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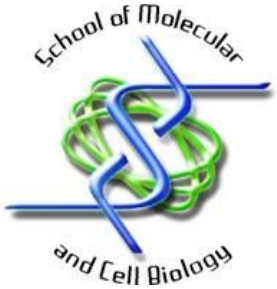
A STUDY OF *PNEUMOCYSTIS* PNEUMONIA IN SOUTH AFRICA

Leigh Anne Davidsson

A thesis submitted to the Faculty of Science, University of the Witwatersrand, in fulfilment of the requirements for the degree of Doctor of Philosophy.





Stockholm, 2011

DECLARATION

I declare that this thesis is my own, unaided work. It is being submitted for the Degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.



_____

7th day of December 2011

ABSTRACT

An increased prevalence of *Pneumocystis jirovecii* with point mutations in the *fas* gene, coding for dihydropteroate synthase (DHPS), has been associated with sulfonamide use. The purpose of this research was to investigate the prevalence of *P. jirovecii* strains containing DHPS polymorphisms in South Africa, and to ascertain their clinical relevance in HIV-positive patients with *Pneumocystis* pneumonia (PCP). A pilot study confirmed the presence of DHPS polymorphisms in 42% of specimens. A subsequent prevalence study confirmed the high prevalence (56%) of mutant *P. jirovecii* in adult patients. A prospective clinical study found that 61% of PCP patients were infected with mutant *P. jirovecii*. The overall in-hospital mortality was 21%. Significantly more patients died in hospital of mutant *P. jirovecii* than those infected with wild-type strains ($P = 0.04$). Mortality at three months among the discharged patients was associated with the wild-type genotype. However, cause of death was unknown. There were no significant associations in patients infected with *P. jirovecii* containing single DHPS mutations versus those infected with the M3 genotype. There was an insignificant trend for patients infected with M3 strains to have lower median $CD4^+$ cell counts versus patients harbouring single mutant strains ($P = 0.06$). Few patients were exposed to sulfonamides and 58% of patients diagnosed with HIV on admission harboured mutant *P. jirovecii*. *P. jirovecii* strains, in a subset of clinical study patients, were characterized by ITS typing. Eleven *bona fide* ITS haplotypes, six ITS1 and nine ITS2 types, were found. Almost half of the patients harboured more than one *P. jirovecii* strain. Eg occurred at a high frequency of 85%, and the presence of the local South African haplotype Eu was confirmed. Other ITS haplotypes detected were: Em, Ec, Eb, Bi, Gg, Ep, Fu_4, Ne and Ai. Two novel ITS1 sequences SA1 and SA2 were detected. There were no obvious associations between ITS haplotype and a particular clinical characteristic or outcome. Eg haplotypes were more often associated with a wild-type DHPS genotype. Inter-human transmission of *P. jirovecii* carrying mutant DHPS genotypes could, at least in part, explain the high prevalence of DHPS mutations in adult HIV-positive South Africans.

To my husband Andreas Davidsson and
to my Lord and Saviour Jesus Christ

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NOMENCLATURE

Ala	Alanine
AIDS	Acquired Immunodeficiency Syndrome
β-TUB	Beta-tubulin
BAL	Bronchoalveolar lavage
bd	Twice a day
bp	Base pair
COPD	Chronic obstructive pulmonary disease
CMV	Cytomegalovirus
CYB	Cytochrome b
DNA	Deoxyribonucleic acid
DHFR	Dihydrofolate reductase
DHPS	Dihydropteroate synthase
EF-3	Elongation factor 3
H&E	Haematoxylin and eosin
HAART	Highly active antiretroviral therapy
HIV	Human immunodeficiency virus
IF	Immunofluorescence
ITS	Internal transcribed spacer
LDH	Lactate dehydrogenase
M1	DHPS mutation one
M2	DHPS mutation two
M3	DHPS mutation three
mtLSU	Mitochondrial large subunit
MSG	Major surface glycoprotein
NICD	National Institute for Communicable Diseases
No.	Number
PABA	Para-aminobenzoic acid
PCP	*Pneumocystis* pneumonia
PCR	Polymerase chain reaction

Pro	Proline
PTB	Pulmonary tuberculosis
qPCR	Quantitative real-time PCR
rRNA	Ribosomal ribonucleic acid
SAM	S-adenosylmethionine
SD	Standard deviation
Ser	Serine
SOD	Superoxide dismutase
TB	Tuberculosis
Thr	Threonine
Trr1	Thioredoxin reductase
TS	Thymidylate synthase
TMP-SMX	Trimethoprim-sulfamethoxazole
WT	Wild-type

LIST OF SYMBOLS

µL	Microliter
µM	Micro Molar
mM	Milli Molar

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INTRODUCTION

1.1 Literature Review

1.1.1 History and Taxonomy

Pneumocystis is an atypical fungus with a worldwide distribution, which opportunistically inhabits the lungs of humans and other mammals. The organism was first discovered over 100 years ago in Brazil by Carlos Chagas in the lungs of guinea pigs, and later in the lungs of a patient who died of trypanosomiasis.[1-3] He was studying *Trypanosoma cruzi* in marmosets and experimentally infected guinea pigs, dogs and rabbits.[4] Chagas incorrectly presumed that *Pneumocystis* was a developmental stage of that parasite. Antonio Carini had, in 1910, also discovered *Pneumocystis* in the lungs of Brazilian rats co-infected with *Trypanosoma lewisi*. He sent tissue samples to the Pasteur Institute in Paris, where the Delanöes, a husband and wife research team, were also studying rats.[5] The Delanöes observed similar cysts in the lungs of sewer rats and in 1912 they realized that *Pneumocystis* was a unique organism unrelated to trypanosomes. They proposed the name *Pneumocystis carinii* in honour of Antonio Carini, and because of the organism's tropism for the lungs and cyst-like shape.[5-7]

The next mention of *Pneumocystis* occurs 30 years later after the Second World War, when premature and malnourished infants in orphanages in Europe were dying of an interstitial pneumonia. It was discovered that *Pneumocystis* was the cause of these epidemics.[8-10] The first case outside of Europe was described in an English infant who died 12 weeks after birth. Her alveoli "were filled with a peculiar foamy or honeycombed material".[11] After the 1950's *Pneumocystis* was associated with pneumonia in children with leukaemia, who were receiving immunosuppressive drugs.[3] Thirty years ago in 1981, the Centers for Disease Control in the USA reported the unusual diagnosis of *Pneumocystis* pneumonia

(PCP) in five young men from Los Angeles.[12] *Pneumocystis* suddenly became a major opportunistic infection in immunocompromised patients with the beginning of the acquired immunodeficiency syndrome (AIDS) epidemic. PCP in non-HIV immunosuppressed patients is becoming increasingly important as these patient numbers rise. This group of patients includes those with haematological and solid organ malignancies, allogeneic bone marrow and solid organ transplant recipients, and patients with autoimmune inflammatory disorders such as Wegener's granulomatosis.[13]

The name *P. carinii* was used for any *Pneumocystis* until it was discovered that *Pneumocystis* species were highly host-specific. As early as 1976, Frenkel *et al* [14] suggested that rat and human *Pneumocystis* should be treated as separate species, based on host specificity and phenotypic differences. It was only in the late eighties that *Pneumocystis* was found to be related to fungi, based on similarities in the nuclear small rRNA subunit.[15-16] DNA analysis also showed the extensive diversity of the *Pneumocystis* genus and a rather lengthy name was adopted for the human form: *P. carinii* formae specialis *hominis* (*P. carinii* f. sp. *hominis*). In 1999, Frenkel again proposed the name *P. jirovecii* for the species that infects humans, and *P. carinii* is now only used to describe the species that occurs in rats.[17-18] In 2006, Redhead *et al* [4] reviewed the classification and nomenclature of *Pneumocystis* and proposed guidelines for the publication of new species according to the International Code of Botanical Nomenclature (ICBN).

Pneumocystis was initially classified as a protozoan parasite on the basis of early morphological observations, and the organism's response to anti-protozoal drugs as opposed to antifungal drugs.[18] Stages in its life cycle were described as cysts and trophozoites and these names are still in use today. However, *Pneumocystis* was found to have staining properties consistent with fungi. A component of the organism's wall is β-1,3-glucan, which is a major component of fungal cell walls. Certain fungal genes were found in *Pneumocystis*, such as the gene for translation elongation factor 3 (EF-3), which is unique to fungal protein synthesis.[19] The enzymes thymidylate synthase (TS) and dihydrofolate reductase exist as two

distinct enzymes in *Pneumocystis*, unlike protozoa where their activities are included in a single bifunctional protein.[20-21] *Pneumocystis* was found to be related to the ascomycetous fungi with *Schizosaccharomyces pombe* as its closest relative species.[22] The current classification of *Pneumocystis* according to the ICBN is as follows:[4,23-24]

Eukaryota
Kingdom: Fungi
Phylum: Ascomycota
Subphylum: Taphrinomycotina
Class: Pneumocystidomycetes
Order: Pneumocystidales
Family: Pneumocystidaceae
Genus: Pneumocystis
Species: *P. jirovecii*, *P. carinii*, *P. murina*, *P. wakefieldiae*

1.1.2 Prevalence and Incidence of *Pneumocystis* Pneumonia

P. jirovecii causes a life-threatening pneumonia in immunocompromised patients and infants less than six months of age. Persons who are immunologically normal usually do not develop clinically important disease.[3] Acute primary infection usually presents in patients previously unaware of their HIV status.[25] Corticosteroid use, neutropenia and immune suppression related to organ transplantation are major non-AIDS predisposing factors for *P. jirovecii* infection.[26] The mortality rate of PCP in HIV-infected adult patients has remained unchanged over the past 20 years, at an average of 12%.[27-28] Morrow *et al*, 2010 reported a high in-hospital case fatality rate of 39% for infants with PCP in South Africa.[29]

The incidence of PCP, which increased dramatically with the advent of the HIV/AIDS pandemic, has decreased in the industrialised world owing to the widespread use of sulfa drug prophylaxis and the introduction of highly active antiretroviral therapy (HAART).[3] Even with ready access to HAART, *P. jirovecii* remains the leading cause of serious opportunistic infection in HIV-

infected patients, particularly those unaware of their HIV status.[27] PCP is an important cause of morbidity and mortality in immunocompromised non-HIV-infected patients in whom its incidence is increasing.[30-31]

Studies from African countries report variable prevalences of PCP in adult HIV/AIDS patients and generally higher rates in children (Table 1.1).[29,32-47] The mean prevalence of PCP in African children calculated from studies in Table 1.1 is 27% (range 10%-49%), and in adults it is 18% (range 4%-43%). Some of these studies were based on autopsy findings, whilst others were hospital-based with various selection criteria and specimen types. Most of the prevalence studies from Africa used only one diagnostic method to detect *P. jirovecii*, and the different diagnostic methods have variable sensitivities for the detection of *P. jirovecii*. Post-mortem lung biopsies of HIV-positive Zimbabwean infants, who died of pneumonia, were tested for *P. jirovecii* using a combination of three different methods. PCR detected most cases, followed closely by cytological staining and the least number of cases were detected by histology.[46] In South Africa, where a limited number of laboratories offer testing for *P. jirovecii*, the vast majority of PCP cases are diagnosed clinically and radiologically. These factors make it difficult to establish prevalence rates for this disease. In South African children infected with HIV, PCP is a major cause of hospitalization and mortality, despite the availability of HAART.[29,41]

Table 1.1 Prevalence of PCP in Sub-Saharan Africa

Country	Reference	Adults/children, HIV status	Study type	Total patients	Percentage prevalence		Concurrent infections with PCP
					PCP	TB	
Botswana	Ansari 2002 [32]	Adults, HIV-positive	Autopsy, died of pulmonary disease, suspected PCP	104	11	40	NS
Botswana	Ansari 2003 [33]	Children ≤13y, HIV-positive	Autopsy, death all causes	35	29	11	NS
Ethiopia	Aderaye 2007 [34]	Adults, HIV-positive	Hospital, , smear negative TB patients	131	30	24	13% (17/131) of all patients had coinfections
Cote d'Ivoire	Lucas 1996 [35]	Children <15m, HIV-positive	Autopsy, death all causes	36	31	0	NS
Kenya	Chakaya 2003 [36]	Adults, HIV-positive	Hospital, smear negative TB patients	51	37	NS	8/19 (42%) PCP patients coinfected with another organism
Malawi	Hargreaves 2001 [37]	Adults, 88% were HIV-positive	Hospital, smear negative TB patients	186	9	NS	2/17 (12%) PCP patients coinfected with PTB
South Africa	Garcia-Jardon 2010 [38]	Adults & children (14% <18 years), HIV-positive	Autopsy, included all patients who died in hospital	86	9	38	NS
South Africa	Morrow 2010 [29]	Children <5m, 61% were HIV-positive	Hospital, patients with acute hypoxic pneumonia	202	21	3	30/43 (70%) PCP patients coinfected with another organism, mainly CMV
South Africa	Murray 2007 [39]	Adult gold miners, HIV-positive,	Autopsy, death all causes	66	14	21	NS
South Africa	Wong 2006 [40]	Adult miners, HIV-positive and negative	Lung autopsy, death all causes	8421	4	NS	107/328 (33%) PCP patients coinfected with another microorganism

Table 1.1 Continued

Country	Reference	Adults/children, HIV status	Study type	Total patients	Percentage prevalence		Concurrent infections with PCP
South Africa	Ruffini 2002 [41]	Children <2y, HIV-positive	Hospital, severe pneumonia	105	49	3	9/51 (18%) PCP patients coinfected with a respiratory viral infection or bacteraemia.
South Africa	Zar 2000 [42]	Children, HIV-positive	Hospital, pneumonia	151	10	NS	NS
South Africa	Mahomed 1999 [43]	Adults, HIV-positive	Hospital, smear negative TB patients with a lower respiratory tract infection	67	43	13	NS
		Adults, renal transplant recipients		36	31	17	
Uganda	Worodria 2003 [44]	Adults, HIV-positive	Hospital, smear negative TB patients	83	39	24	5/32 (16%) PCP patients coinfected with PTB
Zambia	Chintu 2002 [45]	Children <16y, 68% were HIV-positive	Chest autopsy, died of respiratory disease	264	22	20	43/58 (74%) PCP patients coinfected with another lung pathogen
Zimbabwe	Nathoo 2001 [46]	Children <6m, HIV-positive	Post mortem lung biopsy, died of pneumonia	24	67	0	5/16 (31%) PCP patients coinfected with a bacterial pathogen
Zimbabwe	Malin 1995 [47]	Adults, HIV-positive	Hospital, smear negative TB patients, diffuse pneumonia unresponsive to penicillin	64	33	39	6/21 (29%) PCP patients coinfected with PTB

PCP Pneumocystis pneumonia, TB tuberculosis, PTB pulmonary tuberculosis, CMV cytomegalovirus, NS not stated,

§episodes/events

1.1.3 Life Cycle and Transmission

The putative life cycle of *Pneumocystis* is based on ultrastructural observations, as no reliable long-term culture system has been developed (Figure 1.1). The route of human infection is assumed to be airborne via spores, but this infectious stage of the life cycle has yet to be isolated.[48] *Pneumocystis* has a tropism for the lungs and attaches to alveolar epithelial cells. Haploid trophic forms replicate by binary fission during the asexual phase. The fungus proliferates and fills the alveolar spaces, causing lung injury and hypoxia. Matsumoto *et al,* [49] found the presence of synaptonemal complexes, indicating meiotic division, followed by nuclear divisions in *Pneumocystis* infecting rat alveoli. This discovery of a *Pneumocystis* sexual cycle occurring in animals is most unusual for fungi.[50] During the sexual phase two presumptive mating types conjugate, undergo karyogamy, and produce a diploid zygote. The zygote undergoes meiosis and then mitosis to produce eight nuclei. The nuclei are packaged into spores by invagination of the ascus cell membranes. Excystment occurs via protunicate release by unknown mechanisms, and the spores become the vegetative forms that undergo asexual or sexual replication.[48]

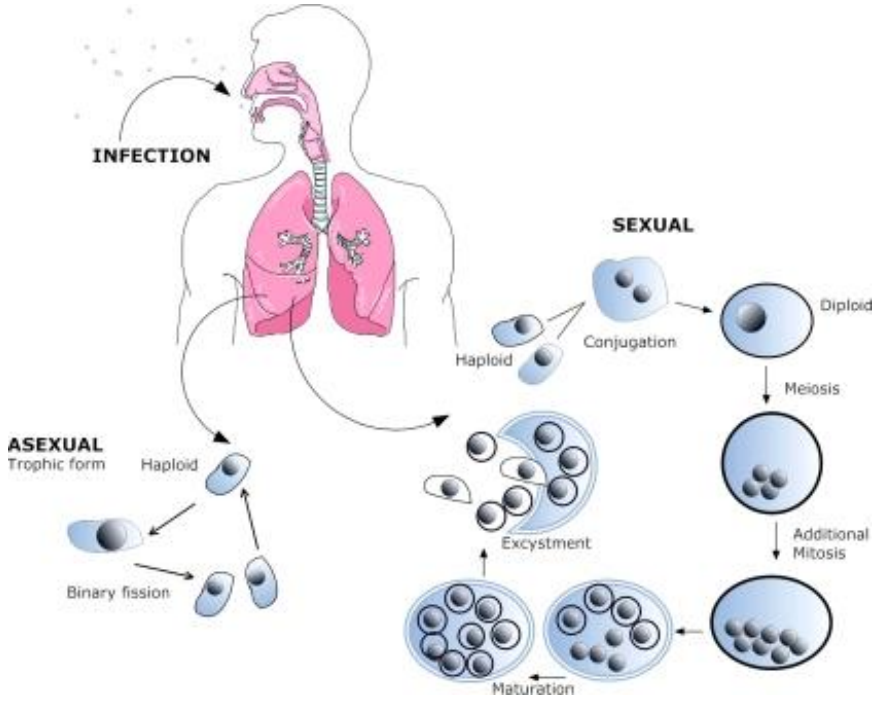

Figure 1.1 Putative life cycle of *Pneumocystis*.[48]

Permission to use this diagram was obtained from the American Society for Microbiology, license number: 2695350073704, 24 June 2011.

P. jirovecii is not a zoonotic disease, as *Pneumocystis* species are highly host-specific. The most likely route of transmission of *P. jirovecii* organisms is person-to-person via the airborne route. Evidence for this is provided by the demonstration of airborne transmission in rodent models, the detection of *P. jirovecii* DNA in exhaled air samples from PCP patients, and cases of nosocomial clustering.[51-53] An outbreak of PCP was described in 16 renal transplant patients attending an outpatient department at a medical centre in The Netherlands [53] The identical ITS haplotype was found in 75% of patients, and researchers concluded that their results were compatible with inter-human transmission or a common environmental source of infection.

Two-thirds of immunocompetent children have been exposed to *Pneumocystis* organisms, demonstrated by the presence of serum antibodies, by the age of four years.[54-55] This indicates that the fungus is ubiquitous, and raises the question of how these infants become exposed at such an early age. It is likely that the spores are the infectious stage in the life cycle with their resistant wall, allowing the fungus to potentially survive in the environment. Cushion *et al* [56] found strong evidence for the spore as the agent of transmission when studying *P. muris* in mice. On the other hand, immunocompetent adults have been proposed as reservoir hosts for *P. jirovecii*, as carriage or colonization in the respiratory tract of healthy individuals has been demonstrated.[57] Asymptomatic carriage of *P. jirovecii* in healthcare workers in close contact with PCP patients has been described.[58] Transplacental transmission of *P. jirovecii* has been demonstrated in rabbits, and vertical transmission was detected in aborted foetuses of immunocompetent women by molecular techniques.[59-60] However, placental transmission of *P. carinii* in rats was not demonstrated.[61] A case of potential transmission from immunocompetent grandparents to an infant was reported from Spain.[62] Both grandparents were colonized with *P. jirovecii*, and the grandmother had a history of rheumatoid arthritis, and the grandfather suffered from chronic bronchitis. Patients with chronic pulmonary diseases, such as chronic obstructive pulmonary disease (COPD), have been shown to be colonized

with *P. jirovecii* and are suggested to be reservoirs of this fungus.[63-64] HIV-infected patients with PCP are a likely source of infection in localities with high prevalence rates. A combination of all of the above acting as potential reservoirs of infection could account for the ubiquitous nature of this fungus.

There has been much debate around reactivation of a latent *P. jirovecii* infection, perhaps acquired as an infant, versus reinfection. Evidence for *de novo* acquisition of *P. jirovecii* with mutant DHPS genotypes has been provided by studies including patients who had never received sulfa drug prophylaxis for PCP.[65-66] In one of these studies, more than half of the patients newly diagnosed with HIV infection had mutant *P. jirovecii*.[66] This suggests recent infection with strains from other patients who were exposed to sulfa medications. Genotyping studies have also supported recent transmission with clusters of PCP cases having a single predominant *P. jirovecii* genotype.[53] Patients with recurrent infections have shown genotype switching in support of this hypothesis.[67]

1.1.4 Host Defence

Pneumocystis organisms attach to alveolar epithelial cells and the alveolar macrophages are the first line of defence to prevent infection. To counter this, *Pneumocystis* organisms reduce the ability of alveolar macrophages to phagocytose, as well as decrease their numbers by inducing apoptosis.[48] Much of the lung damage with a *Pneumocystis* infection is due to inflammation. The immune response to *Pneumocystis* is complex and involves both the humoral and cellular immune systems.[68-69] $CD4^+$ T lymphocytes are essential to clear *P. jirovecii* infection, and most infections occur in immunocompromised patients with lower than 200 cells/mm^3. $CD8^+$ T lymphocytes work together with the $CD4^+$ cells, but play a less significant role in host defence against *Pneumocystis*. $CD8^+$ T lymphocytes have been shown to decrease the severity of PCP.[68] B lymphocytes act as effector cells against *Pneumocystis* infection, and are important in the generation of $CD4^+$ memory T cells.[68-69]

1.1.5 Diagnosis and Clinical Manifestations

1.1.5.1 Clinical Manifestations

PCP patients usually present with dyspnoea (shortness of breath), a non-productive cough and hypoxaemia. PCP is an AIDS-defining infection and HIV-infected patients present subacutely with progressive dyspnoea, low grade or no fever, and malaise. Non-HIV immunosuppressed patients, such as those with malignancies or transplant recipients, present with a more acute illness. These patients have substantial shortness of breath causing severe respiratory distress that frequently requires urgent mechanical ventilation. The clinical presentation in PCP patients can vary and may mimic other infections or disease processes.[3,10,13]

Typical chest radiography shows bilateral, diffuse interstitial infiltrates involving the perihilar regions, with sparing of the apices. However, all types of atypical chest radiographs have been linked with PCP. A high resolution computed tomography scan is more sensitive than chest x-ray and shows the characteristic bilateral groundglass opacities of PCP.[3,10,13]

1.1.5.2 Laboratory Diagnosis

The laboratory diagnosis of *P. jirovecii* can be made within 2 hours on an induced sputum specimen, stained with a fluorescent monoclonal antibody. Treatment based on a confirmed diagnosis, as opposed to empirical therapy, ensures that the patient receives the appropriate medication and potentially toxic medications are avoided. Bronchoalveolar lavage fluid (BAL) is considered the best specimen type for *P. jirovecii* diagnosis, apart from a lung biopsy.

Immunofluorescence (IF) microscopy is commonly used for the laboratory diagnosis of *P. jirovecii*. A UV microscope equipped with the correct wavelength filter is required, as well as an experienced microscopist. Commercial kits are available for either direct or indirect fluorescent staining with monoclonal antibodies. All kits are able to stain the so-called “cyst” forms, but not all kits can

reliably stain the smaller trophic forms. The analytical sensitivity of IF microscopy is high and the specificity is good.[69]

Molecular techniques, such as real-time PCR, are becoming more popular and are often used in conjunction with IF microscopy. Several in-house real-time PCR methods targeting various genes have been developed to replace nested PCR and gel electrophoresis.[69] One of the most common gene targets is that coding for the mitochondrial large subunit (mtLSU). The highly sensitive nature of PCR creates a challenge when interpreting PCR-positive results with low copy numbers and negative IF microscopy results. Distinguishing between colonization and active clinical disease is not easy.

Cytological staining techniques to diagnose *P. jirovecii* are still used in some institutions, as well as in resource-poor settings. Grocott's methenamine silver, toluidine blue O and calcofluor white stains can be used to stain the "cyst-like" forms (Figure 1.2).[13] Giemsa is a non-specific stain that colours all nuclear material red and cytoplasm blue. Trophic forms and "intracystic bodies" are stained with Giemsa, however the cell wall does not take up stain leaving a characteristic halo around the "cyst". Giemsa-stained smears are time-consuming and difficult to read, especially sputum smears that may contain many cells and other microorganisms. The sensitivity and specificity of the different cytological stains varies depending on the specimen type and experience of the microscopist. Cytological stains are generally less sensitive than IF microscopy.[69]

Tissue sections of lung biopsy specimens have long been stained with the non-specific histological stain of haematoxylin and eosin (H&E). The presence of foamy pink exudates filling the alveolar spaces is typical of *P. jirovecii*.[3] The classical Grocott's methenamine silver stain, which was widely used in the past, is mainly reserved for histological specimens.

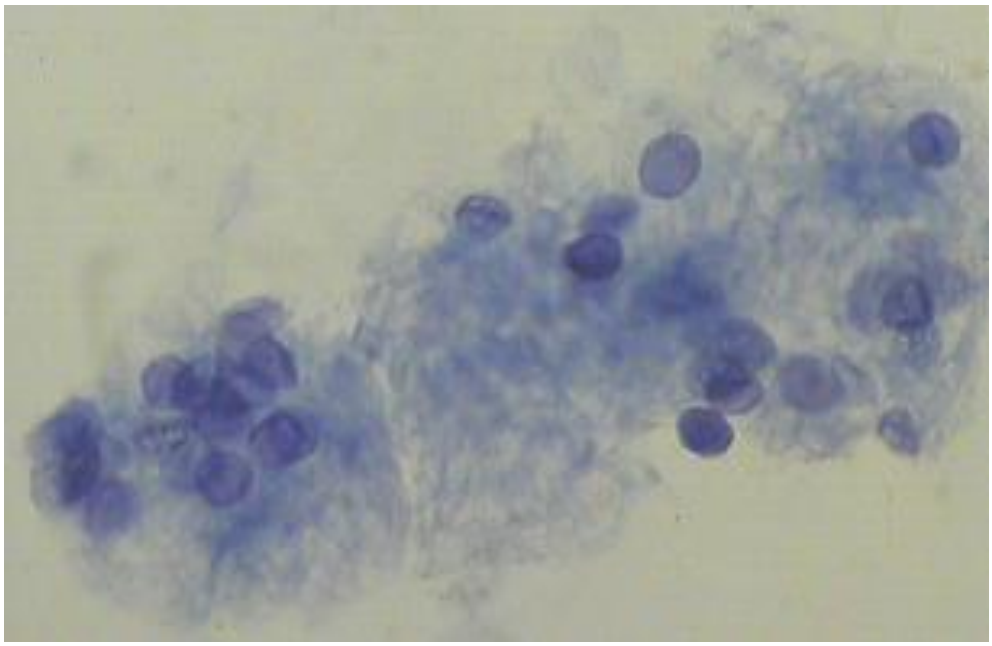

Figure 1.2 *P. jirovecii* stained with toluidine blue O (1000x magnification)

The idea of a quick, cheap, non-invasive serological test for *P. jirovecii* is enticing. Unfortunately, the main disadvantage of the current techniques is the lack of a high degree of specificity. Tests that measure serum lactate dehydrogenase (LDH) and S-adenosylmethionine (SAM) have not yet demonstrated performance characteristics to suggest that they can be useful for the diagnosis of *P. jirovecii*.[70] Elevated serum levels of LDH have been associated with PCP, but are not specific. SAM is essential for methylation reactions and polyamine biosynthesis.[71] *Pneumocystis* was thought to lack the enzyme SAM synthase, and needed to acquire this metabolic intermediate from its human host. Low or undetectable levels of SAM have been reported in PCP patients, with a rise in concentration as the patient recovers.[71] However, de Boer *et al* [72] studied 31 patients, 21 of whom were PCP-positive, and found that SAM levels did not discriminate between patients with and without PCP.

β-glucan, (1→3)-β-D-glucan, is a polysaccharide cell wall component in many fungi, including *Pneumocystis*.[73] This polysaccharide triggers an innate immune

response and can be measured in blood and BAL specimens.[70] In the largest study to date, Sax *et al*, 2011, measured plasma β-glucan levels in a group of 282 HIV-positive patients.[73] They found that PCP patients had higher levels of β-glucan (median 408 pg/ml) compared to those without PCP (median 37 pg/ml), and the test had an analytical sensitivity of 92%. However the specificity was only 65%, which is lower than that for stains of induced sputum. Other invasive fungal infections caused elevated levels of β-glucan. The levels of β-glucan did not correlate with disease severity or response to treatment. The test was cheaper than IF microscopy in their setting.

1.1.6 Management of PCP

Untreated PCP in AIDS patients is almost invariably fatal. Management of the disease in patients with AIDS falls into two categories: therapy for acute primary infection and prophylaxis for those whose HIV infection status is known. Antiretroviral drugs are an integral part of therapy for acute primary PCP.[25] Sulfonamides, usually combined with trimethoprim as in trimethoprim-sulfamethoxazole (TMP-SMX, also known as co-trimoxazole), and dapsone are used for the treatment and prophylaxis of PCP. TMP-SMX is the drug of choice for both the treatment and prophylaxis of mild, moderate and severe PCP. In HIV patients the recommended treatment is trimethoprim 15-20 mg/kg/day plus sulfamethoxazole 75-100 mg/kg/day for 21 days. In non-HIV patients, the duration of treatment can be shortened to 14 days, with decreased risk of relapse.[74] Clinical improvement should occur within 4-7 days.[25] TMP-SMX may cause significant toxicity in up to 40% of patients, but a gradual introduction of TMP-SMX may improve tolerance to the drug.[75] Adjunctive corticosteroids are recommended for patients with moderate to severe PCP.[74]

There are few alternatives for those who cannot tolerate TMP-SMX. Regimens include intravenous pentamidine, clindamycin plus primaquine, trimethoprim plus dapsone and atovaquone suspension.[74] Intravenous pentamidine isethionate is an alternative for patients with severe PCP who are intolerant or unresponsive to TMP-SMX. Aerosolized pentamidine has also been used for the treatment of

active disease, but is associated with unacceptably high relapse rates, as well as the promotion of atypical pulmonary and/or extrapulmonary *P. jirovecii* infection, and is not considered adequate therapy for PCP.[76-77]

PCP prophylaxis has decreased the incidence of the disease and increased the median survival of AIDS patients. It also appears to delay the first AIDS-defining illness by six to 12 months and patients have lower CD4 counts at the time of diagnosis. Prophylaxis is indicated for any HIV-infected adult or adolescent, including pregnant women, with a CD4 count of less than 200 cells/mm^3.[77] TMP-SMX is the recommended first-line treatment for prophylaxis against PCP.[74] Failure of prophylaxis may be due to resistance to the agent of choice, or may reflect factors such as poor compliance or unfavourable pharmacokinetics. It has been suggested that when PCP develops in patients receiving prophylaxis, therapy should be instituted with a different agent, at full dose, rather than the one used for prophylaxis.[25]

1.1.7 DHPS Mutations and Drug Resistance

Sulfonamides inhibit the enzyme dihydropteroate synthase (DHPS) and trimethoprim inhibits dihydrofolate reductase (DHFR), which are essential components of the folate synthesis pathway.[78] *Pneumocystis* organisms require the synthesis of folates, and the role that these two enzymes play in this process of folic acid biosynthesis is illustrated in Figure 1.3. DHPS catalyses the condensation of para-aminobenzoic acid (PABA) and pteridine to form dihydropteroic acid. Further steps in this pathway lead to folic acid synthesis and eventually DNA synthesis. Sulfa drugs are chemical analogues of PABA and bind to DHPS, thus competitively inhibiting the enzyme and folate synthesis (Figure 1.4). DHPS gene mutations are thought to cause structural changes in the enzyme's substrate binding site, which interfere with sulfonamide binding and decrease the affinity to these drugs. Point mutations in the DHPS-encoding gene of other microorganisms such as *Plasmodium falciparum*, *Staphylococcus aureus, Mycobacterium leprae* and *Escherichia coli* have been shown to confer resistance to sulfonamides.[79-83]

In *P. jirovecii*, two nonsynonymous point mutations in the *fas* gene encoding the DHPS enzyme are associated with prior exposure to sulfa drugs.[66-67,84-87] There are concerns about the possible emergence of sulfonamide resistance in this organism. These single nucleotide mutations, at positions 165 and 171, cause amino acid substitutions at positions 55 (threonine to alanine, so called mutation M1) and 57 (proline to serine, mutation M2) in the DHPS protein, respectively. The presence of both mutations is denoted as mutation M3, and the wild-type sequence is commonly accepted as the one with no substitutions (DHPS sequence in GenBank AF139132). As *P. jirovecii* cannot yet be cultured, conventional *in vitro* susceptibility tests cannot be utilized to ascertain drug resistance; therefore, studies of drug resistance in this organism rely on the use of genetic markers and suitable models. Functional complementation of either DHPS-disrupted *E. coli* with mutant *P. jirovecii fas* gene, or FOL1-disrupted *Saccharomyces cerevisiae* with the *fol1* gene mutated at nucleotide positions analogous to 165 and 171 in *fas*, result in loss of susceptibility to sulfamethoxazole and other sulfa-containing drugs.[88-89]

The clinical significance of DHPS mutations in *P. jirovecii* is not fully understood and the few studies that have investigated the clinical effects of these mutations showed conflicting results.[65,67,84,86-87,90-94] These studies used different definitions for patient outcome, as well as different time points for their assessments. Some studies from the developed world have used pooled retrospective data from several countries, as a limited number of clinical studies had adequate sample sizes for the mutant DHPS genotype category. Table 1.2 summarizes results from ten studies that had at least 14 patients in the mutant DHPS category.

An early study from 1999 by Helweg-Larsen *et al*,[67] found a significantly lower three-month survival in patients infected with mutant *P. jirovecii* compared to patients infected with wild-type organisms. In contrast, two other studies [84,93] found no significant association between patient survival and DHPS genotype at four and six weeks respectively. Huang *et al* [90] found an insignificant trend for

patients infected with mutant *P. jirovecii* DHPS to have an increased mortality, whilst Nahimana *et al* [86] had similar in-hospital rates of death for patients with or without mutant *P. jirovecii*. Crothers *et al* [87] found that mortality was not associated with the presence of *P. jirovecii* containing DHPS mutations. Whether the presence of DHPS gene mutations confers clinical resistance to sulfonamides remains unclear and requires further study.

1.1.8 Prevalence of *P. jirovecii* DHPS Mutations

The prevalence of *P. jirovecii* DHPS mutations reported from countries in the developed world ranges widely, from 0% to 81% (Figure 1.5).[65,67,84-87,90-99] Studies from the USA generally report the highest prevalences of *P. jirovecii* DHPS mutations, with an average of 67% calculated from Figure 1.5. European countries by contrast, have much lower prevalences with an average of 17%. The few studies performed in developing countries all report low prevalences of *P. jirovecii* containing DHPS mutations (Table 1.3). A study from 2003, based on a small sample size of 14 patients, reported a prevalence of *P. jirovecii* DHPS mutations in 7% of Zimbabwean HIV-infected adults with PCP. [100] In South Africa, a handful of studies that screened specimens from limited numbers of adults and children have reported low mutation prevalences of up to 13%.[101-103] The apparent low prevalence of DHPS mutations in *P. jirovecii* from developing countries has been assumed to be the result of limited availability of sulfa drug prophylaxis against PCP.

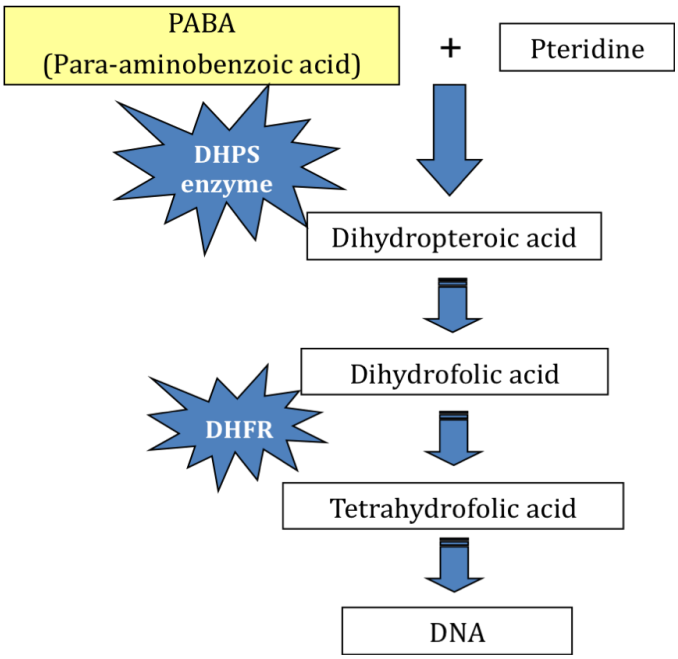

Figure 1.3 Folate synthesis pathway and the roles of the DHPS and DHFR enzymes (adapted from Huang *et al* [104]).
DHPS dihydropteroate synthase, DHFR dihydrofolate reductase

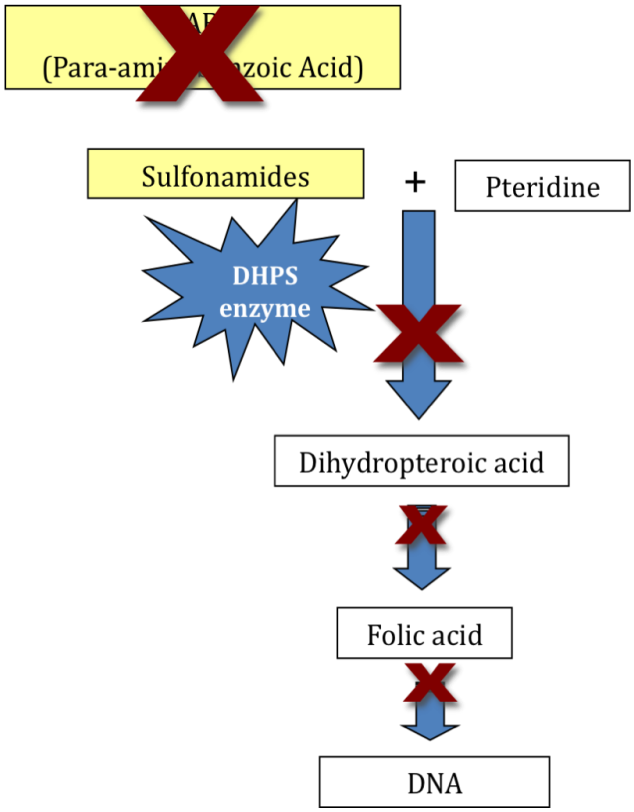

Figure 1.4 Inhibition of the folate synthesis pathway by sulfonamides.
DHPS dihydropteroate synthase

Table 1.2 Results from studies (≥14 patients in the mutant category) investigating the clinical significance of *P. jirovecii* DHPS mutations

No. of patients, country, patient type	DHPS mutations (%), prevalent genotype	Sulfa prophylaxis associated with DHPS mutations	Clinical significance of DHPS mutations	Reference
372[a], UK & USA, HIV+	268[a] (72), not stated	Yes	City of residence associated with presence of DHPS mutations. Insignificant trend for patients with mutant strains to have increased mortality at 6 weeks.	Huang 2006 [90]
215[a], USA, HIV+ adults,	175[a] (81), M3 (113/175)	Yes	Presence of DHPS mutations not associated with mortality at 60 days. Trend for patients with mutant strains to have worse outcomes vs WT.	Crothers 2005 [87]
194, France, HIV+/- adults & children	64 (33), M2 (42/64)	Yes	Suboptimal prophylaxis - failure associated with mutant strains.	Hauser 2010 [91]
158, France, 76% HIV+ adults	57 (36), M2 (36/57)	Yes	Rates of death (in hospital) attributed to PCP similar in patients with or without mutant strains.	Nahimana 2003 [86]
154, Italy, HIV+ adults	14 (9), M1 (6/14)	No	Correlation between DHPS mutants and greater severity of PCP and worse outcomes.	Valerio 2007 [92]
144, Denmark, HIV+ adults	29 (20), M1 (18/29)	Yes	Significantly lower 3-month survival in patients with mutant DHPS strains.	Helweg-Larsen 1999 [67]
136, USA, HIV+ adults	97 (71), M3 (72/97)	N/A	No association between DHPS genotype & death at 6 weeks.	Navin 2001 [93]
98, Spain HIV+ adults	17 (17), M3 (8/17)	Yes	Similar in-hospital, 3-month & 6-month mortality rate regardless of DHPS genotype.	Alvarez-Martinez 2010 [94]
97, USA HIV+ adults	42 (43), M3 (41/42)	Yes	No significant difference in survival at 4 weeks. Patients with mutant strains had significantly increased risk for failure of sulfa/sulfone therapy vs WT.	Kazanjian 2000 [84]
92, France, 90% HIV+	16 (17), M3 (4/16)	No	No difference in outcome according to DHPS genotype.	Latouche 2003 [65]

[a] Episodes or specimens, HIV+ HIV-positive, HIV- HIV-negative, WT wild-type

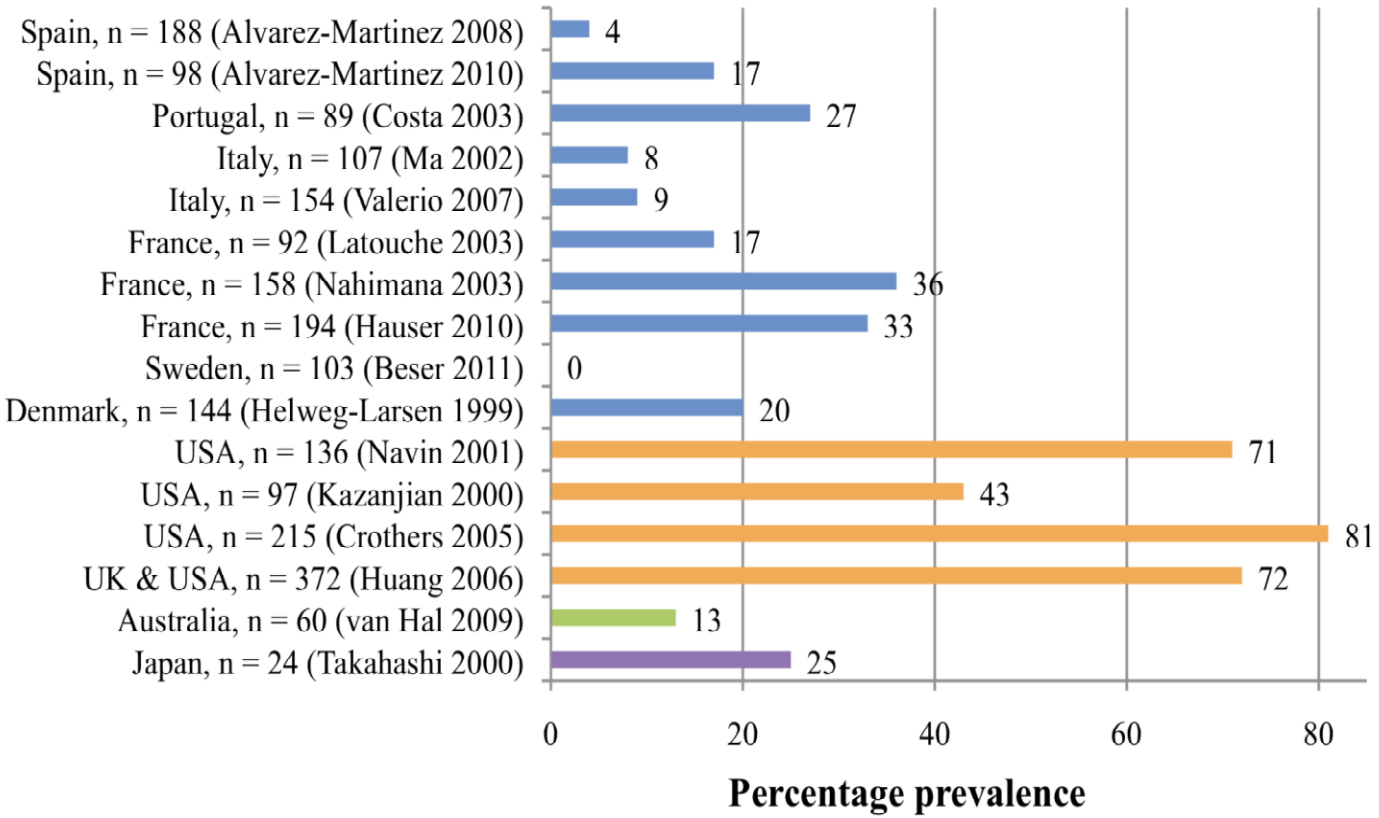

Figure 1.5 Prevalence of DHPS mutations in *P. jirovecii* from studies conducted in developed countries

Table 1.3 Prevalence of *P. jirovecii* containing DHPS mutations from studies conducted in developing countries

Country	Year	Sample size (n = patients)	DHPS mutation prevalence (%)	Reference
South Africa	2008	6	0	Govender 2008 [103]
South Africa	2005	53[a]	1 (2)	Robberts 2005 [102]
South Africa	2004	30	4 (13)	Zar 2004 [101]
Zimbabwe	2003	14	1 (7)	Miller 2003 [100]
Brazil	2006	57	0	Wissmann 2006 [105]
China	2009	10	0	Li 2009 [106]
China	2004	15	1 (7)	Kazanjian 2004 [107]
India	2010	24[b]	1 (4)	Tyagi 2011 [108]
India	2008	5	0	Tyagi 2008 [109]

[a] PCP episodes, not patients

[b] non-HIV-infected patients

1.1.9 Strain Characterization Based on ITS Typing

Conventional microbial typing methods for *Pneumocystis* are not possible, as the organism cannot be cultured for a lengthy period of time. Instead, genetic loci with nucleotide sequence variation have been used for strain characterization, and include the mitochondrial large-subunit rRNA (mt LSU rRNA), the major surface glycoprotein (MSG), beta-tubulin (β-TUB), thymidylate synthase (TS), cytochrome b (CYB), superoxide dismutase (SOD), thioredoxin reductase (Trr1), dihydrofolate reductase (DHFR), the dihydropteroate synthase (DHPS) region of the *fas* gene, and the internal transcribed spacer (ITS) regions in the nuclear ribosomal RNA gene complex. ITS and MSG sequences show marked diversity, but the other loci have been poor candidates for strain typing due to their low sequence diversity.

Characterization of *P. jirovecii* strains in this research study was performed by analysis of sequence variation in the ITS regions in the nuclear rRNA gene complex. The ITS regions show great diversity among *Pneumocystis* strains and provide an informative epidemiological tool for the typing of these strains.[110] There are two ITS regions in the genome of *Pneumocystis*. The ITS1 region is located between the genes encoding the 18S rRNA and the 5.8S rRNA, and the ITS2 region lies between the 5.8S rRNA and the 26S rRNA genes (Figure 1.6). The ITS regions are processed by splicing events during the maturation of the rRNA molecules. Most fungi have multiple copies of rRNA genes and the ITS regions are usually conserved within species, providing a species-specific signature. However, the nuclear ribosomal genes of *Pneumocystis* exist as a single copy, and there is considerable intra-specific sequence variation.[111]



Figure 1.6 Schematic diagram of the nuclear ribosomal gene complex of *Pneumocystis* showing the positions of the non-coding ITS1 and ITS2 regions (based on Lu *et al* [110])

ITS typing methods have been devised by Lee *et al* [112] and Tsolaki *et al*.[113] Different naming systems were devised for the various ITS types, making the comparison and interpretation of their results complicated and sometimes impossible if not enough information is provided in the publications. For this study Lee's system for the naming of ITS haplotypes was used. The uppercase letter represented the ITS1 type and the lowercase letter represented the ITS2 type.[112] At least 100 ITS haplotypes have been described to date, and more than 30 ITS1 types and 40 ITS2 types. The diversity of ITS haplotypes is lower in reality, as some of the haplotypes have been proven to be artifacts generated by the PCR method. Beser *et al*, 2007 showed that chimeric ITS sequences were produced at high rates during PCR amplification of specimens that were co-infected with more than one ITS haplotype.[114] Under standard PCR conditions using *Taq* polymerase, up to 37% of the amplified sequences were hybrid DNA artifacts. By modifying the PCR amplification conditions, the formation if ITS chimeras could be largely abolished and the erroneous establishment of artifactual haplotypes was avoided.

Co-infections with more than one ITS haplotype are common. According to Lee *et al* [112], a sequence found in two or more specimens is considered a distinct haplotype (a combination of ITS1 and ITS2 sequences). Beser *et al* [114] suggested that a single infection is the only true basis for the definition of a *bona fide* *P. jirovecii* ITS haplotype.

The most common ITS haplotype is Eg, which occurs worldwide. Ne, Ai and Eb haplotypes have been reported from many countries.[112,115-117] The ITS haplotype Ne was associated with an outbreak of PCP in renal transplant patients attending an outpatient department at a medical centre in The Netherlands.[53] The same Ne ITS haplotype was found in 75% of patients. A single study from South Africa has investigated the ITS haplotypes in *P. jirovecii* organisms from 19 patients.[118] The global haplotype Eg was most prevalent, and was found in 74% of patients. The second most common haplotype was Gg occuring in 21% of patients. A new

ITS2 type u was described in three patients and was associated with an E in the ITS1. It remains to be seen if Eu is a local *P. jirovecii* ITS haplotype.

1.2 Objectives

The purpose of this research was to investigate the prevalence of *P. jirovecii* strains containing dihydropteroate synthase (DHPS) mutations in South Africa, and to ascertain their clinical significance in HIV-positive patients with *Pneumocystis* pneumonia. The molecular epidemiology of *P. jirovecii* was explored by characterizing the strains circulating in HIV-infected patients.

The research included the following objectives:

1.2.1 To investigate the presence of DHPS polymorphisms in *P. jirovecii* strains in South Africa (Chapter Three).

1.2.2 To determine the prevalence of DHPS polymorphisms in the general *P. jirovecii* population in South Africa (Chapter Four).

1.2.3 To ascertain the clinical significance of *P. jirovecii* DHPS polymorphisms in HIV-infected patients with PCP (Chapter Five).

1.2.4 To characterize the *P. jirovecii* strains circulating in HIV-infected patients and explore potential correlations with DHPS genotype and patient outcome (Chapter Six).

It was hypothesized that the prevalence of DHPS polymorphisms in *P. jirovecii* strains in South Africa would be low, i.e. less than 15%. Two smaller studies from South Africa and a few studies performed in other developing countries, all reported low prevalences of *P. jirovecii* containing DHPS mutations (Table 1.3). It was important to perform a large prevalence study sampling patients from different parts of South Africa, as the overall prevalence of DHPS polymorphisms in the *P. jirovecii* population was unknown.

It was hypothesized that patients infected with *P. jirovecii* containing mutant DHPS genotypes would have poor clinical responses to treatment with sulfa-containing drugs. It was important to perform a clinical study, as the clinical significance of DHPS mutations in *P. jirovecii* is not fully understood and the few studies that have investigated the clinical effects of these mutations showed conflicting results (Table 1.2). If the presence of DHPS gene mutations confers clinical resistance to sulphonamides, then this will have severe implications for the treatment of this disease.

It was hypothesized that the diversity of *P. jirovecii* ITS haplotypes circulating in South Africa would be lower than previously reported [(118)] and that the most common ITS haplotype would be the global Eg haplotype. In addition it was hoped to confirm the presence of the Eu haplotype in the South African *P. jirovecii* population.

1.3 Candidate's role in the studies

The candidate performed all molecular analyses for the prevalence study (Chapter Four) and the ITS characterization study (Chapter Six) at the Swedish Institute for Infectious Disease Control (SMI) in Sweden. The molecular analyses for the clinical study (Chapter Five) were performed both at SMI and at the National Institute for Communicable Diseases (NICD) in South Africa. The candidate had help from a Masters student in South Africa to perform some of the analyses for the clinical study, as well as the cloning of mixed DHPS genotypes. The candidate performed all data analysis and drafted the publications.

C H A P T E R T W O

METHODS AND MATERIALS

2.1 Laboratory Setting

The Parasitology Reference Unit of the National Institute for Communicable Diseases (NICD), in Johannesburg, South Africa, is the largest recipient of referrals for the microbiological confirmation of *P. jirovecii* infection in the country, receiving about 1000 specimens annually. Specimens were processed at the NICD, as well as at the Swedish Institute for Infectious Disease Control (SMI), in Solna, Sweden.

2.2 Hospital Setting

The clinical study in Chapter Five was conducted at Chris Hani Baragwanath Hospital, in Soweto, South Africa. This is one of the largest hospitals in the world with 2 700 beds (maximum 3 200) in current use and serves an estimated population of four million people.

2.3 Specimens and Patient Information

2.3.1 Pilot Study (Chapter Three)

A subset of 79 respiratory specimens from 65 patients were non-randomly chosen for the screening of *P. jirovecii* DHPS polymorphisms. The specimens were received at the National Institute for Communicable Diseases (NICD) in Johannesburg for the routine diagnosis of *P. jirovecii* from April 2003 through September 2004. The majority of specimens were sputa, and two specimens were bronchial washings. All of the specimens were positive for *P. jirovecii* by immunofluorescence (IF) microscopy. The patients were hospitalized in 12 hospitals in four provinces of South Africa: Gauteng (46 patients), North West (11 patients), Eastern Cape (5 patients) and Mpumalanga (3 patients). The mean age

of patients was 34 years (range 17-59 years) and 56% were female. Data concerning the HIV status of patients were not available.

2.3.2 DHPS Prevalence Study (Chapter Four)

Respiratory specimens (n = 932) from patients with suspected PCP (n = 712) were referred to the NICD from 38 public hospitals and clinics in six provinces of South Africa from January 2006 through February 2007. Data regarding patient age, gender and hospital location were extracted retrospectively from a laboratory information system. The mean age of patients was 34 years (SD±13), with a range of one month to 86 years (age was unknown in 81 patients). The female to male ratio was 1.6:1 (gender was unknown in 18 patients). Most patients (n = 491/712, 69%) were from Gauteng Province, and the rest were from the Eastern Cape, Mpumalanga, Free State, Limpopo and Northern Cape Provinces. The majority of specimens received for *P. jirovecii* diagnosis were sputa (n = 839/932, 90%). When dealing with multiple specimens from the same patient and PCP episode, only the first *P. jirovecii*-positive specimen received in the laboratory was included in the analysis. If more than one specimen was taken on the same day, the specimen with the higher fungal load was included in the analysis. Data concerning HIV status, and prophylaxis and/or treatment for PCP were not available for these patients. All procedures followed were in accordance with the ethical standards of the Human Research Ethics Committee (Medical) of the University of the Witwatersrand in Johannesburg (Protocol no. M050320, Appendix G).

2.3.3 Clinical Study (Chapter Five)

Patients admitted to Chris Hani Baragwanath Hospital in Soweto, Johannesburg, South Africa, with clinically and radiologically suspected PCP (n = 268), were prospectively enrolled into the study from March 2005 through June 2009. A sample size calculation was performed based on the results of the laboratory study. A minimum of 250 patients would need to be screened to ensure at least 25-30 patients in the double DHPS mutant M3 category. Informed written consent was obtained from each patient and all procedures followed were in accordance

with the ethical standards of the Human Research Ethics Committee (Medical) of the University of the Witwatersrand in Johannesburg (Protocol no. M040612, Appendix H). At enrolment, patients underwent sputum induction and were interviewed using a standard form (Appendix A). A single respiratory physician, who examined radiological films and completed a standard form for data collection, assessed almost all of the patients. Patients were monitored regularly during their hospital stay and data were recorded on the standard form (Appendix B). Patient treatment regimens were recorded throughout the study period, and the therapy of choice for PCP was co-trimoxazole (15-20 mg/kg/day trimethoprim and 75-100 mg/kg/day sulfamethoxazole for three weeks). Adjunctive corticosteroids (prednisone 40 mg bd for five days, then 40 mg daily for five days, followed by 20 mg daily for 11 days) were given to all patients who were treated for PCP. A telephonic and postal follow-up of discharged patients was conducted to ascertain their outcome at three months after their discharge (Appendices C and D).

Patient features considered for statistical analysis were gender, age, $CD4^+$ cell count, newly diagnosed with HIV on admission, history of previous PCP, sulfa drug exposure, duration of hospital stay, development of pneumothorax, need for mechanical ventilation, respiratory failure and patient outcome.

Sulfa drug exposure was defined as the use of any sulfa-based medication in the four months prior to admission. Patient outcome was classified as follows: died in hospital during the current admission, or discharged and died within three months after discharge, or alive at three months after discharge, or discharged but three-month outcome was unknown.

The respiratory samples were coded and all laboratory analyses were performed blind to the patient and clinical details. When dealing with multiple specimens from the same patient and PCP episode, the first *P. jirovecii*-positive specimen received in the laboratory was included in the analysis. If more than one specimen

was taken on the same day, the specimen with the higher fungal load was included in the analysis.

2.3.4 Strain Characterization (Chapter Six)

The first 40 HIV-positive patients enrolled in the prospective clinical study (Chapter Five) from March 2005 through March 2007, with specimens containing more than 100 *P. jirovecii*-DNA copies, were selected for ITS typing. Respiratory specimens containing less than 100 *P. jirovecii*-DNA copies were excluded, as the nested ITS PCR assay was not sensitive enough to reliably detect DNA in these samples. The specimens comprised 38 induced sputa and two bronchial washings. The respiratory samples were coded and all analyses were performed blind to the clinical details.

2.4 Specimen Processing and Laboratory Diagnosis of *P. jirovecii*

The accuracy of laboratory diagnosis was dependent upon successful sputum induction, as well as the presence of organisms in numbers above the limit of detection for the diagnostic methods. A standard sputum induction protocol was used to train the three study nurses and patient sampling was repeated if the sample volume was inadequate for laboratory diagnosis.

Sputum specimens and other specimen types containing mucus were pre-treated with 0.1% dithiothreitol (Sigma-Aldrich, South Africa). After concentration by centrifugation, microscopic slides were prepared with the sediment. The remaining sediment was washed with phosphate-buffered saline and stored at -70°C prior to DNA extraction. Slides were stained with a direct immunofluorescent antibody (IF) test specific for the detection of *P. jirovecii* cysts (Light Diagnostics, Millipore, Temecula CA), according to the manufacturer's instructions. A positive IF result was defined as a specimen containing at least one cluster of *P. jirovecii* cysts with a characteristic morphology and fluorescent immunostain. An equivocal result was defined as a specimen with suspicious *Pneumocystis*-like cyst morphology, but not meeting the criteria to confirm a positive result. In our setting, IF microscopy had an

analytical sensitivity of 82.4% and specificity of 100% compared with the real-time polymerase chain reaction (qPCR).

2.5 DNA Extraction

2.5.1 Pilot Study (Chapter Three)

DNA was extracted from 50 µL of stored sediment, which was digested with proteinase K at 56°C for 2 hours, prior to extraction using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's tissue protocol. Purified DNA was eluted in 100 µL of sterile water and stored at -20°C.

2.5.2 DHPS Prevalence, Clinical and Strain Characterization Studies (Chapters Four, Five, Six)

Genomic DNA was extracted from 50 µL of sediment, which was digested with proteinase K at 56°C for 2 hours, prior to automated DNA extraction with an M48 BioRobot (Qiagen, Germany) according to the manufacturer's soft tissue protocol. Purified DNA was eluted in 100 µL of sterile water and stored at -20°C.

2.6 Determination of *P. jirovecii* DNA Load

The amount of *P. jirovecii* DNA in specimens was determined by a quantitative real-time polymerase chain reaction (qPCR) targeting a portion of the mitochondrial gene coding for the large ribosomal subunit (mtLSU) of the fungus. This real-time PCR method had a lower limit of detection of 17 target gene copies/µL of extracted DNA. The following primers and probe were used: LSU1: 5´-AAA TAA ATA ATC AGA CTA TGT GCG ATA AGG-3´; LSU2: 5´-GGG AGC TTT AAT TAC TGT TCT GGG-3´; LSUP1: FAM 5´-AGA TAG TCC AAA GGG AAA C-3´TAMRA (Applied Biosystems, Foster City, CA). Amplification reactions were carried out in a total volume of 25 µL containing 1x TaqMan Universal PCR Master Mix, 0.25 µL of a mixture of primers and probe at 20 µM each, 5 µL of template DNA and RNase-free water to complete the volume. Thermal cycling was performed in an Applied Biosystems GeneAmp PCR System 7900 thermocycler as follows: 2 minutes at 50°C, 10 minutes at 95°C, followed by 45 cycles of 15 seconds at 95°C and 1 minute at 60°C.

Quantification was based on extrapolation from standard curves generated by amplification of serial dilutions of a linearized pCR2.1 plasmid containing the *P. jirovecii* DNA fragment defined by primers LSU1 and LSU2.

2.7 Analysis of DHPS Genotypes

2.7.1 Pilot Study (Chapter Three)

Amplification of a 300 bp region of the *fas* gene of *P. jirovecii* spanning the DHPS binding site was performed as previously described by Beard *et al*.[119] In the first round of this PCR, the primers DHPS F1 (5' - CCT GGT ATT AAA CCA GTT TTG CC - 3') and DHPS B_{45} (5' - CAA TTT AAT AAA TTT CTT TCC AAA TAG CAT C-3') were used. In the second round, the primers DHPS A_{HUM} (5' - GCG CCT ACA CAT ATT ATG GCC ATT TTA AAT C-3') and DHPSBN (5'- GGA ACT TTC AAC TTG GCA ACC AC - 3') were used. The primary amplification reaction contained 2 µl of template DNA, 1U of Ampli*Taq* Gold DNA polymerase (PE Applied Biosystems), 125 µM of each deoxynucleotide, 2 mM $MgCl_2$ and 1 µM each of primers in 1x PCR buffer (PE Applied Biosystems) and adjusted with RNase-free water to a total volume of 25 µl. The second amplification reaction contained the same concentration of reagents, and 2 µl of PCR product from the first reaction was included in the mixture. The amplification conditions for the first round were 93°C for 10 minutes, followed by 35 cycles of 92°C for 30 seconds, 52°C for 30 seconds, 72°C for 60 seconds, and a final extension step at 72°C for 5 minutes. The amplification conditions for the second round of PCR were 93°C for 10 minutes, then 35 cycles of 92°C for 30 seconds, 55°C for 30 seconds, and 72°C for 60 seconds, followed by a final extension step at 72°C for 5 minutes. PCR products were visualised by gel electrophoresis on 2% agarose gels. Nested PCR products, encompassing polymorphic nucleotide positions 165A/G and 171C/T, were directly sequenced using the BigDye Terminator sequencing kit (Applied Biosystems, Foster City, CA) and an Applied Biosystems model 310 automated DNA sequencer.

2.7.2 DHPS Prevalence Study (Chapter Four)

Specimens with ≥20 target mtLSU gene copies/μL of extracted DNA were selected for DHPS analysis, as amplification of the single-copy DHPS *fas* gene proved to be less sensitive than the real-time PCR assay. Primers SMIF1: 5'-CAA ATT AGC GTA TCG AAT GAC C-3' and SMIB2: 5'-GCA AAA TTA CAA TCA ACC AAA GTA-3' were used to amplify a 1030 bp region of the DHPS gene. Subsequently, the nested primers SMIF6: 5'-AGC GCC TAC ACA TAT TAT GG-3' and SMIB7: 5'-GTT CTG CAA CCT CAG AAC G-3' were used to amplify a 278 bp region, encompassing polymorphic nucleotide positions ^{165}A/G and ^{171}C/T. The primary amplification reaction contained 5 μl of template DNA, 1.25U of *Taq* polymerase (Invitrogen, Carlsbad CA), 200 μM of each deoxynucleotide, 3 mM $MgCl_2$ and 1 μM each of primers in 1x PCR buffer (Invitrogen, Carlsbad CA), and adjusted with RNase-free water to a total volume of 25 μl. The second amplification reaction contained the same concentration of reagents, but only 1 μl of PCR product from the first reaction was included in the mixture. PCR amplification was performed in a Mastercycler Gradient (Eppendorf AG, Hamburg Germany) using the following conditions for the primary PCR: 94°C for 5 minutes, followed by 35 cycles at 94°C for 30 seconds, 55°C for 1 minute and 72°C for 2 minutes with a final extension at 72°C for 7 minutes. Nested PCR conditions were 94°C for 5 minutes, followed by 35 cycles at 94°C for 30 seconds, 52°C for 30 seconds and 72°C for 1 minute with a final extension at 72°C for 7 minutes. PCR products were visualised by gel electrophoresis on 2% agarose gels. Nested PCR products were sequenced using the primer SMIF6 and BigDye terminator chemistry.

2.7.3 Clinical and Strain Characterization Studies (Chapters Five and Six)

Amplification of a region of the *fas* gene of *P. jirovecii* spanning the DHPS binding site was performed as described in 2.7.2. Nested PCR products were directly sequenced using the primers SMIF6: 5'-AGC GCC TAC ACA TAT TAT GG-3' and SMIB7: 5'-CTG CAA CCT CAG AAC G-3' and BigDye terminator chemistry (Applied Biosystems, Foster City, CA). A second PCR reaction,[119] as described in 2.7.1, was performed on those specimens that did not amplify with

the above set of primers. PCR products from specimens containing a mixture of *P. jirovecii* genotypes were ligated into pCR2.1-TOPO® plasmid vectors from the TOPO TA Cloning Kit (Invitrogen, Carlsbad CA), according to the manufacturer's instructions. Nine to ten independent clones per specimen were sequenced in both directions using the M13 primers and BigDye Terminator chemistry (ABI Prism 310 Genetic Analyzer, PE Applied Biosystems, Foster City, CA).

2.8 Definitions of DHPS Genotypes

The *P. jirovecii* wild-type (WT) genotype was defined as a DNA sequence with no substitutions at positions 165 and 171 in the *fas* gene, i.e. a threonine at DHPS amino acid position 55 and a proline at position 57. The mutant genotype M1 was defined as a sequence with a G substitution at position 165, M2 as a T substitution at position 171, and M3 as having substitutions at both positions. The observed mutations were all non-synonymous changes that resulted in amino acid substitutions at amino acid positions 55 and/or 57. Specimens containing a mixture of mutant and wild-type genotypes were classified in the mutant category.

2.9 Analysis of ITS Haplotypes

2.9.1 ITS PCR

Amplification of the ITS1-5.8S-ITS2 region was performed as previously described by Lee *et al* [112] with modifications to reduce the formation of recombinants by Beser *et al* [114] The primary amplification reaction contained 5 µl of template DNA, 1.25U of *Taq* polymerase (Invitrogen, Carlsbad CA), 200 µM of each deoxynucleotide, 3 mM $MgCl_2$ and 0.8 µM each of primers 1724F2 (5'-AGT TGA TCA AAT TTG GTC ATT TAG AG-3') and ITS2R (5'-CTC GGA CGA GGA TCC TCG CC-3') in 1x PCR buffer (Invitrogen, Carlsbad CA) and adjusted with RNase-free water to a total volume of 25 µl. DNA amplification was carried out under the following conditions: 5 min at 94°C; followed by 10 cycles of 1 min at 94°C, 1 min at 60°C, 4.3 min at 72°C; followed by 30 cycles of 1 min at 94°C, 1 min at 60°C, 1.3 min at 72°C; and a final extension of 15 min at

72°C. The second amplification reaction (nested PCR) contained 2 µl of PCR product from the primary reaction, 1.25U of *Taq* polymerase, 200 µM of each deoxynucleotide, 1.5 mM $MgCl_2$, 2 µM each of primers FX (5'-TTC CGT AGG TGA ACC TGC G-3') and RT2 (5'-CTG ATT TGA GAT TAA AAT TCT TG-3') in 1X PCR buffer and adjusted with RNase-free water to a total volume of 25 µl. Secondary amplification was performed under the following conditions: 5 min at 94°C; followed by 10 cycles of 1 min at 94°C, 1 min at 56°C, 4.3 min at 72°C; followed by 30 cycles of 1 min at 94°C, 1 min at 56°C, 1.3 min at 72°C; and a final extension of 15 min at 72°C. The amplified products were separated by gel electrophoresis on 1.2% agarose gels. Negative controls not containing template DNA were included in all reaction batches to monitor for contamination.

2.9.2 Cloning and Sequencing of ITS PCR Products

Nested PCR products were excised from the agarose gels and purified using a QIAquick Gel Extraction kit (Qiagen, Germany) according to the manufacturer's instructions. The PCR products were ligated into pCR2.1-TOPO® plasmid vectors from the TOPO TA Cloning Kit (Invitrogen, Carlsbad CA) according to the manufacturer's instructions. Bacterial cells transformed by vectors not containing the PCR products expressed LacZ α-peptide and formed blue colonies in the presence of X-gal (Invitrogen, Carlsbad CA). Bacterial cells containing the PCR products did not express the LacZ α-peptide and formed white colonies. Screening of white bacterial colonies containing the PCR insert was performed with M13 vector-specific primers in an amplification reaction mix containing 2.5U of *Taq* polymerase, 200 µM of each deoxynucleotide, 3 mM $MgCl_2$, 0.2 ng/µl of each primer, in 1x PCR buffer and adjusted with RNase-free water to a final volume of 20 µl. Bacterial clones were picked directly into the reaction mix and amplified under the following conditions: 5 min at 94°C; followed by 25 cycles of 30 sec at 94°C, 30 sec at 45°C, 1 min at 72°C; and a final extension for 7 min at 72°C. The presence of cloned inserted PCR products was confirmed by gel electrophoresis on 1.2% agarose gels. Between nine to twelve independent clones per specimen were sequenced in both directions using the M13 primers and BigDye Terminator

chemistry (ABI Prism 310 Genetic Analyzer, PE Applied Biosystems, Foster City, CA).

2.9.3 Determination of ITS Haplotypes

The ITS1-5.8S-ITS2 sequences were edited, aligned and analyzed using the BioEdit Sequence Alignment Editor, version 7.0.4.1.[120] A database was constructed with previously reported ITS sequences and study sequences were aligned with these using ClustalW software and the "best fit" was chosen (Lee *et al* [112] GenBank accession numbers AF013806 to AF013834; Hosoya *et al* [121]; Nimri *et al* [122] GenBank accession numbers AF374238 to AF374265; Matos *et al* [123] GenBank accession number AY390601; Nevez *et al* [124] GenBank accession number AF498265; Robberts *et al* [118] GenBank accession numbers AY328043 to AY328078 and AY330724; Totet *et al* [115] GenBank accession numbers AY135711 and AY135712; Siripattanapipong *et al* [116] GenBank accession numbers AY550105 to AY550109; van Hal *et al* [98] GenBank accession numbers EU442879 to EU442882; Beser *et al* [125] GenBank accession number EU527019 and Gupta *et al* [126] GenBank accession numbers GU228517 to GU228519). Novel sequences were compared to published sequences using the NCBI nucleotide BLAST function.[127] *P. jirovecii* ITS haplotypes were assigned according to the classification system of Lee *et al*. [112] The uppercase letter represented the ITS1 type and the lowercase letter represented the ITS2 type. Variations in the long homopolymeric thymine and adenine tracts were ignored in the determination of ITS type, as these have previously been reported as PCR-induced errors.[113] Sporadic single nucleotide changes were excluded from the analysis of sequence variation, as their significance and origin were uncertain. These sporadic changes were presumed to be PCR-generated errors if they occurred only once and in only one clone from a specimen. The anticipated error rate for the ITS1-5.8S-ITS2 region using *Taq* polymerase is known to be higher than that for introns and exons of the *Trr1* gene (2.6 ±1.2 misincorporations/1000 bp, with 12 558 bp sequenced vs 1.5 ±0.4 misincorporations/1000 bp, with 30 600 bp sequenced).[114]

2.9.4 Nucleotide Sequence Accession Numbers

The sequences of the novel ITS1 E variants SA1 and SA2 were deposited in GenBank with these respective accession numbers JN387046 and JN387047.

2.10 Measures to Prevent Contamination of DNA

Different rooms were used for the initial processing and immunostaining of specimens, DNA extraction, handling of template DNA, addition of PCR product to the nested reaction mix, sequencing steps and gel electrophoresis. A separate pre-PCR laboratory was used to prepare all PCR and sequencing master mixes. DNA was handled in a laminar flow cabinet equipped with a UV lamp for decontamination. Positive and negative controls were included with the DNA extraction process and PCR reactions.

2.11 Data Analysis

All clinical and laboratory investigations were performed blind to each other's results. DNA sequences were analysed using BioEdit Sequence Alignment Editor Software version 7.0.9.0. Statistical analyses were performed with Excel and VassarStats Website for Statistical Computations.[128] The Chi square or Fisher exact tests were used to assess significant associations ($P<0.05$) among categorical variables. Continuous variables were compared using the Mann-Whitney test when data were not distributed normally. A P value of less than 0.05 was considered significant and tests were two-tailed.

C H A P T E R T H R E E

A PILOT STUDY TO INVESTIGATE THE PRESENCE OF DHPS POLYMORPHISMS IN SOUTH AFRICAN *P. JIROVECII* STRAINS

Paper I: Published in 2006.[129] Dini, L., du Plessis, M., Wong, M., Karstaedt, A., Fernandez, V. and Frean, J. Prevalence of DHPS polymorphisms associated with sulfa resistance in South African *Pneumocystis jirovecii* strains, *J. Eukaryot. Microbiol.*, vol. 53(S1), 2006, pp. S110-S111 (Appendix E).

3.1 Introduction

Pneumocystis pneumonia (PCP) is a risk to a substantial proportion of the population of South Africa, in a setting of high HIV/AIDS prevalence, where HAART has been to a large extent unavailable.[130] The South African government initiated the provision of HAART to the public sector in April 2004, but prior to that the HIV epidemic was largely untreated. By the end of 2006, HAART coverage was estimated at 21% of those needing antiretroviral therapy.[131] The prevalence of HIV infection among prenatal clinic attendees in South Africa had risen dramatically from 0.7% in 1990 to 30.2% in 2005.[132] In Gauteng Province, the estimated HIV prevalence in the total population was 14.5% in 2006.[133] In 2007, an estimated 5.7 million people were living with HIV infection out of a total population of 48.5 million, and 350 000 deaths were attributed to AIDS during the same year. [131]

An increased prevalence of DHPS point mutations has been associated with the use of trimethoprim-sulfamethoxazole (TMP-SMX), the drug of choice for the treatment and prophylaxis of PCP.[66-67,84,134-136] This awoke concern for possible drug resistance in fungal strains. Prior to 2004 no studies had been conducted to assess the potential presence of DHPS polymorphisms in *P. jirovecii* strains from South Africa. A single study in 2003, based on a small sample size of 14 patients, had reported a prevalence of *P. jirovecii* DHPS mutations in 7% of Zimbabwean

HIV-infected adults with PCP. [100] Prior to 2003, no other studies investigating *P. jirovecii* DHPS polymorphisms had been published from developing countries.

The aim of this pilot study was to investigate whether DHPS polymorphisms were present in *P. jirovecii* organisms in South Africa.

3.2 Results and Discussion

This is one of the first studies from South Africa investigating DHPS mutations in *P. jirovecii* organisms. A total of 79 respiratory specimens from 65 patients was successfully genotyped at the DHPS locus (Table 3). The prevalence of *P. jirovecii* containing DHPS mutations was 42% (27/65). Two other studies from South Africa with smaller sample sizes were published after we had analyzed our results. They reported much lower prevalences of DHPS mutations, 13% and 4% respectively.[101-102] Robberts *et al* [102] detected a single M3 DHPS genotype in *P. jirovecii* from 53 PCP episodes. The study by Zar *et al* [101] enrolled 30 children from one hospital in the Western Cape Province. They found the M1 DHPS genotype in *P. jirovecii* from two children; the M2 genotype in *P. jirovecii* from one child and another child was infected with *P. jirovecii* containing the M3 genotype.

In this pilot study, *P. jirovecii* strains containing single DHPS mutations, either M1 or M2, were detected in 29% (19/65) of patients, and the double mutation M3 was detected in *P. jirovecii* from 5 patients (8%). The most common DHPS mutation was M1, occurring in 18% (12/65) of *P. jirovecii* isolates. All five patients from the Eastern Cape Province were infected with *P. jirovecii* containing wild-type DHPS genotypes. The other three provinces had patients infected with wild-type and mutant *P. jirovecii* strains. Multiple specimens were available for 13 patients and all except one patient had *P. jirovecii* with identical DHPS genotypes. This patient's first specimen initially contained *P. jirovecii* with a wild-type DHPS genotype, and the second specimen taken two days later contained a mixture of wild-type and M1 DHPS genotypes. Fourteen percent (9/65) of specimens contained mixtures of *P. jirovecii* DHPS genotypes.

A limitation of our pilot study was the non-random selection of specimens, and a larger population-based study would be required to confirm the high prevalence of DHPS mutations in *P. jirovecii* organisms from South Africa.

3.3 Conclusions

This is one of the first studies from South Africa investigating DHPS mutations in *P. jirovecii* organisms. The prevalence of DHPS mutant genotypes was high at 42%, and the most common DHPS mutant genotype was M1. *P. jirovecii* strains containing single DHPS mutations were detected in a third of patients, and mixtures of *P. jirovecii* genotypes were found in 14% of patient specimens. Based on the results of this pilot study, a decision was taken to proceed with a large prevalence study investigating DHPS polymorphisms in the general *P. jirovecii* population (Chapter Four), as well as a clinical study to ascertain their potential significance in patients with PCP (Chapter Five).

Table 3 *P. jirovecii* DHPS genotypes in respiratory specimens from South African patients

DHPS genotype	Nucleotide at position (amino acid)		Proportion of patients (%) n = 65
	165 (55)	171 (57)	
Wild-type (WT)	A (Thr)	C (105)	38/65 (58)
Mutant genotype, total			27/65 (42)
Single mutation: position 165			12/65 (18)
M1 only	G (37)	C (105)	7/65 (11)
M1 + WT	G/A (Ala/Thr)	C (105)	5/65 (8)
Single mutation: position 171			7/65 (11)
M2 only	A (Thr)	T (Ser)	6/65 (9)
M2 + WT	A (Thr)	T/C (Ser/Pro)	1/65 (2)
Double mutation: positions 165 + 171 (M3)	G (37)	T (Ser)	5/65 (8)
Mixed genotypes[a]	G/A (Ala/Thr)	T/C (Ser/Pro)	3/65 (5)

[a] Mixed infections in which the presence of double DHPS mutants and wild-type and/or a mixture of single mutants could not be resolved

C H A P T E R F O U R

THE PREVALENCE OF *P. JIROVECII* DHPS MUTATIONS IN SOUTH AFRICA

Paper II: Published in 2010.[137] Dini, L., du Plessis, M., Frean, J. and Fernandez, V. High prevalence of dihydropteroate synthase mutations in *Pneumocystis jirovecii* isolated from patients with *Pneumocystis* pneumonia in South Africa, *J. Clin. Microbiol.*, vol. 48 no. 6, June 2010, pp. 2016-2021 (Appendix F).

4.1 Introduction

The prevalence of *P. jirovecii* DHPS mutations reported from countries in the developed world ranges widely, from 4% to 81%.[67,86-87,90,92,95,98,138] The few studies performed in developing countries all report low prevalences of *P. jirovecii* containing DHPS mutations.[100,105-109] In South Africa, a handful of studies that screened specimens from limited numbers of adults and children have reported mostly low DHPS mutation rates.[101-103] However, the results of the pilot study (Chapter Three), where 42% of specimens contained *P. jirovecii* with DHPS mutations, suggested that the prevalence of these mutations in the general population could be high.

The aim of this large laboratory-based study was to ascertain the prevalence in South Africa of *P. jirovecii* strains in the population harbouring mutations at positions 165 and 171 in the *fas* gene.

4.2 Results

During the study period, from January 2006 through February 2007, 932 respiratory specimens from 712 patients (93% >15 years of age) with suspected PCP were consecutively received for microbiological confirmation of *P. jirovecii* at the NICD in Johannesburg, South Africa. Immunofluorescence (IF) microscopy was positive for *P. jirovecii* in specimens from 168 (24%) patients. Table 4.1

provides the demographic details of the patients screened for *P. jirovecii* infection using IF microscopy. *P. jirovecii* DNA was detected in all of the IF-positive specimens from 150 patients that were available for assessment of fungal load using quantitative real-time PCR (qPCR). The quantity of *P. jirovecii* DNA measured in the first IF-positive specimen from each of these 150 patients is shown in Table 4.2. Microscopy was deemed equivocal for specimens from 14 patients, and 10 of these yielded positive results with qPCR. Specimens from 530 patients were IF-negative for *P. jirovecii*. Quantitative PCR was performed on a subset of IF-negative specimens from 191 patients that were arbitrarily chosen throughout the year. *P. jirovecii* DNA was detected and quantified in IF-negative specimens from 32 (17%) of these patients.

Amplification of the DHPS gene of *P. jirovecii* was attempted in 201 specimens from 162 patients with a qPCR result of ≥20 target gene copies/μL of extracted DNA. The DHPS locus was successfully genotyped in a total of 183 specimens from 151 patients. Point mutations resulting in amino-acid substitutions Thr55Ala and/or Pro57Ser were detected in *P. jirovecii* from 85 (56%) patients (Table 4.3). Of these, 36 patients (42%) harboured *P. jirovecii* with either mutation Thr55Ala (19 patients, 22%) or Pro57Ser (17 patients, 20%). In specimens from 18 patients (21%) the presence of *P. jirovecii* organisms with both mutations in the DHPS gene could be ascertained. Specimens from 31 patients (36%) contained mixtures of mutant *P. jirovecii* DHPS genotypes, both with and without wild-type, which could not be resolved with direct sequencing. Infections with more than one DHPS genotype were detected in 69% (59/85) of patients harbouring mutant *P. jirovecii*. Multiple respiratory specimens collected during the same PCP episode were available from 23 patients and the majority (17/23, 74%) presented identical DHPS genotypes. There were no significant differences in the frequency of wild-type or mutant DHPS genotypes between genders ($P = 0.39$), age groups ($P = 0.38$) or patients from distinct geographical locations ($P = 0.1$).

4.3 Discussion

The primary aim of this study was to assess the prevalence in South Africa of *P. jirovecii* strains harbouring DHPS mutations previously shown to be associated with exposure to sulfa or sulfone drugs. The number of screened specimens and patients makes this study the largest of its kind conducted to date in the developing world. We found that 56% of PCP patients were infected with mutant *P. jirovecii* strains. This figure stands in contrast to the lower frequencies reported from the African continent and other countries outside of the industrialized world.[100-103,105-107,109] The risk of *P. jirovecii* developing DHPS mutations is higher for PCP patients receiving sulfa prophylaxis than for those not receiving it.[66-67,84-87] A general assumption is that the occurrence of DHPS mutations in developing countries is low due to a less extensive use of sulfa prophylaxis against PCP. Thus, the high frequency of DHPS mutations among PCP patients in South Africa would be indicative of a considerable exposure to sulfa medication, used either prophylactically against PCP or for treatment of other infections. We regard it as unlikely that sulfa (usually TMP-SMX) prophylaxis for PCP alone accounts for the high prevalence of mutant *P. jirovecii* observed in the present investigation. Although information regarding patient HIV status and use of prophylaxis was not available for this laboratory-based study, it is well recognized that a large proportion of HIV-positive patients in South Africa are either unaware of their HIV infection when they present with PCP or have limited access to prophylaxis. A large clinical study at a public hospital in Johannesburg showed that 58% of adult patients presenting with PCP were newly diagnosed with HIV, and only 16% of patients with a previously-confirmed HIV infection were receiving TMP-SMX prophylaxis (see Chapter Five). Furthermore, in a survey of South African primary level health care services for HIV-positive children, two-thirds of public sector clinics did not have a policy in place for the administration of PCP prophylaxis.[139]

The widespread use of sulfa drugs, particularly TMP-SMX, against diseases other than PCP could be contributing to the selection of *P. jirovecii* strains carrying mutations in the DHPS gene. In South Africa, guidelines issued by the

Department of Health have recommended the use of TMP-SMX to treat urinary tract infections, cholera, and acute otitis media at the primary care level, as well as invasive bacterial infections caused by *Shigella* and *Salmonella* species in hospitalised patients.[99] Indications for HIV-positive individuals, apart from PCP prophylaxis and treatment, include prophylaxis against recurrent bacterial infections in children, prophylaxis against bacterial pneumonia, bacteraemia and isosporiasis in adults, as well as prophylaxis and treatment of toxoplasmosis. A 2002 report revealed that 26% of primary health care clinics also administered TMP-SMX for acute respiratory and gastrointestinal tract infections in children,[139] a finding in line with the generalized perception that sulfa-based drugs are often used in primary care as empiric treatment for a variety of conditions including diarrhoea and non-specific respiratory infections in adults and children. Extensive usage of TMP-SMX is known to exert considerable selective pressure on microbial populations and lead to the development of resistance.[140-141] That this is the situation in Gauteng Province and other localities of South Africa is also suggested by the very high rates of TMP-SMX resistance consistently observed in surveillance isolates of pneumococcal and other bacterial species; for example, rates of TMP-SMX resistance in isolates of *Streptococcus pneumoniae*, *Shigella* spp and non-typhoidal salmonellae of over 50%, 83% and 37% respectively, have been reported.[142] Sulfadoxine-pyrimethamine (Fansidar) was used as first-line treatment for malaria in South Africa until 2006; while the number of patients treated (between 1990 and 2006, an average of around 25 000 malaria cases per year) was relatively small in comparison to the burden of HIV-infected individuals, this may have contributed to selective pressure.

The transmission of *P. jirovecii* strains is another factor that may influence the high prevalence of mutant DHPS strains observed in the present investigation. Airborne transmission of *Pneumocystis* spp. from host to host has been demonstrated in rodent models and numerous observations, including several cases of nosocomial clustering, suggest that direct or indirect transmission occurs in humans.[51] Evidence for *de novo* acquisition of *P. jirovecii* with mutant DHPS

genotypes has been provided by studies including patients who had never received sulfa prophylaxis for PCP.[65-66] In one of these studies, more than half of the patients newly diagnosed with HIV infection had mutant *P. jirovecii*.[66] It is possible that inter-human transmission of *P. jirovecii* and concomitant circulation of DHPS genotypes in the population is accelerated in localities of South Africa with high HIV prevalence and large numbers of individuals with some degree of immunosuppression, from subclinical stages to overt AIDS, who are susceptible to colonization by the fungus. It is also possible that mutant strains have an inherent fitness that enables enhanced transmission. Widespread empiric use of TMP-SMX and increased transmission and circulation of strains/genotypes in the population could, at least in part, explain the high prevalence of mutant *P. jirovecii* in settings with limited implementation of PCP prophylaxis.

Lack of clinical surveillance, unavailability of chest radiographic facilities and limited laboratory diagnosis contribute to the scarcity and disparity of information regarding burden of disease and epidemiology of PCP in African settings. In South Africa, collection of bronchoalveolar lavage (BAL) specimens is rare. The few specimens sent for laboratory confirmation are mostly sputa and these are often spontaneously expectorated rather than induced with hypertonic saline.[143-144] Although collection procedure was not specified for the majority of sputum specimen referrals in this study, we estimate that a high proportion were spontaneously expectorated. Clinically, it is accepted that BAL and induced sputum specimens are superior to spontaneously expectorated sputum for the microscopic diagnosis of PCP, in part, because many patients with PCP have a non-productive cough and cannot produce an adequate specimen without undergoing sputum induction. Whilst proper collection of the respiratory sample is of major importance for optimal laboratory diagnosis of PCP, it may also be crucial for the strain/genotype representativity of the specimen and the accurate assessment of mixed-genotype infections. In five out of a total of six cases with discrepant DHPS genotypes, we observed large differences (≥10-fold) in the quantity of *P. jirovecii* DNA measured in specimens from the same PCP episode showing non-identical DHPS genotypes. In some of these cases multiple samples

had been collected within 24 hours or less. This suggests that variable specimen quality, likewise initiation of PCP therapy, may be a cause of inconsistent results in the genotypic analysis of *P. jirovecii* infections.

In South Africa, PCP has been studied more extensively in children and it is now recognised as a common pathogen in HIV-infected infants.[42] In the present study, which predominantly includes specimens from adults, *P. jirovecii* infection was confirmed by IF microscopy in 24% of patients with suspected PCP. By quantitative real-time PCR analysis we could confirm *P. jirovecii* carriage in an additional 17% of patients with clinical PCP suspicion and negative IF microscopy. Detection of *P. jirovecii* was also verified by real-time PCR in specimens from 10 of 14 patients with equivocal microscopy results. Quantitative real-time PCR performed as a single-round amplification in a closed system offers the advantages of increased diagnostic sensitivity and rapidity with a reduced risk of contamination. Quantification of the level of *P. jirovecii* infection or colonization can be useful for monitoring therapy and the improved management of patients.[145] Although increased diagnostic sensitivity carries a higher risk of clinically-false positives, it enhances the chances for early detection of biologically-true positives in individuals with no or incipient clinical manifestations, something that could be of critical importance in localities with high HIV incidence.

This study has certain limitations. Data regarding HIV status, use of sulfa prophylaxis or other medication, and outcome of the patients were not available. These shortcomings, that preclude further analysis of possible underlying associations, expose the type of constraints that are common for laboratory-based studies. However, they do not have any bearing on the central issue of assessing the frequency of DHPS genotypes in *P. jirovecii* strains prevalent in the population. Although the study included patients from a large number of hospitals in South Africa, 57% of the patients with data on *P. jirovecii* DHPS genotypes came from two hospitals in Gauteng Province.

The increased risk of carrying mutant *P. jirovecii* DHPS genotypes in patients receiving sulfa prophylaxis suggests that mutations in the *fas* gene provide a selective advantage so that subpopulations of the fungus may eventually overcome the effects of prophylactic doses of TMP-SMX. The clinical significance of DHPS mutations and in particular whether single polymorphisms or a double mutation have differential effects on response to higher therapeutic doses are questions not yet resolved (see Chapter 5). The appearance of *P. jirovecii* strains with additional mutations and significantly higher levels of tolerance to sulfonamides and/or sulfones is a possibility that cannot be excluded. Examples of cumulative mutations leading to increased antibiotic resistance have been documented for viruses, bacteria and parasitic protozoa.

4.4 Conclusions

The number of screened specimens and patients makes this study the largest of its kind conducted to date in the developing world. *P. jirovecii* infection was confirmed by IF microscopy in 24% of patients with suspected PCP. Colonization by the fungus, characterized by IF-negative microscopy and low gene copy numbers in the real-time PCR, occurred in 17% of patients. The prevalence of infections with *P. jirovecii* containing DHPS mutations in adult South African patients is high at 56%. The results of this prevalence study confirmed the findings of the pilot study in Chapter Three, which reported a prevalence of 42% for *P. jirovecii* DHPS mutations. The frequencies of the different mutant DHPS genotypes were similar. *P. jirovecii* containing single DHPS mutations was found in 42% of patients and strains containing the double mutant M3 genotype were found in 21% of patients. Infections with more than one DHPS genotype were detected in 14% of patients. There were no significant differences in the proportions of wild-type or mutant *P. jirovecii* DHPS genotypes between genders, age groups or patients from distinct geographical locations. Widespread empiric use of TMP-SMX, as well as increased transmission and circulation of mutant DHPS strains/genotypes in the population could account for the high prevalence of mutant *P. jirovecii* in settings with limited implementation of PCP prophylaxis. It was therefore of great importance to proceed with a study to investigate the

potential clinical significance of these mutations in patients infected with this fungus (Chapter Five).

Table 4.1 Comparison of patient demographic data between IF-positive and IF-negative specimens, South Africa

Variables	Patients[a] (n = 712)	*P. jirovecii* immunofluorescence (IF) microscopy result	
		IF-positive (n = 168)	IF-negative (n = 544)
Mean age ±SD (range)	34 ±13 (1m–86y)	33 ±10 (3m–66y)	34 ±13 (1m–86y)
Female:male	1.6:1	1.8:1	1.6:1
Province:			
Gauteng	491	123	368
Eastern Cape	149	28	121
Mpumalanga	53	15	38
Free State	12	2	10
Limpopo	4	0	4
Northern Cape	3	0	3

[a] 93% of patients >15 years of age

m month, y year

Table 4.2 Quantification of *P. jirovecii* DNA in respiratory specimens from patients with suspected *Pneumocystis* pneumonia

Specimens	Patients	Real-time PCR-positive	*P. jirovecii* DNA quantity Median (range)[a]
Sputum			
IF-positive	140	140	3 062 (8 - 291 894)
IF-negative[b]	173	30	7 (1 - 499)
IF equivocal[c]	12	10	151 (9 - 1 549)
Tracheal aspirate			
IF-positive	7	7	1 921 (10 – 180 498)
IF-negative	4	1	1
IF equivocal	1	0	
Bronchoalveolar lavage			
IF-positive	2	2	54 766 (3 – 109 529)
IF-negative	3	0	
Other[d]			
IF-positive	1	1	299
IF-negative	11	1	4
IF equivocal	1	0	

IF immunofluorescence microscopy test

[a] Expressed as target gene copies/µL of extracted DNA. Detailed information regarding handling of specimens and DNA extraction conditions is provided in the methods section.

[b] A set of 205 specimens from 191 patients were arbitrarily chosen for qPCR analysis from the 530 patients with IF-negative results.

[c] Immunofluorescence microscopy was suggestive of *P. jirovecii*, but not conclusive.

[d] Includes 2 bronchial washings, 2 nasopharyngeal aspirates, 1 pleural fluid and 8 samples with unknown specimen types.

Table 4.3 *P. jirovecii* DHPS genotypes in respiratory specimens from South African patients

DHPS genotype	Nucleotide at position (amino acid)		No. patients (%) n = 151
	165 (55)	171 (57)	
Wild-type (WT)	A (Thr)	C (105)	66/151 (44)
Mutant genotype, total			85/151 (56)
Single mutation			36/85 (42)
Position 165 (M1)	G (37)	C (105)	4/85 (5)
M1 + WT	G/A (Ala/Thr)	C (105)	15/85 (18)
Position 171 (M2)	A (Thr)	T (Ser)	7/85 (8)
M2 + WT	A (Thr)	T/C (Ser/Pro)	10/85 (12)
Double mutation			18/85 (21)
Positions 165 + 171 (M3)	G (37)	T (Ser)	15/85 (18)
M3 + M1	G (37)	T/C (Ser/Pro)	1/85 (1)
M3 + M2	G/A (Ala/Thr)	T (Ser)	2/85 (2)
Mixed genotypes[a]	G/A (Ala/Thr)	T/C (Ser/Pro)	31/85 (36)

[a] Mixed infections in which the presence of double mutants and wild-type and/or a mixture of single mutants could not be resolved.

C H A P T E R F I V E

OUTCOME OF HIV-POSITIVE SOUTH AFRICAN PATIENTS WITH PNEUMONIA CAUSED BY *P. JIROVECII* WITH AND WITHOUT DHPS GENE MUTATIONS

Paper III: Manuscript in preparation. Dini, L., Wong, M., Frean, J., du Plessis, M., and Karstaedt, A. Effect of *Pneumocystis jirovecii* dihydropteroate synthase gene mutations on outcome of South African patients with HIV: a prospective study.

5.1 Introduction

After establishing that the prevalence of *P. jirovecii* DHPS mutations corresponding to the DHPS binding site was high (56%) in South Africa (Chapter Four), a large prospective clinical study was launched to investigate the significance of these polymorphisms in patients suffering from PCP. The clinical significance of *P. jirovecii* DHPS mutations is not fully understood and results are conflicting. One study [67] found a significantly lower three-month survival in patients harbouring mutant *P. jirovecii*, whilst two other studies [84,93] found no significant association between patient survival and DHPS genotype at four and six weeks respectively. Huang *et al* [90] found an insignificant trend for patients harbouring mutant *P. jirovecii* DHPS to have an increased mortality, whilst Nahimana *et al* [86] had similar in-hospital rates of death for patients with or without mutant *P. jirovecii*. Crothers *et al* [87] found that mortality was not associated with the presence of DHPS mutations. The majority of clinical studies found an association between sulfa prophylaxis and the presence of DHPS mutations.[66-67,84,86-87,90-91] Whilst two studies, with low numbers of patients infected with mutant *P. jirovecii*, reported no association with sulfa prophylaxis and mutant DHPS genotype. [92,96]

The aim of this large, prospective, clinical study was to ascertain the clinical significance of *P. jirovecii* strains harbouring mutations at positions 165 and 171 in the *fas* gene.

5.2 Results

From March 2005 through June 2009, 306 respiratory specimens from 266 adult patients with clinically and/or radiologically suspected PCP were screened for *P. jirovecii* infection. All patients were confirmed HIV-positive or were clinically strongly suspected of AIDS. Immunofluorescence (IF) microscopy was positive for *P. jirovecii* in specimens from 150 patients (56%). Microscopy was deemed equivocal for specimens from 11 patients; four of which yielded positive results with quantitative real-time PCR (qPCR). *P. jirovecii* DNA was detected by quantitative real-time PCR in specimens from 189 patients (71%), and the median fungal load was 837 target gene copies/µL of extracted DNA (range: 2-704 257).

Prevalence of DHPS genotypes

The DHPS locus was successfully genotyped in specimens from 161 patients (85%). DHPS amplification was not always successful in specimens with fungal loads of less than 170 target gene copies/µL of extracted DNA. Point mutations resulting in amino acid substitutions Thr55Ala and/or Pro57Ser were detected in *P. jirovecii* strains from 98 patients (61%) (Table 5.1). Of these, 57 patients (58%) harboured *P. jirovecii* with single DHPS mutations. The frequency of mutation M1 was 26% (25/98) and M2 was 56% (55/98). The double mutation M3 was present in specimens from 41 patients (42%). *P. jirovecii* infections with more than one DHPS genotype were detected in 47% (75/161) of patients. Specimens containing multiple ambiguous genotypes were cloned and sequenced to resolve the mixtures. Specimens containing mixtures of wild-type and mutant DHPS strains were classified into the mutant category. The proportion of mutant DHPS genotypes stayed relatively stable over the study period, except in 2007 where it dropped to 44% (Figure 5).

Multiple respiratory specimens collected during the same PCP episode were available from 10 patients and six (60%) presented with identical DHPS genotypes (Table 5.2, patients 1 to 10). No genotype switching occurred in the other four patients, but an additional genotype was detected in each case. In two patients the additional DHPS genotype was detected in specimens with a higher fungal load, suggesting that the additional genotype was present at a lower frequency than the original genotypes. DHPS sequences from patient 2 were cloned and sequenced and the additional genotype M2 occurred at a lower frequency than the original genotypes. One patient (patient 11 in Table 5.2) was admitted twice for PCP, with episodes separated by six months. This patient initially presented with a mixture of wild-type, M1 and M2 genotypes, which were resolved by cloning and sequencing of PCR products. During the second admission an M3 genotype was detected in addition to the wild-type genotype.

Association between DHPS mutations and sulfa exposure

Patient demographics, clinical characteristics and outcome are provided in Table 5.3. According to patient interview, only five patients had a previous history of PCP and 26 patients (16%) were exposed to sulfa in the four months prior to admission. There were no significant associations between the presence of DHPS mutations and previous PCP ($P = 0.37$) or exposure to sulfa-based medication ($P = 0.34$). In our study 57 of the 98 patients (58%), who were newly diagnosed with HIV on admission, harboured *P. jirovecii* with mutant DHPS genotypes.

Outcome of patients infected with PCP according to DHPS genotype

Significantly more patients infected with mutant *P. jirovecii* DHPS genotypes died in hospital compared with patients infected with wild-type DHPS genotypes (26/98, 27% versus 8/63, 13%, $P = 0.04$) (Table 5.3). The overall in-hospital mortality was 21% (34/161). The median stay in hospital was 10 days and was similar for patients in the DHPS mutant and wild-type groups. There were no significant associations between DHPS genotype and patient gender, age, $CD4^+$ count, diagnosis of HIV on admission, pneumothorax, need for mechanical ventilation, or respiratory failure. When examining the different mutant DHPS

genotypes, there were no significant associations in patients harbouring *P. jirovecii* with single DHPS mutations (genotypes M1 and M2) compared with those infected with the double mutant M3 genotype (Table 5.4). However, there was an insignificant trend for patients infected with the M3 genotype to have a lower median $CD4^+$ cell count compared to patients harbouring strains with single mutations ($P = 0.06$). In-hospital mortality was similar for patients infected with *P. jirovecii* containing single DHPS mutant genotypes compared with double mutant genotypes (18/57, 32% versus 8/41, 20%).

Patients that survived (79%) were either discharged home well, referred to a TB treatment facility or to a step-down clinic for further treatment. A telephonic and/or postal follow-up was conducted to assess the mortality of patients at three months after discharge, and the response rate was 70% (89/127). When assessing the mortality of only the discharged patients at three months, significantly more patients died of wild-type *P. jirovecii* strains than those infected with mutant strains (9/63, 14% versus 4/98, 4%, $P = 0.02$). The overall mortality rate of the discharged patients at three months was 15% (13/89). When combining in-hospital patient mortality and mortality at three months after discharge, the mortality rate was 38% (47/123) and there were no significant associations with a DHPS genotype ($P = 0.82$), nor with a particular mutant genotype ($P = 0.12$).

5.3 Discussion

This study is unique in the developing world as it was conducted prospectively and has a large sample size, especially in the *P. jirovecii* mutant DHPS category. The primary aim of this study was to assess the clinical significance in South Africa of *P. jirovecii* strains harbouring DHPS mutations previously shown to be associated with exposure to sulfonamide drugs. The majority of patients (61%) harboured mutant *P. jirovecii* strains, a result which is consistent with the prevalence of 56% reported from our previous study [137](Chapter Four). The proportion of mutant DHPS genotypes stayed relatively stable over the study period. The most frequent DHPS mutant genotype was M2, which has a single point mutation at nucleotide position 171 in the *fas* gene, followed by the double

mutant genotype M3, which has single nucleotide point mutations at positions 165 and 171. Multiple *P. jirovecii* infections were found in 47% of patients.

The in-hospital mortality was higher than the mortality of the discharged patients at 3 months. Significantly more patients died in hospital of *P. jirovecii* containing mutant DHPS genotypes than those infected with wild-type strains ($P = 0.04$). Our study is one of the few to show an association between mutant DHPS genotypes and increased in-hospital mortality. A study from Italy with a low prevalence of mutant genotypes also showed a correlation between DHPS mutants and worse outcomes.[92] Crothers *et al* [87] found that patients infected with mutant *P. jirovecii* strains tended to have worse outcomes than those infected with wild-type strains. Helweg-Larsen *et al* [67] found that the presence of DHPS mutations was the most important predictor of mortality.

Interestingly, mortality at three months among the discharged patients was associated with the wild-type DHPS genotype. This result could not have been affected by the relative proportions of DHPS genotypes in the discharged patient group, as more patients were infected with mutant *P. jirovecii* than wild-type (61% versus 39%). However, 30% of discharged patients were lost to follow up and their results may have an effect on the mortality figure. The prevalence of mutant *P. jirovecii* in these patients compared with wild-type was 18% versus 32%. It was not possible to ascertain if the patients died of *P. jirovecii* infection within the three months after discharge. An autopsy study from South Africa showed that one third of patients with *P. jirovecii* infections were co-infected another pathogen.[40] Co-infections with other pathogens contribute to the morbidity and mortality of patients with PCP.

When combining in-hospital mortality and mortality at three months after discharge, there were no significant associations with a particular DHPS genotype. These results appear to be conflicting, but assessing if a patient's outcome is due entirely to the presence or absence of DHPS mutations is very difficult. The assessment of a patient's outcome is complex, because many factors

can affect outcome, such as co-infections with other pathogens, underlying health conditions, severity of disease, and adherence to treatment. Crothers *et al* [87] suggested that mortality in most HIV-infected patients with PCP was related primarily to the underlying severity of illness and the initial severity of PCP, even though they found a trend towards worse outcomes in patients with mutant *P. jirovecii*.

There were no significant associations between the presence of DHPS mutant genotypes and previous PCP or exposure to sulfa medication. However, very few patients (3%) reported a previous PCP episode and only 16% of patients had been exposed to any sulfa-based medication in the four months prior to admission, according to patient recall. One patient who had a previous PCP episode developed an M3 (double mutant) genotype during his second PCP episode six months later. Our data support the hypothesis that *P. jirovecii* strains with mutant DHPS genotypes may be transmitted from person to person, as 58% of the patients diagnosed with HIV on admission harboured *P. jirovecii* with mutant DHPS genotypes. Several studies have reported patients harbouring *P. jirovecii* strains with mutant genotypes, who had not been exposed to sulfa drug prophylaxis.[65-66,91,96]

This study has certain limitations. Some patients could not provide clear information as to their use of sulfa-based medication prior to admission. Patient adherence to treatment could not easily be monitored. The patient outcome results at three months post-discharge may be affected by the fact that 30% of patients (38/127) were lost to follow-up.

5.4 Conclusions

This study is unique in the developing world as it was conducted prospectively and has a large sample size, especially in the mutant DHPS category.

5.4.1 Prevalence of DHPS Genotypes

The majority of patients (61%) harboured mutant *P. jirovecii* strains, a result which is consistent with the 56% reported from the prevalence study (Chapter Four). The proportion of mutant DHPS genotypes stayed relatively stable over the study period. Multiple *P. jirovecii* infections were found in 47% of patients. The most frequent DHPS mutant genotype among patient specimens was M2 followed by M3.

5.4.2 Inter-human Transmission

Few patients were exposed to sulfonamides prior to their admission, and almost 60% of the patients diagnosed with HIV on admission harboured *P. jirovecii* with mutant DHPS genotypes. Inter-human transmission of *P. jirovecii* carrying mutant DHPS genotypes could, at least in part, explain the high prevalence of DHPS mutations in adult HIV-positive South Africans.

5.4.3 Clinical Significance of DHPS Mutant Genotypes

The in-hospital mortality was higher than the mortality of the discharged patients at three months. Significantly more patients died in hospital of *P. jirovecii* containing mutant DHPS genotypes than those infected with wild-type strains ($P = 0.04$). Our study is one of the few to show an association between mutant DHPS genotypes and increased in-hospital mortality. Mortality at three months among the discharged patients was associated with the wild-type DHPS genotype. However, cause of death in these patients was unknown. There were no significant associations in patients harbouring *P. jirovecii* with single DHPS mutations (genotypes M1 and M2) compared with those infected with the double mutant M3 genotype. However, there was an insignificant trend for patients infected with the M3 genotype to have a lower median $CD4^+$ cell count compared to patients harbouring strains with single mutations ($P = 0.06$).

The next chapter (Chapter Six) focuses on a subgroup of patients from this clinical study and characterizes the *P. jirovecii* strains in these patients based on ITS typing.

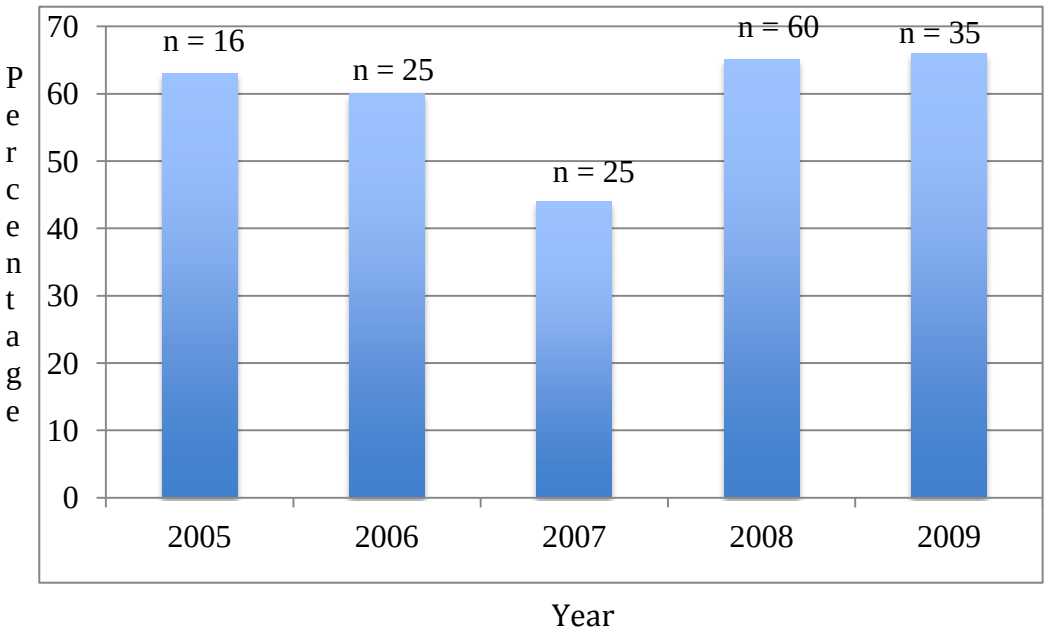

Figure 5 The proportion of mutant DHPS genotypes per year in 161 patients infected with *Pneumocystis jirovecii* (n = total number of patients)

Table 5.1 *P. jirovecii* DHPS genotypes in respiratory specimens from 161 South African patients

DHPS genotype	Nucleotide at position (amino acid)		No. patients (%) n = 161
	165 (55)	171 (57)	
Wild-type (WT)	A (Thr)	C [105]	63/161 (39%)
Mutant genotype, total			98/161 (61%)
Single mutation: position 165			15/98 (15)
M1 only	G [37]	C [105]	0
M1 + WT	G/A (Ala/Thr)	C [105]	15/98 (15)
Single mutation: position 171			39/98 (40)
M2 only	A (Thr)	T (Ser)	11/98 (11)
M2 + WT	A (Thr)	T/C (Ser/Pro)	28/98 (29)
Single mutations: positions 165 + 171			3/98 [140]
M1 + M2 + WT[a]	G/A (Ala/Thr)	T/C (Ser/Pro)	3/98 [140]
Double mutation: positions 165 + 171			41/98 (42)
M3 only	G [37]	T (Ser)	12/98 (12)
M3 + WT[a]	G/A (Ala/Thr)	T/C (Ser/Pro)	12/98 (12)
M3 + M1	G [37]	T/C (Ser/Pro)	2/98 (2)
M3 + M1 + WT[a]	G/A (Ala/Thr)	T/C (Ser/Pro)	2/98 (2)
M3 + M2	G/A (Ala/Thr)	T (Ser)	5/98 (5)
M3 + M2 + WT[a]	G/A (Ala/Thr)	T/C (Ser/Pro)	5/98 (5)
M3 + M1 + M2[a]	G/A (Ala/Thr)	T/C (Ser/Pro)	2/98 (2)
M3 + M1 + M2 + WT[a]	G/A (Ala/Thr)	T/C (Ser/Pro)	1/98 (1)

[a] Mixed genotypes were resolved by cloning and sequencing

WT wild-type, M1 mutation 1, M2 mutation 2, M3 mutation 3

Table 5.2 Comparison of DHPS genotypes in 11 patients with multiple specimens

Patient no.	Specimen	Sampling interval	Fungal load[a]	DHPS genotype	Cloning result[b]
1	1	2 days	33 825	WT	
	2		156	WT	
2	1	2 days	12 270	WT+M3+M2	WTx7, M3x2, M2x1
	2		192 794	WT+M3	WTx8, M3x2
3	1	1 day	2 566	M2	
	2		388	M2+WT	
4	1	1 day	585	WT	
	2		475	WT	
5	1	2 days	873	M3+M2	
	2		45	M3	
6	1	3 days	134	M2+WT	
	2		649	M2+WT	
7	1	2 days	202	WT	
	2		1 085	WT	
8	1	3 days	826	M1+WT	
	2		939	M1+WT	
9	1	2 days	401	WT	
	2		1 705	WT+M2	
10	1	2 days	396	WT	
	2		1 714	WT	
11	1	6 months	8 568	M2+WT+M1	M2x6, WTx3, M1x1
	2		83 596	WT+M3	WTx6, M3x3

[a] Expressed as target gene copies/µL of extracted DNA

[b] Specimens containing mixtures of DHPS genotypes were resolved by cloning and sequencing

Table 5.3 Demographic, clinical characteristics and outcome of 161 PCP patients according to DHPS genotypes

Characteristic	DHPS genotype		*P* value
	Mutant[a] (n = 98)	Wild-type (n = 63)	
Gender: Female, No. of patients (%)	78 (80%)	42 (67%)	0.07[c]
Mean age, years ±SD (range)	36 ±8 (20-58)	34 ±9 (19-56)	0.10[e]
Median $CD4^+$ cell count, cells/mm^3 (range)	18 (1-226)	26 (1-470)	0.20[e]
Newly diagnosed with HIV on admission, No. of patients (%)	57 (58%)	41 (65%)	0.38[c]
History of previous PCP, No. of patients (%)	4 (4%)	1 (2%)	0.65[d]
Sulfa/sulfone exposure[b], No. of patients (%)	18 (18%)	8 (13%)	0.34[c]
Median hospital stay, days (range)	10 (2-42)	10 (3-34)	0.48[e]
Development of pneumothorax, No. of patients (%)	4 (4%)	2 (3%)	1.0[d]
Mechanical ventilation necessary, No. of patients (%)	4 (4%)	2 (3%)	1.0[d]
Respiratory failure, No. of patients (%)	87 (89%)	55 (87%)	0.78[c]
Outcome:			
Death in hospital, No. of patients (%)	26 (27%)	8 (13%)	0.04[c]
Discharged, No. of patients (%)	72 (73%)	55 (87%)	
Discharged, alive after 3 months, No. of patients (%)	50 (51%)	26 (41%)	0.02[c]
Discharged, death within 3 months, No. of patients (%)	4 (4%)	9 (14%)	
3-month outcome unknown, No. of patients (%)	18 (18%)	20 (32%)	

[a] Specimens containing mixtures of wild-type plus mutant DHPS genotype were classified into the mutant category after cloning and sequencing.

[b] Use of any sulfa-based medication in the 4 months prior to admission.

[c] Chi square test, [d] Fisher exact test, [e] Mann-Whitney test, considered significant at the 5% level if $P<0.05$

Table 5.4 Demographic, clinical characteristics and outcome of 98 PCP patients according to mutant DHPS genotype

Characteristic	Single mutation M1 and/or M2 (n = 57)	Double[a] mutation M3 (n = 41)	*P* value
Gender: Female, No. of patients (%)	44 (77%)	34 (83%)	0.49[c]
Mean age, years ±SD (range)	37±8 (21-58)	35±8 (20-54)	0.16[e]
Median $CD4^+$ cell count, cells/mm^3 (range)	20 (3-184)	12 (1-226)	0.06[e]
Newly diagnosed with HIV on admission, No. of patients (%)	37 (65%)	20 (49%)	0.11[c]
History of previous PCP, No. of patients (%)	1 (2%)	3 (7%)	0.30[d]
Sulfa/sulfone exposure[b], No. of patients (%)	9 (16%)	7 (17%)	0.87[c]
Median hospital stay, days (range)	10 (2-26)	9 (2-42)	0.47[e]
Development of pneumothorax, No. of patients (%)	3 (5%)	1 (2%)	0.64[d]
Mechanical ventilation necessary, No. of patients (%)	3 (5%)	1 (2%)	0.64[d]
Respiratory failure, No. of patients (%)	49 (86%)	38 (93%)	0.35[d]
Outcome:			
Death in hospital, No. of patients (%)	18 (32%)	8 (20%)	
Discharged, No. of patients (%)	39 (68%)	33 (80%)	0.18[c]
Discharged, alive after 3 months, No. of patients (%)	25 (44%)	22 (54%)	
Discharged, death within 3 months, No. of patients (%)	4 (7%)	1 (3%)	0.37[d]
Three-month outcome unknown, No. of patients (%)	10 (18%)	10 (24%)	

[a] Specimens containing mixtures of M3+M1/M2 were classified into the M3 category.

[b] Use of any sulfa-based medication in the 4 months prior to admission

[c] Chi square test, [d] Fisher exact test, [e] Mann-Whitney test, considered significant at the 5% level if $P<0.05$

C H A P T E R SIX

STRAIN CHARACTERIZATION OF *P. JIROVECII* BASED ON ITS TYPING

Paper IV: Manuscript in the final stage before submission. Dini, L., Beser, J. and Wong, M. Characterization of *Pneumocystis jirovecii* strains from South Africa based on nucleotide sequence variations in the internal transcribed spacer regions of rRNA genes.

6.1 Introduction

The aim of this component of the research was to characterize the *P. jirovecii* strains present in respiratory specimens from a subset of patients enrolled in the prospective clinical study described in Chapter Five. A secondary aim was to investigate possible correlations between different strains and a particular clinical feature, DHPS genotype or patient outcome. Characterization of *P. jirovecii* strains in this study was performed by analysis of sequence variation in the internal transcribed spacer (ITS) regions in the nuclear rRNA gene complex. The ITS regions show great diversity among *Pneumocystis* strains and provide an informative epidemiological tool for the typing of these strains.[110]

6.2 Results

The ITS1-5.8S-ITS2 region was successfully amplified, cloned and sequenced in specimens from 40 HIV-positive patients. A total of 11 different *bona fide* ITS haplotypes were detected; six ITS1 types and nine ITS2 types, all of which had been previously described. The majority of patients (85%, 34/40) were infected with *P. jirovecii* possessing an Eg ITS haplotype, either singly or in combination with another ITS haplotype (Table 6.1). Eg was present as a single infection in 43% (17/40) of patients. The frequency of multiple ITS haplotype infections in patients was 48% (19/40), which made cloning of PCR products prior to sequencing necessary. At least ten clones per specimen were sequenced in both

directions. The second most common ITS haplotype was Eu, which was found in 13% (5/40) of patients, and occurred as a single infection in 3% (1/40) of them. The Eu haplotype has previously been reported in specimens from South African patients at a frequency of 16%.[118] Three ITS haplotypes, Em, Ec, and Eb, were each found in 10% of patients, and both Ec and Eb occurred as single infections in 3% of patients. Three ITS haplotypes, Bi, Gg and Ep each occurred in 8% (3/40) of patients, but only Gg was found as a single infection in 3% of patients. The ITS haplotype Fu_4, previously reported by Hosoya *et al* [121] in patients from Japan, occurred in 5% (2/40) of patients and was not found as a single infection. The ITS haplotypes Ne and Ai were present in 3% (1/40) of patients and did not occur as single infections. The haplotypes Ei, Au, Eu_4, Fe, Ee, Ng and Cg were considered to be possible recombinant or artifactual haplotypes. This assumption was based on the low frequency of these haplotypes, their unusual association with an ITS1 or ITS2 type, the lack of single infections, and their consistent presence as mixed infections with other accepted more common haplotypes. The generation of artifactual haplotypes by the actual PCR method was proven by Beser *et al*.[114]

Two novel ITS1 sequences, SA1 and SA2, were detected in patient specimens (Table 6.1) and no exact matches were found when the sequences were blasted against published sequences in the NCBI database. Both SA1 and SA2 showed most similarity to ITS1 type E and both were combined with a g in the ITS2 type. Figures 6.2 and 6.3 show SA2 and SA1 aligned with ITS1 type E respectively. SA2 had an AT deletion at position 113 and a T insertion at position 145 in Figure 6.2, and SA1 had an A insertion at position 17 in Figure 6.3. Sporadic single polymorphisms were ignored, as well as variable lengths of homopolymeric thymine tracts at nucleotide positions 66-67. These were presumed to be PCR-generated errors. According to Lee's [112] definition, SA1 and SA2 were not considered as new distinct ITS1 types, as they were not found in two or more specimens. SA1 was identified in eight clones of *P. jirovecii* isolated from a single patient and the other four clones were Eg. SA2 was identified from ten clones of *P. jirovecii* isolated from another patient and the other two clones were Gg.

Table 6.2 describes the *P. jirovecii* ITS haplotypes and their corresponding DHPS genotype that occurred in specimens from each of the 40 study patients. The maximum number of combined ITS haplotypes in a single patient was five, and this particular patient had clones with recombinants that could have arisen from a hidden Ne haplotype (see patient 13 in Table 6.2). Point mutations resulting in amino-acid substitutions Thr55Ala and/or Pro57Ser were detected in *P. jirovecii* strains from 24 patients (60%). *P. jirovecii* with single DHPS mutations were found in 19 patients (48%). The frequency of mutation M1 was 8% (3/40) and M2 was 40% (16/40). The double mutation M3 was present in specimens from 11 patients (28%). Infections with more than one DHPS genotype were detected in 40% (16/40) of patients. Specimens containing mixtures of wild-type and mutant DHPS strains were classified into the mutant category.

Thirty patients had specimens with either a single ITS haplotype or a single DHPS genotype, enabling an analysis of potential associations between them. The prevalence of DHPS genotypes in these 30 patients was 73% wild-type, 27% M2 single mutation, 20% M3 double mutation and 3% M1 single mutation. When comparing ITS haplotype with DHPS genotype, three combinations had a higher than expected prevalence (50%) of mutant genotypes (Table 6.3): Eb with M2, Bi with M3, and Gg with M3.

Patient demographics, clinical characteristics and outcome are provided in Table 6.4. Only 21 patients (53%) were infected with *P. jirovecii* of a single ITS haplotype. There were no obvious associations between ITS haplotype and a particular clinical characteristic or outcome.

6.3 Discussion

The *P. jirovecii* strains present in respiratory specimens from a subset of patients enrolled in the prospective clinical study were characterized by ITS typing. A total of 11 different *bona fide* ITS haplotypes, six ITS1 and nine ITS2 types, were found in *P. jirovecii* isolated from 40 HIV-positive patients in South Africa. As

with the majority of studies from other countries, the global ITS haplotype Eg was most prevalent among *P. jirovecii* infecting HIV-positive patients in South Africa. This haplotype occurred at a high frequency of 85% in the South African isolates, and is the highest reported frequency of Eg to date. This study confirmed the presence of the local South African haplotype Eu. Two ITS haplotypes, Fu_4 and Ep, that had previously only been reported in Japan were also found in South Africa.[121,146] The ITS2 type p was first described in Thailand, where it was linked with the ITS1 types I and R.[116] The frequency of Ne in the South African samples was extremely low and is in keeping with an earlier study from South Africa.[118] The ITS haplotype Ea, previously reported to occur in South Africa was absent in specimens from this study. The two novel ITS1 sequences SA1 and SA2 are likely variations of type E. It remains to be seen if these variants are novel ITS1 types and if their occurrence is local or global.

There were no obvious associations between ITS haplotype and a particular clinical characteristic or patient outcome. However, the groups of patients with a single ITS haplotype infection were often too small to perform statistical analyses. Helweg-Larsen *et al*, 2001 found no association of ITS haplotypes with clinical severity of PCP in 130 patients.[117]

The prevalence of mutant DHPS *P. jirovecii* strains among this group of patients was 60%, which is consistent with the prevalence of 56% reported from our previous study.[137] The most frequent DHPS mutant genotype was M2, which has a single point mutation at nucleotide position 171 in the *fas* gene. Genotype M2 had a higher than expected prevalence in specimens with the *P. jirovecii* ITS haplotype Eb, suggesting a possible association between them. In addition, the double mutant M3, had a higher than expected prevalence in specimens with the ITS haplotypes Bi and Gg. Despite the fact that the vast majority (85%) of specimens contained *P. jirovecii* with an Eg haplotype and 60% of specimens contained a mutant DHPS genotype, Eg strains were more often associated with a wild-type DHPS genotype. This was also the case for the ITS haplotypes Eu, Ec,

Fu_4 and Ep. Meshnick *et al*, 2001 found that Ne, Eg and Ee isolates were more likely to contain DHPS mutations than other ITS haplotypes.[147]

Sporadic single nucleotide changes were presumed to be PCR-generated errors if they occurred only once and in only one clone from a specimen. By ignoring these sporadic nucleotide additions, deletions and substitutions in the cloned sequences, novel ITS types may have been overlooked in this study. However, a strict approach to ITS classification was applied in order to minimize further complications in the field of ITS typing. Minor base changes were ignored in a clone if most of the clones from the specimen were 100 percent similar to a common, accepted ITS type. The usual associations between ITS1 and ITS2 types were considered during the analysis. In addition, the frequencies of previously reported ITS haplotypes were taken into consideration, with less frequent haplotypes being more likely to be recombinants. The occurence of PCR-generated recombinants was minimized by using the modifications suggested by Beser *et al* .[114] The primer concentration was increased two-fold in the first PCR reaction and five-fold in the second PCR reaction, and the time for the elongation step was increased during PCR cycling. We have chosen to report all ITS type combinations, but have marked those that could be artifacts, either PCR-generated or naturally occuring in the population.

The frequency of multiple ITS haplotype infections in patients from this study was 48%, which made cloning of PCR products prior to sequencing essential. Some studies of ITS typing have employed only direct sequencing of PCR products without cloning, despite the fact that Lu *et al* [110] warned that this will not work for specimens with mixed ITS haplotypes. Direct sequencing of ITS PCR products can be performed on specimens containing unambiguous sequences i.e. those with a single *P. jirovecii* ITS haplotype. However, when specimens contain more than one type of *P. jirovecii*, a mixture of sequences are obtained and interpretation of ITS haplotypes is hampered. Thus cloning of PCR products prior to sequencing is required. By sequencing multiple clones it is possible to

detect multiple infections within the same specimen, as well as strains present in lower concentrations.

6.4 Conclusions

A total of 11 different *bona fide* ITS haplotypes, six ITS1 and nine ITS2 types, were found in *P. jirovecii* isolated from 40 HIV-positive patients. There were no obvious associations between ITS haplotype and a particular clinical characteristic or outcome. Almost half of the patients were infected with more than one *P. jirovecii* strain. Eg occurred at a high frequency of 85% among *P. jirovecii* infecting patients. The presence of the local South African haplotype Eu was confirmed. Other ITS haplotypes detected were: Em, Ec, Eb, Bi, Gg, Ep, Fu_4, Ne and Ai. Two novel ITS1 sequences SA1 and SA2 were detected and are likely variations of type E. Eg haplotypes were more often associated with a wild-type DHPS genotype. The following ITS haplotypes had possible associations with a particular DHPS genotype: Eb with M2, Bi with M3, and Gg with M3.

More studies from South Africa and the African continent are needed to characterize and understand the molecular epidemiology of *P. jirovecii*. Specimens from non-HIV immunocompromised patients with PCP should be included in future investigations. A single, standardized ITS typing system is required with simple names for the different ITS haplotypes. An alternative typing system for *Pneumocystis* using multilocus genotyping has shown promise and may be cheaper and less labour-intensive than ITS typing by cloning and sequencing.

Table 6.1 Frequency of *P. jirovecii* ITS haplotypes isolated from 40 HIV-positive patients with PCP in South Africa

ITS haplotype	No. of patients (%) n = 40	No. of patients with a single ITS haplotype infection (%)
Eg	34 (85)	17 (43)
Eu	5 (13)	1 [140]
Em	4 (10)	0
Ec	4 (10)	1 [140]
Eb	4 (10)	1 [140]
Bi	3 (8)	0
Gg	3 (8)	1 [140]
Ep	3 (8)	0
Fu_4	2 (5)	0
Ei^1	2 (5)	0
Ne	1 [140]	0
Ai	1 [140]	0
$SA1^bg$	1 [140]	0
$SA2^bg$	1 [140]	0
Au^a	1 [140]	0
$Eu_4{}^a$	1 [140]	0
Fe^a	1 [140]	0
Ee^a	1 [140]	0
Ng^a	1 [140]	0
Cg^a	1 [140]	0

[a] Possible artifactual haplotype

[b] Novel ITS1 E variants, GenBank accession numbers JN387046 and JN387047

ITS2 type u reported by Robberts *et al*,[118] GenBank accession number AY328054

ITS2 type u_4 reported by Hosoya *et al* [121]

ITS2 type p GenBank accession number AF013836

Table 6.2 Description of *P. jirovecii* ITS haplotypes and corresponding DHPS genotype in specimens from 40 HIV-positive patients with PCP

Patient	ITS haplotype (no. of clones)	DHPS genotype[a]
1	Eb (11); Eg (1)	M2 + WT
2	Eg (11)	WT
3	Ai (6); Eg $^{(140)}$; Eu (2); Au[b] (1)	WT
4	Eg (11)	M3 + WT
5	SA1[d]g (8); Eg (4)	WT
6	Eg (12)	WT
7	Eg (5); Ec (2); Bi (2)	M2 + WT
8	Fu_4 (6); Ec $^{(140)}$; Eu_4[b] $^{(140)}$	WT
9	Eg (11)	M2 + WT
10	Eg (8); Ec (2); Em (1); Eu (1)	M3 + WT
11	Eg (8); Eu $^{(140)}$	M3 + M2 + WT
12	Eg (10)	M2
13	Eb (5); Fu_4 $^{(140)}$; Eg (2); Fe[c] (1); Ee[c] (1)	M2 + WT
14	Em (10); Eg (2)	M3 + M1
15	Ec (11)	M2 + WT
16	Eg (12)	WT
17	Gg (12)	M3
18	Eg (8); Em (4)	WT
19	Eg (10)	M2
20	Eg (8); Ne (1); Ep (1); Ng[b] (2)	M2 + WT
21	Eb (8); Ep $^{(140)}$; Eg (1)	M2 + M3 + WT
22	Eg (10)	M2 + M3 + WT
23	Eg (12)	WT
24	Eg (9); Eu $^{(140)}$	WT
25	Eg (9); Gg $^{(140)}$	WT
26	SA2[d]g (10); Gg (2)	M2 + WT
27	Eg (12)	M2
28	Eu (12)	M2
29	Eg (6); Bi (1); Cg[b] (1); Ei[b] $^{(140)}$	WT
30	Eg (12)	M3
31	Eg (10); Em (1)	M1 +M2 + M3
32	Eb (12)	M2 + WT
33	Eg (12)	WT
34	Eg (12)	WT
35	Bi (8); Eg (2); Ei[b] (2)	M3
36	Eg (11)	WT
37	Eg (9); Ep (1)	WT
38	Eg (12)	M1 + WT
39	Eg (12)	M3
40	Eg (12)	WT

[a] Mixtures of DHPS genotypes were resolved by cloning and sequencing
[b] Possible artifactual haplotype
[c] possible recombinant with a hidden Ne
[d] novel ITS1 E variants, GenBank accession numbers JN387046 and JN387047

Table 6.3 Prevalence of DHPS genotype with corresponding ITS haplotype in specimens from 30 HIV-positive patients with PCP

ITS haplotype + DHPS genotype	DHPS genotype prevalence (%)
Eg + WT	26 (70)
Eg + M2	5 (14)[a]
Eg + M3	5 (14)[a]
Eg + M1	1 (140)
Eu + WT	2 (67)
Eu + M2	1 (33)
Eb + WT	1 (50)[a]
Eb + M2	1 (50)[b]
Ec + WT	2 (67)
Ec + M2	1 (33)
Em + WT	1 (100)
Bi + WT	1 (50)[a]
Bi + M3	1 (50)[b]
Gg + WT	1 (50)[a]
Gg + M3	1 (50)[b]
Ep + WT	1 (100)
Fu_4 + WT	1 (100)
Ai + WT	1 (100)
SA1[c]g + WT	1 (100)

[a] Lower than expected prevalence of DHPS genotype. Expected genotype prevalence: WT 73%, M2 27%, M3 20%, M1 3%

[b] Higher than expected prevalence of DHPS genotype

[c] Novel ITS1 E variant, GenBank accession number JN387046

Table 6.4 Patient demographics and clinical details of 40 HIV-positive patients according to ITS haplotypes

Characteristic	All	Single ITS haplotype infections				
		Eg	Eu	Ec	Eb	Gg
Number of patients (%)	40	17 (43)	1 [(140)]	1 [(140)]	1 [(140)]	1 [(140)]
Gender: Female, No. of patients (%)	32 (80)	14 (82)	1	1	1	1
Mean age, years ±SD (range)	34±10 (20-56)	36±11 (20-56)	40	56	30	29
Median $CD4^+$ cell count, cells/mm^3 (range)	35 (2-175)	27 (2-106)	5	U	29	44
Newly diagnosed with HIV on admission, No. of patients (%)	26 (65)	10 (59)	1	U	0	0
History of previous PCP, No. of patients (%)	3 (8)	1 (6)	0	0	0	1
Median hospital stay, days (range)	10 (2-38)	10 (2-38)	22	5	8	18
Mechanical ventilation necessary, No. of patients (%)	2 (5)	1 (6)	0	0	0	0
Respiratory failure, No. of patients (%)	33 (83)	14 (82)	1	1	0	1
Outcome:						
Death in hospital, No. of patients (%)	10 (25)	5 (29)	1	1	0	0
Discharged, No. of patients (%)	30 (75)	12 (71)	0	0	1	1
Discharged, alive after 3 months, No. of patients (%)	14 (47)	3 (25)			1	1
Discharged, death within 3 months, No. of patients (%)	3 (10)	3 (25)			0	0
3-month outcome unknown, No. of patients (%)	13 (43)	6 (50)			0	0

U unknown

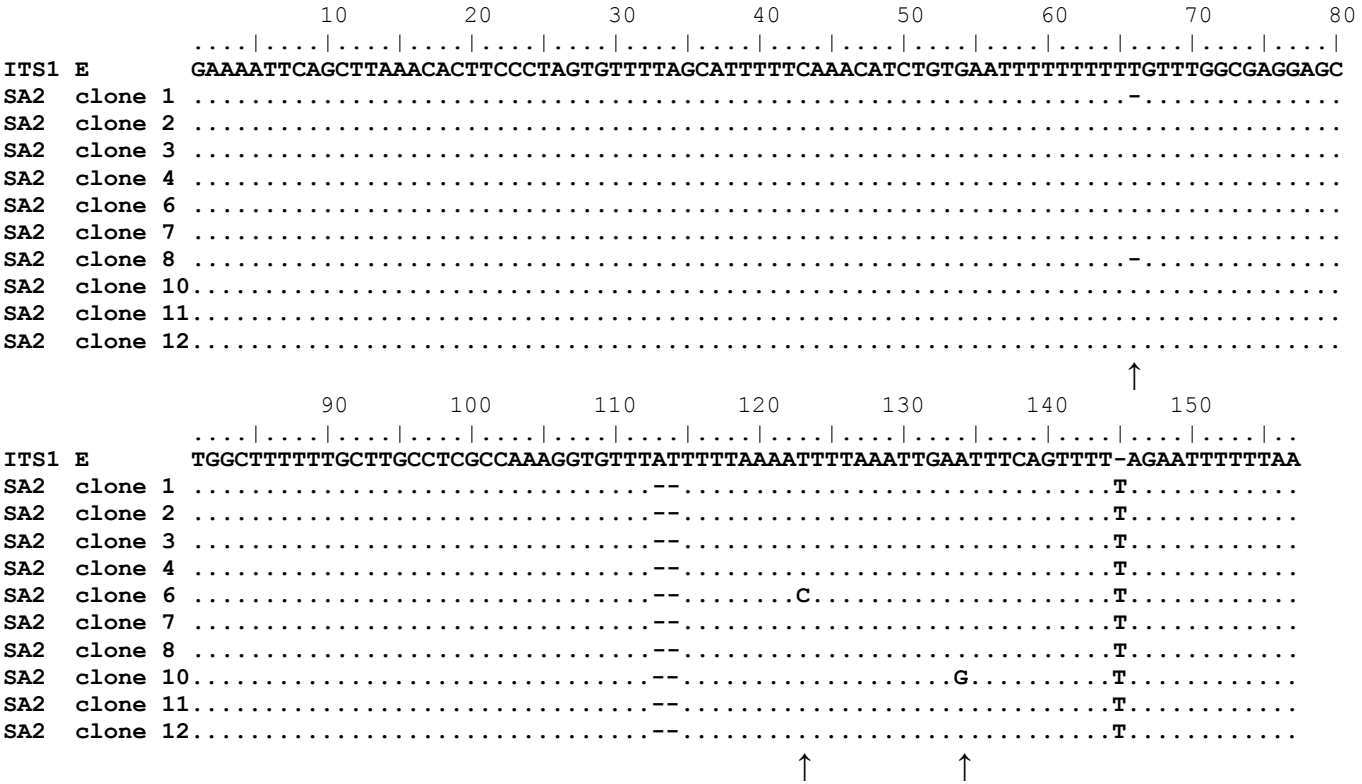

Figure 6.2 The novel ITS1 sequence SA2 identified in ten clones of *P. jirovecii* isolated from a single PCP patient in South Africa. The sequence of SA2 is shown aligned with ITS1 type E, which exhibited the closest homology among established ITS1 types. Sequence E (AF013810) was originally described by Lee *et al* [112]

. nucleotide identical to E sequence

- gaps in sequences, ↑ sporadic polymorphisms presumed to be PCR-generated errors

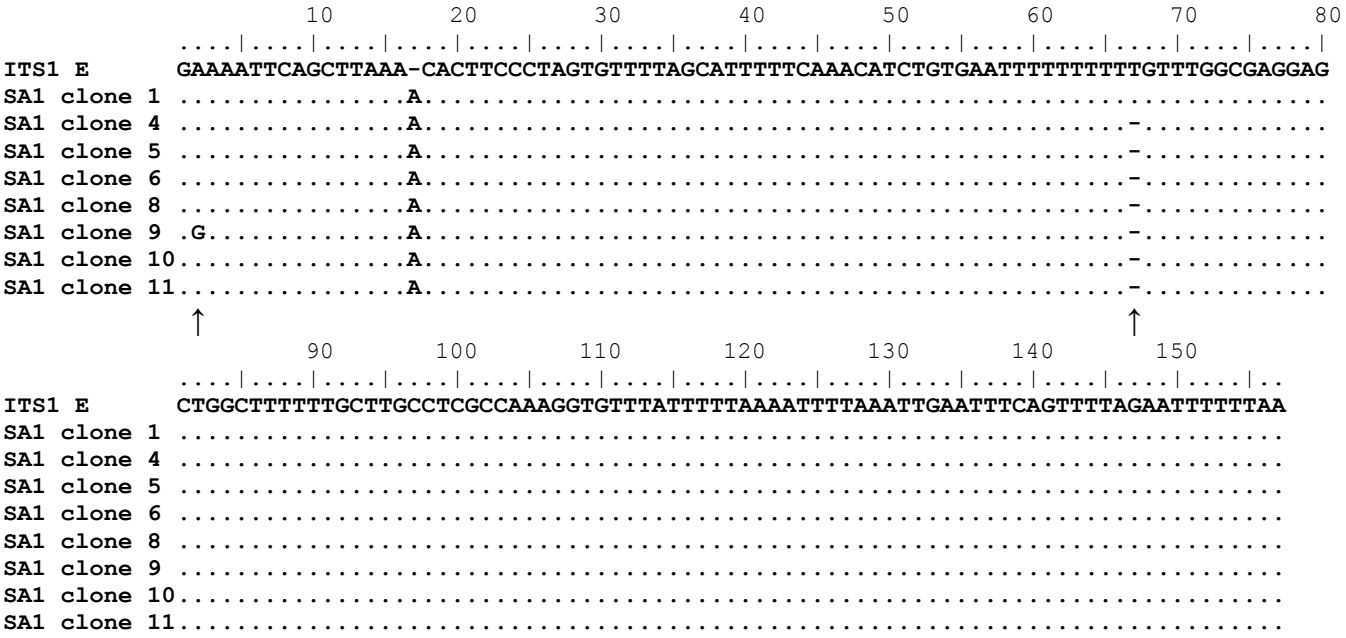

Figure 6.3 The novel ITS1 sequence SA1 identified in eight clones of *P. jirovecii* isolated from a single PCP patient in South Africa. The sequence of SA1 is shown aligned with ITS1 type E, which exhibited the closest homology among established ITS1 types. Sequence E (AF013810) was originally described by Lee *et al* [(112)]

. nucleotide identical to E sequence

- gaps in sequences, ↑ sporadic polymorphisms presumed to be PCR-generated errors

C H A P T E R S E V E N

GENERAL DISCUSSION AND CONCLUSIONS

The purpose of this research was to investigate the prevalence of *P. jirovecii* strains containing DHPS mutations in South Africa, and to ascertain their clinical significance in HIV-positive patients with *Pneumocystis* pneumonia. The molecular epidemiology of *P. jirovecii* was explored by characterizing the strains circulating in HIV-infected patients.

7.1 Prevalence of DHPS Polymorphisms in South Africa

An increased prevalence of *P. jirovecii* with point mutations in the *fas* gene, which codes for the DHPS enzyme, has been associated with the use of sulfonamides. This awoke concern for possible drug resistance in fungal strains. The pilot study, which non-randomly screened *P. jirovecii*-positive specimens from 65 patients from four different provinces in South Africa, confirmed the presence of DHPS polymorphisms in 42% of these specimens (Chapter Three). This is one of the first studies from South Africa investigating DHPS mutations in *P. jirovecii* organisms. Two other studies from South Africa with smaller sample sizes were published after the pilot study results were analyzed. They reported much lower prevalences of DHPS mutations, 13% and 4% respectively.[101-102]

Based on the results of the pilot study (Chapter Three), a decision was taken to proceed with a large prevalence study investigating DHPS polymorphisms in the general *P. jirovecii* population (Chapter Four), as well as a clinical study to ascertain their potential significance in patients with PCP (Chapter Five). The prevalence study was conducted over one year, and the number of screened specimens and patients (n = 712) makes this study the largest of its kind conducted to date in the developing world. *P. jirovecii* infection was confirmed by IF microscopy in 24% of patients with suspected PCP. Colonization by the fungus, characterized by IF-negative microscopy and low gene copy numbers in

the real-time PCR, occurred in 17% of patients. The prevalence of infections with *P. jirovecii* containing DHPS mutations in adult South African patients was high at 56%, and these results confirmed the findings of the pilot study. There were no significant differences in the proportions of wild-type or mutant DHPS genotypes between genders, age groups or patients from distinct geographical locations. Studies from the USA generally report the highest prevalences of *P. jirovecii* DHPS mutations, whilst European countries have much lower prevalences (Figure 1.5). The few studies performed in developing countries have all reported low prevalences of *P. jirovecii* containing DHPS mutations (Table 1.3).

The clinical study is unique in the developing world as it was conducted prospectively and had a large sample size, especially in the mutant DHPS category (Chapter Five). The majority of patients (61%) harboured mutant *P. jirovecii* strains, a result which is consistent with the 56% reported from the prevalence study. The proportion of mutant DHPS genotypes stayed relatively stable over the study period. Multiple *P. jirovecii* infections were found in 47% of patients, compared with 21% in the prevalence study. Figure 7.1 illustrates a comparison of the prevalence of *P. jirovecii* DHPS mutations from the pilot, prevalence and clinical studies.

A comparison of the proportions of *P. jirovecii* DHPS mutant genotypes from the pilot, prevalence and clinical studies is presented in Figure 7.2. M1 was the most common mutant DHPS genotype in the pilot study, whilst the different mutant genotypes had similar frequencies in the prevalence study. The M2 genotype was most frequent in the clinical study, followed by the M3 genotype. *P. jirovecii* strains containing single DHPS mutations, either M1 or M2, were present at similar frequencies in all three studies at an average of 29%. The double mutant M3 genotype was most prevalent in *P. jirovecii* infecting clinical study patients (25% clinical study versus 12% prevalence study versus 8% pilot study). The median frequency of the M3 genotype from all three studies was 12%. *P. jirovecii* infections with more than one DHPS genotype were detected in 47% of patients from the clinical study, 21% from the prevalence study and 14% from the pilot

study.These apparent differences in the frequencies of DHPS mutant genotypes could be explained by different sample populations with local variations in genotype frequencies. The prevalence study had a large sample size from hospitals in six provinces, whilst the clinical study was conducted at a single large hospital in Gauteng Province. Another factor that could affect the distribution of genotypes is the lack of resolution of mixed genotypes in 36% of specimens from the prevalence study. Specimens from the clinical study with mixed ambiguous genotypes were cloned and sequenced to resolve the individual genotypes.

7.2 Clinical Significance of DHPS polymorphisms

The in-hospital mortality was higher than the mortality of the discharged patients at 3 months (21% versus 15%). Significantly more patients died in hospital of *P. jirovecii* containing mutant DHPS genotypes than those infected with wild-type strains (26% versus 8%, $P = 0.04$). Our study is one of the few to show an association between mutant DHPS genotypes and increased in-hospital mortality. A study from Italy with a low prevalence of mutant genotypes also showed a correlation between DHPS mutants and worse outcomes.[92] Crothers *et al* [87] found a trend for patients with mutant strains to have worse outcomes than those with wild-type *P. jirovecii*. Helweg-Larsen *et al* [67] found that the presence of DHPS mutations was the most important predictor of mortality.

Mortality at 3 months among the discharged patients was associated with the wild-type DHPS genotype. However, cause of death in these patients was unknown. There were no significant associations in patients harbouring *P. jirovecii* with single DHPS mutations (genotypes M1 and M2) compared with those infected with the double mutant M3 genotype. However, there was an insignificant trend for patients infected with the M3 genotype to have a lower median $CD4^+$ cell count compared to patients harbouring strains with single mutations ($P = 0.06$).

There were no significant associations between the presence of DHPS mutant genotypes and previous PCP or exposure to sulfa medication. However, very few

patients reported a previous PCP episode and only 16% of patients had been exposed to any sulfa-based medication in the 4 months prior to admission. Almost 60% of the patients diagnosed with HIV on admission harboured *P. jirovecii* with mutant DHPS genotypes. Inter-human transmission of *P. jirovecii* carrying mutant DHPS genotypes could, at least in part, explain the high prevalence of DHPS mutations in adult HIV-positive South Africans.

7.3 Strain Characterization of *P. jirovecii*

The *P. jirovecii* strains present in respiratory specimens from a subset of patients enrolled in the prospective clinical study were characterized by ITS typing.
A total of 11 different *bona fide* ITS haplotypes, six ITS1 and nine ITS2 types, were found in *P. jirovecii* isolated from 40 HIV-positive patients. There were no obvious associations between ITS haplotype and a particular clinical characteristic or outcome. Almost half of the patients were infected with more than one *P. jirovecii* strain. The global ITS haplotype Eg occurred at a high frequency of 85% among *P. jirovecii* infecting patients. The presence of the local South African haplotype Eu was confirmed, and it was the second most common ITS haplotype. Other ITS haplotypes detected were: Em, Ec, Eb, Bi, Gg, Ep, Fu_4, Ne and Ai. Fu_4 and Ep had previously only been reported in Japan. Two novel ITS1 sequences SA1 and SA2 were detected and are likely variations of type E.

The prevalence of mutant DHPS *P. jirovecii* strains among this group of patients was 60%, and the most frequent DHPS mutant genotype was M2. The Eg haplotype was most often associated with a wild-type DHPS genotype. The following ITS haplotypes had possible associations with a particular DHPS genotype: Eb with M2, Bi with M3, and Gg with M3.

7.4 Concluding Remarks

The prevalence of *P. jirovecii* strains containing DHPS polymorphisms among adult HIV-positive South Africans is high. A third of patients were infected with *P. jirovecii* strains containing single DHPS mutations and 12% contained the double mutant M3 genotype. Inter-human transmission of *P. jirovecii* carrying mutant DHPS genotypes could explain, at least in part, this high prevalence.

Significantly more patients died in hospital of *P. jirovecii* containing mutant DHPS genotypes than those infected with wild-type strains. This finding has potentially grave consequences in the light of the high prevalence of DHPS mutations in the general *P. jirovecii* population.

A limited number of *P. jirovecii* strains, characterized by ITS typing, defines the diversity in South Africa. More studies from South Africa and the African continent are needed to characterize and understand the molecular epidemiology of *P. jirovecii*. Specimens from non-HIV immunocompromised patients with PCP should be included in future investigations.

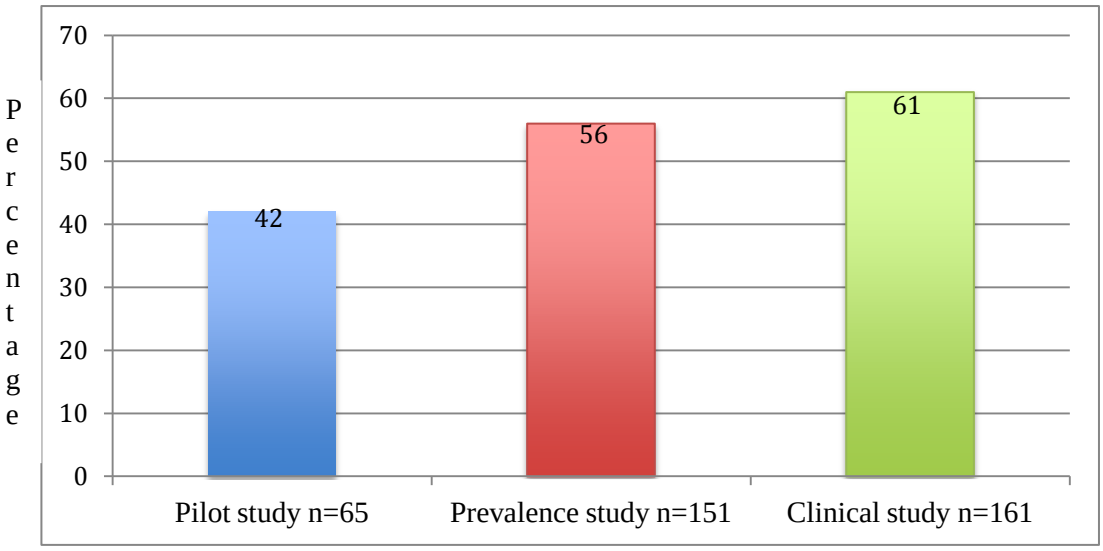

Figure 7.1 A comparison of the prevalence of *P. jirovecii* DHPS mutations from the pilot, prevalence and clinical studies (Chapters Three, Four and Five)

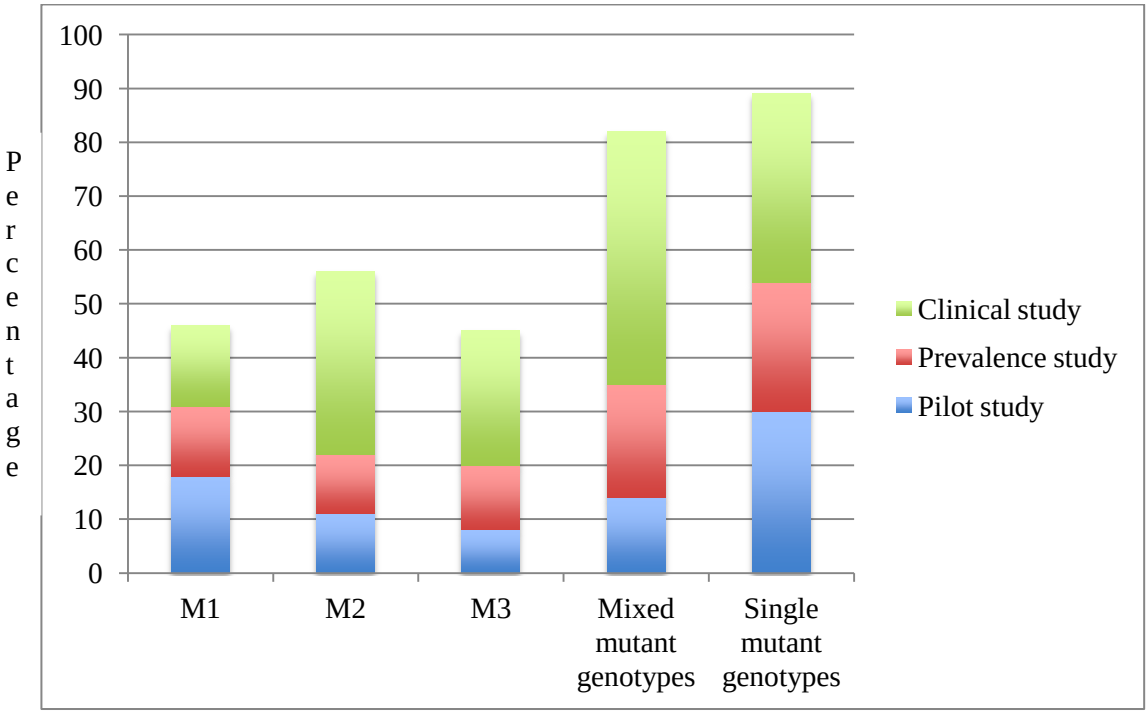

Figure 7.2 A comparison of the proportion of *P. jirovecii* mutant DHPS genotypes from the pilot, prevalence and clinical studies (Chapters Three, Four and Five)

8. APPENDICES A TO H

APPENDIX A

PCP Study, Clinician's Clinical Case Report Form

STUDY NO: _____

HIV status: pos □ neg □ declined to be tested □
Date of first positive HIV test: _____

Previous PCP: no □ yes □ If yes, date: _____
Previous pneumonia or RTI: no □ yes □ If yes, date: _____
Bactrim prophylaxis in last 3 mo: no □ yes □ If yes, dose: _____

HAART: no □ yes □ If yes, date started: _____
Drugs and dose:
□ d4T/stavudine/Zerit _____
□ 3TC/lamivudine_____
□ efavirenz/Stocrin _____
□ nevirapine/Viramune _____
□ zidovudine/AZT _____
□ ddI/Videx _____
□ lopinavir + ritonavir/Kaletra _____

Previous TB: no □ yes □ If yes, when? _____
Previous medical/surgical history: _____
_____
_____
_____

Medication in last 3 mo:_____

_____
_____

Smoker: no □ yes □

SYMPTOMS
Dyspnoea: no □ yes □ If yes, duration: _____

Cough: no □ yes □ Productive □ Non-productive □ Duration: _____

Fever: no □ yes □ If yes, duration: _____

Chest pain: no □ yes □ Right □ Left □ Duration: _____

STUDY NO: _____

EXAMINATION:

Temp: _____

BP: _____

Pulse: _____

RR: _____

Oral thrush : □ yes □ no

Chest auscultation:

_____

_____

Other significant clinical findings :

_____

_____

_____

_____

_____

_____

STUDY NO: _____

INVESTIGATIONS

Blood investigations:

	Date	Result
FBC		
U&E		
CD4		

ABG: Date: _____ FiO_2 _____
pH _____ HCO_3 _____
pCO_2 _____ BE _____
pO_2 _____ sat _____

CXR: Date: _____ No. _____

_____

_____

Sputum:

	Date	Result
MC&S		
TB auramine		
TB culture		

STUDY NO: _____

Investigations for PCP:
e.g. sputum, induced sputum, bronchial washings, BAL, transbronchial biopsy:

Specimen	Date	Result

If induced sputum was performed: by wall O_2 □ by portable nebulizer □

TREATMENT

O_2: 24% □ 28% □ 35% □ 40% □ 60% □ polymask □

Co-trimoxazole:

Route	Dose	Start date	End date

Steroids:

Route	Dose	Start date	End date

Other drugs:

Route	Dose	Start date	End date

DAILY PROGRESS

Day	Date	Temp	BP	Pulse	RR	O_2 sat on room air	O_2 sat on oxygen (%)

COMPLICATIONS

Respiratory failure: no □ yes □

Pneumothorax: no □ Yes □ Spontaneous □ Iatrogenic □
Right □ Left □ Date: _____
Rx: O_2 □ Heimlich valve □ IC drain □

Sulpha allergy: no □ yes □

Other:

_____

_____

_____

_____

OUTCOME

Discharged □ Date: _____ Follow-up appointment: _____

Died □ Date: _____

Excluded □ Date: _____

APPENDIX B

PCP Study Nurse's Clinical Case Report Form

Informed consent obtained: yes □ no □
If yes, check that a study number has been assigned to this patient and proceed with the interview.

STUDY NO: _____

Interviewer's name:_____

Date of admission:_____

Ward:_____

Patient's name:_____

Age: _____

Gender: M □ F □

Hospital no:_____

Weight: _____

Height: _____

Address:
_____

_____
Telephone no:_____

Leigh Dini will detach this page once the form is complete and keep it secure.

STUDY NO: _____

HIV status: pos □ neg □ declined to be tested □
Date of first positive HIV test: _____

Previous PCP: no □ yes □ If yes, date: _____
Previous pneumonia or RTI: no □ yes □ If yes, date: _____
Bactrim prophylaxis in last 3 mo: no □ yes □ If yes, dose: _____

HAART: no □ yes □ If yes, date started: _____
Drugs and dose:
□ d4T/stavudine/Zerit _____
□ 3TC/lamivudine_____
□ efavirenz/Stocrin _____
□ nevirapine/Viramune _____
□ zidovudine/AZT _____
□ ddI/Videx _____
□ lopinavir + ritonavir/Kaletra _____

Previous TB: no □ yes □ If yes, when? _____

Previous medical/surgical history: _____
_____
_____
_____

Medication in last 3 mo:_____
_____
_____

Smoker: no □ yes □

SYMPTOMS

Dyspnoea: no □ yes □ If yes, duration: _____
Cough: no □ yes □ Productive □ Non-productive □ Duration: _____
Fever: no □ yes □ If yes, duration: _____
Chest pain: no □ yes □ Right □ Left □ Duration: _____

STUDY NO: _____

Investigations for PCP:
e.g. sputum, induced sputum, bronchial washings, BAL, transbronchial biopsy:

Specimen	Date	Result

If induced sputum was performed: by wall O_2 □ by portable nebulizer □

DAILY PROGRESS

Day	Date	Temp	BP	Pulse	RR	O_2 sat on room air	O_2 sat on oxygen (%)

OUTCOME

Discharged □ Date: _____ Follow-up appointment: _____

Died □ Date: _____

Excluded □ Date: _____

APPENDIX C

PCP Study, Telephonic Questionnaire

Patient name:_____ Date discharged:_____

Months after discharge:_____STUDY NO:_____

This form is to be completed about 4 months after a patient with PcP has been discharged from hospital

Name of interviewer:_____

Introduction: Introduce yourself and explain why you are calling and that you would like to ask a few questions about the patient's health and the medications they are taking.

	Date	Telephone number called	Reached someone?		Name of person reached	Relationship to patient
1st attempt			Yes	No		
2nd attempt			Yes	No		
3rd attempt			Yes	No		
(if you can't make contact after 3rd attempt please record the patient as lost to follow-up)						

Please complete the form once you've made contact with the patient or secondary contact

1. Are you speaking to the participant directly?.....☐
   0=No 1=Yes
   (If yes: cross through questions 2 through 9 and skip to question 10)

If you are speaking to the secondary contact: (explain that the participant is part of a research study and that you would like to contact him or her)

2. Is there a phone number to reach the participant directly?.....☐
   0=No 1=Yes

3. What is that number?.....☐☐☐☐☐☐☐☐☐☐
   (If number is provided: please call the patient and complete the questionnaire starting with question 10. If the number is not provided: please ask the contact questions 4-9)

If the contact number for the participant is not provided, then ask the following questions

4. How is the participant's general health status?
   _____
   _____
   _____

5. Is the participant still alive? ……☐
   0=No 1=Yes 2=Not sure/don't know
   (If yes: the form is completed. Thank the contact for their help. If no: ask questions 6-9)

If the participant has died

6. Approximately when did the participant pass away?.....☐☐/☐☐/☐☐☐☐

7. Where did the participant pass away?.....☐
   1=At home 2=Hospital 3=Hospice 4=Other
   If other specify_____

8. Did the participant have pneumonia when he or she passed away? ……☐
   0=No 1=Yes 2=Not sure/don't know

9. Can you tell me more about how he or she passed away?
   _____
   _____
   _____

If you've answered this question: the form is completed. Please thank the contact for their help.

If you are speaking to the participant directly

10. How is your health generally?
    _____
    _____
    _____
    _____

11. Have you had any health problems since your discharge from Chris-Hani Baragwanath Hospital?.....☐
    0=No 1=Yes 2=Not sure/don't know
    (If no: skip to question 13)

12. Tell me more about the health problems you have had
    _____
    _____

13. Have you had pneumonia since you were discharged?……...……..…....☐
    0=No 1=Yes 2=Don't know/Not sure

14. Have you had PcP since you were discharged? …….....……..….....☐
    0=No 1=Yes 2=Don't know/Not sure

15. Are you taking Bactrim (also called Nucotrim) drugs at the moment?.....☐
    0=No 1=Yes 2=Not sure/don't know

16. Have you had TB since you were discharged?.....☐
    0=No 1=Yes 2=Not sure/don't know

17. Have you been given ART (antiretroviral) drugs?.....☐
    0=No 1=Yes 2=Not sure/don't know

Thank you for answering my questions and I appreciate your time.

APPENDIX D

PCP Study, Postal Questionnaire

Hello, my name is Doctor Michelle Wong from Chris Hani Baragwanath Hospital. I am trying to find one of patients that I treated for a respiratory illness. Please can you help me to find this patient by answering a few questions?

My patient's name is: *type in patient's name.*

1. Do you know my patient? Yes □ No □

2. Do you have a contact telephone number for my patient? Yes □ No □
   If yes, please write the telephone number here:_____

3. Do you have an address for my patient? Yes □ No □
   If yes, please write the address here:_____
   _____

4. Do you know if my patient is alive □, has passed away □ or unknown □?
   If he/she has passed away, do you know the date of death? _____

Please could you send this form back to me in the enclosed envelope (I have already paid for the postage)?

Thank you very much for helping me.

Best regards

Dr Michelle Wong and Enrolled nurse Prudence Seboka
(Tel. 011 933 9168)

APPENDIX E

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Prevalence of DHPS Polymorphisms Associated with Sulfa Resistance in South African *Pneumocystis jirovecii* Strains

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Table 1. Estimated HIV prevalence in South Africa[a].

Geographic location	Population (million)	Estimated HIV-infected people (million)	HIV prevalence: prenatal clinic (range)[b]	HIV prevalence: total population (%)
South Africa	46.90	5.54	30.2% (29.1%–31.2%)	11.2
Gauteng Province	9.02	1.40	29.6% (27.8%–31.5%)	14.5
Western Cape Province	4.65	0.26	13.1% (8.5%–17.7%)	5.4

[a]Data from Crewe-Brown et al. (2004) and Department of Health (2006).
[b]Data from calendar year 2005; 95% confidence interval (CI).

IT is estimated that 5–6 million South Africans, including some of the growing number of AIDS orphans (over one million to date), are at risk for developing *Pneumocystis* pneumonia (PcP) at some point in their lives, especially as the availability of highly active antiretroviral therapy (HAART) is limited (UNAIDS/UNICEF/USAID 2004). The prevalence of HIV among prenatal clinic attendees in South Africa has risen dramatically from 0.7% in 1990 to 30.2% in 2005 (Table 1; Department of Health 2006). *Pneumocystis jirovecii* was the most common pathogen isolated from HIV-infected children hospitalized for pneumonia during a study at Chris Hani Baragwanath Hospital (Madhi et al. 2003). This hospital is one of the largest in the world with 2,700 beds (maximum 3,200) in current use and serves an estimated population of four million people. Trimethoprim-sulfamethoxazole (TMP-SMX) is the drug of choice for treatment and prophylaxis of PcP. An increased rate of point mutations at amino acid residues 55 (M1) and 57 (M2) in the *P. jirovecii* dihydropteroate synthase (DHPS) gene have been linked to sulfa prophylaxis (Helweg-Larsen et al. 1999; Huang et al. 2000; Kazanjian et al. 2000; Ma, Borio, and Masur 1999; Nahimana et al. 2003; Zingale et al. 2003). Dihydropteroate synthase mutations have been shown to confer resistance to sulfa compounds in some bacteria and protozoan parasites (Brooks et al. 1994; Dallas et al. 1992). The general aims of this project are to (1) estimate the burden of *P. jirovecii* infections in South African adults and children, (2) increase awareness of PcP in Africa, and (3) assess the prevalence of DHPS mutations in a South African *P. jirovecii* population as part of this clinical study. An initial pilot study was conducted to determine the rate of DHPS mutations in *P. jirovecii* strains circulating in the population. The majority of specimens were from HIV-positive adults in the Gauteng province. Specimen types included sputum, bronchial washings, and bronchoalveolar lavage. Strains detected in routine diagnostic respiratory specimens were screened for the presence of M1 and M2 DHPS polymorphisms. In the current prospective clinical study we are investigating the prevalence of *P. jirovecii* DHPS mutations in patients with PcP and its correlation with sulfa prophylaxis and clinical outcome.

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MATERIALS AND METHODS

The study sites are three government hospitals in Gauteng Province: Chris Hani Baragwanath Hospital (primary site), Tambo Memorial Hospital and Helen Joseph Hospital. HIV-positive adult inpatients with clinically and radiologically suspected PcP are enrolled. Patients undergo sputum induction and a standardized interview on admission, and are monitored daily until discharged. *Pneumocystis jirovecii* is detected by direct immunofluorescent antibody microscopy. DNA is extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) and a fragment of the DHPS gene is amplified by PCR using a previously described method (Beard et al. 2000). Cleaned PCR products are sequenced directly.

RESULTS AND DISCUSSION

The prevalence of DHPS mutations in the initial pilot study of 79 respiratory specimens was 38%. M1 mutations were detected in 15.2% of specimens, M2 mutations in 11.4%, a mixture of M1 and M2 in 11.4% and mixed wild type and mutants in 11.4%. The prevalence rate of mutations in the clinical study to date is 63.6%; 31.8% of specimens contained an M2 mutation, 31.8% had a mixture of M1 and M2 and 50% were mixed wild type and mutant.

Forty-four induced sputum specimens were obtained from 41 patients and laboratory-confirmed *Pneumocystis* infection was found in 54% (22/41) of patients (17 females, five males). Median age was 29.5 yr (range 23–56) and 95% (21/22) were confirmed to be HIV-positive (one patient declined testing, but had clinical symptoms consistent with HIV infection). The median CD4 count (20/22) was 24×10^6/L (range $2–99 \times 10^6$) and none of the patients were receiving HAART. One patient who was receiving sulfa prophylaxis had a wild-type *Pneumocystis* genotype. One patient that was previously treated empirically for PcP had a mixed M1/M2 genotype. Three patients were co-infected with *Mycobacterium tuberculosis*; one patient had cytomegalovirus (CMV) pneumonitis; and one patient had Kaposi's sarcoma. Five patients died, three of intractable respiratory failure despite therapy for PcP; one patient after being re-admitted a week after discharge with possible Guillain–Barré syndrome; and one death was probably related to *M. avium* bacteraemia.

A high prevalence of *P. jirovecii* DHPS polymorphisms was found in Gauteng Province, South Africa, which contrasts with two studies from the Western Cape Province that found much lower rates of 1.9% (Robberts et al. 2005) and 13.3% (Zar et al. 2004), respectively. However, these studies are not directly comparable, as the later study (Zar et al. 2004) included only children and the current study so far only has adults. Provincial HIV prevalence rates show geographic variations in HIV prevalence, which may account for the considerable geographical differences in the prevalence of DHPS mutations (Table 1). Until now very few patients have received PcP prophylaxis and the general use of sulfa drugs in the population is not known. However, data on pneumococcal isolates shows a steadily increasing rate of TMP-SMX resistance from 2000 to 2004 (Crewe-Brown et al. 2004). We hypothesize that the general use of sulfa drugs is exerting a selective pressure on *P. jirovecii* strains containing DHPS mutations in South Africa.

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APPENDIX F

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High Prevalence of Dihydropteroate Synthase Mutations in *Pneumocystis jirovecii* Isolated from Patients with *Pneumocystis* Pneumonia in South Africa▽

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Pneumocystis jirovecii* pneumonia (PCP) is an important cause of morbidity and mortality in immunocompromised patients. Sulfa-containing drugs are used for the treatment and prophylaxis of PCP. Mutations in the *P. jirovecii fas* gene, which encodes dihydropteroate synthase (DHPS), are associated with prior exposure to sulfa drugs, and their appearance suggests the emergence of variants with reduced sulfa susceptibility. The present study examined the prevalence of DHPS mutations in *P. jirovecii* strains isolated from South African patients with PCP. *P. jirovecii* infection was investigated by immunofluorescence microscopy and quantitative real-time PCR with respiratory specimens from 712 patients (93% of whom were >15 years of age) with suspected PCP consecutively received for the detection of *P. jirovecii* over 1 year. PCR amplification and sequencing of the DHPS *fas* gene was attempted with DNA from the *P. jirovecii*-positive samples. *P. jirovecii* infection was confirmed by immunofluorescence microscopy in 168/712 (24%) of the patients. Carriage of the fungus was revealed by real-time PCR in 17% of the patients with negative microscopy results. The *P. jirovecii fas* gene was successfully amplified from specimens from 151 patients and sequenced. Mutations resulting in the Thr55Ala and/or Pro57Ser amino acid substitution were detected in *P. jirovecii* strains from 85/151 (56%) patients. The high frequency of PCP episodes with *P. jirovecii* harboring DHPS mutations in South Africa indicates that populations of this fungus are evolving under the considerable selective pressure exerted by sulfa-containing antibiotics. These results, similar to previous observations of sulfa drug resistance in bacterial populations, underscore the importance of the rational use of sulfa medications either prophylactically against PCP or for the treatment of other infections.

Pneumocystis pneumonia (PCP), a major opportunistic infection in immunocompromised patients, is caused by the fungus *Pneumocystis jirovecii*. The incidence of PCP, which increased dramatically with the advent of the HIV/AIDS pandemic, has decreased in the industrialized world owing to the widespread use of sulfa drug prophylaxis and the introduction of highly active antiretroviral therapy (HAART). However, PCP remains an important cause of morbidity and mortality in HIV/AIDS patients, as well as in immunocompromised non-HIV-infected patients, in whom its incidence is increasing (17, 35). In South Africa, which has a population of 48.5 million, an estimated 5.7 million people were living with HIV in 2007, and 350,000 deaths were attributed to AIDS during the same year (20). The South African government initiated the provision of HAART to the public sector in April 2004, but prior to that, the HIV epidemic was largely untreated. By the end of 2006, the rate of HAART coverage was estimated to be 21% among those needing antiretroviral therapy (20). Studies from African countries report variable incidences of PCP in adult patients with HIV/AIDS and generally higher rates in children (1, 3, 27, 28, 43, 47, 49). In South Africa, where a limited number of laboratories offer testing for *P. jirovecii*, the vast majority of PCP cases are diagnosed clinically and radiologically.

Sulfonamides, usually combined with trimethoprim, as in trimethoprim-sulfamethoxazole (TMP-SMX), and dapsone are used for the treatment and prophylaxis of PCP. There are few alternative drugs for the treatment of this infection. Sulfonamides inhibit the enzyme dihydropteroate synthase (DHPS), an essential component of the folate synthesis pathway (36). In *P. jirovecii*, two nonsynonymous point mutations in the *fas* gene, which encodes the DHPS enzyme, are associated with prior exposure to sulfa drugs (5, 15, 16, 22, 26, 32), and concerns have been raised about the possible emergence of resistance to sulfa drugs (38). These mutations, at nucleotide positions 165 and 171, cause the amino acid substitutions Thr55Ala and Pro57Ser in the DHPS protein, respectively. Point mutations in the DHPS-encoding genes of microorganisms such as *Plasmodium falciparum*, *Staphylococcus aureus*, *Mycobacterium leprae*, and *Escherichia coli* have been shown to confer resistance to sulfonamides (6, 14, 21, 45). As *P. jirovecii* cannot yet be cultured, conventional *in vitro* susceptibility tests cannot be utilized; therefore, studies of drug resistance in this organism rely on the use of genetic markers and suitable models. Functional complementation of either DHPS-disrupted *E.*

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coli with a mutant *P. jirovecii fas* gene or FOL1-disrupted *Saccharomyces cerevisiae* with the *fol1* gene mutated at positions analogous to positions 165 and 171 in *fas* results in the loss of susceptibility to sulfamethoxazole and other sulfa-containing drugs (19, 29).

The prevalence of *P. jirovecii* DHPS mutations reported from countries in the developed world ranges widely, from 4% to 81% (2, 5, 15, 18, 32, 39, 41, 42). In South Africa, a few studies that screened specimens from limited numbers of adults and children have reported mostly low mutation rates (8, 12, 34, 48). Here we present the results of a large laboratory-based study aimed at ascertaining the prevalence in South Africa of *P. jirovecii* strains harboring mutations at positions 165 and 171 in the *fas* gene.

MATERIALS AND METHODS

Laboratory setting. The Parasitology Reference Unit of the National Institute for Communicable Diseases (NICD), in Johannesburg, South Africa, is the largest recipient of referrals for the microbiological confirmation of *P. jirovecii* infection in the country, receiving about 1,000 specimens annually.

Clinical specimens and patient information. Respiratory specimens (n = 932) from patients with suspected PCP (n = 712) were referred to the NICD from 38 public hospitals and clinics in six provinces of South Africa from January 2006 through February 2007. Data regarding patient age, gender, and hospital location were retrospectively extracted from a laboratory information system. The mean age of the patients was 34 ± 13 years, with the age range being 1 month to 86 years (the ages of 81 patients were unknown). The ratio of females to males was 1.6:1 (the genders of 18 patients were unknown). The majority of patients (n = 491/712, 69%) were from Gauteng Province; and the rest were from the Eastern Cape, Mpumalanga, Free State, Limpopo, and Northern Cape Provinces. The majority of specimens received for the detection of *P. jirovecii* were sputa (n = 839/932, 90%). When multiple specimens from the same patient and PCP episode were provided, only the first *P. jirovecii*-positive specimen received in the laboratory was included in the analysis. If more than one specimen was taken on the same day, the specimen with the higher fungal load was included in the analysis. Data concerning HIV status and prophylaxis and/or treatment for PCP were not available for these patients. All procedures followed were in accordance with the ethical standards of the Human Research Ethics Committee (Medical) of the University of the Witwatersrand in Johannesburg (protocol no. M050320).

Specimen processing and laboratory detection of *P. jirovecii*. Sputum specimens and other specimen types containing mucus were pretreated with 0.1% dithiothreitol (Sigma-Aldrich, South Africa). After centrifugation, microscopic slides were prepared with the sediment. The remaining sediment was washed with phosphate-buffered saline and stored at −70°C prior to DNA extraction. The slides were stained with a direct fluorescent antibody (for immunofluorescence [IF] microscopy) specific for the detection of *P. jirovecii* cysts (Light Diagnostics, Millipore, Temecula, CA), according to the manufacturer's instructions. A positive IF microscopy result was defined as the presence in a specimen of at least one cluster of *P. jirovecii* cysts with the characteristic morphology and fluorescent immunostaining pattern. An equivocal result was defined as the presence in a specimen of a cyst that had a suspicious *Pneumocystis*-like cyst morphology but that did not meet the criteria for confirmation of a positive result.

DNA extraction. DNA was extracted from 50 μl of sediment, which was digested with proteinase K at 56°C for 2 h, prior to automated DNA extraction with an M48 BioRobot apparatus (Qiagen, Germany), according to the manufacturer's protocol for soft tissue. The purified DNA was eluted in 100 μl of sterile water and stored at −20°C.

Determination of *P. jirovecii* DNA load. The amount of *P. jirovecii* DNA in specimens was determined by a quantitative real-time PCR (qPCR) targeting the mitochondrial gene coding for the large ribosomal subunit (mtLSU) of the fungus. The following primers and probe were used: LSU1 (5′-AAA TAA ATA ATC AGA CTA TGT GCG ATA AGG-3′), LSU2 (5′-GGG AGC TTT AAT TAC TGT TCT GGG-3′), and LSUP1 (6-carboxyfluorescein–5′-AGA TAG TCC AAA GGG AAA C-3′–6-carboxytetramethylrhodamine) (Applied Biosystems, Foster City, CA). The amplification reactions were carried out in a total volume of 25 μl containing 1× TaqMan universal PCR master mixture, 0.25 μl of a mixture of primers and probe at 20 μM each, 5 μl of template DNA, and RNase-free water to complete the volume. Thermal cycling was performed in an Applied Biosystems GeneAmp PCR system 7900 thermocycler, as follows: 2 min at 50°C and 10 min at 95°C, followed by 45 cycles of 15 s at 95°C and 1 min at 60°C. Quantification was based on extrapolation from standard curves generated by amplification of serial dilutions of linearized plasmid pCR2.1 containing the *P. jirovecii* DNA fragment defined by primers LSU1 and LSU2.

Analysis of DHPS genotypes. Specimens with ≥20 target mtLSU gene copies/μl of extracted DNA were selected for DHPS analysis, as the amplification of the single-copy DHPS *fas* gene proved to be less sensitive than the real-time PCR assay. Primers SMIF1 (5′-CAA ATT AGC GTA TCG AAT GAC C-3′) and SMIB2 (5′-GCA AAA TTA CAA TCA ACC AAA GTA-3′) were used to amplify a 1,030-bp region of the DHPS gene. Subsequently, nested primers SMIF6 (5′-AGC GCC TAC ACA TAT TAT GG-3′) and SMIB7 (5′-GTT CTG CAA CCT CAG AAC G-3′) were used to amplify a 278-bp region encompassing polymorphic nucleotide positions ^{165}A/G and ^{171}C/T. PCR amplification was performed in a Mastercycler gradient apparatus (Eppendorf AG, Hamburg, Germany) by using the following conditions for the primary PCR: 94°C for 5 min, followed by 35 cycles at 94°C for 30 s, 55°C for 1 min, and 72°C for 2 min and a final extension at 72°C for 7 min. The nested PCR conditions were 94°C for 5 min, followed by 35 cycles at 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min and a final extension at 72°C for 7 min. The PCR products were visualized by gel electrophoresis on 2% agarose gels. The nested PCR products were sequenced by using primer SMIF6 and BigDye Terminator chemistry.

Measures taken to prevent contamination of DNA. Different laboratories were used for the initial processing and immunostaining of the specimens, DNA extraction, addition of the PCR product to the nested reaction mixture, and gel electrophoresis. A laboratory free of *Pneumocystis* DNA was used to prepare all PCR master mixtures. The DNA was handled in a laminar flow cabinet equipped with a UV lamp for decontamination. Positive and negative controls were included with the DNA extraction process and PCRs.

Data analysis. The DNA sequences were analyzed with BioEdit sequence alignment editor software (version 7.0.9.0). Statistical analyses were performed with the Excel program. The chi-square test was used to assess significant ($P < 0.05$) associations among categorical variables.

RESULTS

During the study period, from January 2006 through February 2007, 932 respiratory specimens from 712 patients (93% of whom were >15 years of age) with suspected PCP were consecutively received for the microbiological confirmation of PCP at the NICD in Johannesburg, South Africa. IF microscopy was positive for *P. jirovecii* in specimens from 168 (24%) patients. *P. jirovecii* DNA was detected in all of the IF microscopy-positive specimens from 150 patients that were available for assessment of the fungal load by qPCR. The quantity of *P. jirovecii* DNA measured in the first IF microscopy-positive specimen from each of these 150 patients is shown in Table 1. The IF microscopy result was deemed equivocal for specimens from 14 patients, and 10 of these yielded positive results by qPCR (Table 1). Specimens from 530 patients were IF microscopy negative for *P. jirovecii*. Quantitative PCR was performed with a subset of IF microscopy-negative specimens from 191 patients that were arbitrarily chosen throughout the year. *P. jirovecii* DNA was detected in IF microscopy-negative specimens from 32 (17%) of these patients and quantified (Table 1).

Amplification of the DHPS gene of *P. jirovecii* was attempted in 201 specimens from 162 patients with a qPCR result of ≥20 target gene copies/μl of extracted DNA. The DHPS locus was successfully genotyped in a total of 183 specimens from 151 patients. Point mutations resulting in the Thr55Ala and/or Pro57Ser amino acid substitution were detected in *P. jirovecii* isolates from 85 (56%) patients (Table 2). Of these, 36 patients (42%) harbored *P. jirovecii* isolates with either the Thr55Ala mutation (19 patients, 22%) or the Pro57Ser mutation (17 patients, 20%). In specimens from 18

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TABLE 1. Quantification of *P. jirovecii* DNA in respiratory specimens from South African patients with suspected *Pneumocystis* pneumonia

Specimen and IF microscopy result	No. of patients	No. of patients qPCR positive	Median (range) *P. jirovecii* DNA quantity[a]
Sputum			
Positive	140	140	3,062 (8–291,894)
Negative[b]	173	30	7 (1–499)
Equivocal[c]	12	10	151 (9–1,549)
Tracheal aspirate			
Positive	7	7	1,921 (10–180,498)
Negative	4	1	0.8
Equivocal	1	0	
Bronchoalveolar lavage			
Positive	2	2	54,766 (3–109,529)
Negative	3	0	
Other[d]			
Positive	1	1	299
Negative	11	1	4
Equivocal	1	0	

[a] Expressed as the target number of gene copies/μl of extracted DNA. Detailed information regarding the handling of specimens and DNA extraction conditions is provided in Materials and Methods.

[b] A set of 205 specimens from 191 patients from among the 530 patients with IF microscopy-negative results was arbitrarily chosen for qPCR analysis.

[c] Immunofluorescence microscopy was suggestive of *P. jirovecii*, but the result was not conclusive.

[d] Includes two bronchial washings, two nasopharyngeal aspirates, one pleural fluid specimen, and eight samples of unknown specimen types.

TABLE 2. *Pneumocystis jirovecii* DHPS genotypes in respiratory specimens from South African patients

DHPS genotype	Nucleotide at position (amino acid):		No. of patients positive/total no. of patients (%) (n = 151)
	165 (55)	171 (57)	
Wild type	A (Thr)	C (Pro)	66/151 (44)
Mutant genotype, total			85/151 (56)
Single mutation			36/85 (42)
Position 165 (M1)	G (Ala)	C (Pro)	4/85 (5)
M1 + wild type	G/A (Ala/Thr)	C (Pro)	15/85 (18)
Position 171 (M2)	A (Thr)	T (Ser)	7/85 (8)
M2 + wild type	A (Thr)	T/C (Ser/Pro)	10/85 (12)
Double mutation			18/85 (21)
Positions 165 + 171 (M3)	G (Ala)	T (Ser)	15/85 (18)
M3 + M1	G (Ala)	T/C (Ser/Pro)	1/85 (1)
M3 + M2	G/A (Ala/Thr)	T (Ser)	2/85 (2)
Mixed genotypes[a]	G/A (Ala/Thr)	T/C (Ser/Pro)	31/85 (36)

[a] Mixed infections in which the presence of mutants with double mutations and the wild type and/or a mixture of mutants with single mutations could not be resolved.

patients (21%), the presence of *P. jirovecii* organisms with both mutations in the DHPS gene could be ascertained. Specimens from 31 patients (36%) contained mixtures of mutant *P. jirovecii* DHPS genotypes, both with and without the wild-type genotype, which could not be resolved by direct sequencing. *P. jirovecii* infections with more than one DHPS genotype were detected in 69% (59/85) of patients harboring mutant *P. jirovecii* strains. Multiple respiratory specimens collected during the same PCP episode were available from 23 patients, and strains in the majority of the specimens (17/23, 74%) presented identical DHPS genotypes. There were no significant differences in the frequency of wild-type or mutant DHPS genotypes between genders ($P = 0.39$), age groups ($P = 0.38$), or patients from distinct geographical locations ($P = 0.1$).

DISCUSSION

The primary aim of the present study was to assess the prevalence in South Africa of *P. jirovecii* strains harboring DHPS mutations previously shown to be associated with exposure to sulfa or sulfone drugs. The number of specimens and patients screened makes this study the largest of its kind conducted in the developing world to date. We found that 56% of patients with PCP were infected with mutant *P. jirovecii* strains. This proportion stands in contrast to the lower frequencies reported from the African continent and other countries outside the industrialized world (8, 12, 23, 25, 30, 34, 40, 46, 48). The risk that *P. jirovecii* will develop mutations in the DHPS gene is higher for patients with PCP receiving sulfa drug prophylaxis than for those not receiving it (5, 15, 16, 22, 26, 32, 38). A general assumption is that the frequency of occurrence of mutations in the DHPS gene in developing countries is low due to the less extensive use of sulfa drug prophylaxis against PCP. Thus, the high frequency of mutations in the DHPS gene among strains from PCP patients in South Africa would be indicative of a considerable exposure to sulfa medication used either prophylactically against PCP or for the treatment of other infections. We regard it as unlikely that sulfa drug (usually TMP-SMX) prophylaxis for PCP alone accounts for the high prevalence of mutant *P. jirovecii* strains observed in the present investigation. Although information regarding the HIV infection status of the patients and the use of prophylaxis was not available for this laboratory-based study, it is well recognized that a large proportion of HIV-positive patients in South Africa are either unaware of their HIV infection when they present with PCP or have limited access to prophylaxis. During a large clinical study in progress at a public hospital in Johannesburg, interim results showed that 58% of adult patients presenting with PCP were newly diagnosed with HIV infection and that only 27% of patients with a previously confirmed HIV infection were receiving TMP-SMX prophylaxis (M. Wong, personal communication). Furthermore, in a survey of South African primary-level health care services for HIV-positive children, two-thirds of the public-sector clinics did not have in place a policy for the administration of PCP prophylaxis (11).

The widespread use of sulfa drugs, particularly TMP-SMX, against diseases other than PCP could be contributing to the selection of *P. jirovecii* strains carrying mutations in the DHPS gene. In South Africa, guidelines issued by the Department of Health have recommended the use of TMP-SMX to treat urinary tract infections, cholera, and acute otitis media at the

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primary-care level, as well as invasive bacterial infections caused by *Shigella* and *Salmonella* species in hospitalized patients (37). Indications for HIV-positive individuals, apart from PCP prophylaxis and treatment, include prophylaxis against recurrent bacterial infections in children; prophylaxis against bacterial pneumonia, bacteremia, and isosporiasis in adults; as well as the prophylaxis and treatment of toxoplasmosis. A 2002 report revealed that 26% of primary health care clinics also administered TMP-SMX for the treatment of acute respiratory and gastrointestinal tract infections in children (11), a finding in line with the generalized perception that sulfa-based drugs are often used in primary care as empirical treatment for a variety of conditions, including diarrhea and nonspecific respiratory infections in adults and children. The extensive usage of TMP-SMX is known to exert considerable selective pressure on microbial populations and lead to the development of resistance (4, 9). That this is the situation in Gauteng Province and other localities of South Africa is also suggested by the very high rates of TMP-SMX resistance consistently observed in surveillance isolates of pneumococcal and other bacterial species; for example, rates of TMP-SMX resistance in isolates of *Streptococcus pneumoniae*, *Shigella* spp., and nontyphoidal salmonellae of over 50%, 83%, and 37%, respectively, have been reported (13, 44). Sulfadoxine-pyrimethamine (Fansidar) was used as the first-line treatment for malaria in South Africa until 2006; while the number of patients treated (between 1990 and 2006, an average of about 25,000 malaria cases per year) was relatively small in comparison to the burden of HIV-infected individuals, this may have contributed to selective pressure.

The transmission of *P. jirovecii* strains is another factor that may influence the high prevalence of mutant DHPS strains observed in the present investigation. The airborne transmission of *Pneumocystis* spp. from host to host has been demonstrated in rodent models; and numerous observations, including several cases of nosocomial clustering, suggest that direct or indirect transmission occurs in humans (33). Evidence for the *de novo* acquisition of *P. jirovecii* with mutant DHPS genotypes has been provided by studies that included patients who had never received sulfa drug prophylaxis for PCP (16, 24). In one of those studies, more than half of the patients newly diagnosed with HIV infection harbored mutant *P. jirovecii* strains (16). It is possible that the interhuman transmission of *P. jirovecii* and the concomitant circulation of DHPS genotypes in the population are accelerated in localities of South Africa with a high prevalence of HIV infection and large numbers of individuals with some degree of immunosuppression, from subclinical stages to overt AIDS, who are susceptible to colonization by the fungus. The widespread empirical use of TMP-SMX and the rates of increased transmission and circulation of strains/genotypes in the population could explain, at least in part, the high prevalence of mutant *P. jirovecii* strains in settings in which the implementation of PCP prophylaxis is limited.

A lack of clinical surveillance, the lack of availability of chest radiographic facilities, and limited laboratory diagnostic capabilities contribute to the scarcity and disparity of information regarding the burden of disease and the epidemiology of PCP in African settings. In South Africa, the collection of bronchoalveolar lavage (BAL) specimens is rare. The few specimens sent for laboratory confirmation are mostly sputa, and these are often spontaneously expectorated rather than induced with hypertonic saline (7, 10). Although the collection procedure was not specified for the majority of sputum specimen referrals in this study, we estimate that a high proportion of specimens were spontaneously expectorated. Clinically, it is accepted that BAL fluid and induced sputum specimens are superior to spontaneously expectorated sputum for the microscopic diagnosis of PCP, in part because many patients with PCP have a nonproductive cough and cannot produce an adequate specimen without undergoing sputum induction. While the proper collection of the respiratory sample is of major importance for the optimal laboratory diagnosis of PCP, it may also be crucial for the strain/genotype representativity of the specimen and the accurate assessment of mixed-genotype infections. In five of a total of six cases with discrepant DHPS genotypes, we observed large differences (≥10-fold) in the quantity of *P. jirovecii* DNA measured in specimens from the same PCP episode caused by strains showing nonidentical DHPS genotypes. In some of these cases, multiple samples had been collected within 24 h or less. This suggests that variable specimen quality, as well as initiation of PCP therapy, may be a cause of inconsistent results in the genotypic analysis of *P. jirovecii* infections.

In South Africa, PCP has been studied more extensively in children, and *P. jirovecii* is now recognized as a common pathogen in HIV-infected infants (49). In the present study, which predominantly included specimens from adults, *P. jirovecii* infection was confirmed by IF microscopy in 24% of patients with suspected PCP. By quantitative real-time PCR analysis, we could confirm *P. jirovecii* carriage in an additional 17% of patients with a clinical suspicion of PCP and negative IF microscopy results. The detection of *P. jirovecii* was also verified by real-time PCR in specimens from 10 of 14 patients with equivocal microscopy results. Quantitative real-time PCR performed in a closed system offers the advantages of increased diagnostic sensitivity and rapidity with a reduced risk of contamination. Quantification of the level of *P. jirovecii* infection or colonization can be useful for the monitoring of therapy and the improved management of patients (31). Although increased diagnostic sensitivity carries a higher risk of clinically false-positive results, it enhances the chances for the early detection of biologically true-positive results for individuals with no or incipient clinical manifestations, something that could be of critical importance in localities with a high incidence of HIV infection.

This study has certain limitations. Data regarding HIV infection status, the use of sulfa drug prophylaxis or some other medication, and the outcomes for the patients were not available. These shortcomings, which preclude further analysis of possible underlying associations, expose the types of constraints that are common for laboratory-based studies. However, they do not have any bearing on the central issue of assessing the frequency of DHPS genotypes in *P. jirovecii* strains prevalent in the population. Although the study included patients from a large number of hospitals in South Africa, 57% of the patients for whom data on *P. jirovecii* DHPS genotypes were available came from two hospitals in Gauteng Province.

The increased risk that patients receiving sulfa drug prophy-

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laxis carry mutant *P. jirovecii* DHPS genotypes suggests that mutations in the *fas* gene provide a selective advantage so that subpopulations of the fungus may eventually overcome the effects of prophylactic doses of TMP-SMX. The clinical significance of DHPS mutations and, in particular, whether single polymorphisms or double mutations have differential effects on the response to higher therapeutic doses are questions not yet resolved. The appearance of *P. jirovecii* strains with additional mutations and significantly higher levels of tolerance to sulfonamides and/or sulfones is a possibility that cannot be excluded. Examples of cumulative mutations leading to increased antibiotic resistance have been documented for viruses, bacteria, and parasitic protozoa.

In summary, the results of the present investigation show that the frequency of infections with *P. jirovecii* strains harboring DHPS mutations is high in adult patients in South Africa. This finding suggests the existence of considerable antimicrobial pressure favoring the establishment in the population of *P. jirovecii* strains with reduced sensitivities to sulfa drugs. The most likely source of this selective pressure is the utilization of TMP-SMX, both prophylactically against PCP and for the treatment of other infections. The importance of regular monitoring for DHPS gene polymorphisms and the rational use of sulfa medications is underscored.

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APPENDIX G

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R.14/49 Du Plessis

CLEARANCE CERTIFICATE	**PROTOCOL NUMBER M010320**
PROJECT	Characterisation of Pneumocystis jiroveci Strains in South Africa
INVESTIGATORS	Dr M Du Plessis
DEPARTMENT	RMPRU
DATE CONSIDERED	05.04.01
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 05.05.20 **CHAIRPERSON** .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Prof J Preen

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10005, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

APPENDIX H

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Dini

CLEARANCE CERTIFICATE	PROTOCOL NUMBER M040967
PROJECT	Survey and Management of Drug Resistant Pneumocystis jirovecci Pneumonia in Gauteng South Africa
INVESTIGATORS	Mrs L Dini
DEPARTMENT	Parasitology Reference Unit
DATE CONSIDERED	04.06.25
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 04.06.28 CHAIRPERSON ..... (Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Prof J Feary

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and ONE COPY returned to the Secretary at Room 10005, 10th Floor, Senate House, University.

I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. I agree to a completion of a yearly progress report.

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

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