of calcofluor white may be limited [41]. The performance of conventional stains has been superseded by immunofluorescence (IF) microscopy, using anti-*P. jiroveci* monoclonal antibodies [15,16]. However, in the majority of studies only the ascus form was targeted and a combination of stain and/or IF kit to detect both ascus and trophic forms is recommended. IF kits that detect both forms are available (e.g. Monofluo™ *Pneumocystis jirovecii* IFA or Merifluor *Pneumocystis* kits) [15].

In a comparison of four staining methods, sensitivities were 73.8%, 76.9%, 48.4%, and 90.8%, for calcofluor white, Grocott-Gomori, Diff-Quik (modified Wright’s-Giemsa) and Merifluor *Pneumocystis*, respectively [42]. The sensitivity of the Diff-Quik method was significantly lower than the other methods. For conventional stains, the corresponding specificity was >99%, whereas for the IF antibody assay (Merifluor *Pneumocystis* kit) it was 94.7%, significantly lower than the other methods [42]. The authors concluded that the Merifluor *Pneumocystis* kit was a useful screen to exclude PCP, but the specificity/PPV was insufficient to confirm disease. However, the positive likelihood ratio (LR+ve, less affected by prevalence) for the Merifluor *Pneumocystis* kit was 17.1, and subsequent positive results are associated with PCP.
Conversely, none of the non-IF methods generated a negative likelihood ratio (LR-ve) ≤0.1, and cannot be used to exclude disease confidently.

For microscopic approaches, a primary screen with a highly sensitive IF method, confirmed by a secondary specific method is recommended [15,16]. A summary of the comparative performance of various microscopic staining and fluorescent techniques for the diagnosis of PcP is shown in Table 2 [43–45]. It is important to consider the influence of specimen type and quality on assay performance. There is no standardized approach to sampling the respiratory tract and protocols will vary across centers affecting the quality of BAL and sputa. When comparing both IF and conventional staining on sputum and BAL, the sensitivity was lower when testing sputum across all assays [45]. In a meta-analysis involving seven studies with 160 cases and 162 controls, the sensitivity and specificity of staining and IF of induced sputum was determined using BAL testing as a reference [46]. Overall sensitivity and specificity when testing induced sputum was 55.5% and 98.6%, respectively. However, the sensitivity when testing induced sputum was significantly higher when tested with IF (67.1%) than with conventional staining (43.1%) [46].

2.6. (1-3)-β-D-glucan

The use of assays to detect (1-3)-β-D-glucan (BDG) is now widely accepted and permits the testing of easily obtainable serum/plasma specimens. Clinical trials of BDG performance for the diagnosis of PcP are lacking, but various meta-analyses of clinical evaluations exist (Table 3) [47–49]. Overall, sensitivity is high and BDG negativity can be used to exclude PcP, although false negatives have been noted [50]. Specificity is suboptimal (<90%) [47–49]. A BDG-positive result alone cannot be considered diagnostic of PcP, due to the assays broad detection range coupled with a patient cohort that may be susceptible to other fungal pathogens. The result should be interpreted along with radiological findings together with a PcP-specific assay. Specificity will also be affected by noninfective factors, such as potential sources of false positivity [51]. For the diagnosis of PcP, there was no difference in the overall accuracy of BDG assays developed by different manufacturers [48]. In one meta-analysis, BDG performance when testing samples from HIV-infected versus non-HIV-infected patients was comparable, although in a more recent study sensitivity was deemed to be significantly lower in the non-HIV-infected population (HIV infected: 92% versus non-HIV infected: 85%), potentially a result of the greater burden of organism seen in HIV infected PcP [48,49].

BDG assays utilize a single positivity threshold for the detection of invasive fungal disease and it is not possible to confidently determine organism-specific fungal etiology on the strength of positivity. However, for cases of PcP, it is not unusual to see positivity greater than the upper limit of the assay (e.g., Fungitell >500 pg/ml), even in the absence of IF staining of respiratory samples [55,56]. In the study of Damiani et al., the median Fungitell BDG concentration across 17 cases of PcP was 1945 pg/ml (range: 122–8000 pg/ml), with 10 of the cases generating concentrations >500 pg/ml, and 14 cases with concentrations >300 pg/ml [57]. Both control and Pneumocystis colonized patients had BDG concentrations below 90 pg/ml. Differentiation of colonization from infection was also possible using the Beta-Glucan test Wako™ (colonization: 49 pg/ml versus infection: 173 pg/ml) [58]. Compared to the Fungitell assay, the overall BDG concentrations generated by the Wako assay were lower for all categories of infection, potentially reflecting the differences in reaction kinetics and subsequent positivity thresholds and highlighting the necessity to independently validate different kits. When testing serum by the Fungitell assay, using a positivity threshold of 300 pg/ml the sensitivity, specificity, LR+ve and LR-ve were 91%, 92%, 11.4, and 0.1, respectively, indicating that the assay could be used to both confirm and exclude disease [34].

With Pneumocystis primarily infecting the respiratory tract, a limitation of BDG is poor clinical utility when testing respiratory samples. Candida species are common commensals of the mucosal membranes and airway colonization by other fungi is possible, as such the presence of elevated BDG concentrations

<table>
<thead>
<tr>
<th>Reference</th>
<th>Assay</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
<th>LR+ve</th>
<th>LR-ve</th>
<th>DOR</th>
</tr>
</thead>
<tbody>
<tr>
<td>[42]</td>
<td>CW</td>
<td>73.8%</td>
<td>99.6%</td>
<td>98.0%</td>
<td>93.4%</td>
<td>184.5</td>
<td>0.26</td>
<td>709.6</td>
</tr>
<tr>
<td></td>
<td>MF</td>
<td>90.8%</td>
<td>94.7%</td>
<td>82.9%</td>
<td>97.5%</td>
<td>17.1</td>
<td>0.007</td>
<td>176.3</td>
</tr>
<tr>
<td></td>
<td>DQ</td>
<td>48.4%</td>
<td>99.6%</td>
<td>96.9%</td>
<td>88.0%</td>
<td>121.0</td>
<td>0.51</td>
<td>237.3</td>
</tr>
<tr>
<td></td>
<td>GMS</td>
<td>76.9%</td>
<td>92.2%</td>
<td>96.2%</td>
<td>94.2%</td>
<td>96.2</td>
<td>0.23</td>
<td>418.3</td>
</tr>
<tr>
<td>[43]</td>
<td>CB</td>
<td>74.3%</td>
<td>99.6%</td>
<td>92.9%</td>
<td>98.0%</td>
<td>165.6</td>
<td>0.3</td>
<td>552.0</td>
</tr>
<tr>
<td></td>
<td>MoF</td>
<td>60.0%</td>
<td>99.3%</td>
<td>87.5%</td>
<td>96.9%</td>
<td>89.2</td>
<td>0.4</td>
<td>223.0</td>
</tr>
<tr>
<td></td>
<td>Giemsa</td>
<td>34.6%</td>
<td>100%</td>
<td>100%</td>
<td>95.1%</td>
<td>&gt;346a</td>
<td>0.7</td>
<td>&gt;494.3a</td>
</tr>
<tr>
<td></td>
<td>GMS</td>
<td>50%</td>
<td>100%</td>
<td>100%</td>
<td>96.5%</td>
<td>&gt;500a</td>
<td>0.5</td>
<td>&gt;1000a</td>
</tr>
<tr>
<td></td>
<td>Giemsa</td>
<td>50%</td>
<td>100%</td>
<td>100%</td>
<td>96.5%</td>
<td>&gt;500a</td>
<td>0.5</td>
<td>&gt;1000a</td>
</tr>
<tr>
<td>[45]b</td>
<td>MoF</td>
<td>93.1%</td>
<td>100%</td>
<td>100%</td>
<td>95.5%</td>
<td>&gt;931a</td>
<td>0.07</td>
<td>&gt;13300a</td>
</tr>
<tr>
<td></td>
<td>DQ</td>
<td>87.9%</td>
<td>97.6%</td>
<td>98.1%</td>
<td>85.4%</td>
<td>36.6</td>
<td>0.12</td>
<td>305.0</td>
</tr>
<tr>
<td></td>
<td>GMS</td>
<td>89.7%</td>
<td>95.2%</td>
<td>96.3%</td>
<td>97.0%</td>
<td>18.7</td>
<td>0.11</td>
<td>170.0</td>
</tr>
<tr>
<td></td>
<td>PCIF</td>
<td>94.8%</td>
<td>88.1%</td>
<td>91.7%</td>
<td>92.5%</td>
<td>8.0</td>
<td>0.06</td>
<td>133.3</td>
</tr>
</tbody>
</table>

Values have been generated using a specificity of 99.9% to overcome...

Results represent combined induced sputum and bronchoalveolar lavage fluid testing

CW: Calcofluor white; GMS: Grocott-Gomori methamine silver; MF: Merifluor Pneumocystis; CB: Calcofluor blue; DQ: Diff-Quik; MoF: Monofluo™ Pneumocystis jirovecii; PCIF: P. carinii IF kit.

When interpreting the results the influence of incorporation bias on performance parameters should be considered, as in many studies the results, particularly in combination with the others tests have been used to define cases and controls.
are not indicative of disease, and could be misleading in symptomatic patients. In one study the specificity of BDG testing of BAL samples was only 68%, compared to 92% when testing serum and reproducibility was poor with only 5.9% of retested BAL samples confirming the earlier result [56]. Even when using higher positivity thresholds BDG, specificity when testing BAL fluid remained compromised (241 pg/ml: 39%; 783 pg/ml: 79%) [34]. While there has been a successful attempt to differentiate PcP infected from colonized/uninfected patients based on BDG concentration, others have found receiver operator characteristic curve analysis to be of limited use in defining BDG BAL threshold [34, 56,59].

2.7. Molecular investigations

The use of molecular-based tests for the diagnosis of PcP continues to be described with too many studies to be discussed individually, although summarized in the guidelines for the diagnosis of PcP in hematology patients [16]. While the focus on development of local assays provides technological diversity, it prevents methodological standardization, which remains limited, and can affect the outcomes of meta-analyses. Nevertheless, meta-analyses determining the performance of PcP PCR show excellent performance for diagnosis (LR+ve ≥10), but more so the exclusion of PcP (NPV: ≥99%, LR−ve: ≤0.03) (Table 4) [52–54].

Subgroup analysis using microscopy as the reference standard showed the specificity of PCR (Se: 97%; Sp: 93%) was not significantly affected, although specificity was increased to 96% when using reference standards other than microscopy to define cases [52]. Comparison of performance in HIV-infected and non-HIV-infected cohorts was similar [54]. When testing BAL, the sensitivity and specificity were 100% and 87%, respectively. When induced sputa were incorporated sensitivity was 97% and specificity was 93% [54]. Comparison of PCR performance when testing BAL with oropharyngeal wash fluid (OW) showed OW to have significantly lower sensitivity (76%) but higher specificity (93%), indicating that the PCR detection of Pneumocystis in the upper airways is a good indicator PcP (LR+ve 10.4, compared to 8.0 in BAL) [54]. While PcP PCR negativity when testing BAL fluid appears to provide the ability to confidently exclude PcP, false negatives associated with a mutation in the large subunit mitochondrial rRNA has been noted [60]. As with all molecular-based assay, surveillance for genetic drift is required, but complicated by the lack of surveillance cultures. The use of nasopharyngeal aspirates cannot be used to exclude PcP, but may provide a useful adjunct diagnostic test in combination with other markers (e.g. BDG) [61].

From a technical perspective, the use of commercial kits for cell wall disruption reduced specificity [54]. Conversely, using a commercial kit for overall nucleic acid extraction improved specificity and arguably, it is not necessary to perform excessive cell wall disruption for efficient nucleic acid extraction from Pneumocystis [54]. PCR amplification of the ITS region was associated with improved sensitivity, but along with amplifying the large subunit mitochondrial rRNA, decreased specificity [54]. The use of nested-PCR provided significantly lower specificity which could be attributed to its potential to detect subclinical levels of Pneumocystis, although could also be an effect of the contamination prone process [53]. Nowadays, the use of conventional PCR amplification systems has been superseded by real-time (quantitative) PCR platforms associated with improved specificity, but also have been used to differentiate Pneumocystis infection from colonization using the fungal burden based on the PCR cycle threshold (Ct) [54,62–64]. When interpreting the significance of the burden, the underlying condition of the patient and quality of sample must be considered when trying to distinguish actual infection from possible colonization. For example, in one study using a real-time a Ct of 27 was associated with 100% specificity for the diagnosis of PcP in HIV-infected patients, with positivity earlier than this threshold (≤27 cycles) excluding the possibility of colonization. Yet the optimal Ct in non-HIV-infected patients was 31 cycles and associated specificity was only 80%, so Pneumocystis colonization remained a possibility (20%) [62]. Conversely, an upper Ct of 35 cycles generated a sensitivity of 80% and 1/5 PcP non-HIV-infected cases would be missed. When setting thresholds to confirm or exclude disease, it is critical that specificity and sensitivity are ≥95%, respectively. Otherwise the utility of the assay is compromised, with cases potentially missed and infection and colonization potentially indistinguishable, generating results of limited clinical value.

Table 3. The performance of (1–3)-β-D-Glucan Testing for the diagnosis of Pneumocystis pneumonia as determined by meta-analyses.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Study</th>
<th>Karageorgopoulou [47]</th>
<th>Onishi [48]</th>
<th>Li [49]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cases/Total (n/N)</td>
<td>357/2080</td>
<td>286/2331</td>
<td>433/2195</td>
<td></td>
</tr>
<tr>
<td>Sensitivity (%)</td>
<td>94.8</td>
<td>95.5</td>
<td>90.8</td>
<td></td>
</tr>
<tr>
<td>Specificity (%)</td>
<td>86.3</td>
<td>84.3</td>
<td>78.1</td>
<td></td>
</tr>
<tr>
<td>PPV (%)</td>
<td>54.3</td>
<td>46.0</td>
<td>50.5</td>
<td></td>
</tr>
<tr>
<td>NPV (%)</td>
<td>99.0</td>
<td>99.3</td>
<td>97.2</td>
<td></td>
</tr>
<tr>
<td>LR +ve</td>
<td>6.9</td>
<td>6.1</td>
<td>4.1</td>
<td></td>
</tr>
<tr>
<td>LR −ve</td>
<td>0.06</td>
<td>0.05</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td>DOR</td>
<td>115</td>
<td>122</td>
<td>34.2</td>
<td></td>
</tr>
</tbody>
</table>

PPV: positive predictive value; NPV: negative predictive value; LR +ve: positive likelihood ratio; LR −ve: negative likelihood ratio; DOR: diagnostics odds ratio. The table contains data determined for range of susceptible patients testing with various BDG assays.

Table 4. The performance of PCR for the diagnosis of Pneumocystis pneumonia as determined by meta-analyses.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Study</th>
<th>Summah [52]</th>
<th>Fan [53]</th>
<th>Lu [54]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cases/Total (n/N)</td>
<td>506/2330</td>
<td>606/1793</td>
<td>416/2505</td>
<td></td>
</tr>
<tr>
<td>Sensitivity (%)</td>
<td>97</td>
<td>98</td>
<td>99</td>
<td></td>
</tr>
<tr>
<td>Specificity (%)</td>
<td>94</td>
<td>91</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>PPV (%)</td>
<td>82</td>
<td>85</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td>NPV (%)</td>
<td>99</td>
<td>99</td>
<td>&gt;99</td>
<td></td>
</tr>
<tr>
<td>LR +ve</td>
<td>16.2</td>
<td>10.9</td>
<td>9.9</td>
<td></td>
</tr>
<tr>
<td>LR −ve</td>
<td>0.03</td>
<td>0.02</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>DOR</td>
<td>540</td>
<td>545</td>
<td>990</td>
<td></td>
</tr>
</tbody>
</table>

The table provides the performance for PcP PCR when testing both HIV-infected and non-HIV-infected patients, when testing upper and lower respiratory tract specimens, and is irrespective of differing technical details.

Due to incomplete information the case and total population were calculated using sample numbers.

PPV: positive predictive value; NPV: negative predictive value; LR +ve: positive likelihood ratio; LR −ve: negative likelihood ratio; DOR: diagnostics odds ratio.
When interpreting low-level PcP PCR positives (Ct >35) in an attempt to differentiate infection from colonization, it is important to determine both the quality of sampling but also understand the presentation of clinical disease in non-HIV-infected patients, where a low fungal burden may generate significant immune response during infection. Theoretically, human DNA can be used as a surrogate for assessing sample quality. Low levels of human DNA could represent poor sample quality, with low-level PcP PCR positives representative of inadequate sampling rather than colonization, but the latter remains a possibility. Conversely, if a large quantity of human DNA is present but the PcP burden is low it could represent a strong immune response, particularly relevant in the non-HIV-infected population. When considering either of these scenarios it remains impossible to exclude colonization. Clinical interpretation including the possibility, or even exclusion of alternative diagnoses, and using combination testing (see below) to enhance confidence in a diagnosis of PcP may be required. For reference, it is essential to know the typical burden of human DNA in respiratory samples and it is also required that sampling is standardized, which for BAL remains highly variable. The sampling of the upper respiratory tract is less variable and has been associated with greater specificity [54].

Given the broad range of available PcP PCR assays, it may be wise for centers to incorporate commercially manufactured and standardized tests that have developed an understanding of how to interpret, in particular low-level positives. In a comparative study of three commercial assays (Pneumocystis jirovecii (carinii) – FRT PCR Kit (AmpliSens), MycAssay Pneumocystis (Myconostica) and real-time PCR Pneumocystis jirovecii (Bio-Evolution)), the sensitivity and specificity when testing proven/probable PcP was 100%, 100%, 95% and 83%, 93% and 100%, respectively, and sample concordance between the Amplisens and MycAssay was excellent (Kappa: 0.85) [65]. One interesting concept is the development of a commercial real-time PCR for both the detection of organism and dihydropteroate synthase (DHPS) point mutations associated with resistance to sulfa-based drugs such as sulfamethoxazole and dapsone, used for both prophylaxis and treatment of PcP [66]. Using a positivity threshold of 32 cycles, the sensitivity and specificity of the PneumoGenius® assay were 70% and 82%, respectively. Performance may have been affected by the classification of disease based on clinical findings in high risk hosts responding to PcP therapy, but missing a mycological criterion. Nevertheless, the assay was able to screen for sulfadiazine direct from 89 samples and showed a 4.5% resistance rate [66].

With more than 60 types of P. jirovecii identified, it was initially proposed that 30% of PcP cases were infected with multiple types, the ability to investigate transmission and clusters has been hampered by the difficulty in cultivating Pneumocystis [67,68]. More recent research utilizing next-generation sequencing deemed PcP was caused by a mixture of different genotypes and not a single strain, further complicating cluster investigation [69]. Molecular-based methods can also be used to determine the epidemiology and transmission of infection and to investigate potential outbreak scenarios and multi-locus sequence typing and multilocus real-time mutation frequencies have been used [11,70,71].

2.8. Combination testing

While the reference standard for the diagnosis of PcP remains microscopic evidence, usually IF, within a respiratory specimen its limited sensitivity cannot be used to exclude disease [14-16]. The question remains whether by combining more sensitive tests specificity of diagnosis can be improved, while maintaining confidence in exclusion. In the adult hematological population, current guidelines suggest a diagnostic algorithm involving real-time PCR and IF testing of BAL in patients with a clinical suspicion of disease. If both are positive, a diagnosis of PCR is confirmed and vice versa [16]. If PCR is positive, but IF negative, diagnosis is made if high burdens are detected. For low burdens, additional BDG testing is recommended. If PCR is negative but IF positive then this is considered technically inconsistent and the quality of either result is questioned [16]. This begs the question why IF is still being performed, rather than being replaced with PCR in combination with BDG testing. In a study comparing circulating biomarkers with PcP lung burden 96% (25/26) of patients that were BAL PcP PCR positive, but IF negative were also positive by BDG; as were all (10/10) patients that were BAL PcP PCR and IF positive [72]. Conversely, 29% (10/34) of PcP PCR and IF negative were BDG positive, although 15/34 were diagnosed with proven/probable invasive aspergillosis. Given the panfungal nature of BDG, it makes sense to perform a primary investigation using PcP PCR on BAL, and if positive confirm the results when the pulmonary burden is low with BDG testing [57]. When BAL samples are not available, BDG testing of serum is recommended where negativity can be used to exclude PcP, but positivity should be confirmed by PCR (or IF) testing of less invasive respiratory samples [16]. However, given the current limitations of PCR (possible detection of colonization) and BDG (detection of other fungal species) testing, there may be a preference to combine PCR with microscopy to confirm or exclude PcP when testing deeper respiratory specimens [16,73].

The combination of BDG testing in association with LDH levels permits a fully noninvasive sampling regime and has been successfully evaluated for the diagnosis of PcP. When using optimal thresholds (BDG: 400 pg/ml; LDH: 350U/l), specificity was 84% [74]. A further serological biomarker multicenter study evaluated BDG, LDH, Krebs von den Lungen-6 antigen (KL-6, a potential marker of interstitial pneumonitis) and S-adenosyl methionine (SAM, a metabolic intermediate possibly exogenously required by Pneumocystis) to aid in the diagnosis of PcP [75]. The best overall performance was by combining BDG with KL-6 (Se: 94% Sp: 90%). Although sensitivity was slightly higher when combining BDG with LDH, specificity was compromised (Sensitivity: 97%; Specificity: 72%). For all these approaches, it could be argued that the absence of organism specific assay compromises confidence in diagnosis, and incorporating a Pneumocystis specific PCR is required. If this is the case then the combination of PCR/BDG is preferable to using another nonspecific serological biomarker.

3. Management

PcP can run a fulminant course, particularly in non-HIV-infected individuals and early treatment improves prognosis.
Table 5. Recommendations for the prophylaxis of adult patients at risk of Pneumocystis pneumonia.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Prophylaxis Population</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1) CD4 count &lt;200 cells/µl</td>
<td>ALL</td>
<td></td>
<td>(1) All SOT, especially lung transplant</td>
</tr>
<tr>
<td>(2) CD4 cell &lt;14%</td>
<td>(2) allogeneic HSCT, steroids (&gt;20 mg/day/4 weeks)</td>
<td></td>
<td>(2) Increasing immunosuppression to prevent graft rejection</td>
</tr>
<tr>
<td>(3) CD4 count 200–250 cells/µl in the absence of regular 3-month CD4 monitoring</td>
<td>(3) Alemtuzumab</td>
<td></td>
<td>(3) Recurrent or chronic CMV infection</td>
</tr>
<tr>
<td>(4) Not patients receiving pyrimethamine/sulfadiazine for toxoplasmosis</td>
<td>(4) Fludarabine/cyclophosphamide /rituximab</td>
<td></td>
<td>(4) Prolonged course of corticosteroids (&gt;20 mg for ≥2 weeks)</td>
</tr>
<tr>
<td></td>
<td>Optional: Lymphoma with R-CHOP14 or escalated BEACOPP, nucleoside analogs, radiotherapy for brain tumors/metastasis with steroids</td>
<td></td>
<td>(5) Prolonged neutropenia</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(6) Episodes of autoimmune disease</td>
</tr>
<tr>
<td><strong>Duration</strong></td>
<td>Until CD4 count ≥200 cells/µl for &gt;3 months</td>
<td></td>
<td>A minimum 6–12 months post-transplant for all SOT recipients. Patients with lung or small bowel grafts or those prior PcP or chronic CMV disease may require lifelong prophylaxis</td>
</tr>
<tr>
<td><strong>Therapy</strong>&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Front line: Trimethoprim/sulfamethoxazole one single-strength (80 mg TMP/400 mg SMX) daily or one double-strength tablet (160 mg TMP/800 mg SMX) daily. Second line: Trimethoprim/sulfamethoxazole one double-strength tablet (160 mg TMP/800 mg SMX) three times per week. Dapsone (50 mg twice daily). Dapsone (200 mg) + pyrimethamine (75 mg) + leucovorin (25 mg) weekly. Dapsone (50 mg daily) + pyrimethamine (50 mg weekly) + leucovorin (25 mg weekly). Pentamidine aerosols (300 mg per month). Atovaquone 1500 mg daily</td>
<td>Front line: Trimethoprim/sulfamethoxazole one single-strength (80 mg TMP/400 mg SMX)/day or double strength tablet (160 mg TMP/800 mg SMX)/day or three per week. Second line: Dapsone (50 mg twice daily). Pentamidine aerosols (300 mg per month).</td>
<td>Front line: Trimethoprim/sulfamethoxazole one single-strength (80 mg TMP/400 mg SMX)/day or double strength tablet (160 mg TMP/800 mg SMX)/day or three per week. Second line: Dapsone (50–100 mg once a day)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Where possible, only the recommendation receiving an ‘A’ grading or the preferred drug of choice have been listed.

Table 6. Recommendations for the treatment of adult patients diagnosed with Pneumocystis pneumonia.

<table>
<thead>
<tr>
<th>Recommendation</th>
<th>CDC, NIH, HIVMA/IDSA (HIV) [12]</th>
<th>ECIL (hematology) [18]</th>
<th>American Society of Transplantation (SOT) [14]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Targeted Treatment</strong></td>
<td></td>
<td></td>
<td>All SOT with suspected/diagnosed PcP</td>
</tr>
<tr>
<td><strong>Population</strong></td>
<td>HIV/AIDS patients with suspected/diagnosed PcP</td>
<td>Hematological malignancy, solid cancer, solid organ transplant, autoimmune/inflammatory conditions with suspected/diagnosed PcP</td>
<td>At least 14 days, extended to 21 days for severe cases</td>
</tr>
<tr>
<td><strong>Duration</strong></td>
<td>3 weeks</td>
<td>A minimum of 14 days</td>
<td>Frontline: Trimethoprim/sulfamethoxazole (15–20 mg/kg TMP; 75–100 mg/kg SMX per day)</td>
</tr>
<tr>
<td><strong>Therapy&lt;sup&gt;a&lt;/sup&gt;</strong></td>
<td>Frontline: Trimethoprim/sulfamethoxazole (15–20 mg/kg TMP; 75–100 mg/kg SMX per day). For moderate-to-severe disease (i.e. hypoxemia) adjunctive corticosteroids should be used. Second line for severe disease: Primaquine and clindamycin (30 mg/600mgx3) per day. Second line for mild/moderate disease: Dapsone (100 mg daily) + trimethoprim (15 mg daily) + Atovaquone (750 mg BID).</td>
<td>Frontline: Trimethoprim/sulfamethoxazole (15–20 kg TMP; 75–100 mg/kg SMX per day) with TMP administered by IV every 6–8 h. For hypoxemic patients potentially in combination with 40–60 mg of prednisolone (twice daily). Second line: IV Pentamidine (Initially 4 mg/kg/day over 1–2 h). Recipients of pancreas/islet transplants should receive an alternative second-line therapy.</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Where possible, only the recommendation receiving an ‘A’ grading or the preferred drug of choice have been listed.
Disease can be stratified according to mild, moderate, or severe, depending on presenting symptoms, oxygen saturation and chest radiographic changes. Requirement for mechanical ventilation and vasopressors is a poor prognostic feature.

Clinicians should commence antimicrobials on the basis of clinical suspicion and before diagnostic investigations have been performed. Increasingly, sensitive molecular and biomarker detection is picking up patients who have only minimal symptoms or who are asymptomatic and this can present a diagnostic dilemma. Prophylaxis of at-risk patients is also considered a mainstay of management. Guidelines for the prophylaxis and treatment have been developed for different groups and are summarized in Tables 5 and 6.

Although included within the fungal kingdom on the basis of cell wall composition and structure combined with nucleotide sequence similarity, *Pneumocystis jirovecii* is not susceptible to polyene and azole antifungal drugs, due to the absence of ergosterol from its cell wall. The different morphological forms also show varying susceptibility to other drugs with *in vitro* inhibition of the cyst but not trophic forms by echinocandins [76]. Trimethoprim, sulfa drugs, and pentamidine form the main stays of treatment. Corticosteroids are of proven benefit in HIV-infected individuals with disease, but a beneficial role has not been established for other patient groups. The most effective way of preventing PnP in people living with HIV is by immune-reconstitution, through the administration of effective antiretroviral therapy. Prophylaxis should be administered until immune reconstitution has been achieved.

### 3.1. Prophylaxis

Recommendations for prophylaxis are comprehensively reviewed in the ECIL guidelines, although this focuses on patients with hematological malignancies and undergoing SCT [13].

Prophylaxis is recommended in risk groups that include HIV-infected patients with CD4 counts less than 200 cells/mm [3], transplant patients, and patients with high-risk hematological malignancies, as well as a growing number of patients receiving disease-modifying drugs and aggressive chemotherapeutic regimens for an array of inflammatory and malignant diseases [77]. This last group is increasing rapidly and includes patients receiving TNF blockade (infliximab, adalimumab, etanercept), CD-52 antibodies (alemtuzumab), B cell blockade (Rituximab) and selective T cell blockade, in addition to antipurine drugs, bendamustine, nucleoside analogs and high-dose steroids for prolonged periods [78].

Cotrimoxazole remains the drug of choice for both prophylaxis and treatment. Systematic review and meta-analysis have shown significant benefit in preventing PnP and reducing PnP-related mortality, although the trials analyzed focused on hematological malignancy and solid organ transplant patients and tended to be small and of poor quality [79]. The benefit in HIV-infected populations is well documented and the effect on survival is compelling, but there are few data in other groups of patients. Prophylaxis is still not universally used in hematological patients receiving rituximab, despite recommendations for prophylaxis in rheumatoid arthritis [77].

A variety of different prophylactic regimens of cotrimoxazole have been used, daily, alternative day, and thrice weekly have all been used and the optimum regimen in different patient groups has not been determined. ECIL guidelines recommend either one single strength tablet (480 mg) daily or one double strength (960 mg) table three times a week [13].

Intolerance of cotrimoxazole and adverse events (including, rashes and marrow suppression) are relatively frequent and may necessitate use of second-line agents. Inhaled pentamidine, dapsone, and atovaquone have all been used effectively but are considered inferior to cotrimoxazole, and should only be used after careful consideration. In a randomized control trial comparing cotrimoxazole versus pentamidine as primary *Pneumocystis* prophylaxis in HIV-infected patients, there were no breakthrough cases associated with the use of cotrimoxazole compared a rate of 8.4% when using pentamidine, although there were more side effects associated with the use of cotrimoxazole [80]. In second randomized controlled trial comparing the use of inhaled cotrimoxazole versus inhaled pentamidine as secondary prophylaxis in HIV-infected patients, the risk of PnP recurrence was 3.3 (95% CI: 1.7–6.2) times higher in the pentamidine group (P < 0.001) [81]. A randomized control trial of cotrimoxazole, dapsone plus pyrimethamine and pentamidine used as primary prophylaxis in HIV-infected patients, showed the lowest number of breakthrough cases associated with the prophylactic use of cotrimoxazole, although this did not reach statistical significance [82].

It may be possible to reintroduce cotrimoxazole when adverse events resolve.

Inhaled pentamidine has the advantage that it is administered monthly but requires a jet nebulizer and side-room facilities for effective and safe administration. Dapsone can trigger methemoglobinemia in susceptible individuals and patients should be screened for glucose-6-phosphate dehydrogenase deficiency before use. Other serious side effects include a potentially fatal idiosyncratic dapsone-hypersensitivity syndrome causing fever, skin rash, eosinophilia, and major organ dysfunction. Atovaquone is generally better tolerated and probably as effective as the other second-line agents. Use tends to be limited by higher drug acquisition costs.

### 3.2. Treatment

Recommendations are comprehensively reviewed in the ECIL guidelines although this focuses on patients without HIV disease [17].

High-dose cotrimoxazole is the treatment of choice given intravenously at 20 mg/kg/day in 2–4 divided doses. For severe disease, primaquine plus clindamycin is used for intolerant and refractory cases. Intravenous pentamidine has also been used, but experience is limited. In a randomized control trial of pentamidine versus cotrimoxazole for the treatment of PnP in HIV-infected patients, there was a 25% (95% CI: 5–45, P: 0.03) improvement in survival without respiratory support associated with completion of treatment of cotrimoxazole (86%) compared with pentamidine (61%) [83]. For mild-to-moderate disease, atovaquone may be used second line.
There is little evidence for the use of echinocandins alone or in combination with other drugs.

Adjunctive corticosteroids (50–80 mg daily) have established benefit in severe disease in patients with HIV, but use in other patients should be considered on a case-to-case basis.

Treatment durations of 14–21 days are recommended depending on response and severity of disease. Patients can be slow to respond and may actually deteriorate clinically in the first few days of treatment. Assessment of failure to respond cannot be made confidently during the first week of treatment.

4. Conclusions

With the incidence of PcP increasing through infection in high-risk non-HIV-infected patients, it is essential that every effort is made to optimize the diagnosis of PcP. While the development of culture-based methods is a breakthrough in the field, they come at a time when reliance on culture to attain a microbiological diagnosis is less and the role of PcP culture more suited to the academic scenario. Non-mycological laboratory markers and clinical presentation although satisfactory to initiate therapy in high-risk individuals do not provide a definitive diagnosis. Diagnosis by IF remains the reference standard, but the development of non-culture-based strategies has aided the diagnosis of other fungal diseases (e.g. invasive aspergillosis) and the combination of PcP PCR along with BDG testing may be suitable alternative, especially given the low incidence of disease. With both prophylaxis and treatment based on the primary use of cotrimoxazole, the emergence of resistance to sulfa-based drugs is of concern, and in the absence of culture, molecular techniques are the only route available to identifying resistance in *Pneumocystis*.

5. Expert commentary

A weakness in the diagnosis of PcP remains the resistance to move away from microscopic based diagnosis. It is accepted that sensitivity is far from optimal and false negatives will occur, but this conflicts with the low incidence of disease that dictates testing to be used to exclude disease, with subsequent sensitivity paramout. For a disease where recent incidences across HIV, hematology and solid organ transplantation were approximately 1% or less, the pretest probability of not having PcP is approximately 99% and it is far easier to use a highly sensitive (≥95%) test to confidently exclude disease than a highly specific (≥95%) assay to confirm it [19–21]. For example, for a disease with a prevalence of 1% and an assay with a good sensitivity and specificity of 90% the posttest probability of disease associated with a positive result is 8.3%, whereas the posttest probability of no disease associated with a negative result is 99.9%. Increasing specificity to 95% and 99%, respectively, increases the posttest probability of disease, when the assay is positive to 15.4% and 47.6%, respectively. So even with an excellent specificity of 99% it is more likely that the patient does not have disease. If this applied specifically to PcP and typical performance of PCR and IF microscopy then it is clear that even though IF provides a greater degree of diagnostic confidence it is still not infallible, and its lower sensitivity limits its application to exclude disease.

For PCR to take over as the reference method for PcP diagnosis standardization is required and commercially produced kits, international collaborative efforts of the Fungal PCR initiative and external quality control exercises (Quality Control for Molecular diagnosis (QCMD)) will assist this process.

With a reliance on testing lower respiratory tract specimens (e.g. BAL), the testing for PcP will always be balanced against the risk of obtaining the sample (e.g. during thrombocytopenia). Consequently, clinical diagnosis, based on risk factors, symptoms and response to therapy, will occur, but in cases not responding to therapy, this could reflect a pneumonia caused by a different etiology or possibly a case of treatment-resistant PcP. Moving away from testing BAL specimens to less-invasive specimens, such as upper respiratory samples or even blood, alleviates the clinical pressure and also removes the need to standardize bronchoscopy, which varies considerably between centers and impacts on test performance and interpretation. It is unlikely that a single noninvasive test will be able to provide both a diagnosis and the ability to exclude disease, but combining PCR of upper respiratory tract specimens with BDG testing of serum may do so. Currently, large-scale performance data is limited but the ability to offer this noninvasive approach will surely appeal to clinicians and it is hoped there will be sufficient evidence in the near future to confirm the applicability of this strategy.

6. Five-year view

Within the next five years, diagnosis of PcP will become less reliant on IF, with the potential for IF to become obsolete. The standardization of PCR through the efforts of the Fungal PCR initiative and through commercial development coupled with increasing prospective information on the performance of real-time PCR will provide greater understanding of interpretation of low-level PCR positives, across a range of patient populations. Combining PCR with BDG will further reduce the requirement for IF diagnosis. The development of syndromic testing using multiplex molecular methods may allow PcP to be detected alongside a range of other respiratory pathogens (e.g. Abbott iRIDICA) in a single assay. Whether BDG could be combined with *Pneumocystis*-specific immunology (antibodies or antigen) and provide a totally serological approach is yet to be proved. Although antibody ELISA tests targeting the major surface glycoproteins (Msg A, Msg B and Msg C) in *Pneumocystis* have shown promise, there is very little in the way of standardization and commercialization [84]. There is also the problem of positivity in healthy individuals who have been exposed to *Pneumocystis*, and as antibody levels peak almost a month post recovery, whether significant antibody positivity will occur too late to be clinically useful [84–86].

The application of next-generation sequencing (NGS) in relation to *Pneumocystis* is limited by the lack of culture. The ability to perform cell culture may alleviate the problem and...
should be focused on strains of Pneumocystis which are resistant to treatment to identify new molecular mechanisms of resistance. NGS may also provide further insights into transmission and sources of infection, allowing improved infection control measures to be applied. By combining direct PCR testing of nucleic acid extracted from respiratory specimens, NGS can provide enhanced broad-range diagnosis in symptomatic patients, but also an understanding of the respiratory microbiome and the prevalence of Pneumocystis colonization in asymptomatic individual.

From a clinical perspective, it is likely that the population at risk of PcP will expand with cases diagnosed in novel cohorts and the application of resistance monitoring is likely to become a standard procedure and has already been trialed in Europe where anthropophilic transmission and suboptimal prophylaxis were identified as risk factors [87].

Key issues

- The population of patients at risk from PcP is growing and changing. While the incidence of disease in the HIV infected cohort may be reducing due to successful anti-retroviral therapy, the incidence in other populations (Haematology, particularly conditions affecting lymphocyte count and function; solid organ transplant recipients, including renal transplants; solid malignancy; rheumatoid conditions; pre-existing chronic lung conditions; patients with connective tissue disorders and those receiving immuno-modulatory therapies) is increasing.
- In high risk patients, clinical presentation and radiology is sufficient for initiating empirical therapy but should not be used as definitive diagnosis, and on commencing therapy every effort should be made to achieve an organism specific mycological diagnosis.
- The reference method for the diagnosis of PcP is the microscopic examination of respiratory samples, preferably BAL fluid, with immuno-fluorescent staining, using anti-Pneumocystis antibodies targeting both ascus and trophic forms.
- Negative microscopy cannot be used to exclude PcP, but given the low incidence of disease exclusion of disease is a sensible use of mycological testing. As Both PcP PCR of BAL and BDG of serum/plasma have a high sensitivity they can be used to exclude PcP when negative, and meet this requirement.
- BDG testing of serum and plasma is very sensitive (>90%), but not sufficiently specific and given the broad detection range coupled with the susceptibility of the at-risk patient population it should be combined with an organism specific test. The BDG testing of respiratory samples is not recommended and adds very little to testing serum/plasma.
- Standardisation of PcP PCR methodology would be beneficial, although meta-analyses of current methodology provide high (≥90%) sensitivity and specificity when testing BAL fluid. PCP PCR sensitivity is reduced when testing upper respiratory samples, although specificity is increased. Commercial PcP PCR tests will assist in methodological standardisation and have the ability to identify genetic markers associated with resistance to sulfa-based therapy direct from the specimen. Molecular based methods can be used to identify origin of infection, transmission routes, outbreaks situations as well as epidemiology and evolution of the organism.
- Combination testing, involving IF microscopy and PCR on BAL, or in the absence of BAL, BDG on serum/plasma and PCR/IF on an upper respiratory sample is recommended. Albeit there is a strong argument for combining PCR and BDG alone.
- Guidelines for the prophylaxis and treatment of PcP in HIV, solid organ transplantation, haematology and rheumatoid conditions are available.

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Declaration of interest

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References

Papers of special note have been highlighted as either of interest (●) or of considerable interest (★★) to readers.

10. Yianniakis EP, Boswell TC. Systematic review of outbreaks of Pneumocystis jiroveci pneumonia: evidence that P. jiroveci is a

- Excellent review of outbreaks and their potential impact.


- Up-to-date review of diagnosis of PcP, principally applied to hematology patients but likely applicable to all cohorts. Provides an excellent tabular review of PCR technology and provides a combined diagnostic algorithm.


- Article highlighting the potential overlooked burden of fungal disease.


- Provides a systematic explanation of radiological review of pulmonary complications in cancer. Excellent background for the non-radiologist.


- Up-to-date review of diagnosis of PcP, principally applied to hematology patients but likely applicable to all cohorts. Provides an excellent tabular review of PCR technology and provides a combined diagnostic algorithm.


- Article highlighting the potential overlooked burden of fungal disease.
67. The development of commercial assays will help standardize PCR methodology, but PneumoGenius® assay has the added benefit of being able to detect common single-nucleotide polymorphisms that provide resistance to sulfaphenicol therapies.
71. Study utilizing next-generation sequencing to show that Pcp is usually caused by multiple strains.


