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Chapter 68
Drug Resistance in *Pneumocystis jirovecii*

Jannik Helweg-Larsen, Thomas Benfield, Joseph Kovacs, and Henry Masur

1 Introduction

Pneumocystis jirovecii (previously known as *Pneumocystis carinii*) is an opportunistic fungus that causes pneumonia, Pneumocystis pneumonia (PCP), in immunocompromised individuals. Before 1982, PCP was relatively rare and primarily diagnosed among patients with congenital immunodeficiencies, and patients receiving potent immunosuppressive therapy as part of an antineoplastic regimen. However, with the AIDS pandemic PCP emerged as the most common AIDS-defining diagnosis in industrialized countries. The peak incidence of PCP was observed in the late 1980s and early 1990s. Subsequently, there has been a decline in the incidence of PCP because of the widespread introduction of PCP chemoprophylaxis and the introduction of increasingly potent HIV-1 antiretroviral regimens. However, PCP remains a serious opportunistic infection among heavily immunosuppressed patients who are not receiving appropriate chemoprophylaxis.

2 The Organism

Pneumocystis were identified early in the last century in guinea pigs by Chagas and in rat lungs by Carini (1, 2). These investigators mistakenly considered the organisms as a new form of *Trypanozoma cruzi*. In 1912, Pneumocystis was recognized as a new species and named in honor of Carini (3). Pneumocystis was first described in humans in 1942 by two Dutch investigators, van der Meer and Brug, who described it in three cases: a 3-month-old infant with congenital heart disease and in 2 of 104 autopsy cases – a 4-month-old infant and a 21-year-old adult (4). However, *Pneumocystis* was first established as a human pathogen when Jirovec in 1952 identified the organism as the cause of interstitial plasma cell pneumonia among premature or malnourished infants in orphanages (5).

For most of the twentieth century, *Pneumocystis* was considered as a protozoon and single species based on its morphologic features, its resistance to classical antifungal agents and the effectiveness of certain drugs used to treat protozoan infections. However, in 1988, based on the work by Edman and colleagues (6), phylogenetic analysis of ribosomal RNA (rRNA) sequences and observations of genome size placed *P. carinii* in the fungal kingdom. Functional and phylogenetic comparisons of several other genes have since confirmed its position (7–9). Phylogenetic data suggest that *Pneumocystis* is an ancient organism without any close relatives. It has been suggested that the *Pneumocystis* species represent an early divergent line in the fungal kingdom, which may have branched coincident with the bifurcation of the basidiomycete and ascomycete lineages. The organism has recently been placed in a group of fungi entitled the Archiascomycetes (10). In contrast to most other fungi, *Pneumocystis* possesses only one copy of the nuclear ribosomal RNA locus, has a fragile cell wall and contains little or no ergosterol (11).

Pneumocystis organisms have been identified in most mammalian species in which it has been searched for. Genetic and antigenic analyses have shown that *Pneumocystis* includes a broad family of organisms, with species specificity among its mammalian hosts (11–13). Remarkably, the level of genetic divergence between *Pneumocystis* organisms infecting different mammals is greater than the degree of divergence observed between certain fungi classified as distinct species (14, 15). Phylogenetic comparisons of DNA sequences in organisms from 18 different nonhuman primate species have demonstrated that sequence divergence correlates with the phylogenetic difference between the host species, which suggests that *Pneumocystis* species have evolved together with their hosts (16).

In 1994, an interim trinomial name change was adopted with the name *P. carinii* f.sp. *hominis* for *Pneumocystis* infecting humans and *P. carinii* f.sp. *carinii* for one of the two species infecting rats (17). Subsequently, in 2002,

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because of the recognition of its genetic and functional distinctness, the organism infecting humans was renamed *Pneumocystis jirovecii,* in honor of Otto Jirovec, who was among the first to describe the microbe in humans (18–20).

3 Transmission and Infection

Since *P. jirovecii* organism cannot be cultured in vitro, knowledge about its biology has been difficult to obtain. However, the development of molecular and immunological techniques has permitted considerable insight into this organism and on how it interacts with its various animal hosts. Antibody and PCR findings indicate that primary infection with *P. jirovecii* happens in early childhood with a uniform high incidence in all geographic areas, and suggest that *P. jirovecii* organisms are ubiquitous. Its environmental source is, however, unknown. Organisms may be coming from inanimate environmental sources, or may be spread by healthy humans. Studies have not conclusively demonstrated the environmental niche.

It was previously though that the infection was carried life-long and that clinical infection was a result of reactivation in immunocompromised hosts. PCR findings have questioned this view and support a more complex picture of transmission and infection.

When the organism is obtained initially as a primary infection, it is not clear whether an immunocompetent host develops a transient disease. Various investigators have proposed that primary infection might correlate with the development of upper or lower respiratory manifestations, or with the development of sudden infant death syndrome (21–23). Following primary infection, the presumption, based on murine models, has been that the organism becomes latent, later manifesting clinically if the patient becomes profoundly immunosuppressed.

More recent data, however, suggests that human hosts can be infected with more than one strain of *Pneumocystis jirovecii*, raising the possibility that infection can be acquired on multiple occasions, leading to latency with a variety of distinct organisms (24). The clinical disease PCP may, therefore, occur as a reactivation of a prior latent organism, or as a result of recent acquisition of an airborne pathogen.

As noted above, the environmental source of *Pneumocystis* has not been identified. Since most infants acquire antibody against *Pneumocystis* during the first year of life, the organism must be ubiquitous. Whether the organism is being shed into the environment regularly by healthy hosts, or whether the organism is introduced into the environment from an inanimate environmental source such as trees or grass is unknown. However, nonhuman animals are not the source, because, as mentioned above, each animal species is infected with a different strain of *Pneumocystis*, and there is no cross-species infection that has been identified.

Pneumocystis has specific tropism for the lung, where it exists in the alveoli. In rare cases organism have been detected in other organs, but it seldom causes disease at extrapulmonary sites. After inhalation, the organism attaches tightly to the surface of type I alveolar cells (25). Adherence is primarily mediated by the major surface glycoprotein (MSG) (26, 27). This protein is the most abundant antigen on the surface of *Pneumocystis* and is encoded by a multicopy gene family. MSG represents a family of proteins that are highly polymorphic, repeated and distributed among all the chromosomes of *Pneumocystis*. MSG shows high level of antigenic variation by switching the expression of multiple MSG genes, with a system that resembles the antigenic system used for antigenic variation in *Trypanozoma cruzi* (28, 29). It is likely that this antigenic variation in MSG serves for avoiding the host immune response. There is no detailed knowledge of the life cycle and the mode of replication has not been definitely established, but both asexual and sexual life cycles have been proposed (30, 31). Recently, several genes, which in other fungi are involved in mating, pheromone responsiveness, and responses to environmental changes, have been demonstrated in *Pneumocystis*, suggesting that the organism has a sexual replication cycle that responds to environmental changes in the lung (32, 33).

4 Drug Treatment

The major drug classes used for treatment and prophylaxis of PCP include antifolate drugs, diamines, atovaquone, and macrolides (Tables 1 and 2). Most traditional antifungal agents have no activity against *Pneumocystis*. As *Pneumocystis* was originally believed to be a protozoon, initial drug testing focused on drugs with activity against protozoan infections.

In 1958, pentamidine isethionate was the first drug used to successfully treat PCP (34). In the 1960s, the combination of sulfadoxine and pyrimethamine was used for the prevention of epidemic infantile pneumocystosis in Iran (35). In 1966, Rifkind treated two patients with sulfadiazine and pyrimethamine; both patients died, but two patients were successfully treated 4 years later (36). Between 1974 and 1977, studies led by Hughes et al. established that the combination of trimethoprim–sulfamethoxazole (TMP–SMX) is effective for both treatment and prophylaxis of murine and then human PCP (37–39). TMP–SMX is as effective as intravenous pentamidine for therapy, and is still the treatment of choice. Additionally, TMP–SMX is the most effective chemoprophylaxis for PCP, and therefore the standard for prevention.

Table 1 Regimens for prophylaxis against Pneumocystis pneumonia

Drug	Oral or aerosol dose
First choice	
Trimethoprim–sulfamethoxazole	1 DS or SS daily
Alternatives	
Trimethoprim–sulfamethoxazole	1 DS three times per week
Dapsone	50 mg twice daily or 100 mg twice weekly
Dapsone with	50 mg daily
Pyrimethamine plus	50 mg weekly
Leucovorin	25 mg weekly
Dapsone with	200 mg weekly
pyrimethamine plus	75 mg weekly
Leucovorin	25 mg weekly
Pentamidine aerosolized	300 mg monthly via nebulizer system
Atovaquone	1,500 mg daily
[a]Pyrimethamine plus	25–75 mg qd
Sulfadiazine	0.5–2.0 g q6h

DS: double strength = 800 mg sulfamethoxazole, 160 mg trimethoprim; SS: single strength = 400 mg sulfamethoxazole, 80 mg trimethoprim.
[a]This regimen only for use in case of concurrent toxoplasmosis

Other drugs have proven activity for therapy, including sulfadiazine plus pyrimethamine, atovaquone, clindamycin plus pyrimethamine, trimetrexate, dapsone and aerosolized pentamidine. Not all drugs that are effective for therapy are also effective for chemoprophylaxis. Dapsone, dapsone–trimethoprim, atovaquone and aerosolized pentamidine are also effective for prophylaxis. Intravenous pentamidine and clindamycin–primaquine have not been shown to be effective for chemoprophylaxis. There are other drugs that have in vitro activity or anecdotal anti-PCP activity in humans and could have a role in managing human disease if all other alternatives were not feasible. These include azithromycin, doxycycline, and caspofungin.

5 Prophylaxis

Among HIV-infected patients, the occurrence of PCP is closely related to the CD4 count: With lower CD4 counts, the risk of PCP increases. While a count of 200 cells/mm^3 is often used as an indicator or susceptibility, HIV-infected patients do in fact develop PCP at counts higher than 200 cells/mm^3, although at a frequency lower than that at 200, 100, or 50 cells/mm^3.

Patients with congenital immunodeficiencies, particularly X-linked immunodeficiency with hyper-immunoglobulin M and SCID, patients receiving long-term and high-dose corticosteroid therapy, and patients receiving certain chemotherapeutic regimens for cancer therapy or transplantation are at the risk of developing PCP. Interestingly, some chemotherapeutic agents such as fludarabine or antithymocyte globulin produce a much higher risk of PCP than other regimens (40–43). In patients without HIV, CD4 counts are not a reliable marker of susceptibility. Several studies have shown that the occurrence of PCP is not as predictable with these markers in diseases unrelated to HIV (42).

Systemic chemoprophylaxis against PCP was introduced by Dutz in Iran in the early 1950s. He showed that outbreaks of PCP could be aborted with the use of sulfadoxine plus pyrimethamine (44). Hughes et al. followed this observation with a classic study of children with acute lymphocytic leukemia (ALL); they showed that PCP could be virtually eliminated by TMP–SMX prophylaxis (39) Subsequently this prophylaxis was used for other populations of cancer and transplant recipients with a very high success rate. With the advent of the AIDS epidemic, PCP prophylaxis was used sporadically in the 1980s. After the publication of a convincing study by Fischl et al., PCP prophylaxis became a standard of care for HIV-infected patients with CD4 counts less than 200 cells/mm^3 in 1989 (45). The identification of additional risk factors for the development of PCP has led to expanded recommendations for the use of PCP chemoprophylaxis – details are provided in Table 3. HIV-1 infected patients with oral candidiasis or a CD4 count less than 200 cells/μL, should be offered primary prophylaxis. Secondary prophylaxis should be offered to all patients following an episode of PCP. In HIV patients receiving prophylaxis; prophylaxis can safely be interrupted if immune function is improved above a CD4 count of 200 cells/μL for at least 3 months following antiretroviral therapy. If the patient subsequently fails antiretroviral therapy and the CD4 declines to below 200 cells/μL, prophylaxis should be restarted.

In non-HIV infected individuals, conditions such as organ transplantation, high-dose steroid treatment and/or high-dose chemotherapy may confer a high risk of PCP. Prophylaxis should be offered as shown in Table 3. Several prophylactic regimens are available. The most efficient, cheap and widely used regimen is daily TMP–SMX. TMP–SMX prophylaxis is relatively well tolerated by most non-HIV patients; in contrast, HIV patients have a high frequency of adverse effects, in particular rash and myelosuppression. Before the advent of antiretroviral therapy, 50% of patients experienced an adverse effect after 12 months of prophylaxis with double-strength TMP–SMX (160/800 mg), and half would have switched to other types of prophylaxis after 3 years (46). Fortunately, 80/400 mg TMP–SMX daily appears to be equally effective and is associated with fewer side effects than 160/800 mg daily (47). Because of its efficacy, ease of administration and cost, every effort should be tried to maintain patients at risk of PCP on TMP–SMX. For patients, who have reacted to TMP–SMX, it has been shown to be safe to reintroduce TMP–SMX by dose escalation (48, 49). A variety of dosing regimens can be used with similar efficacy. Tolerability may improve with the lower dose or the intermittent regimens.

Table 2 Drug regimens for the treatment of PCP

Drug	Route	Dose	Toxicity	Advantages	Disadvantages
First choice					
Trimethoprim–sulfamethoxazole	By mouth	2 DS every 8 h	Rash and fever Anemia and neutropenia Hyperkalemia	Superior efficacy Inexpensive Oral and iv	Rash common
	Intravenous	Trimethoprim 5 mg/kg with sulfamethoxazole 20 mg/kg every 8 h	Hepatitis Nephritis Anaphylactoid reaction	Bacterial and anti-toxoplasmosis activity	
Alternatives					
Dapsone plus trimethoprim	By mouth	100 mg daily	Rash, nausea and vomiting and fever Methemoglobinemia, leukopenia and haemolytic anemia	Inexpensive	No iv formulation
	By mouth	320 mg every 8 h	Liver function abnormalities; headache Dapsone may cause hemolysis in patients with G-6PD		
Clindamycin plus primaquine	By mouth, intravenous	300–450 mg every 6 h 30 mg daily	*Clostridium difficile* diarrhea, nausea and vomiting. Primaquine may cause hemolysis in patients with G-6PD deficiency		No iv formulation for primaquine
Pentamidine	Intravenous	4 mg/kg day	High incidence of adverse effects, particularly hypoglycemia and nephrotoxicity Pancreatitis and IDDM. Hypotension with short infusion time Pancytopenia Q-T prolongation	Highly effective	Toxicity common. Only iv formulation
Atovaquone	By mouth	750 mg twice daily	Rash, nausea, diarrhea and headache (20%) Fever, increased transaminases and neutropenia	Well tolerated	Expensive Useful for mild disease
Adjunctive therapy Prednisone in patients with room air pAO_2 < 70 mmhg (9.3 kPa)	By mouth, intravenous	40 mg twice daily for 5 days 40 mg daily, days 6 through 11 20 mg daily, days 12 through 21 while on anti-PCP therapy	Standard of care for moderate or severe disease	Metabolic problems, especially glucose and electrolyte changes	

6 Treatment of PCP

Untreated PCP is invariably fatal. In the beginning of the HIV epidemic, the mortality rate of PCP was reported to be 30–40% (50, 51), increasing to 70–90% among patients who progressed to respiratory failure (52). Over the past decade, mortality rates have dropped to 5–15% (53–58). This appears to be a consequence of earlier recognition of the infection, the introduction of adjuvant corticosteroids to patients with moderate-to-severe PCP as defined by a PaO_2 of less than

Table 3 Recommendations for PCP prophylaxis and risk identification in selected diseases

Disease	Risk identification	Duration of prophylaxis	Comment
HIV-1 infection CD4 cell count<200 Oropharyngeal candidiasis CD4 cell count <14% Prior AIDS-defining illness	Prior PCP	Lifelong unless CD4 count >200 × >3 months due to ART	Prophylaxis improves survival Restart prophylaxis if CD4 count falls to < 200 despite ART
Organ transplantation			
Kidney Lung Heart/Liver Autologous BMT Allogenic BMT Rejection Graft versus Host disease	Depends on intensity of immunosuppression and occurrence of graft versus host disease or rejection	General: minimum 6 month after transplantation: At least 6 months Indefinitely 6-12 month 6–12 month Minimum 1 year Reinstate Reinstate	Need for PCP prophylaxis determined by clinical experience. CD4 count is not a reliable predictor
Malignancy			
Acute lymphoblastic leukemia (ALL)	During and subsequent to combination chemotherapy	During severe immunosuppression Continue during maintenance therapy for childhood ALL	Need for PCP prophylaxis determined by clinical experience with each chemotherapeutic regimen. CD4 count is not a reliable predictor
Chronic lymphatic leukemia (CLL)	Treatment with Fludarabine or Alemtuzumab (Campath, anti-CD52)	Minimum 2 months after discontinuation or until CD4>200	
Lymphoma	Certain chemotherapeutic regimens e.g. PROMACE-CYTABOM	3–6 month post chemotherapy	

BMT bone marrow transplantation; ART antiretroviral therapy

70 mmHg, better diagnostic and therapeutic abilities related to concomitant processes, and improved ICU supportive measures.

The importance of educating patients to seek medical attention early, when symptoms are still mild, must be an emphasis of patient management programs. Both patients and health care professionals must recognize that mild symptoms such as dyspnea, cough, or low-grade fever can be the initial manifestation of PCP, especially in patients with CD4+ T lymphocyte counts below 200 cells/mm^3. Thus, clinicians should not wait for all the features of PCP to be present, or for the chest radiograph to be abnormal, before initiating a workup for PCP. Moreover, once there is a high suspicion therapy should be instituted promptly if the diagnostic procedures will be delayed.

The choice of specific chemotherapy is also important. The most potent drugs for PCP treatment are antifolate drugs, which act by blocking de novo synthesis of folates through inhibition of dihydroperoate synthase (DHPS) or dihydrofolate reductase (DHFR) (Fig. 1).

DHPS catalyzes the condensation of *p*-aminobenzoic acid (PABA) and hydroxymethyl dihydropterin–pryophospate to produce dihydropteroate, which is later converted to dihydrofolate by dihydrofolate synthase. Subsequently, dihydrofolate is reduced by dihydrofolate reductase (DHFR) into tetrahydrofolate. Sulfa drugs are structural analogs of PABA and inhibit DHPS.

The earliest clinical trials to treat PCP were performed with sulfadiazine plus pyrimethamine on the assumption that these drugs would have synergistic action against pneumocystis, as against plasmodia. When the commercial combination of sulfamethoxazole plus trimethoprim was developed to treat bacterial infections, this preparation was assessed for PCP therapy and prophylaxis since commercial sponsorship of studies could be obtained. At that time, there was no knowledge about the relative potency of various sulfonamide preparations against pneumocystis, nor was there information about the relative potency of various DHFR inhibitors. Subsequently, it was found that sulfamethoxazole is probably as potent as any of the other commercially available sulfonamide preparations as discussed below. However, trimethoprim is not as potent as other available DHFR inhibitors, as also described below.

In Table 2, drug treatment options for PCP are listed together with the most important advantages and toxicities of each drug regimen. During the 1980s several trials investigated

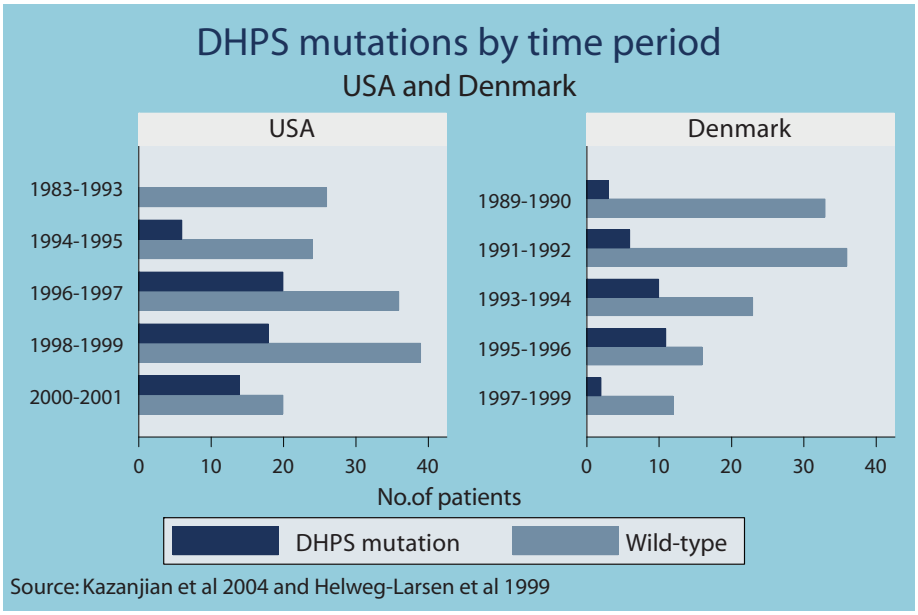

Fig. 1 Emergence of DHPS since 1989, worldwide

the efficacy of TMP–SMX compared to pentamidine (59–62). In the only noncrossover trial ($n = 70$) (61), TMP–SMX was associated with a better survival than pentamidine. However, when all the trials are considered, TMP–SMX and pentamidine appear to have roughly comparable efficacy (59). Drug toxicity occurs in 24–57% of HIV-infected patients treated with TMP–SMX (63).

Adverse effects generally occur after 7 days of therapy and most commonly include rash, fever and leukopenia. Hepatotoxicity characterized by elevated transaminases also occurs. There are cases of sulfamethoxazole-induced interstitial nephritis, renal calculus formation, anaphylactoid reactions and pancreatitis reported. Trimethroprim can be associated with hyperkalemia. These toxicities are usually not life threatening, although fatal cases of Stevens–Johnson syndrome have occurred.

Pentamidine is associated with a high frequency of toxicities, some of which are treatment-limiting. Early experiences with rapid infusions of pentamidine were associated with hypotension and death, so this route of administration was abandoned. Intramusuclar injections were better tolerated in terms of blood pressure, but they caused a high frequency of sterile abscesses. Therapy was then administered by slow intravenous infusion, which is the best tolerated route. Inhaled pentamidine has been used for therapy, and is well tolerated, but efficacy is poor. Pentamidine is nephrotoxic and causes predictable glomerular and tubular damage to the kidney. Pentamidine is toxic to the pancreas; its initial effects cause a surge of insulin release that often manifests as hypoglycemia. Hypoglycemia can occur days or weeks after starting therapy, and may occur many days after stopping therapy. Leukopenia can also occur. Pentamidine prolongs the QT interval, and cases of torsades de pointe have been reported. Treatment-limiting toxicities with pentamidine treatment occur in 13–80% of patients.

Alternatives for the therapy to TMP–SMX and pentamidine include dapsone–pyrimethamine, clindamycin–primaquine, and atovaquone (Table 2). Trimetrexate has activity, but is no longer commercially available. Dapsone has not been studied as a single drug and thus should not be used alone for treatment. Dapsone–trimethoprim is effective, however, and probably has potency that is comparable to TMP–SMX. However, since this combination does not come as a fixed-dose combination, is only available orally, and cross-reacts with sulfa in 50% of allergic patients, this regimen does not offer many advantages over TMP–SMX.

Clindamycin–primaquine appears to work on a metabolic pathway different from that of TMP–SMX. Two comparative trials of clindamycin/primaquine with TMP–SMX in moderate-to-severe PCP demonstrated apparent equivalence for clindamycin–primaquine, but both trials were underpowered (64, 65). Clindamycin causes a relatively high incidence of hepatitis, rash and diarrhea in HIV-infected patients. Primaquine can only be given orally.

Atovaquone is well tolerated and acts on a metabolic pathway different from that of TMP–SMX. However, this drug is also only available orally, and does not appear to be as potent as TMP–SMX (66). This is a good alternative to TMP–SMX for patients with mild disease who cannot tolerate TMP–SMX.

Efficacy of dapsone–pyrimethamine has only been demonstrated for mild-to-moderate PCP and for atovaquone only for mild PCP (64, 66–68). Both must be administered orally.

The optimal duration of therapy for PCP has never been properly tested. Usual recommendations are that

HIV-negative patients should receive 2 weeks and HIV-positive patients three weeks of drug treatment.

Many patients experience progressive oxygen desaturation during the first 4–5 days of therapy. This deterioration appears to be caused by the drug-induced death of *Pneumocystis* organisms with exacerbation of alveolar inflammation. This inflammation can be reduced by corticosteroids. Four randomized, controlled trials demonstrated that corticosteroids could reduce mortality in patients with moderate or severe disease (69–72). On the basis of these results, adjunctive steroids are now recommended for all patients with severe disease ($PaO_s < 70$ mmgh).

7 Sulfonamide Resistance

The widespread use of TMP–SMX and dapsone for therapy and prophylaxis of PCP among HIV patients has led to the concern that sulfa (sulfonamide or sulfone) resistance could develop in *P. jirovecii*.

In many pathogenic bacteria and parasites, resistance to sulfonamides has increased as a consequence of selective pressure, and has limited the efficacy of sulfonamides (73). Widespread use of sulfa drugs for malaria and bacterial infection in Africa has produced high rates of resistance in *P. falciparum* and many bacterial species (74). In San Francisco, the increasing use of PCP prophylaxis among HIV patients led to a marked increase in trimethoprim–sulfamethoxazole resistance among isolates of *Staphylococcus aureus* and seven genera of Enterobacteriaceae (75). In a retrospective study, trimethoprim–sulfamethoxazole resistance was more than twice as likely in blood culture isolates from HIV patients receiving trimethoprim–sulfamethoxazole compared to patients not receiving this prophylaxis (76).

In pathogens such as *Escherichia coli, Neisseria meningitidis, Mycobacterium leprae* and *Plasmodium falciparum*, sulfonamide resistance is caused by mutations in the primary sequence of the DHPS gene (77–79). The mutations that confer resistance are localized within a highly conserved active site of the DHPS protein. In *Pneumocystis*, the DHPS protein is part of a trifunctional protein along with dihydroneopterin aldolase and hydroxymethyldihydropterin pyrophosphokinase, that together are encoded by the multidomain *FAS* gene (80).

In 1997, Lane and co-workers were the first to identify non-synonymous (resulting in changes in the encoded amino acid) DHPS mutations in *Pneumocystis jirovecii* (81). The most frequent DHPS mutations occur at nucleotide positions 165 and 171, which lead to an amino acid change at positions 55 (Thr to Ala) and 57 (Pro to Ser). The homologous Thr and Pro are highly conserved across species, including *Pneumocystis* infecting other hosts. Thus, these variants appear to represent true mutations rather than allelic polymorphisms. Either mutation can occur alone. The Th55 is homologous to Thr62 of *E. coli* DHPS, which based on its crystal structure, binds the pterin substrate. It is hypothesized that the Thr55Ala and Pro57Ser affect the position of Arg56 (whose homologue in *E. coli* is involved in binding pterin as well as sulfa drugs), decreasing its ability to bind sulfa drugs and resulting in a consequent reduction in sulfa drug sensitivity (82, 83).

However, frequently both mutations are seen in the same isolate. While the association with sulfa exposure is consistent with the concept that these mutations represent resistance that developed under drug pressure, documenting resistance is very difficult partly because *Pneumocystis* cannot be cultured, and partly because functional enzymes (recombinant or native) are unavailable.

Recently, *Saccharomyces cerevisiae* has been used as a model to study *P. jirovecii* DHPS resistance. The DHPS enzyme of *S. cerevisiae* has high functional and genetic similarity to the DHPS of *P. jirovecii*. This enzyme from Saccharomyces is also trifunctional. By site-directed mutagenesis, the in vitro effects of mutations identical to the DHPS mutations in *P. jirovecii* can be investigated. Using this model, two recent studies reported that the double DHPS mutations Thr55Ala and Pro57Ser result in an absolute requirement for PABA, consistent with resistance being associated with altered substrate binding (84, 85). Interestingly, the single mutation Pro57Ser conferred resistance to sulfadoxine, which is supported by clinical observations suggesting a specific association of this mutation with sulfadoxine resistance in PCP (84). However, one study showed an increase in sensitivity of the double mutations to sulfamethoxazole, suggesting that this approach may not accurately reflect the effect of these mutations in *P. jirovecii*.

Several clinical studies have investigated the frequency and significance of DHPS mutations in *P. jirovecii*. Table 4 provides a summary of the studies reporting frequencies of mutations in sulfa-exposed and sulfa-unexposed patients. Although the studies vary considerably in size (13–158 patients) and in definitions of sulfa exposure, a clear association between previous exposure to sulfa drugs (primarily for prophylaxis rather than therapy) and DHPS mutations has been shown in all studies. Large geographical variation in the prevalence of DHPS mutations has been reported, ranging from 7 to 69% of isolates. In the US, the incidence of mutations was lower in Indianapolis and Denver compared to San Francisco, where one study reported that more than 80% of patients were infected with mutant strains (86). Wide variations have also been observed in studies from Europe with a particularly low incidence in Italy; in one study, an 8% frequency of mutations was found among 107 HIV patients between 1994 and 2001 (87). Mutations have rarely been found in clinical isolates obtained prior to the early 1990s, but seem to have increased in frequency recently, presumably as a consequence of increasing selective pressure caused by the

Table 4 Prevalence of DHPS mutations and association with sulfa exposure

Study	Country (year)	Number of DHPS mutations/no. of PCP episodes	DHPS mutations/ sulfa exposed	DHPS mutations/ no sulfa exposure	Risk ratio (95%CI)
Santos (112)	France (1993–1998)	11/20 (55%)	5/5 (100%)	3/12 (25%)	4. 0 (1.5–10.7)
Helweg-Larsen et al. (90)	Denmark (1989–1999)	31/152 (20%)	18/29 (62%)	13/123 (11%)	5.87 (3.26–10.57)
Ma et al. (99)	USA (1985–1998)	16/37 (43%)	11/16 (69%)	3/15 (20%)	3.44 (1.19–9.97)
Huang et al. (113)[a]	USA (1996–1999)	76/111 (69%)	57/71 (80%)	19/40 (48%)	1.69 (1.20–2.39)
Ma et al. (87)	Italy (1994–2001)	9/107 (8%)	6/31	3/76	4.90 (1.31–18.38)
Beard et al. (114)[a]	USA (1995–1998)	152/220 (69%)	np	np	Na
Takahashi et al. (115)	Japan (1994–1999)	6/24 (25%)	2/3 (33%)	4/24 (19%)	4.00 (1.20–13.28)
Costa et al. (116)	Portugal (1994–2001)	24/89 (27%)	5/16 (31%)	19/73	1.20 (0.53–2.73)
Visconti et al. (117)	Italy (1992–1997)	7/20 (35%)	3/4	3/14	3.50 (1.11–11.07)
Zingale et al. (118)	Italy (1996–2002)	25/64 (39%)	21/29	4/35	6.34 (2.45–16.37)
Nahimana et al. (94)	France (1993–1996)	57/158 (36%)	25/29	32/129	3.48 (2.49–4.85)
Kazanjian et al. (89)	USA (1983–2001)	58/145 (40%)	38/56	20/89	3.02 (1.97–4.62)
Kazanjian et al. (89)	China (1998–2001)	0/15	0/0	1/15	Na
Totet et al. (119)	France (1996–2001)	0/13	0/0	2/13	Na
Crothers et al. (120)	USA (1997–2002)	175215	65/72	110/143	2.12 (1.05–4.27)
Valerio et al. (121)	Italy (1994–2004)	14/154 (9%)	4/38	10/116	1.22 (0.41–3.67)

Treatment according to current or previous exposure to sulfone drugs (trimethoprim–sulfamethoxazole, dapsone or sulfadiazine) at diagnosis of PCP. *F* France; *DK* Denmark; *I* Italy; *P* Portugal; *Np* not provided; *Na* not applicable

[a]Overlap of patients in these studies

widespread use of sulfa drugs for prophylaxis (they were widely used for treatment in the 1980s) of PCP (88–90), Fig. 1. Importantly, DHPS mutations have also been increasingly found in patients without any previous exposure to sulfa drugs, suggesting person-to-person spread of mutant strains.

On the basis of a genetic analysis of multiple loci, it appears that the mutations arose independently in multiple strains of *Pneumocystis* (91). In a genotype study of 13 European HIV patients with recurrent episodes of PCP, a switch from wild-type to mutant DHPS occurred in five of seven patients who had a recurrence of the otherwise same molecular type of *P. jirovecii* (92). All patients had received treatment or secondary prophylaxis with trimethoprim–sulfamethoxazole or dapsone. These findings suggest that DHPS mutants may be selected in vivo (within a given patient) under the pressure of trimethoprim–sulfamethoxazole or dapsone. The emergence of DHPS mutations appears to be specific for *P. jirovecii* because only wild-type *Pneumocystis* DHPS has been found in other primate species (93).

The clinical significance of DHPS mutations, specifically with regard to response to prophylaxis and therapy using a sulfa-based regimen (primarily trimethoprim–sulfamethoxazole or dapsone), has been controversial. Several studies have reported a significant association of DHPS mutations with failure of low-dose sulfa prophylaxis (Table 4). However, the extent to which this association reflects actual drug resistance or failure to comply with prescribed prophylaxis is unknown. Hence, in spite of the emergence of mutant DHPS strains, current clinical experience supports the efficacy of trimethoprim–sulfamethoxazole prophylaxis when taken regularly. However, there is evidence to suggest a contributory role for DHPS mutations in breakthrough PCP in patients using alternative sulfa prophylaxis. Hauser et al. found a significant association with the failure of pyrimethamine–sulfadoxine prophylaxis and the Pro57Ser mutation: all the 14 patients failing this type of prophylaxis harboured this mutation (94). Further, a relatively high number of prophylaxis failures associated with DHPS mutations have been described in patients receiving dapsone prophylaxis. Thus, available data currently suggest that DHPS mutations contribute to low-level sulfa resistance, and may be the most important in failure of second-line sulfa prophylaxis. However, the major reason for PCP breakthrough continues to be the poor adherence to chemoprophylaxis (95).

Studies assessing the impact of DHPS mutations on response to therapeutic, high-dose trimethoprim–sulfamethoxazole have been conflicting, as shown in Fig. 2. While initial case reports suggested that patients with mutant DHPS strains had increased risk of failing sulfa therapy or prophylaxis (96), subsequent studies have not supported such a conclusion. A Danish study of 152 patients with AIDS-related PCP found that the presence of DHPS mutations was an independent predictor of decreased 3-month survival, when compared to patients harboring wild-type DHPS (90). However, two more recent studies have found either no effect or a trend for lower death rate when comparing patients with DHPS mutation to wild-type (86, 94). There are several possible reasons for the discrepancy between the studies, including methodological differences in the definitions of survival endpoints or prophylaxis and treatment failures, or other confounding factors related to the difficulties in assessing

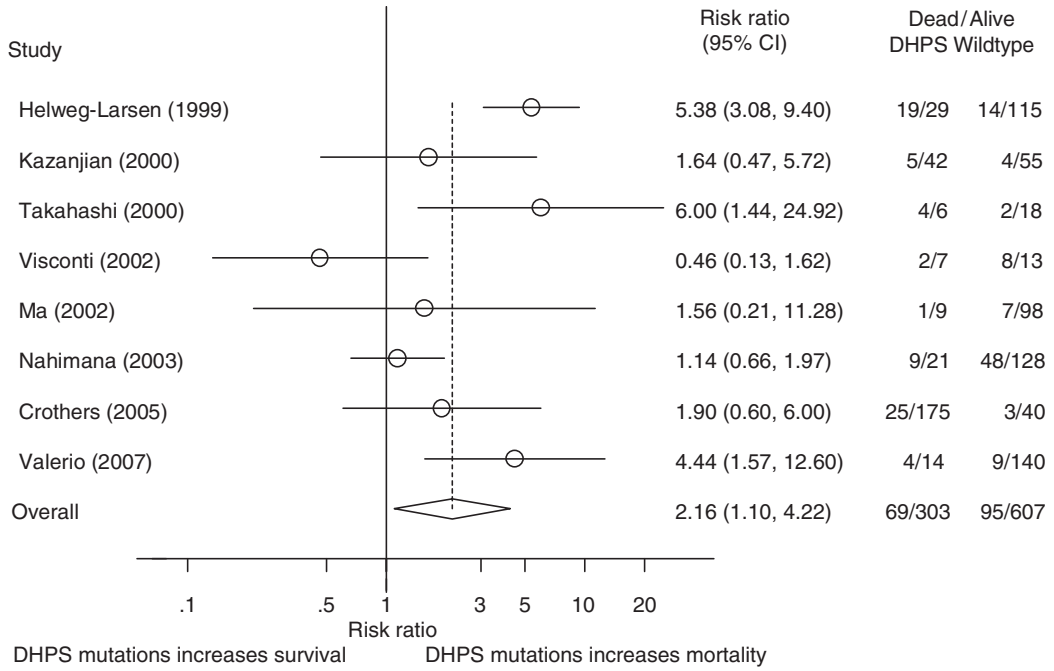

Fig. 2 Risk of deaths with DHPS mutation compared to wild-type in published observation studies. Forest plot of DHPS mutations and survival in HIV-positive patients. DerSimonian random effects analysis

clinical resistance (see **Box 1**). Moreover, even in studies reporting an association of DHPS mutations with failure of sulfa therapy, the majority of patients with mutant DHPS strains have been successfully treated with trimethoprim–sulfamethoxazole or dapsone–trimethoprim. These observations suggest that the currently identified DHPS mutations may confer only low-level sulfa resistance, allowing PCP to occur in the setting of prophylactic doses of sulfa drugs, that is overcome by the higher doses used for therapy. Given that *Pneumocystis* has already demonstrated an ability to mutate under antibiotic pressure, a major concern is that additional mutations may develop that produce high-level resistance.

8 DHFR Resistance

The diaminopyrimidines, trimethoprim and pyrimethamine, are competitive inhibitors of dihydrofolate reductase (DHFR), which catalyzes the reduction of the biologically inactive 7,8-dihyfrofolate to the active 5,6,7,8-tetrahydrofolate in the presence of NADPH and is essential for the biosynthesis of purine/pyrimidine nucleotides, thymidylate and certain amino acids. They are used in combination with sulfonamides.

Interestingly, in animal models trimethoprim does not add any potency to sulfonamides, and thus may not be contributing at all to the anti-PCP efficacy of TMP–SMX (97). The amino acid sequence of DHFR from *P. jirovecii* differs from rat-derived *P. carinii* by 38%. Recently, Ma and Kovacs evaluated the activity of DHFR inhibitors by using a yeast assay expressing *P. jirovecii* DHFR and observed that the human *Pneumocystis*-derived DHFR had ~10-fold increase in sensitivity to trimetrexate and trimethoprim compared to rat *Pneumocystis*-derived DHFR. For the human *Pneumocystis*-derived DHFR yeast strain, trimethoprim and pyrimethamine were both weak inhibitors, with IC_{50}s in the micromolar range; trimetrexate was about 10- and 40-fold more potent than trimethoprim and pyrimethamine, respectively (Table 5). Given that trimetrexate is much more potent against PCP than trimethoprim in vitro, the combination of trimetrexate and sulfamethoxazole may be a more potent combination than trimethoprim plus sulfamethoxazole. However, there are currently no clinical data to support this.

In several bacterial and parasitic species, resistance to DHFR inhibitors has emerged as a consequence of selective pressure by DHFR inhibitors. In this way, resistance of *P. falciparum* and *P. vivax* to pyrimethamine has emerged and is now widespread (98). However, despite the widespread use of trimethoprim in combination with sulfamethoxazole for the prevention and treatment of PCP, only relatively few DHFR mutations have been identified in *Pneumocystis* DHFR (99–102). Ma et al. detected only a single synonymous DHFR mutation in specimens obtained from 32 patients, of whom 22 had previous exposure to TMP–SMX therapy or prophylaxis (99). Takahashi et al. found four mutations in *P. jirovecii*

Box 1 Limitations to the study of drug resistance in *Pneumocystis*

Compared to other pathogenic fungi, the study of drug resistance in *P. jirovecii* has been and continues to be difficult. In spite of many attempts there exists no in vitro culture system for propagation of *Pneumocystis*. The absence of a culture system precludes standard susceptibility testing and has greatly limited the understanding of many fundamental aspects of the organism and impeded investigations into the mechanisms of drug resistance. Since the knowledge of the metabolic pathways is limited, most drug development has been empiric and the currently available treatment options for PCP have been unchanged during the last 15 years. Experimental systems have mainly relied on immunosuppressed animal, in particular the rat model of *Pneumocystis*.

Another problem is that no consistent definition of clinical failure exists. In other fungal infections, clinical resistance is classically defined as the persistence or progression despite the administration of appropriate antimicrobial treatment. However, this definition is problematic when applied to PCP. First, persistence of *Pneumocystis* organisms may happen in spite of a successful treatment response. Studies using repeat bronchoscopy during and immediately after successful treatment of PCP have shown that clearance of organisms is slow, with approximately half of patients still harboring *Pneumocystis* at the end of three weeks of treatment in spite of a successful treatment response (122–125). Although infection is eventually cleared and the viability of organisms detected at the end of treatment is uncertain, it is clear that the detection of organisms during or at the end of treatment cannot be interpreted as a proxy for resistance. Second, host inflammatory response, rather than resistance to antimicrobial drug treatment, may cause an apparent absence of response to treatment. PCP is characterized by marked pulmonary inflammation that in severe cases results in alveolar damage and respiratory failure. Although an efficient immune response is required to control the infection, it has also been demonstrated that an excessive inflammatory response, rather than direct effects of *Pneumocystis* organisms, is crucial for the pulmonary injury (126, 127). Therefore, a severe inflammatory response with respiratory distress, rather than drug resistance, may cause treatment failure. Third, treatment of PCP is associated with a high incidence of adverse effects including fever. In clinical practice, it may be difficult to know whether a slow treatment response with continuing fever is caused by the infection or by the treatment. Given the difficulties in defining clinical failure, reported failure rates for primary trimethoprim–sulfamethoxazole treatment in AIDS patients have varied considerably, ranging from 10 to 40% of cases (38, 53, 55).

In addition, the contribution of nonadherence in presumed failure of prophylaxis may be difficult to assess. The most important reason for prophylaxis failure continues to be nonadherence to prescribed prophylaxis (95, 128, 129). Clinical resistance has been investigated by genotyping of *P. jirovecii* isolates from patients who develop PCP in spite of prescribed chemoprophylaxis. However, in most studies assessment of adherence to prophylaxis has been based on chart reviews, which may fail to disclose nonadherence to a drug regimen. The likelihood of developing *P. jirovecii* resistance within a patient is likely to be higher with inadequate or interrupted dosing. Hence, in theory resistance mutations could be markers of poor adherence, rather than the direct cause of treatment failure.

Table 5 Inhibitory concentrations (50%, IC_{50}) of DHFR inhibitors from a yeast complementation assay

DHFR inhibitor	IC_{50} (nM) Human-derived *P. jiroveci* DHFR	Rat-derived *P. carinii* DHFR
Trimethoprim	5,700	81,000
Pyrimethamine	20,500	33,200
Trimetrexate Adapted (94)	490	4,200

DHFR from 27 patients, of whom only three had previous exposure to TMP–SMX (100). Two of these mutations were nonsynonymous and were not associated with prior exposure to TMP–SMX. In both studies, patients were successfully treated with TMP–SMZ. Nahimana et al. documented nonsynonymous substitutions in 9 of 15 patients receiving a DHFR inhibitor as part of their prophylactic regimen compared to 2 of 18 not receiving a DHFR inhibitor (101). Interestingly, 5 of 7 patients receiving pyrimethamine had nonsynonymous substitutions, suggesting a greater selective pressure of this drug. A South African study found nonsynonymous DHFR mutations in samples obtained between 2001 and 2003 in 3 of 27 patients. None had long-term exposure to TMP–SMX before developing PCP (102). Finally, Matos and coworkers from Portugal recently reported a 27% rate of DHFR mutations in 128 PCP episodes, without association to failure of PCP prophylaxis (103).

In conclusion, although several studies have reported DHFR mutations, there is so far no evidence that the widespread use of trimethoprim or pyrimethamine have caused emergence of clinical significant resistance to DHFR inhibitors.

8.1 Atovaquone

Atovaquone (2-[*trans*-4-(4´-chlorphenyl)cyclohexyl]-3-hydroxy-1,4-hydroxynaphthoquinone) is used to prevent and treat disease caused by *P. jirovecii*, *Plasmodium* spp., *Toxoplasma gondii* and *Bebesia* spp. (104). Atovaquone is structurally similar to the mitochondrial protein ubiquinone (coenzyme Q), and competitively binds to the cytochrome bc_1 complex. The bc_1 complex catalyzes electron transfer from ubiquinone to cytochrome c and thereby proton translocation across the mitochondrial membrane resulting in the generation of ATP. Binding of atovaquone to the ubiquinol oxidation pocket of the bc_1 complex and the Rieske iron–sulphur protein disrupts electron transport and leads to collapse of the mitochondrial membrane potential (105). Eventually, this presumably results in the depletion of ATP within *Pneumocystis* and leads to killing of the organism (106). Mutations of the cytochrome b gene have been identified in *Plasmodium* spp., *Toxoplasma gondii* and *Pneumocystis*. In vitro studies of Plasmodium and Toxoplasma show that these mutations confer resistance to atovaquone. Since *Pneumocystis* cannot be propagated in vitro, similar susceptibility testing cannot be done. In vitro studies of the *Saccharomyces cerevisiae* cytochrome bc_1 complex and atovaquone have demonstrated binding to the ubiquitol pocket. Introduction of mutations near the binding pocket led to decreased activity of atovaquone (105). Introduction of seven mutations observed in isolates of *Pneumocystis* from atovaquone-experienced patients into *S. cervisiae* cytochrome b increased the inhibitory concentration from 25 to >500 nM (107, 108).

Results from two clinical studies have been published. In the first, sequencing of the cytochrome b gene of *Pneumocystis* from ten patients showed sequence variations in four patients (109). Three of four patients receiving atovaquone as prophylaxis demonstrated such variations. Notably, two of them had nonsynonymous changes leading to amino acid substitutions within the ubiquitol pocket. Similar mutations in other microorganisms are associated with resistance to atovaquone. One patient, who had not received atovaquone prophylaxis, had a synonymous change that did not confer any change in amino acid sequence. In the second study, a nested case-control study, significantly more patients who previously had been exposed to atovaquone (5 of 15 patients) had mutations compared to unexposed patients (3 of 45) (110). Five different mutations near the ubiquitol pocket were described bringing the total number to seven. The high number of mutations is unusual but may be explained by a higher mutational rate and impaired proofreading of mitochondrial genes. Survival from PCP did not differ between patients with or without mutations. Overall, these findings are consistent with the development of atovaquone resistance after selective pressure is exerted.

9 Pentamidine and Clindamycine–Primaquine

Pentamidine and clindamycine–primaquine are used for prevention and treatment of PCP, but possible resistance mechanisms have yet to be discovered and reported.

10 Conclusion

In spite of the inability to culture the organisms, it is now clear that mutations involved in sulfa and atovaquone drug resistance have emerged in *P. jirovecii* as a result of selective pressure by the widespread use of PCP prophylaxis. Currently, the clinical effect of the described mutations seems modest. DHPS mutations at codon 55 and 57 are implicated in the failure of low-dose sulfaprophylaxis, but there is no firm evidence that DHPS mutations result in significant resistance to high-dose sulfa therapy. However, it is possible that if additional mutations arise, then high-level sulfa resistance could emerge and lead to diminished efficacy of TMP–SMX. This would lead to the loss of the most efficient and inexpensive therapy for PCP.

The increasing HIV epidemic and use of TMP–SMX in the third world may significantly increase the risk for the development of high-level resistance. Therefore, investigations into the mechanisms of drug resistance and identification of new molecular targets are continuing. A promising advance will be the completion of the *Pneumocystis* Genome Project, which was initiated in 1997. Complete physical maps and gene sequences are being determined for the genomes of *P. carinii* (111). These data will be crucial for further understanding of the infection and will enable identification of new polymorphic regions and drug targets and may eventually also lead to the development of a culture system.

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