Title: Diagnosis and Management of *Pneumocytis jirovecii* infection

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ABSTRACT

Pneumocystis jirovecii is a ubiquitous fungus causing pneumonia in humans. Diagnosis was hampered by the inability to culture the organism, with diagnosis based on microscopic examination of respiratory samples. Performance of microscopy was improved by immunofluorescent (IF) testing using monocloncal antibodies targeting both cyst and trophic forms. Although microscopy is specific, with positivity used to define disease, poor sensitivity meant negativity could not exclude it.

New assays can assist in the diagnosis. PCR has permitted testing of respiratory samples other than bronchoalveolar lavage (BAL), easing sampling pressures. PCR has greater sensitivity than IF but questions remain as to the significance of low level positivity, in respect to colonisation versus disease associated with low fungal burdens. Conversely, PCR negativity in BAL samples can exclude disease, provided sampling is adequate. The presence of 1-3-β-D-Glucan in serum is also a useful biomarker, providing high sensitivity. However, 1-3-β-D-Glucan is not specific to *Pneumocystis jirovecii* pneumonia (PCP), and definitive thresholds for PCP are not available. Combination testing has the potential to both diagnose and exclude PCP. Recommendations on prophylactic and therapeutic management will be discussed with reference to new guidelines for PCP.

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 anti-retroviral therapy and the use of prophylaxis. Most HIV associated cases of PCP now occur in patients with undiagnosed HIV. [4, 5] There is an increasing population of susceptible non-HIV patients, including those with solid malignancies, solid organ transplant and haematopoietic stem cell transplant recipients, patients receiving immuno-suppressive therapies for auto-immune and inflammatory conditions and those with genetic primary immune deficiency disorders.[6] A national study over the decade 2000-2010 showed an annual average increase in incidence of 9%, and the largest cohort associated with PCP were those suffering from underlying haematological 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 pre-existing lung disease. [5, 7]

Children are exposed to *Pneumocystis* at early age, between the ages of 2-4 years old over 80% of children will have generated antibodies. Reactivation of latent infection was a presumed source of infection in susceptible hosts later in life.[8, 9] However, several documented PCP outbreaks confirm anthropophilic transmission, likely by airborne dispersal. Furthermore, typing revealed that infection was associated with place of diagnosis rather than place of birth. [10] Increased risk for developing PCP is associated with immuno-suppression, 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 extra-pulmonary disease, potentially associated with any organ, a rare manifestation. Symptoms are generally non-specific, including fever, non-productive cough, worsening chest pain, shortness of breath (especially on exertion), with the severity of symptoms often greater in non-HIV patients. [11] In mild cases initial examination may appear normal, although under exertion heart rate and oxygenation levels may become abnormal. [11] In HIV+ patients the onset of symptoms can be indolent often delaying diagnosis by weeks, whereas in non-HIV patients PCP presentation is acute, often fulminant, particularly after corticosteroid administration.[12] The mortality rate in HIV+ patients ranges from 17-30%, whereas in non-HIV patients are higher ranging from 28-53%. [12]

Given the non-specific 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. [13] 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 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 aetiological specificity. Recent guidelines for the diagnosis and management of PCP are available but the evidence is lacking or weak in many areas. [11-17]

DIAGNOSIS

Understanding test formats

The incidence of a disease influences utility of diagnostic tests, and can determine the optimal testing strategy in different clinical settings. Before ordering any test, clinicians should decide how the test result (positive or negative) would affect the management of their patient. If both outcomes are the same then the test has no clinical value. Clinicians often focus on a purely diagnostic approach, but many tests are better suited to exclude a diagnosis, avoiding the need for unnecessary therapy. 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 pre-test probability of disease is small compared to the pre-test probability of not having disease. 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 multi-disciplinary interpretation of results. Different sample types, for example upper and lower respiratory tract specimens and even blood samples, may shift the emphasis of the result from sensitivity/NPV to high specificity/positive predictive value (PPV).

Radiological investigations

Chest radiography (CXR) may be normal during the early stages of disease, but can worsen rapidly, particularly in the non-HIV population. [11, 12, 17] Computerised tomography (CT) scans are more sensitive than conventional radiographic techniques, providing evidence of infection even during the early stages of disease in non-HIV patients, and there is a role for CT despite CXR negativity. [18, 19] CXR typically presents with bilateral, diffuse interstitial infiltrates that progress to bilateral consolidations. [11, 18, 20]

CT generally demonstrates bilateral, symmetric patchy ground-glass attenuation. Consolidations may be present in mid or late stages of disease. [12, 18] Other findings include nodules, cysts, pneumothoraces, upper lobe localization, linear opacities and septal thickening. [11, 12, 20, 21] Cavitation, intra-thoracic adenopathy and pleural effusions are less likely. [11, 16, 21] The radiological presentation of PCP is not specific and can imitate other pathogens (e.g. bacterial pneumonia). [22] Radiology cannot provide an aetiological diagnosis, but may be used to initiate empirical 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. [23-25] CT has also been used to determine the severity and prognosis of PCP infection.[18, 21]

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. [26] Lymphocyte function in addition to absolute numbers may also be significant and the role of recent immunosuppressant drugs and other biological response modifying agents should be considered.

Hypoxaemia will vary depending on the severity of disease and HIV status, and regularly presents as a mild and severe reduction arterial oxygen in HIV+ and non-HIV patients, respectively. [11, 12] Serum lactate dehydrogenase (LDH) elevation is a suggestive marker, with levels >500ml/dL associated with PCP. [11] 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+ population. In a study of LDH in performance in HIV+ and non-HIV cohorts the sensitivity and specificity were 100%/47% and 63%/43%, respectively, showing that within HIV+ cohort a negative result could be used to confidently exclude disease, but positivity required confirmatory testing. [27] The use of procalcitonin serum concentration to differentiate PCP from other respiratory infections and/or colonisation is not clear. [28-30]

Clinical factors have also been used to predict mortality. In large observational cohort study of 451 HIV+ patients five significant predictors (Age, recent intravenous drug use, total bilirubin, serum albumin and alveolar-aterial oxygen gradient) were determined through multivariate analysis and incorporated into model to predict PCP mortality. [31]

Conventional Techniques – Culture

The difficulty in culturing *Pneumocystis* has hindered both diagnosis and research and development. Several methods using various co-culture cell lines were described but failed to attain widespread use. [32] Most attempts have used rat-models and subsequently *P. carinii* not *P. jirovecii*. In 1999, *P. carinii* initially isolated from rat lung was cultured using continuous axenic cultivation. [33] This complex technique has been successfully applied to the recovery of *P. carinii* from lungs and BAL fluid of rats and used to investigate life-cycle, but has limited use in routine diagnostics. [34-36]

In 2014, the first successful cultivation and propagation of *P. jirovecii* direct from BAL was achieved using a three-dimensional air-liquid interface culture system formed by CuFi-8 respiratory epithelial cell line. [32] 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.

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 both ascus and trophic forms but do not stain the cell wall. Toluidine blue is a generic stain for nucleic acids and polysaccharides, while calcofluor white stains chitin and cellulose, neither is specific for *Pneumocystis*. The performance of conventional stains has been superseded by IF microscopy using anti-*P. jirovecii* monoclonal antibodies. [12] 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). [12]

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. [37] 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. [37] 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 (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 ≤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. [12] A summary of the comparative performance of various microscopic staining and fluorescent techniques for the diagnosis of PCP is shown in table 2. [38-40]

It is important to consider the influence of specimen type and quality on assay performance. There is no standardised approach to sampling the respiratory tract and protocols will vary across centres 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. [40] 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. [41] Overall sensitivity and specificity when testing induced sputum was 55.5% and 98.6%, respectively, although the sensitivity when IF testing (67.1%) was significantly greater than conventional staining (43.1%). [41]

(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). [42-44] Overall, sensitivity is high and BDG negativity can be used to exclude PCP, although false negatives have been noted.[45] Specificity is suboptimal (<90%).[42-44] 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 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 non-infective factors such as potential sources of false positivity.[46] For the diagnosis of PCP there was no difference in the overall accuracy of BDG assays developed by different manufacturers. [43] In one meta-analysis, BDG performance when testing samples from HIV+ versus HIV- patients was comparable, although in a more recent study sensitivity was deemed to be significantly lower in the non-HIV population (HIV+: 92% versus HIV-: 85%), potentially a result of the greater burden of organism seen in HIV+ PCP. [43, 44]

BDG assays utilise a single positivity threshold for the detection of invasive fungal disease and it is not possible to confidently determine organism specific fungal aetiology based 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 >500pg/ml), even in the absence of IF staining of respiratory samples. [47, 48] In the study of Damiani *et al*. the median Fungitell BDG concentration across 17 cases of PCP was 1945pg/ml (range: 122-8000pg/ml), with 10 of the cases generating concentrations >500pg/ml, and 14 cases with concentrations >300pg/ml. [49] Both control and *Pneumocystis* colonised patients had BDG concentrations below 90pg/ml. Differentiation of colonisation from infection was also possible using the Beta-Glucan test Wako™ (colonisation: 49pg/ml versus infection: 173pg/ml).[50] 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 300pg/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.[30]

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 colonisation by other fungi is possible the presence of elevated BDG concentrations 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. [48] Even when using higher positivity thresholds BDG specificity when testing BAL fluid remained compromised (241pg/ml: 39%; 783pg/ml 79%).[30] While there has been a successful attempt to differentiate PCP infected from colonised/uninfected patients based on BDG concentration. Others have found receiver operator characteristic curve analysis to be of limited use in defining BDG BAL threshold. [30,48,51]

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. [13] While the focus on development of local assays provides technological diversity, it prevents methodological standardisation, 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]

Sub-group analysis using microscopy as the reference standard showed performance (Se: 97%, Sp: 93%) comparable to the combined population, whereas specificity was increased to 96% when using other reference standards.[52] Comparison of performance in HIV+ and HIV- cohorts was similar. [54] When testing BAL the sensitivity and specificity were 100% and 87%, respectively, but when induced sputa were incorporated sensitivity was 97% and specificity was 93%. [54] Comparison of performance when PCR testing BAL fluid 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 sub-unit mitochondrial rRNA has been noted and as with all molecular based assay surveillance for genetic drift is required, but complicated by the lack of surveillance cultures. [55] 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). [56]

From a technical perspective, the use of commercial kits for cell wall disruption and nucleic acid extraction affected specificity, while targeting the ITS region for PCR amplification improved sensitivity, but, along with targeting the large sub-unit mitochondrial rRNA, decreased specificity. [54] The use of nested-PCR provided significantly lower specificity which could be attributed to its potential to detect sub-clinical 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 that are associated with improved specificity but also have been used to differentiate *Pneumocystis* infection from colonisation. [54, 57-59] When interpreting the significance of the burden the underlying condition of the patient and quality of sample must be considered. For example, in one study using a real-time PCR cycle threshold (Ct) of 27 was associated with 100% specificity for the diagnosis of PCP in HIV+ patients, yet the optimal Ct in HIV- patients was 31 cycles and associated specificity was 80%. [57] Conversely, an upper Ct of 35 cycles generated a sensitivity of 80% and 1/5 PCP HIV- 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 and results of limited clinical value.

When interpreting low level PCP PCR positives (Ct >35) it is important to determine both the quality of sampling and also understand the presentation of clinical disease in HIV-patients with a low fungal burden but significant immune response. Theoretically, human DNA can be used as a surrogate for sample assessment. Low levels of human DNA could represent poor sample quality, whereas if a large quantity is present it could represent a strong immune response. For reference it is essential to know the typical burden of human DNA in respiratory samples and it is also requires that sampling is standardised, 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 centres to incorporate commercially manufactured and standardised 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 were excellent (Kappa: 0.85).[60] 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.[61] 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 sulfa-resistance direct from 89 samples and showed a 4.5% resistance rate.[61]

With more than 60 types of *P. Jirovecii* identified and approximately 30% of PCP cases infected with multiple types, the ability to investigate transmission and clusters has been hampered by the difficulty in cultivating *Pneumocystis.*[62, 63] 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 multi-locus real-time mutation frequencies have been used.[10, 64, 65]

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.[12, 13, 17] The question remains whether by combining more sensitive tests specificity of diagnosis can be improved while maintaining confidence in exclusion. In the adult haematology 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.[13] 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. [13] This begs the questioned 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% of (25/26) 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. [66] 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, and if positive confirm, dependent on pulmonary burden, with BDG testing. [49] 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. [13]

The combination of BDG testing in association with LDH levels permits a fully non-invasive sampling regime and has been successfully evaluated for the diagnosis of PCP. When using optimal thresholds (BDG: 400pg/ml; LDH: 350U/l) specificity was 84%. [67] A further serological biomarker multi-centre 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. [68] 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 non-specific serological biomarker.

MANAGEMENT

PCP can run a fulminant course, particularly in HIV negative individuals and early treatment improves prognosis. 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 some 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 Table 5.

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 ascospores (cyst) but not trophic forms by echinocandins. Trimethoprim, sulfa drugs and pentamidine form the main stays of treatment. Corticosteroids are of proven benefit in HIV positive individuals with disease but a beneficial role has not been established for other patient groups. The most effective way of preventing PCP in people living with HIV is by immune-reconstitution through the administration of effective anti-retroviral therapy. Prophylaxis should be administered until immune reconstitution has been achieved.

Prophylaxis

Recommendations for prophylaxis are comprehensively reviewed in the ECIL guideline although this focuses on patients with haematological malignancies and undergoing SCT.[16]

Prophylaxis is recommended in risk groups that include HIV positive patients with CD4 counts less than 200 cells/mm^3, transplant patients, and patients with high-risk haematological 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. [69] This last group is increasing rapidly and includes patients receiving TNF blockade (infliximab, adalimumab, etanercept), anti-IL1 therapies (alemtuzamab). B-cell blockade (Rituximab) and selective T cell blockade in addition to anti-purine drugs, bendamustine, nucleoside analogues and high-dose steroids for prolonged periods. [70]

Cotrimoxazole remains the drug of choice for both prophylaxis and treatment. Systematic review and meta-analysis have shown significant benefit in preventing PCP and reducing PCP related mortality although the trials analysed focused on haematological malignancy and solid organ transplant patients and tended to be small and of poor quality. [71] The benefit in HIV populations is well documented and the effect on survival is compelling but there are few data in other groups of patients particularly those receiving disease modifying drugs. Prophylaxis is still not universally used in haematological patients receiving rituximab despite recommendations for prophylaxis in rheumatoid arthritis. [69]

A variety of different prophylactic regimens of cotrimaxazole 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 (480mg) daily or one double strength (960mg) table three times a week. [16]

Intolerance of cotrimaxazole 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 be used effectively but are considered inferior to cotrimaxazole on the basis of largely retrospective comparisons and should only be used after careful consideration.

It may be possible to reintroduce cotrimaxazole 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 methaemoglobinaemia 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.

Treatment

Recommendations are comprehensively reviewed in the ECIL guideline although this focuses on patients without HIV disease.[15]

High dose cotrimoxazole is the treatment of choice given intravenously at 20mg/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 confined to case reports. For mile to moderate disease, atovaquone may be used second-line. The use of echinocandins is not recommended.

Adjunctive corticosteroids (50-80mg 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.

CONCLUSIONS

With the incidence of PcP increasing through infection in high-risk non-HIV-infected patients, it is essential that ever 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-culturebased 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.

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 be used to exclude disease, with subsequent sensitivity paramount. 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.

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 individuals.

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 trialled 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 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 incidence of disease exclusion of disease is a sensible use of mycological testing. Both PCP PCR of BAL and BDG of serum/plasma can be used to exclude PCP when negative.

- 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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Table 1. A summary of risk factors for *Pneumocystis* pneumonia.

Underlying condition	Risk Factor
HIV/AIDS[a]	CD4 count <200cells/μl, CD4 cell percentage <14%, Previous PCP, Oral Candidiasis, Higher HIV burden, Ongoing bacterial pneumonia.
Haematological malignancy[b]	CD4 count <200cells/μl, Lymphocytopenia, Immuno-suppression to prevent rejection of allogeneic haematopoietic SCT. For autologous SCT patients receiving purine analogues 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).

Solid-organ transplantation[c]	CD4 count <200cells/µl, Corticosteroids, Anti-lymphocyte therapy, Mycophenolate mofetil, Calcineurin inhibitors, CMV disease, Graft rejection, Prolonged neutropenia, Exposure to cases of PCP.
Inflammatory Disease	Administration of multiple (≥3) immunomodulatory medications, including: Calcineurin inhibitors and/or anti-TNF therapy. Corticosteroids.

[a] Information collated from CDC, NIH, HIVMA/IDSA guidelines (11)

[b] Information collated from ECIL guidelines (16)

[c] Information collated from American Society of Transplantation guidelines (17)

Table 2. Studies comparing the performance of various microscopic staining and fluorescent kits for the detection of PCP. When interpreting results the influence of incorporation bias on performance parameters should be considered, as in many studies the results, particularly in combination with the other tests have been used to define cases and controls.

Reference	Assay	Performance Parameter						
		Sensitivity	Specificity	PPV	NPV	LR+ve	LR-ve	DOR
37	CW	73.8%	99.6%	98.0%	93.4%	184.5	0.26	709.6
	MF	90.8%	94.7%	81.9%	97.5%	17.1	0.097	176.3
	DQ	48.4%	99.6%	96.9%	88.0%	121.0	0.51	237.3
	GMS	76.9%	99.2%	96.2%	94.2%	96.2	0.23	418.3
38	CB	74.3%	99.6%	92.9%	98.0%	165.6	0.3	552.0
	MoF	60.0%	99.3%	87.5%	96.9%	89.2	0.4	223.0
	Giemsa	34.6%	100%	100%	95.1%	>346[a]	0.7	>494.3[a]
39	GMS	50%	100%	100%	96.5%	>500[a]	0.5	>1000[a]
	Giemsa	50%	100%	100%	96.5%	>500[a]	0.5	>1000[a]
40[b]	MoF	93.1%	100%	100%	95.5%	>931[a]	0.07	>13300[a]
	DQ	87.9%	97.6%	98.1%	85.4%	36.6	0.12	305.0
	GMS	89.7%	95.2%	96.3%	87.0%	18.7	0.11	170.0
	PCIF	94.8%	88.1%	91.7%	92.5%	8.0	0.06	133.3

[a] Values have been generated using a specificity of 99.9% to overcome ∞

[b] Results represent combined induced sputum and bronchoalveolar lavage fluid testing

Key:

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

Table 3. The performance of (1-3)-β-D-Glucan Testing for the diagnosis of *Pneumocystis* pneumonia as determined by meta-analyses. The table contains data determined for range of susceptible patients testing with various BDG assays.

Parameter	Study		
	Karageorgopoulos (40)	Onishi (41)	Li (42)
Cases/Total (n/N)	357/2080	286/2331	433/2195
Sensitivity (%)	94.8	95.5	90.8
Specificity (%)	86.3	84.3	78.1
PPV (%)	54.3	46.0	50.5
NPV (%)	99.0	99.3	97.2
LR +ve	6.9	6.1	4.1
LR -ve	0.06	0.05	0.12
DOR	115	122	34.2

Key:
PPV: Positive predictive value
NPV: Negative predictive value
LR +ve: Positive likelihood ratio
LR -ve: Negative likelihood ratio
DOR: Diagnostics Odds ratio

Table 4 The performance of PCR for the diagnosis of *Pneumocystis* pneumonia as determined by meta-analyses. The table provides the performance for PCP PCR when testing both HIV+ and HIV- patients, when testing upper and lower respiratory tract specimens, and is irrespective of differing technical details.

Parameter	Study		
	Summah (50)[a]	Fan (51)	Lu (52)
Cases/Total (n/N)	506/2330	606/1793	416/2505
Sensitivity (%)	97	98	99
Specificity (%)	94	91	90
PPV (%)	82	85	66
NPV (%)	99	99	>99
LR +ve	16.2	10.9	9.9
LR -ve	0.03	0.02	0.01
DOR	540	545	990

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

Key:

PPV: Positive predictive value
NPV: Negative predictive value
LR +ve: Positive likelihood ratio
LR -ve: Negative likelihood ratio
DOR: Diagnostics Odds ratio

Table 5. Therapeutic Recommendations for the management of *Pneumocystis* pneumonia in adults

Recommendation		Guidelines (population)		
		CDC, NIH, HIVMA/IDSA (HIV) [11]	ECIL (Haematology) [14, 16]	American Society of Transplantation (SOT) [(17)]
Prophylaxis	Population	1) CD4 count <200cells/µl 2) CD4 cell <14% 3) CD4 count 200-250 cells/µl in the absence of regular 3 month CD4 monitoring 4) Not patients receiving pyrimethamine/sulfadiazine for toxoplasmosis	1) ALL 2) allogeneic HSCT, steroids (>20mg/day/4 weeks) 3) Alemtuzumab 4) Fludarabine/cyclophosphamide /rituximbab Optional: Lymphoma with R-CHOP14 or escalated BEACOPP, nucleoside analogues, radiotherapy for brain tumours/metastasis with steroids	1) All SOT, especially lung transplant 2) Increasing immuno-suppression to prevent graft rejection 3) Recurrent or chronic CMV infection 4) Prolonged course of corticosteroids (>20mg for ≥ 2weeks) 5) Prolonged neutropenia 6) Episodes of autoimmune disease
	Duration	Until CD4 count ≥200 cells/µl for > 3 months	1) Induction to end of maintenance 2) Engraftment for at least 6 months until immuno-competent	A minimum 6-12 months post-transplant for all SOT recipients. Patients with lung or small bowel

		3) More than 6 months post completion 4) Minimum of 6 months post completion	grafts or those prior PCP or chronic CMV disease may require lifelong prophylaxis
Therapy [a]	Front line: Trimethoprim/sulfamethoxazole one single-strength (80mg TMP/400mg SMX) daily or one double strength tablet (160mg TMP/800mg SMX)/daily. Second line: Trimethoprim/sulfamethoxazole one double strength tablet (160mg TMP/800mg SMX) three times per week Dapsone (50mg twice daily) Dapsone (200mg) + pyrimethamine (75mg) + leucovorin (25mg) weekly Dapsone (50mg daily) +	Front line: Trimethoprim/sulfamethoxazole one single-strength (80mg TMP/400mg SMX)/day or double strength tablet (160mg TMP/800mg SMX)/day or three per week. Second line: Dapsone (50mg twice daily) Pentamidine aerosols (300mg per month)	Front line: Trimethoprim/sulfamethoxazole one single-strength (80mg TMP/400mg SMX)/day or double strength tablet (160mg TMP/800mg SMX)/day or three per week. Second line: Dapsone (50-100mg once a day)

		pyrimethamine (50mg weekly) + leucovorin (25mg weekly) Pentamidine aerosols (300mg per month) Atovaquone 1500mg daily		
Targeted Treatment	Population	HIV/AIDS patients with suspected/diagnosed PCP	Haematological malignancy, solid cancer, solid organ transplant, autoimmune/inflammatory conditions with suspected/diagnosed PCP	All SOT with suspected/diagnosed PCP
	Duration	3 weeks	A minimum of 14 days	At least 14 days, extended to 21 days for severe cases
	Therapy [a]	Frontline: Trimethoprim/sulfamethoxazole (15-20mg/kg TMP; 75-100mg/kg SMX per day) For moderate to severe disease (i.e. hypoxemia) adjunctive corticosteroids should be used	Frontline: Trimethoprim/sulfamethoxazole (15-20mg/kg TMP; 75-100mg/kg SMX per day)	Frontline: Trimethoprim/sulfamethoxazole (15-20mg/kg TMP; 75-100mg/kg SMX per day) with TMP administered by IV every 6-8h. For hypoxemic patients potentially in combination with 40-60mg of prednisolone (twice daily)

	Second line for severe disease: Primaquine and clindamycin (30mg/(600mgx3)) per day Pentamidine IV (4mg/kg/day) Second line for mild/moderate disease: Dapsone (100mg daily) + trimethoprim (15mg daily) Atovaquone (750mg BID)	Second line: Primaquine and clindamycin (30mg/(600mgx3)) per day Pentamidine IV (4mg/kg/day)	Second line: IV Pentamidine (Initially 4mg/kg/day over 1-2h) Recipients of pancreas/islet transplants should receive an alternative second line therapy.

[a] Where possible only the recommendation receiving an "A" grading or the preferred drug of choice have been listed.