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Chapter I

Pneumocystis jirovecii Pneumonia

Enrique J. Calderón[1], José Manuel Varela[1], Isabelle Durand-Joly[2] and Eduardo Dei-Cas[2]

Instituto de Biomedicina de Sevilla and CIBER de Epidemiología y Salud Pública, Internal Medicine Service, Virgen del Rocío University Hospital. Seville, Spain[1]

Parasitology-Mycology Service (EA3609), Biology & Pathology Centre, UDSL, Univ. Lille Nord de France, Lille-2 University Hospital Centre & IFR-142 Institut Pasteur de Lille, France[2]

Abstract

Pneumocystis jirovecii (formerly *Pneumocystis carinii* sp. f. *hominis*) is an unusual fungus exhibiting pulmonary tropism and a highly defined host specificity. It is generally regarded as an opportunistic microorganism causing severe and often fatal pneumonia in AIDS patients. However, with the currently rising number of patients receiving immunosuppressive therapies for malignancies, allogeneic organ transplantations and autoimmune diseases, *Pneumocystis* pneumonia is becoming more and more recognized in non-HIV immunosuppressed individuals. The clinical presentation in HIV-infected patients may differ from that in other immunocompromised patients and its diagnosis continues to be challenging because no combination of symptoms, signs, blood chemistries, or radiographic findings is specific of *Pneumocystis* pneumonia. In addition, as *P. jirovecii* cannot be grown in culture from clinical specimens, the diagnosis of *Pneumocystis* pneumonia continues to rely on the microscopic demonstration of the characteristic organisms using conventional cytochemical or immunofluorescence staining in respiratory samples. These methods are useful when the organism burden is relatively high but they are insufficient for reliable detection when there is a small parasite load. Therefore, in an attempt to improve diagnosis of *Pneumocystis* pneumonia, more sensitive molecular techniques such as conventional and quantitative PCR have been developed. Using molecular technique mutations in both the gene encoding dihydropteroate synthetase, the target enzyme of sulfonamides, and the gene encoding cytochrome B, conferring potential atovaquone resistance, have been demonstrated.

However, their clinical relevance on treatment failure has not yet been determined. Co-trimoxazole, an association of trimethoprim and sulfamethoxazole, pentamidine isethionate or atovaquone has been extensively prescribed for the prophylaxis and therapy of *Pneumocystis* pneumonia.

Nevertheless, co-trimoxazole is currently regarded as the drug of choice for prophylaxis and therapy of any form or severity of *Pneumocystis* pneumonia. Looming on the horizon is the specter of resistance to co-trimoxazole and atovaquone, but there are few options for other alternative treatments. A prompt appropriate therapy is probably the most crucial factor in improving the prognosis of this devastating pneumonia for which care providers must continue to maintain a high index of suspicion in immunocompromised patients at risk. The management of *Pneumocystis* pneumonia remains a major challenge for all physicians caring for immunosuppressed patients.

Introduction and Historical Perspective

Pneumocystis jirovecii, previously known as *Pneumocystis carinii* sp. f. *hominis* [1], is an atypical fungus exhibiting pulmonary tropism and a highly defined host specificity. This microorganism causes opportunistic infection, particularly pneumonia, in patients who have impaired immunity. The general term for clinical disease caused by *Pneumocystis* is pneumocystosis.

Pneumocytsis was originally identified in 1909 by Carlos Chagas in the lungs of guinea pigs that were inoculated with the blood of trypanomiasis patients. Therefore, he erroneously thought that this organism was part of the life cycle of *Trypanosoma cruzi*. One year later, Antonio Carini made a similar description in the lungs of rats infected by *Trypanosoma lewisi*. It was not until 1912 that the Delanoës working at the Pasteur Institute in Paris recognized that *Pneumocystis* in rats represented a unique species and suggested naming the new microorganism *P. carinii* in honor of Antonio Carinii [2].

For seven decades, most investigators thought *Pneumocystis* organisms to be protozoans because they do not look much like fungi base on the histological characteristics of its trophozoite and cyst life forms, fail to grow much in culture, and are not eliminated from patients by treatment with the usual antifungal agents. By contrast, drugs, such as trimethoprim-sulfamethoxazole and pentamidine, which are often useful in treating protozoan infections, are also active against *Pneumocystis*.

Throughout this time *P. carinii* has been regarded as a single protozoan organism capable of infecting a wide variety of animal species [3]. This idea lasted until 1988 when DNA studies were able to identify it as an atypical fungus close to the family of *Aschomycetos* [4]. Subsequent studies using molecular techniques allowed knowing other aspects, as it is a ubiquitous fungus with pulmonary tropism, which colonizes only mammals and that have a high specificity for the host (stenoxenism). In this way, it has been shown in cross-infection experiments that the species of *Pneumocystis* is specific to each type of mammal, with no transmission among mammals of different species [5]. Therefore, human pneumocystosis is not a zoonotic disease, and this notion has important implications for the epidemiology of human-derived *Pneumocystis*. These findings have recently determined the modification of the nomenclature of *Pneumocystis* that colonize and cause infection in humans, formerly known as *P. carinii sp. f. hominis*, and has now been renamed *P. jirovecii* [6], leaving the end of *P. carinii* to the cause of infection in rats.

Pneumocystis is generally regarded as an opportunistic microorganism causing serious pneumonia in immunocompromised patients, especially in those with AIDS. However, *Pneumocystis* was first identified as a human-pathogen in premature or malnourished infants suffering from interstitial plasma cell pneumonia in European countries around World War II, occasionally occurring in epidemics [2,3]. Since then *Pneumocystis* pneumonia (PcP) had only been reported infrequently in individuals with malignancies and solid organ transplantations until the human immunodeficiency virus (HIV) pandemia turned PcP into a major medical and public health problem in the 1980s [2]. During the 1990s, the introduction of highly active antiretroviral therapy (HAART) for HIV infection and *Pneumocystis* chemoprophylaxis reduced the frequency of PcP. Although at the beginning of this century, the incidence of pneumonia caused by this microorganism among subjects with HIV infection has decreased in developed countries, the prevalence of AIDS-related PcP in developing countries remains high and poorly controlled. AIDS-related PcP continues to be a devastating illness among subjects unaware of their HIV infection, persons without access to antiretroviral therapy, among patients who are intolerant or non-adherent, and in occasional cases of failure of prophylaxis [4]. For theses reasons, PcP still remains considered as a principal AIDS-defining illness [7].

Presently, interest in *P. jirovecii* infection goes beyond AIDS patients since with the rising number of patients receiving immunosuppressive therapies for autoimmune diseases, malignancies, allogeneic bone marrow or solid organ transplantations, PcP is more and more recognized in non-HIV immunosuppressed patients [5,6,8]. Underlying conditions associated with PcP in HIV-negative patients include hematologic or solid malignancies, allograft transplantation, autoimmune inflammatory disorders (mainly Wegener granulomatosis and systemic lupus erythematosus), inflammatory bowel disease, protein-calorie malnutrition, and congenital immunodeficiency disorders [5,6,8-12]. Lately, PcP has been reported in patients undergone treatment with new biological tumor necrosis factor-alpha antagonist agents (adalimumab, infliximab, etanercept) and anti-CD20 monoclonal antibody, rituximab [13-16].

However, despite advances in laboratory technology, the diagnosis of PcP continues to be challenging [17]. PcP may be difficult to diagnose owing to nonspecific symptoms and signs, the use of chemoprophylaxis and simultaneous infection with multiple organisms in an immunocompromised individual [18]. On the other hand, few treatment options exist for patients with PcP. Thus, management of PcP remains a major challenge to all physicians caring for these patients.

Life-Cycle

The complete life cycles of any of the species of *Pneumocystis* are not known, but presumably, all resemble the others in the genus. Many investigators have attempted to cultivate *Pneumocystis* using a variety of techniques, but have had limited success, impeding studies of *Pneumocystis*. Pneumonia models in immune-suppressed animals remain the main source of organisms for laboratory studies, yet these approaches have numerous inherent difficulties. Studies of the life cycle of *Pneumocystis* have been based mainly on light and electron microscopic analysis of forms seen in infected lungs or short-term cultures [19] There are two predominant morphologic life cycle forms of *Pneumocystis*, the trophic form

(1-4 μm) and the cystic form (8-10 μm) with three intermediate cyst stages (early, intermediate, and late precysts).

All stages are found in lungs but the trophozoite stage is the vegetative state that predominates over the cystic form during infection by approximately 10:1. During infection, most trophic forms are haploid and it has been hypothesized that trophic forms can conjugate and develop into cysts. The mature cysts contain eight intracystic nuclei (figure 1). It has been suggested that trophic forms originate from the intracystic nuclei of the mature cyst as its ruptures and then undergo vegetative growth or conjugation to re-form the cysts forms. It is further proposed that they may also undergo asexual reproduction through haploid mitosis and binary fission. In an infected host, *Pneumocystis* exists almost exclusively within lung alveoli. The trophic forms attach to the alveolar epithelium trough interdigitation of their membranes. This adherence is characterized by close apposition of the cell surface without fusion of the membranes and strongly promotes proliferation of the organism. *Pneumocystis* maintains an extracellular existence within alveoli, and probably obtains essential nutrients from the alveolar fluid or living cells. The adherence of *Pneumocystis* also inhibits the growth of lung epithelial cells. Although organism attachment to alveoli epithelial cells is essential for *Pneumocystis* infection and propagation, invasion of host cells is uncommon and extrapulmonary pneumocystosis occurs only in the setting of severe immunosuppression.

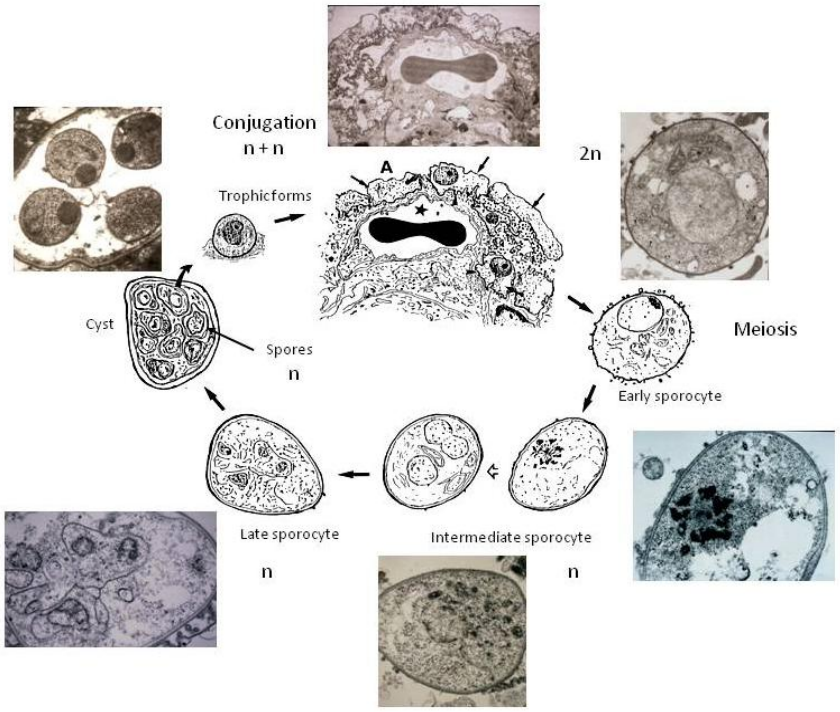


Figure 1. A hypothetical Pneumocystis life cycle illustrated by transmission electron micrographs and corresponding interpretation drawings of organisms developing in mammalian lungs. Mononuclear thin-walled trophic forms (small arrows) are attached to type 1 epithelial alveolar cells. An alveolar capillary vessel was indicated (star). Following conjugation (n+n), trophic forms could evolve into early sporocyte (2n), in which synaptonemal complexes evidenced meiosis. While an electron-lucent layer develops in intermediate sporocytes, mitotic nuclear divisions proceed. An additional mitotic replication leads to a thick-walled late sporocyte containing eight haploid (n) nuclei. In the mature cyst, the eight haploid (n) spores are

fully formed. These forms are able to leave the cyst and subsequently attach to type I alveolar cells. A: alveolar space. (Modified from: Aliouat-Denis et al. Mem Inst Oswaldo Cruz. 2009; 104:419-26. [19]).

Clinical Symptoms and Radiological Findings

Patients with PcP often develop dyspnea, which increases over time; cough productive of clear sputum or nonproductive cough; low grade or no fever; malaise, and sometimes chest tightness or pain. However, the clinical picture in individual patients is variable and many infectious and non-infectious processes can present identically. Also, the general hallmarks of this disease such as fever, shortness of breath, and diffuse infiltrates do not invariably occur, especially early in the course while the disease is mild [18,20,21]. Acute dyspnea with pleuritic chest pain may indicate the development of a pneumothorax, which has been presented in 2% to 4% of patients [22].

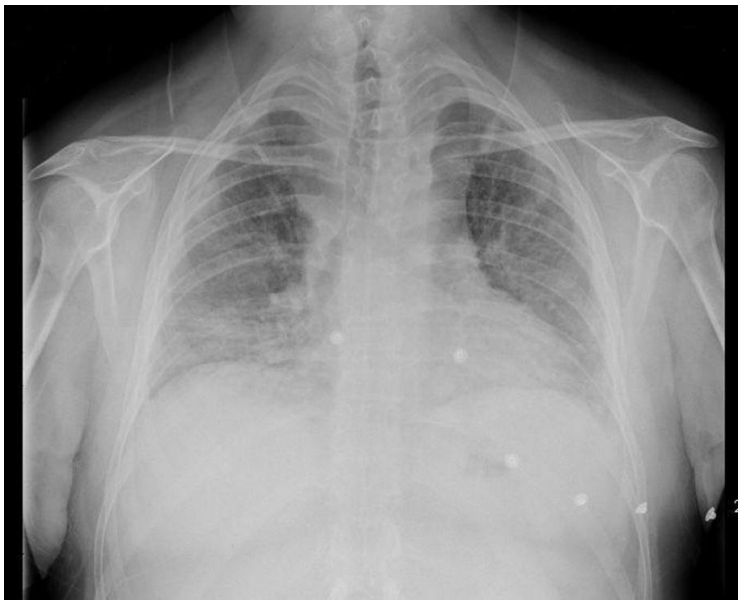
In patients infected with HIV, PcP is a common AIDS-defining illness and occurs most frequently in subjects with a CD4+ count less than 200 cells per cubic millimeter. The clinical course is subacute onset with progressive dyspnea, a nonproductive cough, malaise, and low-grade fever. A more acute illness with symptoms including a cough productive with purulent sputum should suggest an alternate infectious diagnosis, such as bacterial pneumonia or tuberculosis.

Non-HIV immunosuppressed patients usually have a more rapid onset than those infected with HIV. PcP usually has a subacute presentation with more insidious involvement in patients with HIV infection than in non-HIV immunosuppressed patients where PcP is much more likely to be an acute illness causing severe respiratory distress that frequently requires mechanical ventilation within the first several days [23,24]. In children, the symptoms of PcP can often be quite subtle, with an increased respiratory rate heralding the first sign of respiratory tract involvement. After a gradual onset, patients present progressive dyspnea, cyanosis, anorexia, weight-loss, and diarrhea whereas cough and fever can be absent [25].

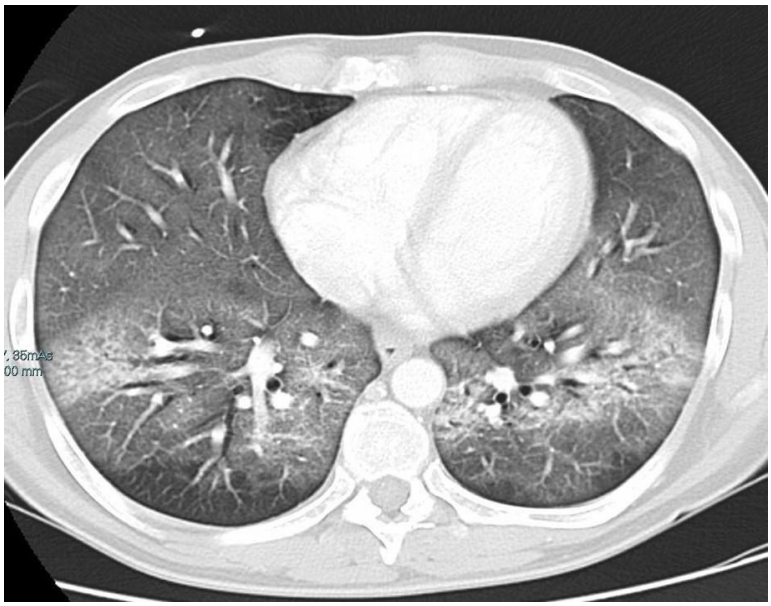
In all cases, a high index of suspicion and a thorough history are key factors in early detection of PcP. Physical examination may reveal tachypnea, tachycardia, and cyanosis. Lung auscultation usually reveals few abnormalities with dry cackles or rhonchi present in less than 50% of patients. Individuals with PcP can be hypoxemic with respiratory alkalosis but can also have normal alveolar-arterial gradients if identified early in the natural history of their disease. Elevated serum levels of lactate dehydrogenase (LDH) have been related with PcP and probably reflects lung parenchymal damage but is not specific. In general, laboratory abnormalities are less severe in HIV-infected patients than in non-HIV immunosuppressed patients [5].

Classic chest radiographic features of PcP, in patients with and without HIV infection, are bilateral, symmetric, fine reticular interstitial infiltrates involving the perihilar areas (figure 2a), becoming more homogenous and diffuse as the severity of the infection increases [18]. However, almost every conceivable radiographic presentation has been linked to PcP, including asymmetrical infiltrates, nodular densities, cavitary lesions, lymphadenopaties, pleural effusions, pneumatoceles, and pneumothorax. Patients who receive aerosolized pentamidine have an increased frequency of upper-lobe infiltrates, pneumothorax, or cystic lesions. Early in the course of PcP, the chest radiograph may be normal in up to 25% of cases [26]. A high-resolution computed tomography scan is more sensitive than a chest radiograph and it may reveal changes suggestive of PcP (figure 2b), as extensive ground-glass

attenuation or cystic lesions predominating in perihilar areas, even then chest radiographic findings are normal [27]. While such findings are suggestive, they are not diagnostic. However, a negative high-resolution computed tomography scan may allow exclusion of PcP in such patients.



2a



2b

Figure 2. Radiographic findings of Pneumocystis pneumonia. (2a) Chest x-ray of a Pneumocystis pneumonia in a patient with brain neoplasm revealing diffuse infiltrations in both lung fields. (2b) Chest high-resolution CT scan of a patient with renal transplantation showing diffuse ground glass opacities and thickened alveolar septum in both lungs.

Immunorestitution disease (IRD) is defined as an acute symptomatic or paradoxical deterioration of a (most probably) preexisting infection that is temporally related to the recovery of the immune system and it is due to immunopathological damage associated with the reversal of immunosuppressive processes. PcP manifesting as a form of IRD has been described in both HIV and non-HIV immunosuppressed patients [28-30]. Among HIV-infected patients, PcP manifesting acutely during the initiation of HAART is a well-recognized phenomenon [31]. AIDS-related PcP patients seem to be at risk of clinical deterioration due to IRD if antiretroviral therapy is started within one to two weeks after the initiation of treatment for PcP [31,32]. The onset of clinical deterioration is associated with an increase in the CD4 lymphocyte count and a reduction in the HIV viral load [31,32].

In non-HIV immunosuppressed patients, the clinical symptoms of PcP may be unmasked during the reversal of immunosuppression, often at the time when the dose of steroids is tapered or when the endogenous steroid production is reduced [33,34]. Rapid reduction of immunosuppressive therapy has been implicated as a predisposing factor for the development of PcP in non-HIV immunosuppressed patients. In this group of patients, PcP manifesting as IRD often runs an acute and fulminant course, with nonspecific lesions on chest radiographs and high lymphocyte counts. This atypical presentation can delay the diagnosis of PcP if physicians do not have a high index of suspicion [32].

Extrapulmonary manifestations of *P. jirovecii* infection (extrapulmonary pneumocystosis) are distinctly unusual. Extrapulmonary pneumocystosis has been reported primarily among HIV-infected patients, particularly those who receive aerosolized pentamidine for prophylaxis of PcP. Mainly, during the terminal stage of HIV-related disease *Pneumocystis* organisms may disseminate from the lungs to other organs where they induce secondary visceral lesions. However, at times pulmonary infection may not be apparent when extrapulmonary lesions are detected. For HIV-infected patients, extrapulmonary pneumocystosis limited to the choroid layer or ear (external auditory canal or middle ear) has a better prognosis, with good response to specific treatment, than disseminated pneumocystosis in multiple noncontiguous sites. Disseminated pneumocystosis is usually clinically evident, with symptoms related to the affected organs. Lymph nodes, spleen, kidneys, liver, thyroid, and bone marrow are the most commonly infected organs, but microorganisms have also been found in the brain, pancreas, skin, heart, muscle, and other organs [35]. Lesions are frequently nodular and may contain necrotic material or calcification. Extrapulmonary pneumocystosis in solid organs appears on the computed tomography scan as focal, hypodense lesions with well-defined borders and central or peripheral calcification [26]. Non-HIV-associated extrapulmonary pneumocystosis has been rarely reported. In the described cases, disseminated disease often occurred immediately premortem and extrapulmonary pneumocystosis was not clinically evident [35].

In all cases, the clinical diagnosis is complicated because no combination of symptoms, signs, blood chemistries, or radiographic findings is specific of *Pneumocystis* infection. As such, identification of *Pneumocystis* organisms or its DNA in a clinically relevant sample is required to make a diagnosis.

Diagnosis

The single most important diagnostic tool for *Pneumocystis* infection is a high clinical suspicion. In the right clinical setting, an immunosuppressed patient with new onset of dyspnea or new symptoms of pneumonia, with or without radiological findings, should prompt further evaluation, particularly if they are not receiving chemoprophylaxis.

Laboratory Diagnosis of PCP

Microscopic Detection of Pneumocystis

P. jirovecii organisms are usually detected in bronchoalveolar lavage fluids (BALF), induced sputum (IS) samples, or lung biopsy specimens by means of light microscopy (figure 3), immunofluorescence, or molecular methods. No in vitro system for obtaining routinely *Pneumocystis* isolates from patients is available. Using light microscopy, parasites, especially mature cysts, can be detected using phase contrast or Nomarski interference contrast on wet smears. However, microbiologists now detect these parasites on air-dried smears stained by toluidine blue O (TBO), Gomori-Grocott's methenamine silver nitrate (GMS), or methanol-Giemsa methods [36,37].

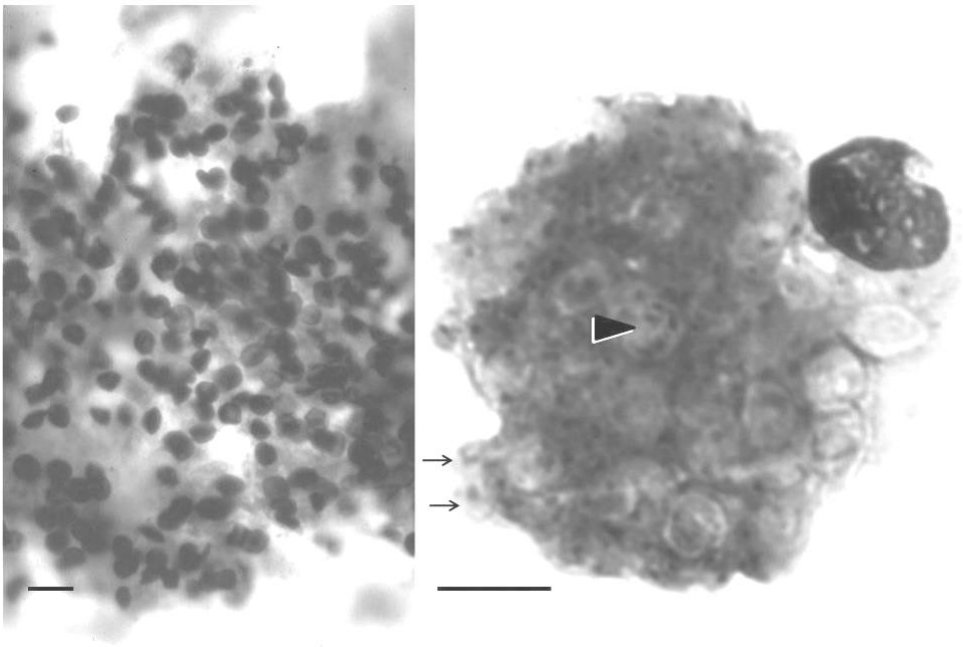


Figure 3. Pneumocystis organisms in cytospin smears of human bronchoalveolar lavage fluid samples. Left: clustered cystic forms stained with Gomori–Grocott's methenamine silver nitrate. Right: Pneumocystis organisms stained with methanol–Giemsa stain: clustered trophic, sporocytic, and cystic forms. A mature cyst containing many spores is quite visible (arrowhead). The cell wall of cystic and sporocytic forms appears as a clear, thin peripheral halo. An alveolar macrophage may also be observed (top right). Bar= 10 µm.

TBO, cresyl violet, and GMS have a good affinity for components of the cyst wall [38]. Thus, TBO stains the cell walls of cystic forms metachromatically in reddish violet and GMS in dark brown. Silver particles deposit on the glucan-rich electron-lucent middle layer of the cyst wall; in contrast, only little silver deposition was recorded in the electron-dense, unique layer of the thin trophic form's cell wall, as shown by ultrastructural studies [39].

TBO or GMS stains facilitate rapid parasite detection, even at low magnification, in all kinds of clinical specimens. However, these dyes also stain the cell wall of yeasts or other fungi. For this reason, a good strategy to identify *Pneumocystis* organisms accurately in clinical specimens is to systematically associate the examination of both TBO- or GMS-stained smears and methanol-Giemsa–stained smears from the same specimen (table 1). Actually, methanol-Giemsa (or other equivalent panoptical Giemsa-like stains) makes it possible, on the one hand, to distinguish *Pneumocystis* organisms from other microorganism and, on the other hand, to identify the different *Pneumocystis* life-cycle stages (figure 3). In fact, Giemsa and other stains with similar cytological affinities, such as Diff Quick or RAL-555, cause the parasite nuclei to stain pinkish purple and the cytoplasm to stain blue [40,41]. They do not stain cystic or sporocytic walls, which appear like a clear peripheral halo around cystic forms. These polychrome stains make it possible accurately to distinguish *Pneumocystis* trophic or cystic forms from other fungi and also from host cells or cell debris. On the whole, the biggest advantage of methanol-Giemsa or Giemsa-like stain methods consists in staining trophic forms and sporocytes (figure 3), which remain unidentified in TBO- or GMS-stained smears [41].

In order to detect *Pneumocystis* organisms in histological sections from lung or other organs, pathologists target usually the cystic forms, since trophic ones are uneasily identifiable in paraffin-embedded tissues. Therefore, they use GMS and, less frequently, TBO staining procedures adapted to tissue sections. Trophic forms can however be identified in epon-embedded semi-thin sections stained with toluidine blue or other stains [41,42]. Furthermore, *Pneumocystis*-specific fluorescein, phosphatase or peroxidase-labeled monoclonal antibodies available from many suppliers may help to identify *Pneumocystis* organisms in BALF, IS or tissue samples (table 1).

Efficiency and cost-effectiveness of the different microscopic stains evoked here vary according to the experience of groups, technical protocols, local incidence of PcP and other factors [43] (table 1). It is generally accepted, however, that association of methods that stain the cystic cell wall (e.g. TBO or GMS) with panoptical techniques (methanol-Giemsa or analogous staining methods) is usually required [44,45]. Moreover, it is usually recognized that specific antibody staining is mainly helpful to detect *Pneumocystis* organisms in non-BALF smears (e.g. IS, expectorated sputum, gastric wash) and to clarify conflicting light microscopic observations [17,46-48]. Finally, it must be remembered that the actual PcP diagnostic currently relies on microscopic detection of *Pneumocystis* cysts and/or trophic forms on stained respiratory samples [17], and that bronchoalveolar lavage is usually regarded as a gold standard procedure, with reported sensitivities ranging from 90% to 98% [49,50].

Table 1. Laboratory diagnostic methods for Pneumocystis pneumonia

Technique	Suitable kind of sample	Needed experience	Sensitivity	Specificity	Advantages	Drawbacks	Recommended combination with:
Microscopy: PC/IC	BALF wet smear	very good	Variable	good	rapidity	needs confirmation by other methods	panoptical stain
GMS/TBO	BALF air-dried cytospin smear or biopsy (histological section)	average	High	average	cost; rapidity	false positive (poor experienced staffs); identifies only the cystic stages	panoptical stain
Panoptical stains*	BALF air-dried cytospin smear	very good	Average	very high	cost; rapidity; identify all Pneumocystis stages	limited sensitivity (poor experienced staffs)	GMS/TBO
FL Mab	BALF, IS or sputum air-dried cytospin smear	good	High	good	good sensitivity/ specificity	cost; time-consuming	-
IP/AP Mab	biopsy (histological section), air-dried cytospin smear	good	Good	good	good specificity	cost; time-consuming	-

PCR	BALF, IS, OW, NPA, biopsy	average	very high	very high	Helpful in HIV-negative patients; rapidity (real-time PCR assays); non-invasive sampling; genotyping	cost; positive in colonized patients	-
BG	serum	average	Good	low	rapidity; post-therapeutic control	positive in other deep fungal infections	other tests
KL-6	serum	average	Good	low	-	positive in other pulmonary infections	
Serum Pneumocystis antibody assay	serum	average	depending on antigen and assay	depending on antigen and assay	helpful in epidemiology studies	positive in people without PcP	other tests

*Giemsa or Giemsa-like stains.

BALF: Bronchoalveolar lavage fluid; BG: serum beta-1,3-glucan; FL Mab: fluorescein-labeled Pneumocystis monoclonal antibody; GMS: Grocott-methenamine silver stain; IP/AP Mab: immuneperoxidase/alkaline-phosphatase labeled monoclonal antibody; IS: induced sputum; PC/IC: phase contrast/interference contrast; TBO; toluidine blue stain. KL-6: Mucin like glycoprotein.

Molecular Detection of Pneumocystis

Many *Pneumocystis* PCR assays were developed in the last two decades. PCR tools were revealed as highly efficacious to amplify *Pneumocystis* DNA from diverse kinds of clinical specimens (BALF, IS, expectorated sputum, oropharyngeal, or nasopharyngeal wash samples, biopsy specimens) (figure 4) [51-56]. In the clinical laboratory, the use of molecular methods is mainly warranted to increase the sensitivity of *P. jirovecii* detection in clinical specimens in order to establish earlier PcP diagnosis, detecting low parasite rates, mainly in non-HIV infected patients with PcP, and detecting *Pneumocystis* DNA in noninvasive samples [54,57] (table 1). Moreover, PCR assays followed by direct sequencing or other strategies were used for typing *Pneumocystis* isolates in order to identify parasite strains and to explore correlation between specific genotypes and virulence, transmissibility or drug susceptibility. PCR, especially nested PCR assays applied to noninvasive samples, have also been used to detect *Pneumocystis* colonization either in susceptible individuals or in apparently healthy people, including healthcare staffs in hospitals [55,58,59].

For PcP diagnosis in humans, conventional or real-time PCR assays based on the amplification of the large subunit of mitochondrial ribosomal DNA (mtLSUrDNA) [51,60] are the most commonly used, but many other sequences have been targeted (Major Surface Glycoprotein, Internal Transcribed Spacers, Thymidylate Synthase, Dihydrofolate Reductase, heat-shock protein 70, etc.) [54,61,62]. Comparative evaluating studies are not easy to perform because of different clinical contexts, sampling methods, laboratory reagents or technical strategies used for DNA extraction, amplification or analysis of results [54].

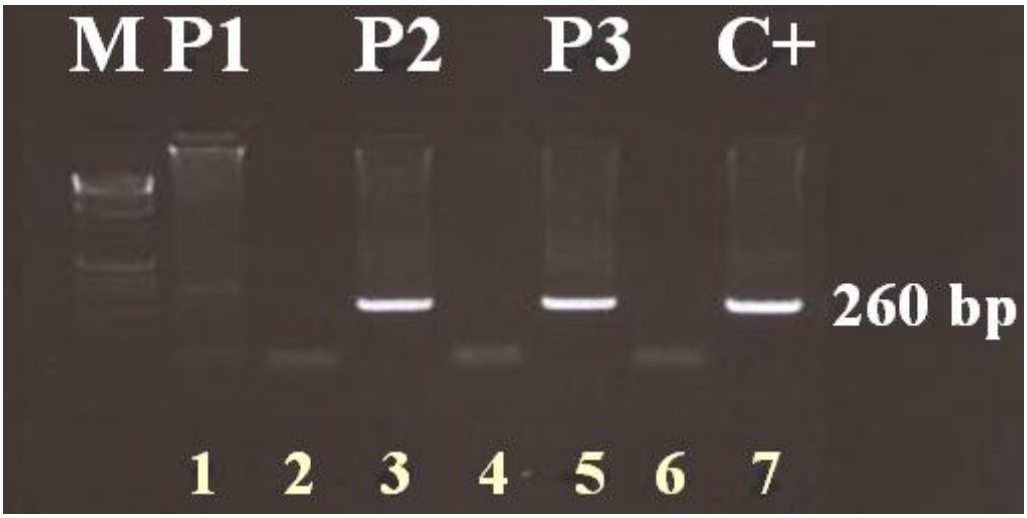


Figure 4. Nested PCR (mtLSU rRNA region) results. M: molecular mass marker. Lane 1 (P1) negative specimen. Lanes 3 and 5 (P2, P3) positive specimens of oral wash in cystic fibrosis patients. Lane 7 (C+) positive control. Lanes 2, 4 and 6 negative controls (water).

In general, conventional, or real-time *Pneumocystis* PCR assays, have represented a significant advance in PcP laboratory diagnosis. Actually, highly sensitive and specific PCR tools, especially real-time PCR assays, improved the clinical diagnosis of PcP allowing an

accurate, early diagnosis of *Pneumocystis* infection [54], which should lead to a decreased duration from onset of symptoms to treatment. This period has a recognized impact on prognosis since PcP-associated respiratory failure requiring mechanic ventilation entails significant mortality [63]. In addition, PCR assay may reveal PcP in patients with negative microscopic test. For instance, among 62 HIV-negative patients with clinical PcP diagnosed in the Lille University Hospital between 1998 and 2001, 30 patients (48%) had positive PCR results with negative microscopic tests [64].

Notably, molecular techniques play a significant role when they are applied to noninvasive specimens as IS, oropharyngeal wash (OW, obtained by gargling 10 ml of 0.9% NaCl for >60 seconds) [57,65-67] or nasopharyngeal aspirates (NPA) [68]. When DNA sequences used as primers or probes have been adequately defined, the analytical specificity of *Pneumocystis*-PCR assays applied to noninvasive or to BALF samples should usually be 100% [54,61,66]. With regard to sensitivity, *Pneumocystis*-mtLSUrDNA PCR showed high analytical sensitivity for the detection of *Pneumocystis* organisms on BALF samples from AIDS patients, with a detection threshold of 0.5–1 organism/μl^{-1} [61]. The sensitivity of PCR assays applied to OW (or other noninvasive samples) is certainly lower (<80%) [60,65,69] than that of PCR on BALF samples (>95%) [68]. However, OW can be easily repeated in order to monitor the evolution of infection and, potentially, the therapeutic response [60].

A significant problem of *Pneumocystis* PCR assays is raised by *Pneumocystis* colonization [70]. Actually, a positive PCR result associated with a negative microscopic test may result from either *Pneumocystis* colonization or PcP. In common practice, this difficulty is often solved with careful clinical, radiological and laboratory assessment of the patient's pathological condition, as is usually done with other infectious diseases, especially when their agents are opportunistic pathogens. However, the alternative of quantifying parasite rates was also explored [71]. Thus, a quantitative real-time PCR assay that targeted the *Pneumocystis* Major Surface Glycoprotein (MSG) multigene family was applied to OW samples, and revealed significant differences between PcP patients and *Pneumocystis* colonized subjects in the number of MSG copies. The authors suggested a cutoff value of 50 MSG gene fragment copies/tube for distinguishing between the two conditions [71]. However, quantitative PCR results seemed difficult to use in the field. The main problem was the inability to control the volume of the sample. Another difficulty relates to the kind of patients. Actually, it seems difficult to apply the same cutoff to AIDS patients, patients with other underlying diseases, or individuals receiving chemoprophylaxis against *Pneumocystis*.

There is no formal agreement about an unequivocal definition of *Pneumocystis* colonization. The notion may however be characterized by clinical and experimental observations. In clinical practice, the diagnosis of *Pneumocystis* colonization or subclinical carriage is usually retained when *Pneumocystis* DNA is detected by PCR methods in respiratory samples from immunodepressed or immunocompetent subjects without symptoms or signs of *Pneumocystis* infection, and who do not progress to PcP [72]. In these subjects, *Pneumocystis* organisms are only exceptionally detected by microscopy [73]. Interestingly, recent experimental data strengthened the biological significance of *Pneumocystis* colonization [74]. They demonstrated that *Pneumocystis* organisms can replicate in the lungs of immunocompetent carriers, stimulate an antibody response and be efficiently transmitted by airborne route to either naive immunocompetent hosts, who will develop a primary infection, or to immunosuppressed hosts, who may then develop PcP [74]. In addition, there is evidence to suggest that beyond PcP, *Pneumocystis* colonization may induce local or

systemic inflammation, a condition that could aggravate chronic pulmonary diseases. For instance, *P. jirovecii* pulmonary carriage in patients with chronic obstructive pulmonary disease (COPD) could favor the progression of this disease [72,75], which is a major cause of death worldwide [76].

Efforts have been made to associate specific *P. jirovecii* genotypes with virulence, drug susceptibility or other medically important biological properties of parasite strains. Some studies reported some correlation between polymorphism and clinical features [65,77,78]. Polymorphism of internal transcribed spacer (ITS1/ITS2) sequences was quite frequently used and more than 30 ITS1 genotypes and 40 ITS2 genotypes with more than 90 haplotypes (combinations of ITS1 and ITS2 types) have been reported [79]. It was shown that a high proportion of ITS recombinants, detected under standard conditions, would be chimeras generated during the genotyping process mainly during elongation [80]. However, this difficulty could be overcome by combining several measures (adding a proofreading polymerase, extending the elongation time, increasing melting temperature, decreasing the number of cycles) [80].

Most polymorphism studies targeted mutations of the *P. jirovecii* dihydropteroate synthase (DHPS) gene, which could potentially be linked with sulfa resistance. Regarding this issue, and since effective *P. jirovecii* culture systems are unavailable, several groups have assessed putative trimethoprim-sulfamethoxazole drug resistance by detecting *Pneumocystis* DHPS mutations. Indeed nonsynonymous DHPS point mutations at nucleotide positions 165 and 171 entail an amino acid change at positions 55 (Thr to Ala) and/or 57 (Pro to Ser) [81]. Such mutations confer resistance to sulfa drugs in other organisms, including *Escherichia coli*, *Streptococcus pneumoniae* and *Plasmodium falciparum*. The *P. jirovecii* DHPS mutant form has also been shown to be more resistant to sulfamethoxazole in a *Saccharomyces cerevisiae* model [82], but it is still uncertain if *Pneumocystis* DHPS mutations lead to drug resistance in patients [83,84]. Such mutations were shown to be associated with the use of trimethoprim-sulfamethoxazole or dapsone (two DHPS inhibitors), the duration of sulfa or dapsone prophylaxis and with geographic areas in which sulfamethoxazole or dapsone were commonly used for PcP prophylaxis [84-86]. However, results of studies searching specifically to establish an association between the presence of *P. jirovecii* DHPS mutations and clinical outcomes, such as treatment failure or death, are contradictory [84,87-91]. Outstandingly, most PcP patients carrying *P. jirovecii* isolates with DHPS mutations responded well to trimethoprim-sulfamethoxazole (TMP-SMX)treatment and survived probably because these mutations may confer a low-level of resistance to sulfa-drugs that is overcome by high drug concentration achieved in lung tissues by sulfamethoxazole [84,92,93].

Other Laboratory Diagnostic Methods

a) Beta-D-glucan assay

β-1,3-glucan (BG) is the main structural component of the cell wall of all fungi, including *Pneumocystis* cysts [94]. Interestingly, high serum BG levels have been reported in patients with PcP [95-97]. Consistently, such levels decreased with effective anti-*Pneumocystis* treatment [96]. Serum BG appeared therefore as a good marker of *Pneumocystis* infection.

The potential utility of this assay was illustrated in a retrospective case-control study of 295 patients with suspected PcP comparing BG with microscopic examination of BAL. The BG assay had a sensitivity of 92% and a specificity of 86% for detecting PcP for a cut-off level of 31.1 pg/ml [98].

In a recent study, it has been observed that BG levels in non-HIV patients with PcP are lower than in HIV patients. This could be attributed to the fact that HIV patients have greater numbers of microorganisms than non-HIV patients [97].

However, BG levels could not be correlated with PcP prognosis, and false positive results could exceed more than 30% [97]. False positive results were reported in patients undergoing bacterial septicemia, hemodialysis with cellulose dialysis membranes, treatment with immunoglobulin, glucan-containing antitumor drugs, amoxicillin-clavulanate, piperacillin-tazobactam or contact with gauze or surgical sponges containing BG [99]. Furthermore, since invasive fungal infections also induce an increase of serum BG, the test should often be associated with laboratory assays aiming at detecting such infections [95]. At least four BG assays allowing assessing serum BG levels in clinical laboratories are available: Fungitec G, Wako and B-G Star, which are used in Japan; Fungitell, which is used in Europe and USA [99].

These preliminary studies suggest that in the right clinical setting serum BG may provide a useful noninvasive diagnostic adjunct for patients with *Pneumocystis* infection. However, additional information is necessary to address the general specificity of BG in diagnosing PcP versus other fungal infections in diverse immune-suppressed patient populations and to differentiate among patients with PcP and patients with *Pneumocystis* colonization.

b) *S*-adenosylmethionine (SAM)

Some observations suggested that S-adenosylmethionine (SAM), which is a universal methyl donor synthesized from methionine and ATP by SAM synthetase, could stimulate *Pneumocystis* in vitro growth [100]. Since SAM was depleted from both the culture medium and the plasma of rats with PcP, it was hypothesized that *Pneumocystis* cells could scavenge SAM from host fluids due to the lack of SAM synthetase [100]. Consistently, plasma SAM levels were found to be low in patients with PcP and to increase gradually with treatment [101,102]. These findings strengthened the idea of using plasma SAM levels as a non-invasive PcP diagnostic method. However, recent data showed that SAM-related issues could be more complex than previously thought. Firstly, differences in SAM levels between laboratories could be influenced by the method of measurement. Thus, Wang and colleagues using Chromatography Tandem Mass Spectrometry found generally higher plasma SAM levels than those reported before [103]. The same group was unable to distinguish patients with acute PcP from the ones without PcP based on plasma SAM levels, though these levels increased significantly with effective anti-*Pneumocystis* treatment. Indeed, the concern needs to be further explored because fasting status, dietary intake of methionine, and other medications can affect plasma SAM concentration [103]. Secondly, and contrary to the results of previous works [100], *P. carinii*, *P. murina* and *P. jirovecii* have genes that encoded SAM synthetase (Sam1) [104]. Moreover, the corresponding *Sam1* mRNA is transcribed, and the protein, which is enzymatically active, was immuno-localized in *P. murina* cells. Such data suggest strongly that the *Pneumocystis* species do not depend on an exogenous source of SAM to survive [104].

c) KL-6

KL-6 is a mucin-like glycoprotein expressed on type II pneumocytes and bronchiolar epithelial cells. This serological marker has been found in elevated levels in several studies in patients with PcP. However, the reported false-positive rate and level of detection were not as good as for the BG assay [97,98]. Recent investigations indicant that JKL-6 is more a generalized marker for alveolar epithelial injury [105] and can also be detected in non-fungal infections such as *Legionella*, severe tuberculosis and respiratory syncythial virus bronchiolitis, and even in noninfectious interstitial lung disease [106-108]. Therefore, the KL-6 elevation in PcP is thought to be related to lung damage and regeneration of epithelium lining and cannot be used as a specified marker of *Pneumocystis* infection.

d) Serological tests

Serum antibody detection constitutes an adjunctive strategy currently used to diagnose systemic fungal infections, even in immunodepressed patients. However, this strategy was rarely used for PcP diagnosis because healthy subjects frequently have significant levels of the serum *Pneumocystis* antibody. Moreover, the antibody response against *Pneumocystis* infection is currently highly variable and the results reported by diverse groups are contradictory [109]. In contrast, *Pneumocystis* antibody assays, especially those using recombinant *Pneumocystis* antigens, constitute an interesting tool in epidemiology [110].

Management Strategies for PCP

There is no universally agreed approach on the initial management of patients with suspected PcP. Many institutions treat patients with suspected PcP empirically, while others pursue a definitive microbiological diagnosis [63]. In the absence of prospective studies comparing various management and diagnostic strategies, the specific approach to a patient with suspected PcP is often based on the incidence of PcP and clinician and institutional preferences and experiences [17,63]. Since PcP can be rapidly progressive and the mortality rate remains high, particularly among non-HIV immunosuppressed patients, early therapy is essential [8-10].

Identification of patients having mild, moderate or severe PcP disease guides the choice of drug for the treatment, as well as to decide if adjuvant corticosteroids are indicated (table 2) [111]. In AIDS-related PcP, the typical duration of therapy is at least 21 days because of the risk for relapse with shorter treatment duration. However, in patients with PcP without HIV-infection two weeks of treatment is usually adequate, even though treatment should be individualized and extended if recovery is prolonged [10,23,112,113]. There are no randomized trial data indicating when specific anti-*Pneumocystis* therapy should be modified because of inadequate response [114,115]. In the absence of corticosteroid therapy, early and reversible deterioration within the first 3–5 days of therapy is typical. Patients generally improved after 4 to 8 days of therapy. Therefore, changes in treatment due to lack of efficacy should rarely be made prior to 4 to 8 days and noninfectious processes, as congestive heart

failure or pulmonary emboli, or concurrent infections should be ruled out previously [112,113].

Table 2. Grading of severity of Pneumocystis pneumonia

	Mild	Moderate	Severe
Symptoms and signs	Dyspnoea on exertion, with or without cough and sweats	Dyspnoea on minimal exertion and occasionally at rest. Cough and fever	Dyspnoea and tachypnoea at rest. Persistent fever and cough
Arterial oxygen tension (PaO2) at rest	> 11.0 kPa (82.7 mmHg)	8.0 to 11.0 kPa (60-82.7 mmHg)	< 8.0 kPa (60 mmHg)
Arterial oxygen saturation (SaO2) at rest	> 96%	91 to 96%	< 91%
Chest radiograph	Normal, or minor perihiliar shadowing	Diffuse interstitial shadowing	Extension interstitial shadowing with or without diffuse alveolar shadowing

Modified of Miller RF, et al. J. Antimicrob. Chemother. 1996; 37 (Suppl B): 33-53 [135

Although the overall prognosis of patients whose degree of hypoxemia requires intensive care unit (ICU) admission or mechanical ventilation remains poor, survival in up to 50% of patients requiring ventilatory support has been reported. Patients with reasonable functional status and severe PcP should be offered ICU admission or mechanical ventilation [112].

Treatment

The recommended treatment of PcP has remained unchanged for many years, being Co-trimoxazole, an association of trimethoprim and sulfamethoxazole, the drug of choice as first line of treatment. Regarding which agent is preferred for the second line of choice, data are limited (table 3).

Drug related toxicities are increasing in HIV-infected patients and organ transplant recipients. Because of the potential for additive or synergistic toxicities associated with anti-*Pneumocystis* and antiretroviral therapies, certain health-care providers delay initiation of HAART until after the completion of anti-*Pneumocystis* therapy, or until at least 2 weeks after initiating anti-*Pneumocystis* therapy, despite some suggestion of potential benefit of early HAART in the treatment of patients with AIDS-related opportunistic infections [112,116]. In order to correctly manage PcP, it is important to distinguish between progressive PcP, drug toxicity, and concomitant infection if clinical deterioration is detected.

Table 3. Drug therapy for treatment of Pneumocystis pneumonia in adults according to severity

	Moderate to severe Pneumocystis pneumonia		
Therapeutic use	Drug	Dose	Route
First line	Trimethoprim-Sulfamethoxazole	15-20 mg/Kg daily divided into 3 or 4 doses 75-100 mg/Kg daily divided into 3 or 4 doses	Intravenous
Second line	Primaquine plus Clindamycin	30 mg daily 600-900 mg three times daily	Oral Intravenous
Second line	Pentamidine	4 mg/Kg daily (3 mg/Kg if toxicities)	Intravenous
Salvage therapy	Trimetrexate plus Leucovorin	45 mg/m^2 daily 20 mg/m^2 four times daily	Intravenous Intravenous or oral
Adjunctive therapy	Prednisone Methylprednisolone	Days 1–5: 80 mg daily divided into 2 doses Days 6–10: 40 mg daily Days 11–21: 20 mg daily 75% of prednisone dose	Oral Intravenous
	Mild to moderate Pneumocystis pneumonia		
First line	Trimethoprim-Sulfamethoxazole	15-20 mg/Kg daily divided into 3 doses 75-100 mg/Kg daily divided into 3 doses	Oral
Second line	Dapsone plus Trimethoprim	100 mg daily 15-20 mg/Kg daily divided into 3 doses	Oral Oral or intravenous
Second line	Primaquine plus Clindamycin	15-30 mg daily 300-450 mg 3 or 4 times daily	Oral Oral
Second line	Atovaquone	750 mg two times daily	Oral with food

Trimethoprim-Sulfamethoxazole (TMP-SMX)

TMP and SMX target sequential steps in the folate synthesis pathway. TMP inhibits dihydrofolate reductase and SMX inhibits dihydrotperoate synthetase. TMP-SMX is the treatment of choice for PcP in all patients who tolerate this drug, and it achieves the most rapid clinical response of the anti-*Pneumocystis* agents [112,117]. The recommended dose of TMP-SMX for adults (or children aged > 2 months) is 15 to 20 mg/kg/day of TMP and 75 to 100 mg/kg/day of SMX intravenously every 6 or 8 hours. With renal dysfunction, dosing must be reduced. The bioavailability of TMP-SMX from oral therapy is comparable to parenteral administration [112,118].

Patients, who have PcP despite the use of TMP-SMX prophylaxis, are usually successfully treated with TMP-SMX. In this way, the presence of mutations in the DHPS gene of *P. jirovecii* has been associated with resistance to sulfa drugs, although the clinical

outcome is uncertain [84,91,119]. Drug related toxicities from TMP-SMX are greater than that from therapy with other anti-*Pneumocystis* agents. The side effects of TMP-SMX are: rash (30-55%), (including Stevens-Johnson syndrome), fever (30-40%), leukopenia (30-40%), hepatitis (20%), thrombocytopenia (15%), azotemia (1-5%), and hyperkaliemia [120-122]. Nephrotoxicity occurs frequently in the renal transplantation recipient receiving full-dose of TMP-SMX. Liver transplant recipients are particularly susceptible to TMP-SMX toxicity. Leucovorin to prevent myelosuppression is not recommended because of its uncertain efficacy and higher rate of failure [112].

Pentamidine

Pentamidine is an aromatic diamidine that has broad-spectrum anti-protozoal activity. This drug inhibits metabolism of *P* amino benzoic acid, interferes with anaerobic glycolysis, inhibits oxidative phosphorylation, and impairs nucleic acid and protein synthesis. It was the first drug reported to treat PcP successfully and subsequent reports have confirmed the efficacy of intravenous pentamidine. Although intravenous pentamidine has been recommended as the main alternative to TMP-SMX for moderate to severe PcP [121], a recent study has found a greater risk of death when pentamidine was used as first and second-line therapy for HIV-associated PcP as compared with TMP-SMX and clindamycin-primaquine [117]. These findings could be due to toxicities related to pentamidine and the absence of an antibacterial effect, in contrast to TMP-SMX or clindamycin-primaquine, which might act against concomitant bacterial co-infection [117].

Pentamidine for children and adults is administered once a day at 4 mg/kg (maximum 300 mg daily) intravenously, infused slowly 1 to 2 hr in 5% glucose; due to its toxicity, the dose can be reduced to 3 mg/kg. Aerosolized pentamidine should not be used because of limited efficacy and more frequent relapse, and intramuscular administration is not used due to the related complications [123]. Side effects of pentamidine include azotemia, pancreatitis, hypo- or hyperglycemia, pancytopenia, electrolyte abnormalities, cardiac dysrhythmia and renal dysfunction [123,124]. Pentamidine should be avoided in pancreas transplant recipients due to the potential for islet cell necrosis.

Clindamycin-Primaquine

Clindamycin is a lincosamide antibiotic used to treat infections with anaerobic bacteria but can also be used to treat some protozoan diseases. Primaquine is an 8-aminoquinoline anti-protozoan agent. This combination is effective in adult patients with mild to moderate PcP, but data for children are not available [125,126]. Clindamycin is given at 600 to 900 mg intravenously or 300-450 mg orally every 6 to 8 hours and primaquine is given at 15 to 30 mg/day given orally. Clindamycin component can be administered intravenously in severe cases; primaquine is only available orally. Recently, clindamycin-primaquine appeared superior to pentamidine as second-line therapy for PcP in patients failing or developing toxicity with TMP-SMX [117]. Side effects of clindamycin include rash, anemia, neutropenia and the development of *Clostridium difficile* colitis. The main toxicity of primaquine is

methemoglobinemia, thus, patients should be tested for glucose-6-phosphate dehydrogenase deficiency before administration of primaquine [113].

Dapsone

Dapsone is a sulfone drug that inhibits DHPS and it is used as an alternative therapeutic regimen for mild-to-moderate PcP. Dapsone must be taken with TMP [127]. Although this association might have similar efficacy and fewer side effects than TMP-SMX, it is less recommended due to the number of pills. The dosage of dapsone for adolescents and adults is 100 mg orally once daily (among children aged < 13 years, 2 mg/kg/day). The dosage of TMP for children and adults taken orally is 15 mg/kg/day divided into three doses [112,118]. The most common adverse effects associated to dapsone are methemoglobinemia and hemolysis, especially in those with glucose-6-phosphate dehydrogenase deficiency. Thus, patients should be tested for glucose-6-phosphate dehydrogenase deficiency [113].

Atovaquone

Atovaquone is a unique naphthoquinone that targets the cytochome B complex and, thus, inhibits mitochondrial electron transport. This drug was developed clinically in the 1990s and it is available only as oral agent. It is used as a second-line agent for treatment of mild to moderate PcP if TMP-SMX cannot be used. The standard dosing regimen for adults is atovaquone 750 mg orally twice a day with food for increasing gastrointestinal absorption (30-40 mg/kg/day for children < 3 months and > 24 months of age; between 3-24 months of age, 45 mg/kg/day are required) [118,127]. Mutations of the cytochrome *b* gene have occurred in atovaquone-resistant isolates of *Pneumocystis*, but the clinical significance of gene mutations has not been determined [129]. The advantages of atovaquone include oral administration and fewer side effects. Disadvantages are its high cost and its bioavailability, although it has been improved with the micronized suspension formulation [128]. The most frequently reported adverse effects are rash, nausea, diarrhea, elevation of liver enzyme levels and headache. Atovaquone does not cause bone marrow suppression [113].

Trimetrexate

Trimetrexate is an analogue of methotrexate that is an inhibitor of dihydrofolate reductase, and *in vitro* it is 1500 times more potent than trimethoprim [130,131]. This drug is effective for treating PcP but is available only in an intravenous formulation. Because this drug also inhibits human folate metabolism, leucovorin must be administered concomitantly to prevent cytopenias [113]. A clinical trial showed that trimetrexate is less effective but better tolerated than TMP-SMX against AIDS-related PcP [132]. Trimetrexate with folinic acid have been approved for use in patients with moderately severe PcP, however, it is no longer available commercially. The dosage recommended for treatment of PcP is trimetrexate, 45 mg/m^2 intravenously once daily, plus leucovorin 20 mg/m^2 orally or

intravenously four times daily [132]. Leucovorin therapy must extend for 72 hours past the last dose of trimetrexate. For adults, trimetrexate may alternatively be dosed on a mg/kg basis, depending on the patient's body weight: <50 kg, 1.5 mg/kg; 50-80 kg, 1.2 mg/kg, and >80 kg, 1.0 mg/kg. Also, leucovorin may be dosed on a mg/kg basis (<50 kg, 0.6 mg/kg, and >50 kg 0.5 mg/kg) administered every 6 hours. Despite the suggestion that leucovorin impairs the efficacy of TMP-SMX, there is no indication that the coadministration of leucovorin impairs the efficacy of trimetrexate for PcP [113]. In some cases, trimetrexate plus leucovorin could be used as salvage treatment for PcP [133].

Adjunctive Therapies

The use of corticosteroids may reduce pulmonary inflammation response caused by the lysis of *Pneumocystis* in the lung after initiating treatment of PcP. Corticosteroids have been related with a significant benefit in terms of preventing deterioration in oxygenation in the first seven days of therapy, mortality, and reduction of intubations in AIDS patients [134]. Corticosteroids are indicated in HIV-infected patients with a moderate-to-severe PcP, who have hypoxemia (the partial pressure of arterial oxygen less than 70 mm Hg with the patient breathing room air or an alveolar-arteriolar gradient greater than 35). In these cases, corticosteroids should be administered as early as possible within 72 hours after starting anti-*Pneumocystis* therapy [18,112]. Recommended doses are shown in table 3.

In non-HIV infected patients with PcP there are no randomized clinical trials about the use of adjunctive corticosteroids and data are far less clear. Moreover, non-HIV immunocompromised patients constitute a heterogeneous group of patients and most of them have been on corticosteroid at the time they developed PcP. Therefore, the recommendations of adjunctive corticosteroids therapy in non-HIV patient must be individualized. In patients with severe PcP a dose of 60 mg or more of prednisone daily resulted in a better outcome than lower doses of prednisone [135].

Novel Agents

Novel agents undergoing clinical investigation include echinocandins and pneumocandins, which target synthesis of beta 1,3 glucan, a cell wall compound of Pneumocystis and other fungi.

The sordarin family, probably the most active anti-*Pneumocystis* molecules, inhibits protein synthesis in fungi by stabilizing the ribosome/EF2 complex. This mode of action contrasts with a typical antifungal, which targets the cell membrane. Some sordarin derivatives have shown excellent in-vitro and in-animal model activities against a wide range of pathogenic fungi which include *Pneumocystis,* but until now, no clinical trials have been started [136,137].

Caspofungin is an echinochandin that acts on the cell wall by inhibiting β-1,3-glucan synthesis and it has been approved for several fungal infections such as the *Candida* and *Aspergillus* species. Caspofungin has shown activity against *Pneumocystis* in experimental animal models and it has strong activity on cyst forms and weak activity on trophic forms [138]. Because TMP-SMX affects only the trophic forms, it has been suggested that the

association of TMP-SMX and caspofungin, by fully inhibiting the organism life cycle, may provide a synergistic activity against *Pneumocystis.* Cases of PcP have been reported where the association of caspofungin and TMP-SMX achieved a complete cure of PcP [139,140]. However, this promising therapeutic approach needs to be assessed by controlled clinical trials.

Prognosis

Despite treatment, mortality from PcP still remains high. Several studies highlight that mortality rates are declining in patients with PcP. However, in other studies, PcP has remained the leading cause of death among those not receiving or failing to comply with HAART or PcP prophylaxis. Predictors of mortality include older age, recent injection drug use, increased total bilirrubin, low serum albumin, and alveolar-arterial oxygen gradient >50 mm Hg [141.

Non-HIV patients present more acutely with fulminate respiratory failure associated with fever and dry cough and frequently require mechanical ventilation. Most studies demonstrate a worse survival (51-80%) in non-HIV patients compared with AIDS patients (86-92%) [142]. As PcP is a severe infection with a high mortality rate, prevention is essential in the groups at risk.

Prophylaxis Regimens for PCP

Many studies have demonstrated that PcP can largely be prevented by administration of chemoprophylaxis to susceptible individuals [11,143-146]. According with the American Thoracic Society recommendations both patients infected with HIV and non-HIV immunosuppressed patients need to receive prophylaxis to prevent disease depending on specific risks to the patient's immune system [147]. Recommendations for chemoprophylaxis should be based on weighing the efficacy against the risk of adverse events, the risk of developments of antimicrobial resistance, and the cost of the intervention [10]. Medications recommended for chemoprophylaxis against PcP are listed in table 4.

Primary Prophylaxis

The majority of recommendations are based on studies performed in HIV-infected patients. Guidelines recommend starting primary prophylaxis against PcP in HIV-infected adolescents and adults, including pregnant patients, and patients under HAART, when the CD4 cell count is less than 200 cells/mm^3 or the patient has a history of oropharyngeal candidiasis. Patients with a CD4 cell percentage of <14% or a history of an AIDS-defining illness should be considered for chemoprophylaxis [112]. Prophylaxis recommendations for HIV-infected children are age-based. Chemoprophylaxis should be provided for children 6 years or older based on adults guidelines, for children aged 1 to 5 years if CD4 counts are less

than 500 cells/mm^3 or CD4 percentage is less than 15%, and for all HIV-infected infants younger than 12 months [116].

Table 4. Prophylaxis regimens for Pneumocystis pneumonia

Drug	Dose for adults	Dose for children	Route	Comments
Trimethoprim-Sulfamethoxazole	160/800 mg (DS tablet) per day or 3 times per week 80/400 mg (SS tablet) per day	150/750 mg/m^2 body surface area (max: 320/1600 mg) as single or 2 divided doses 3 times per week	Oral	First choice Weekly regimen is recommended if daily therapy in not tolerated
Dapsone	100 mg per day	2 mg/Kg body weight (max: 100 g) per day 4 mg/Kg body weight (max: 200 g) per week	Oral	Alternative choice Ensure patient does not have Glucose-6 phosphate dehydrogenase deficiency
Pentamidine	300 mg per month	300 mg per month (aged ≥ 5 years)	Aerosol	Alternative choice
Atovaquone	1500 mg per day	30-45 mg/Kg body weight according to age per day	Oral	Alternative choice Take with high-fat meals for maximal absorption
Dapsona + Pyrimethamine + Leucovorin	50 mg per day 50 mg per week 25 mg per week		Oral Oral Oral	Alternative choice Ensure patient does not have Glucose-6 phosphate dehydrogenase deficiency Effective in preventing toxoplasmosis
Dapsona + Pyrimethamine + Leucovorin	200 mg per week 75 mg per week 25 mg per week		Oral Oral Oral	Alternative choice Ensure patient does not have Glucose-6 phosphate dehydrogenase deficiency Effective in preventing toxoplasmosis

Although immunosuppressed HIV-negative patient studies about PcP prophylaxis are limited, a meta-analysis has recently confirmed that prophylaxis with TMP-SMX significantly reduced PcP infections and PcP-related mortality in these patients [143]. Daily systemic administration of corticosteroid is the second most common reason for developing PcP after HIV infection [148-150]. For this reason, administration of chemoprophylaxis to patients who are receiving at least 20 mg of prednisone per day for at least one month has been suggested [10,24]. However, this approach would unnecessarily expose patients to drug side-effects and could potentially encourage drug resistance. As an alternative, it has been suggested that a CD4 cell count of less than 200 cells/mm^3 might indicate the use of PcP

prophylaxis in patients who are receiving long-term corticosteroid treatment, although this test is not nearly as sensitive or specific as it is in HIV-infected individuals [149]. In this sense, CD4 cell count could be monitored to determine when to introduce a primary chemoprophylaxis:

- after one month of immunosuppression in patients who are in treatment with steroid dosage greater than 15 mg prednisolone or equivalent per day,
- corticosteroid treatment proposed for more than 3 months or
- total lymphocyte count less than 600 cells/mm^3

However, prospective investigation is required to validate this preventive strategy [10,149].

For non-HIV immunosuppressed patients, there is no reliable laboratory marker for susceptibility. In fact, the benefit of chemoprophylaxis should be balanced with the risk of severe adverse events, and depends on the attack rate of PcP [10,150]. In this sense, it becomes clear that chemoprophylaxis for PcP should be considered when the risk for PcP in adults is higher than 3.5% (among children a much lower risk would probably warrant prophylaxis because adverse events are infrequent) and continued as long as the immunosuppressive condition remains active [143]. Such rates of risk are seen in recipients of solid organ or allogeneic bone marrow during the first 6 months after transplant or after treatment of rejection episodes and, for the latter, throughout the period of immune-suppression, as well as in patients with acute lymphoblastic leukemia and Wegener granulomatosis [143,144]. Available data have led experts to recommend prophylaxis in patients with connective tissue diseases who receive chronic corticosteroid therapy combined with another immunosuppressive drug as well as in patients with systemic lupus erythematosus or Wegener granulomatosis during the first year of treatment, particularly when they have lymphopenia or renal failure [10].

TMP-SMX is the recommended prophylactic agent in both HIV-infected and uninfected immunosuppressed patients, because of its high efficacy, relative safety, low cost, and broad antimicrobial spectrum [10,11,112,144]. TMP–SMX is also effective in preventing *Toxoplasma gondi*, *Isospora belli*, *Cyclospora cayetanensis* and some bacterial infections such as, *Streptococcus pneumoniae*, *Salmonella*, *Haemophilus*, *Staphylococcus*, and common gram-negative gastrointestinal and urinary pathogens [11]. Either one single-strength tablet daily or one double-strength tablet daily are the preferred regimens, but the first regimen might be better tolerated than the second [112]. An alternative can be one double-strength tablet three times per week [10,112]. TMP-SMX at a dose of one double-strength tablet daily confers cross-protection against toxoplasmosis and selected common respiratory bacterial infections. Lower doses of TMP-SMX also likely confer such protection [112,144].

For patients who have an adverse reaction that is not life threatening, prophylaxis with TMP-SMX should be reinstituted. These patients might better tolerate reintroduction of the drug with a gradual increase in dose or reintroduction of TMP-SMX at a reduced dose or frequency [112]. If TMP-SMX is not tolerated, a second choice would be dapsone given 100 mg daily, dapsone 50 mg daily plus pyrimethamine 50 mg weekly plus leucovorin 25 mg weekly or dapsone 200 plus pyrimethamine 75 mg plus leucovorin 25 mg weekly, aerosolized pentamidine 300 mg monthly administered by an ultrasonic or jet-nebulizer, and atovaquone

1500 mg daily [11,112]. Dapsone is effective and inexpensive but associated with more serious adverse effects than atovaquone [146]. Atovaquone is effective, safe and it is effective against *Toxoplasma gondii* but it is more expensive [11]. The widespread concept that TMP-SMX is contraindicated for prophylaxis in patients treated with methotrexate might be obsolete because the safety of one single-strength tablet daily or one double-strength tablet thrice weekly has been proved in clinical studies [151,152]. However, these patients need to receive folate supplementation, and blood counts and liver-function tests should be closely monitored [10].

Primary prophylaxis should be discontinued for HIV-infected adult and adolescent patients who have responded to HAART with an increase in CD4 counts higher than 200 cells/mm^3 during more than 3 months [153]. Prophylaxis should be reintroduced if the CD4 cell count decreases to less than 200 cells/mm^3. Concerning immunosuppressed non-HIV-infected patients, data are limited and the optimal duration of chemoprophylaxis is still undecided, although probably the length of prophylaxis should continue as long as the immunosuppressive conditions remains active [10,12,150].

Secondary Prophylaxis

HIV-infected adult and adolescent patients who have developed previous episodes of PcP should receive secondary prophylaxis [18]. Chemoprophylaxis should be discontinued for adult and adolescent patients when the CD4 cell count increases to more than 200 cells/mm^3 for a period of 3 months because of HAART [153]. Prophylaxis should be reintroduced if the CD4 count decreases again to less than 200 cells/mm^3. If PcP recurs at a CD4 count higher than 200 cells/mm^3, continuing PcP prophylaxis for life would be prudent [112].

The risk for recurrence of PcP is undefined in non-HIV immunosuppressed patients and recommendations of secondary prophylaxis have not been established. Alternatives would be to monitor patients closely in order to detect any recurrence or to place patients on secondary chemoprophylaxis throughout the period of susceptibility as long as the immunosuppressive condition persists [10].

Conclusion

Pneumocystis jirovecii is an atypical fungus that causes PcP in HIV-infected individuals and immunosuppressed patients. PcP is today still a major cause of morbidity and mortality among immunocompromised persons, especially those with AIDS, and constitutes a worldwide problem to public health. While the incidence of PcP among HIV infected individuals has decreased in developed countries, the prevalence of AIDS-related PcP in developing countries remains high and poorly controlled. Currently, with the rising number of patients receiving immunosuppressive therapies for malignancies, allogeneic organ transplantations and autoimmune diseases, PcP is being recognized more and more in non-HIV-immunosuppressed individuals in developed countries. The epidemiology of this infection is only beginning to be understood. The accumulating evidence suggests that *P. jirovecii* is a highly infectious organism with low virulence that takes advantage of hosts as

temporary reservoirs of infection. In this sense, colonization with *P. jirovecii* (that is infection without disease) has recently gained attention as an important issue for understanding the complete cycle of human *Pneumocystis* infection. The clinical presentation in HIV-infected patients may differ from that in other immunosuppressed patients and its diagnosis continues to be challenging. Clinicians must be familiar with its presentation and management because mild cases are sometimes difficult to diagnose. Co-trimoxazole is the most effective medication for its prevention and treatment but other alternative medications are also available. Future clinical research should also include studying the transmission and epidemiology of PcP in populations worldwide, improving the diagnosis of PcP, improving regimens for prophylaxis and treatment in various patient populations, and determining the significance of the DHPS mutations in various populations and in different geographic locations. Furthermore, the threat of emerging resistance to available anti-*Pneumocystis* drugs highlights the need to continue to investigate the biology of this organism in the hope of developing novel treatment strategies.

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