GENETIC INVESTIGATIONS OF *PNEUMOCYSTIS JIROVECII*: DETECTION, COTRIMOXAZOLE RESISTANCE AND POPULATION STRUCTURE

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DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Signature……. Date……

ABSTRACT

Pneumocystis jirovecii is a significant contributor to the burden of disease in immunocompromised patients. The polymerase chain reaction (PCR) is more sensitive and specific than microscopy. Cotrimoxazole prophylactic breakthrough and treatment failures have been reported, and associated with mutations at codons 55 and 57 of *P. jirovecii* dihydropteroate synthase (DHPS). No phylogenetic or population genetic models have been successful in elucidating *P. jirovecii* intraspecies strain relatedness.

Aims: 1) Compare detection rates of nine PCR techniques and immunofluorescence microscopy (IF); 2) Determine the extent of co-infecting pathogens associated with Pneumocystis Pneumonia (PcP); 3) Determine local *P. jirovecii* ITS1-5.8S-ITS2 rDNA strain types, and model lineage evolution employing a coalescent-theory based statistical parsimony network analysis; 4) Investigate the possible emergence of cotrimoxazole-resistant strains

Methods: PCR was evaluated on clinical specimens employing: ITS nested; DHPS single and nested; DHFR nested; major surface glycoprotein (MSG) heminested; mitochondrial large subunit rRNA (mtLSUrRNA) single and nested; 18S rRNA one-tube nested, and real-time 5S rRNA PCR. Retrospective analysis of co-infecting pathogens seen in PcP patients was conducted. ITS regions were amplified, cloned and sequenced. Statistical parsimony was applied for coalescence based network genotype analysis. DHPS genome walking was attempted and DHPS and DHFR primer annealing sites explored. Amplified DHPS and DHFR genes were cloned and sequenced.

Results: Most sensitive PCR technique was mtLSUrRNA nested followed by 5S real-time PCR. A poor correlation exist between mtLSUrRNA PCR and IF. Review of clinical records suggested a high rate of false-positive IF results. *P. jirovecii* was detected in 4.3% *M. tuberculosis*-positive HIV-positive, and 2.5% *M. tuberculosis*-positive HIV-negative patients. *P. jirovecii* was detected in 45% HIV-negative

patients. The most prevalent ITS type was Eg. Four new combinations: Eo, Je, Ge, No; 11 new ITS1 and 13 new ITS2 sequences were identified. A new ITS2 type was detected in three patients and designated u. More than one strain type was detected in 15/19 patients. Analysis of 5.8SrDNA region revealed 13 clones containing 1-2 nucleotide polymorphisms. Of 85 mtLSUrRNA PCR-positive specimens, currently employed primers amplified DHPS and DHFR genes from 53 and 27 specimens, respectively. Newly designed DHPS primers increased detection in 3 / 28 previously DHPS-negative mtLSUrRNA-positive specimens. Of 56 DHPS genes amplified and sequenced, one contained the double mutation (Thr55Aa; Pro57Ser). DHFR Ala67Val was detected in three specimens and a new DHFR genotype (Arg59Gly; C278T) was demonstrated.

Conclusions: The study emphasises the need to evaluate PCR primers against local strains. It is recommended that mtLSUrRNA PCR be performed in parallel to IF and discordant results resolved with clinical evaluation. Co-infection with *P. jirovecii* and *M. tuberculosis* occurs in South Africa, and treatment for both pathogens is recommended when demonstrated by the laboratory. ITS genotyping employing statistical parsimony network analysis suggests type Eg as major ancestral haplotype, and supports recombination contributing to strain diversity worldwide. DHPS mutations may signal emergence of resistance to cotrimoxazole in South Africa, however, low sensitivity of primers limits surveillance efforts.

OPSOMMING

P. jirovecii dra grootendeels by tot die morbiditeit van imuunonderdrukte pasiënte. Die polimerase ketting reaksie (PKR) is meer sensitief en spesifiek as mikroskopie. Mutasies in dihidropteroaat sintase (DHPS) by kodons 55 en 57, word geassosieer met deurbraak van profilakse en onsuksesvolle behandeling met cotrimoxazole. Geen filogenetiese of populasie genetiese modelle was al suksesvol om *P. jirovecii* intraspesie verwantskappe aan te toon nie.

Doelwitte: 1) Vergelyk die deteksie syfers van nege PKR tegnieke en immunofluoressensie mikroskopie (IF); 2) Bepaal die omvang van ko-infektiewe patogene op *Pneumocystis* pneumonie; 3) Bepaal die plaaslike *P. jirovecii* ITS1-5.8S-ITS2 rDNA stam tipes, en ontleed dié evolusie deur samevloei gebaseerde statistiese enkelvoudige netwerk analise; 4) Ondersoek die moontlike ontwikkeling van cotrimoxazole weerstandbiedendheid.

Metodes: PKR was ge-evalueer op kliniese monsters deur: ITS dubbel; DHPS dubbel en enkel; DHFR dubbel; MSG halfdubbel; mtLSUrRNA enkel en dubbel; 18S rRNA enkelbuis dubbel; en in-tyd 5S rRNA PKR. Ko-infektiewe patogene was retrospektief beskryf. ITS gebiede was geamplifiseer, gekloneer en onderwerp aan DNA volgorde bepaling. Statisties enkelvoudige samevloei gebaseerde netwerk genotiep analise was onderneem. DHPS genomiese wandeling was onderneem en DHPS en DHFR peiler aanhegtings ondersoek. Geamplifiseerde DHPS en DHFR gene was gekloneer en DNA volgordes bepaal.

Resultate: Die mees sensitiewe PKR tegniek was mtLSUrRNA dubbel, gevolg deur 5S in-tyd PKR. ’n Swak korrelasie was gesien tussen mtLSUrRNA PKR en IF. Ondersoek van kliniese verslae het ’n hoë voorkoms van fals positiewe IF resultate getoon. *P. jirovecii* was opgespoor in 4.3% *M. tuberculosis*-positiewe MIV-positiewe, en 2.5% *M. tuberculosis*-positiewe MIV-negatiewe pasiënte. *P. jirovecii* was geïdentifiseer in 45% MIV-negatiewe pasiënte. Die mees prevalente ITS stam tipe was Eg. Vier nuwe kombinasies: Eo, Je, Ge, No; 11 nuwe ITS1 en 13 nuwe ITS2

tipes was geïdentifiseer. ’n Nuwe ITS2 tipe, genoem “u”, was ontdek in drie pasiënte. Meer as een ITS stam tipe was opgespoor in 15/19 pasiënte. Dertien nuwe 5.8SrDNA DNA volgordes was ontdek. DHPS was geamplifiseer uit slegs 53/85 mtLSUrRNA-positiewe monsters. So ook was DHFR uit slegs 27/85 mtLSUrRNA-positiewe monsters geamplifiseer. Drie van 28 monsters wat mtLSUrRNA-positief maar DHPS-negatief was, is deur nuwe DHPS peilers geamplifiseer. Van 56 DHPS gene is slegs een monster met die dubbel mutasie (Thr55Ala; Pro57Ser) geïdentifiseer. Die DHFR Ala67Val mutasie was in drie pasiënte geïdentifiseer. ’n Nuwe DHFR genotipe (Arg59Gly; C278T) was ontdek.

Gevolgtrekkings: Hierdie studie beklemtoon die noodsaaklikheid om PKR peilers teen plaaslike stamme te ondersoek. Dit word aanbeveel dat mtLSUrRNA PKR saam met IF uitgevoer word, en dat onversoenbare resultate deur kliniese gegewens beslis word. Ko-infeksie van *P. jirovecii* en *M. tuberculosis* kom voor en behandeling vir albei patogene word aanbeveel indien dit deur die laboratorium aangedui word. ITS genotipering en aanwending van statistiese enkelvoudige netwerk analise dui aan dat Eg die hoof voorganger stam tipe is. Die analise ondersteun die voorkoms van rekombinasie wat kan bydra tot stam diversiteit. Die identifisering van DHPS mutasies verteenwoordig die ontwikkeling van weerstandbiedendheid tot cotrimoxazole in Suid-Afrika, maar die lae sensitiwiteit van huidige peilers beperk die ondersoek daarvan.

DEDICATION

My parents

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LIST OF ABBREVIATIONS

3’ ETS	3’ external transcribed spacer
5’ ETS	5’ external transcribed spacer
AIDS	Acquired immunodeficiency syndrome
Ala	Alanine
AP1	Adaptor primer 1
AP2	Adaptor primer 2
Arg	Arginine
AROM	Aromatic amino acid synthesis
ATP	Adenosine triphosphate
BAL	Bronchoalveolar lavage
bp	Base pairs
Bx	Biopsy
CD	Cluster of differentiation
CMV	Cytomegalovirus
Cys	Cystine
DHF	Dihydrofolate
DHFR	Dihydrofolate reductase
DHFS	Dihydrofolate synthase
DHP	Dihydropteroate
DHPP	2-amino-4-hydroxy-6-hydroxymethyl-7,8-dihydropterinine
DHPPK	hydroxymethyldihydropterin pyrophosphokinase
DHPPP	Dihydropteroate pyrophosphokinase
DHPS	Dihydropteroate synthase
DNA	Deoxyribonucleic acid
dNTP’s	deoxynucletide triphosphates
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra-acetic acid
ES	Expansion segment
f. sp.	*formae speciales*
fas	Folic acid synthesis

GSP1	Gene specific primer 1
GSP2	Gene specific primer 2
h	Hours
HAART	Highly active antiretroviral therapy
HIV	Human immunodeficiency virus
IF	Immunofluorescence
Indel	Insertion / deletion
IPTG	Isopropyl-β-D-thiogalactopyranoside
ITS	Internal transcribed spacer regions of the ribosomal RNA
LB	Luria Bertani
min	Minutes
MRCA	Most recent common ancestor
MSG	Major surface glycoprotein
mtLSUrRNA	Mitochondrial large subunit ribosomal RNA
mtSSUrRNA	Mitochondrial small subunit ribosomal RNA
n	Nested
Oac	Acetate
*p*ABA	*para*-Amino benzoic acid
PcP	*Pneumocystis* pneumonia
PCR	Polymerase chain reaction
PET	Formalin-fixed paraffin-wax embedded tissue
Pro	Proline
Pwo	*Pyrococcus woessii*
r	correlation coefficient
rDNA	Ribosomal DNA
rRNA	Ribosomal ribonucleic acid
RSV	Respiratory cyncytial virus
s	Single
sec	Seconds
SSCP	Single strand conformational polymorphism
Taq	*Thermus aquaticus*
TASP	Tracheal aspirate

TB	Pulmonary tuberculosis
THF	Tetrahydrofolate
Thr	Threonine
Tm	Melting temperature
TMP-SMX	Trimethoprim-sulphamethoxazole
TS	Thymidylate synthase
Tyr	Tyrosine
USA	United States of America
Val	Valine
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

CHAPTER 1

INTRODUCTION

1.1 *PNEUMOCYSTIS JIROVECII* PATHOGENESIS AND EPIDEMIOLOGY

1.1.1 Historical overview

The first description of what turned out to be *Pneumocystis* was by Carlos Ribeiro Justiniano Chagas [Chagas, 1909, cited in Dutz, 1970]. In 1909, while working at the Oswaldo Cruz Institute, Rio de Janeiro, he investigated a new disease of railroad workers which was subsequently called Chagas' disease. While working on this disease Chagas, as part of his investigations, examined the lungs of a 3-month-old child and described what he thought was the sexual form (schizont) of *Schizotrypanosoma*, containing 8 'leishmanial' bodies within one capsule [Chagas, 1909, cited in Dutz, 1970]. Subsequent investigations of rats, monkeys and other free-living species in Africa and South America initially confirmed Chagas' findings of the presence of this so-called schizont of *Schizotrypanosoma* [Carini, *et al*., 1910,

cited in Dutz, 1970]. Slides of infected material were requested by Mesnil and Laversan at the Institute Pasteur in Paris, from Carini at the Institute Pasteur in Brazil. At the Paris Instritute, Dr. and Mrs. Delanoe reviewed Carini's slides, and together with their own investigations on sewer rats at the time, concluded in 1912 that the entity identified as a sexual form of *Schizotrypanosoma* was in fact an entirely different organism. As they received their original slides from Carini they proposed the name *Pneumocystis carinii* [Delanoe & Delanoe, 1912, cited in Dutz, 1970]. Maciel and Carini confirmed this [Carini & Maciel, 1914, cited in Dutz, 1970], so did Chagas who withdrew his original claim of the reported schizont [Chagas, 1913, cited in Dutz, 1970]. However, the question of human pathogenicity of *Pneumocystis carinii* rested for a generation.

In 1938 Ammich differentiated a plasmacellular pneumonia from the conventional "pneumonia alba" and "syphilitic lung", with peculiar honeycombed exudates in the alveoli [Ammich, 1938, cited in Dutz, 1970]. This type of pneumonia was associated with *P. carinii* for the first time in 1942 in the Netherlands by Van der Meer and Brug, however, an aetiologic relationship was not established [van der Meer & Brug, 1942, cited in Dutz, 1970]. The first epidemics of *Pneumocystis* were reported in countries of central Europe in the late 1940's where marasmic and premature infants in orphanages were infected [Vaněk & Jirovec, 1952, cited in Dutz, 1970]. It was from these epidemics that, in 1952, Jirovec and Vaněk established the aetiologic relationship of *P. carinii* / *P. jirovecii* (section 1.1.2) to interstitial plasmacellular pneumonia of premature or marasmic orphans. [Vaněk & Jirovec, 1952, cited in Dutz, 1970]. The European orphanage epidemics abated when food rationing was abolished, and life returned to normal. However, after 1954, sporadic infections caused by *Pneumocystis* were reported from countries all over the world [Burke & Good, 1973]. The association of infections with *Pneumocystis* in immunocompromised patients became known in 1964 [Hill, *et al*., 1964], and by 1973 a high index of suspicion of *Pneumocystis* infection was advised in the following categories of patients with pneumonia: premature infants, marasmic and malnourished children, patients with congenital immunodeficiency (humoral and cell mediated) or acquired immunodeficiency such as diseases related to malignancies,

immunosuppressive therapies (particularly adrenocorticosteroids, methotrexate), and transplant recipients [Burke & Good, 1973].

In the early 1980s reports of a syndrome characterized by opportunistic infections, uncommon in the general population emerged. This was the emergence of acquired immunodeficiency syndrome (AIDS). *Pneumocystis* pneumonia (PcP) became the most prevalent opportunistic disease in patients with AIDS, and lethal if not treated correctly [Gottlieb, *et al*., 1981; Anon., 1983]. In 1983 PcP was responsible for the death of the first two patients diagnosed with AIDS in South Africa [Ras, *et al.*, 1983]. Since the introduction of highly active antiretroviral therapy (HAART) the incidence of PcP in the developed world seems to be declining, however, in the developing world it is on the increase.

1.1.2 Taxonomy

Classification of the organism was debated for many years. The proponents for protozoa argued that the structure of the merozoites, inability to grow on fungal media, absence of ergosterol, clinical response to antiprotozoan drugs such as pentamidine, and insensitivity to antifungal agents indicated that it was a protozoan [Dutz, 1970]. The proponents of the fungal theory based their arguments on staining characteristics and electron microscopic appearance of the capsule [Dutz 1970]. Most authors considered *Pneumocystis* to be a protozoan until 1988. Phylogenetic analysis of the 16S, 5.8S and 26S ribosomal RNA (rRNA) and other gene sequences revealed *Pneumocystis* to be a member of the Fungi [Edman, *et al.*, 1988; Liu, *et al.*, 1992] and related to the Ascomycetes with its nearest extant relative being Scizos*accharomyces pombe* [Ma, *et al.*, 2001; Cushion, 2004]. However, analysis of the 5S rRNA placed *Pneumocystis* closer to the Amoeba or Myxomycota, rather than Ascomycota [Watanabe, *et al.*, 1989]. *Pneumocystis* also possesses a fungal characteristic in that the dihydrofolate reductase (DHFR) gene is neither physically nor genetically linked to thymidylate synthase (TS). Absence of the bifunctional DHFR-TS supports the fungal argument as protozoa have the bifunctional enzyme

[Edman, *et al.*, 1989b]. It is now widely accepted that *Pneumocystis* is classified within the Fungal kingdom [Edman, *et al.*, 1988; Liu, *et al.*, 1992; Ma, *et al.*, 2001].

Pneumocystis has been identified in a variety of mammals including old and new world monkeys, rodents, domestic dogs, and lagomorphs [Botha & van Rensburg, 1979; Guillot, *et al.*, 1999; Laakkonen, *et al.*, 1999; Furuta, *et al.*, 2001; Guillot, *et al.*, 2001; Laakkonen, *et al.*, 2001; Hugot, *et al.*, 2003]. As isolates from different host species appeared stenoxenic and failed to cross-infect different hosts [Durand-Joly, *et al.*, 2002], it prompted classification to be based on the host species. Thus came about the trinomial nomenclature such that *Pneumocystis carinii* was divided into "*formae speciales*" or f. sp. followed by an indication of the host species: *P. carinii* f. sp *hominis* for the human derived pathogen, *P. carinii* f. sp *carinii* and *P. carinii* f. sp *ratti,* both for distinct rat isolates. This was done in accordance to the International Code of Botanical Nomenclature (since the realisation of it being a fungus) [Anon, 1994]. With the progress in genetic analysis of these special forms, it was decided that the isolates from the various host species differ from one another sufficiently to warrant the award of species status to the host specific isolates [Stringer, *et al.*, 1997; Stringer, *et al.*, 2001]. Because the Czech parasitologist Otto Jirovec was credited for associating *Pneumocystis* with plasmacellular pneumonia [Vaněk & Jirovec, 1952, cited in Dutz, 1970], the human pathogen is now named *P. jirovecii* and one of the two distinct rat isolates retained the name *P. carinii* in recognition of Carini's original work [Stringer, *et al.*, 2002; Cushion, 2004]. *P. jirovecii* has been in published use since 1999 [Frenkel, 1999; Stringer, *et al.*, 2002] and its continuation will be decided in 2005 at a conference to be convened by the International Code of Botanical Nomenclature. For the purposes of international conformity, the name *P. jirovecii* will be used throughout this dissertation.

1.1.3 Life cycle and pathogenesis

Three morphologic stages of *Pneumocystis* have been observed in the mammalian lung: the primary proliferative form is known as the trophic stage and the intermediary between the trophic form and the cyst is the precyst. Haploid trophic forms can undergo either binary fission to yield more haploid daughter cells, or such haploid trophic forms can mate, resulting in diploid trophic forms. Synaptonemal complexes have been observed in these diploid stages and suggest meiosis. Such meiotic divisions (two meiotic cycles) lead to the formation of a precyst that undergoes mitosis, with the resultant eight haploid spores contained in a cyst. Spores actively exit the cyst (spore case), and continue the cycle [Yoshida, *et al.*, 1989; Cushion, *et al.*, 1997; Cushion, 2004].

P. jirovecii trophic forms attach to type I alveolar epithelial cells. The *Pneumocystis* major surface glycoprotein (MSG) binds to fibronectin [Nakamura, *et al.*, 1998] and epithelial cell surface glycoproteins via a mannose-dependent mechanism [Limper, *et al.*, 1991]. Vitronectin and laminin bind in a similar manner [Limper, *et al.*, 1993]. As the infection evolves and progresses, the alveolar-epithelial membrane barrier becomes disrupted, resulting in epithelial cell death [Nakamura, *et al.*, 1998]. In addition, *Pneumocystis* releases degradative enzymes such as serine proteases that add to cellular disruption [Breite, *et al.*, 1993]. The result of the tissue destruction is a reduced forced vital capacity, forced expiratory volume in one second, and diffusing capability for carbon monoxide [Nelsing, *et al.*, 1995]. Pathogenesis is enhanced by type II alveolar epithelial cells as they secrete surfactant proteins (particularly surfactant protein A) which bind to MSG and in so doing mask *Pneumocystis* antigens from immunological recognition [Sternberg, *et al.*, 1993].

1.1.4 Clinical disease manifestations

P. jirovecii infection in debilitated infants presents with an interstitial plasma cell pneumonia. The disease begins insidiously with symptoms of poor feeding and

progresses gradually to overt respiratory distress and cyanosis. In the compromised adult patient PcP is associated with shortness of breath, fever, and a nonproductive cough. Sputum is produced occasionally but haemoptysis is rare. PcP in human immunodefiency virus (HIV)-positive patients usually occurs as a more subtle disease with symptoms lasting weeks to months before acute disease presents. On physical examination of patients with PcP, tachypnoea and tachycardia is found in the severely ill. Children may become cyanosed with flaring of the nasal alae. Radiologically a diffuse bilateral infiltrate is observed extending from the perihilar regions [Walzer, 2000]. Clinical presentation differs between HIV-positive and –negative patients. PcP in HIV-negative patients has been associated with a shorter duration of symptoms (5 days vs. 28 days), the patients are more likely to be febrile (92% vs. 76%), have a higher median respiratory rate (30 vs. 23.4 breaths/min), lower median room arterial oxygen tension (52 vs. 69 mmHg), and a higher room air alveolar-arterial oxygen gradient (59 vs. 41 mmHg) [Kovacs, *et al.*, 1984].

1.1.5 Epidemiology

The environmental reservoir of *P. jirovecii* has not been identified; however questions have been raised concerning possible human carriers as the reservoir. Employing DNA amplification techniques, *P. jirovecii* has been detected in some 10% - 51% of HIV-positive and 5.8% - 32% of HIV-negative patients without clinical evidence of pneumonia [Nevez, *et al.*, 1997; Nevez, *et al.*, 1999b; Sing, *et al.*, 1999; Hauser, *et al.*, 2000; Nevez, *et al.*, 2001; Vargas, *et al.*, 2001; Wakefield, *et al.*, 2003; Morris, *et al.*, 2004]. In addition to these sensitive techniques, *P. jirovecii* have been detected by immunofluorescence in post mortem lung biopsies of HIV-positive and –negative patients diagnosed with diseases other than pneumonia. [Nevez, *et al.*, 1999a]. Vargas *et al*. reported detecting *P. jirovecii* DNA in 24 of 74 HIV-negative infants presenting with upper or lower respiratory tract infections (excluding PcP), implicating infants as a reservoir in the transmission cycle [Vargas, *et al.*, 2001]. Further implication of infants has been the detection of *P. jirovecii* DNA in 25% of immunocompetent infants presenting with bronchiolitis [Nevez, *et al.*, 2001]. Patients

with cystic fibrosis may also add to the human reservoir pool as 7.5% have had *P. jirovecii* DNA detected by the polymerase chain reaction (PCR) [Sing, *et al.*, 2001]. However, HIV-positive patients seem to have the highest rates of colonisation (42 / 91); HIV-positive patients from San Francisco and Cincinnati appear to be at greater risk for carriage compared to similar patients in Los Angeles, suggesting a geographic association with risk of colonisation [Morris, *et al.*, 2004]. Molecular epidemiologic studies have indicated that *P. jirovecii* strains identified in adults with active PcP can be found a) in paediatric PcP patients and b) as transient colonisers in immunocompetent infants; again lending support to the possibility that infants may constitute a major reservoir of infectious *Pneumocystis* [Totet, *et al.*, 2003a; Totet, *et al.*, 2003b].

The precise mode of transmission and infectivity of *P. jirovecii* is not clear. There have been reports of transmission of *P. jirovecii* from a mother to her 4.5-week-old infant [Miller, *et al.*, 2002] and from PcP patients to health care workers [Vargas, *et al.*, 2000; Miller, *et al.*, 2001]. These, and reports of outbreaks in transplant units, suggest possible airborne transmission of the fungus [Hennequin, *et al.*, 1995]. Analysis of rural air by spore traps in rural Oxfordshire (far removed from hospitals or laboratories), air samples collected from animal facilities housing *P. carinii*-infected rats, air filtrations collected in wards housing PcP patients, and air samples collected in ward corridors 25m from the patient rooms have yielded DNA of *P. carinii*, *P. ratti* and *P. jirovecii* [Wakefield, 1996; Ollson, *et al.*, 1998]. Studies on the survival and infectivity of *P. carinii* have established that the airborne form of the fungus trapped in airblower prefilters retains viability and infectivity for at least 5 months between -80 ºC and room temperature. It was suggested that at least one life cycle stage of *P. carinii* is dormant and survives outside the mammalian host, and that infection can be initiated by a form that remains viable and infective for long periods in the environment [Kaneshiro & Maiorano, 1996].

Seroepidemiological investigations in the USA and Chile have shown that more than 85% of infants under 2½ years of age have antibodies to *Pneumocystis*, 20% of whom had no evidence of clinical disease [Peglow, *et al.*, 1990; Vargas, *et al.*, 2001].

Serologic studies have shown that *Pneumocystis* has a worldwide distribution although prevalence to specific antigens has geographic variability [Smulian, *et al.*, 1993].

With the emergence of AIDS, *P. jirovecii* has become the most prevalent pulmonary pathogen and causes the most common life-threatening infection (52% primary diagnoses) in these patients [Anon., 1983]. Even with the introduction of highly active antiretroviral therapy (HAART) and chemoprophylaxis *P. jirovecii* remains a major cause of infection in immunocompromised patients in industrialised countries [Wallace, *et al.*, 1997; Calderón, *et al.*, 2004], accounting for up to 35% of all cases of pneumonia in HIV-positive patients admitted to hospital [Rimland, *et al.*, 2002].

PcP in AIDS patients in Africa was initially described as being rare [Elvin, *et al.*, 1989]. Total absence or incidences of less than 10% have been reported [Elvin, *et al.*, 1989; Carme, *et al.*, 1991; Abouya, *et al.*, 1992]. Based on these findings recommendations were made to stop cotrimoxazole prophylaxis in HIV-positive patients in Cote d'Ivoire [Abouya, *et al.*, 1992]. Either through advances in diagnostic strategies or a real increase in incidence, recent reports claim a much higher incidence of PcP, particularly in HIV-positive children. Since 1999, incidence rates in HIV-positive patients of between 43% and 48% have been reported from African studies. [Mahomed 1999, *et al.*; Ruffini, *et al.*, 2002].

1.1.6 Co-infection

Worldwide, PcP and pulmonary tuberculosis (TB), caused by *Mycobacterium tuberculosis*, are the primary lung infections associated with HIV. Their respective prevalence, however, varies geographically. *P. jirovecii* is the most frequent cause of opportunistic pneumonia in the USA and Europe, while *M. tuberculosis* seems to be the main pulmonary pathogen in African HIV-positive patients [Orlovic, *et al.*, 2001]. Dual pulmonary infections with these pathogens have been reported in up to 66% of cases from Africa [Atzori, *et al.*, 1993]. It has been suggested that a surprisingly high

frequency of such co-infections may occur in TB hyper-endemic areas [Orlovic, *et al.*, 2001]. Orlovic *et al*. reported the co-existence of PcP and TB in 9.9% of induced sputum specimens obtained from HIV-positive patients [Orlovic, *et al.*, 2001]. Similarly, Aderaye *et al*. reported the detection of *P. jirovecii* in Ethiopian patients with proven TB in 13.5% HIV-positive and 4.3% HIV-negative patients, respectively. These investigators reported a prevalence of PcP in 30.3% of HIV-positive patients with suspected TB [Aderaye, *et al.*, 2003].

Earlier reports of low incidences of PcP in African countries may have been the result of a low index of suspicion, and poor laboratory techniques. Clinical differentiation between PcP and TB can be difficult as the clinical presentation and radiological appearances may be similar [Hargreaves, *et al.*, 2001; Rennert, *et al.*, 2002]. Malin *et al*. reported pulmonary tuberculosis to be the diagnosis most frequently given to HIV-infected patients with abnormal chest radiography findings consistent with PcP [Malin, *et al.*, 1995].

In addition to TB, rates of co-infection with other pathogens range from 20 – 70% [McLeod, *et al.*, 1989; Hsiao, *et al.*, 1997]. It has been reported that patients with PcP living in developing countries are at high risk of developing co-infections with various pathogens, notably Cytomegalovirus (CMV), *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Cryptococcus neoformans* [McLeod, *et al.*, 1989; Hsiao, *et al.*, 1997].

1.2 LABORATORY DIAGNOSIS

1.2.1 Microscopic detection

Culture confirmation of the presence of *P. jirovecii* is still not possible and diagnosis relies on the microscopic detection of the cysts or trophic forms of the fungus in respiratory fluids such as bronchoalveolar lavage fluids (BAL), tracheal aspirates, induced sputum or from tissues obtained from transbronchial or open lung biopsies.

Staining techniques can be classified as either specific for the sporecase wall such as methenamine silver, immunofluorescence and calcofluor white, and stains that are specific for the trophic forms such as Giemsa and Diff-Quick [Blumenfeld, *et al.*, 1988; Beselski, *et al.*, 1990]. Grocott's methenamine silver stain has traditionally been used to identify the cystic stage while Giemsa is used to stain the trophic stage. These staining techniques are inherently nonspecific. Newer generation stains such as Diff-Quick and Fungifluor have a higher specificity than the older generation stains [Procop, *et al.*, 2004] but immunofluorescence (IF) has subsequently increased the specificity and sensitivity of diagnostic staining [Stratton, *et al.*, 1991; Alvarez, *et al.*, 1997]. Besides the lack of sensitivity and specificity of staining techniques, these tests have other inadequacies such as inability of a single stain to detect all the morphologic stages of the fungus, and observer skill. In addition, it has been shown that staining procedures, which rely on the presence of the cyst wall, lack the ability to differentiate viable cysts from cysts that are empty remnants after successful antibiotic treatment or clearance [Leigh, *et al.*, 1993; Roux, *et al.*, 1994; Armbruster, *et al.*, 1995; Liebovitz, *et al.*, 1995]. Immunofluorescent stains are unable to identify trophic stages of the fungus that lack the antigens present on the cyst wall [Leigh, *et al.*, 1993]. It has been shown that staining methods specific for the cyst wall are not effective when patients are treated with inhaled pentamidine because the structure of the organism may be changed and are therefore missed [Armbruster, *et al.*, 1995].

1.2.2 Polymerase chain reaction

Early diagnosis of PcP in immunocompromised patients, a life threatening complication if not treated correctly, may lower associated morbidity and mortality. The PCR has been reported to be superior in the detection of *P. jirovecii* as it has been shown to be more sensitive (10^4 – 10^6 times as sensitive) and more specific than staining methods [Leigh, *et al.*, 1993]. A number of reports have been published on the PCR for the detection of *P. jirovecii* in clinical samples [Wakefield, *et al.*, 1990a; Wakefield, *et al.*, 1990b; Kitada, *et al.*, 1991; Tamburrini, *et al.*, 1993; Honda, *et al.*, 1994; Lee, *et al.*, 1994; Chouaid, *et al.*, 1995; Moonens, *et al.*, 1995; Skøt, *et*

al., 1995; Mathis, *et al.*, 1997; Rabodonirina, *et al.*, 1997; Ribes, *et al.*, 1997; Huang, *et al.*, 1999; Sandhu, *et al.*, 1999; Sing, *et al.*, 2000; Torres, *et al.*, 2000; Helweg-Larsen, *et al.*, 2002; Kasolo, *et al.*, 2002; Lishimpi, *et al.*, 2002]. The major advantages of the PCR are the ability to detect low numbers of organisms (sensitivity) and method specificity. Studies have indicated that the PCR can rapidly convert from positive to negative once specific treatment for PcP is initiated, and could be a good indicator of treatment response. Pertaining to this, anomalous results were demonstrated on comparing IF with PCR [Armbruster, *et al.*, 1995].

The first report of a PCR for clinical detection of *P. jirovecii* in BAL was by Wakefield *et al*., who employed primers directed at the mitochondrial large subunit ribosomal RNA (mtLSUrRNA) [Wakefield, *et al.*, 1990a]. The sensitivities of various PCR techniques have been reported to be very high, with detection limits for a single round PCR reported as 0.5 – 10 cysts / reaction, and nested PCR 1 – 3 cysts / reaction, depending on the primers used [Tamburrini, *et al.*, 1993; De Luca, *et al.*, 1995; Lu, *et al.*, 1995; Rabodonirina, *et al.*, 1997; Ribes, *et al.*, 1997]. A number of loci on *P. jirovecii* chromosomes have been characterised and evaluated for potential PCR diagnostic purposes. They include: dihydropteroate synthase (DHPS), dihydrofolate reductase (DHFR), thymidylate synthase (TS), internal transcribed spacer regions of the rRNA (ITS), 5S rRNA, 18S rRNA, major surface glycoprotein (MSG), mitochondrial large subunit rRNA (mtLSUrRNA) and mitochondrial small subunit rRNA (mtSSUrRNA) [Wakefield, *et al.*, 1990a; Kitada, *et al.*, 1991; Tamburrini, *et al.*, 1993; Honda, *et al.*, 1994; Lee, *et al.*, 1994; Chouaid, *et al.*, 1995; De Luca, *et al.*, 1995; Moonens, *et al.*, 1995; Lu, *et al.*, 1995; Skøt, *et al.*, 1995; Lane, *et al.*, 1997; Mathis, *et al.*, 1997; Rabodonirina, *et al.*, 1997; Ribes, *et al.*, 1997; Huang, *et al.*, 1999; Sandhu, *et al.*, 1999; Sing, *et al.*, 2000; Torres, *et al.*, 2000; Helweg-Larsen, *et al.*, 2002; Kasolo, *et al.*, 2002; Lishimpi, *et al.*, 2002]. More recently real-time PCR has been described as being a sensitive and specific technique with the potential for quantitative analysis to differentiate colonisation from infection [Kaiser, *et al.*, 2001; Palladino, *et al.*, 2001; Larsen, *et al.*, 2002a; Larsen, *et al.*, 2002b; Larsen, *et al.*, 2004]. On employing different PCR primers and gene targets varying sensitivities and specificities have been reported [De Luca, *et al.*,

1995; Lu, *et al.*, 1995]. Lu *et al*. investigated different PCR reactions targeting 6 regions: ITS nested, 18S ribosomal RNA nested, mtLSUrRNA single, TS single, 5S rRNA single, and DHFR single. The nested PCR reactions (ITS and 18S rRNA) both yielded sensitivities of 100%, however, 18S rRNA PCR amplified DNA from *Saccharomyces cerevisiae* and *Cryptococcus neoformans*. Of the single round PCR reactions mtLSUrRNA yielded a sensitivity of 87%, TS 60%, 5S rRNA 33% and DHFR 23%. TS primers also amplified DNA from *Candida albicans* and *C. neoformans*. The reason for the varying sensitivity of the primers / targets was suggested to be target copy number and ease of denaturation [Lu, *et al.*, 1995]. Huang *et al*. developed a heminested PCR targeting the multicopy MSG gene and reported superior sensitivity to that of mtLSUrRNA single PCR [Huang, *et al.*, 1999]. For the various PCR methods, results indicate sensitivities ranging from 50% - 100% and specificities from 79.3% - 100% [De Luca, *et al.*, 1995; Moonens, *et al.*, 1995; Skøt, *et al.*, 1995; Mathis, *et al.*, 1997; Ribes, *et al.*, 1997; Huang, *et al.*, 1999].

Although PCR based methods are considered generally more sensitive and specific than staining, results may not be available for several hours. Cytochemical staining on the other hand does provide a rapid diagnosis. Recently, real-time PCR has been reported as being a rapid PCR technique with significantly reduced cycling times and without the need for post-amplification detection of amplicons by time-consuming agarose gel electrophoresis. A closed system is ensured and the risk of amplicon contamination dramatically reduced [Palladino, *et al.*, 2001].

Real-time PCR studies employing *P. jirovecii* MSG directed primers have been associated with reports of sensitivities and specificities of between 88% - 100% and 85% - 98.6%, respectively [Flori, *et al.*, 2004; Larsen, *et al.*, 2004]. Real-time PCR employing the DHFR gene primers have resulted in the detection of <5 copies of a DHFR-plasmid construct standard / tube [Larsen, *et al.*, 2002b]. Quantitative interpretations have led to cut-off values being introduced in order to differentiate between colonisation and active infection [Larsen, *et al.*, 2002b; Flori, *et al.*, 2004; Larsen, *et al.*, 2004]. Larsen *et al*., employing *P. jirovecii* MSG real-time PCR on oral washings suggested a cut-off value of 50 copies / tube; ≥50 copies being diagnostic

of active infection and <50 copies supportive of diagnosis but further investigations required [Larsen, *et al.*, 2004]. Flori *et al*. however, suggested a cut-off value of 10^3 copies / tube; >10^4 copies being diagnostic of infection with 10^3 – 10^4 considered a "grey zone" and <10^3 copies being considered chronic carrier / at an early stage of infection development [Flori, *et al.*, 2004]. Both these studies, employing the same PCR targets (MSG) and their characteristic cut-off values, have been able to increase real-time PCR specificity values from 85% to 100% and from 84.9% to 98.6%, respectively [Flori, *et al.*, 2004; Larsen, *et al.*, 2004]. However, Flori *et al*. demonstrated for both mtLSUrRNA single-round standard PCR and MSG real-time PCR 100% sensitivity but 84.9% specificity for real-time PCR vs. 87% specificity for standard PCR [Flori, *et al.*, 2004].

1.3 GENOTYPING AND COALESCENT PHYLOGENETIC ANALYSIS

1.3.1 Strain typing

To establish a genotyping technique to differentiate strains among infected individuals various genomic regions of *P. jirovecii* have been investigated for possible polymorphisms. Seven housekeeping genes have been investigated for potential use in strain typing. The TS gene showed no genetic diversity in 13 specimens collected from patients with PcP. However, this locus could be of use for differentiating strains from different hosts [Latouche, *et al.*, 1997a]. 5S rRNA variations have been detected to the extent that 6 variants were obtained from 20 specimens collected from patients with PcP [Latouche, *et al.*, 1997a]. The aromatic aminoacid synthesis (AROM) locus yielded only two nucleotide polymorphic positions and therefore has limited use for strain differentiation [Tsolaki, *et al.*, 1998]. Similarly, the mtSSUrRNA yielded only few variants [Tsolaki, *et al.*, 1998]. Single strand conformational polymorphism (SSCP) analysis of the β-tubulin and intron of the 26S rRNA genes displayed a limited number of patterns, again suggestive of low genetic diversity [Hauser, *et al.*, 1997; Nahimana, *et al.*, 2000]. MtLSUrRNA has been reported to have sufficient variation for strain typing [Lee, *et al.*, 1993; Latouche, *et al.*, 1997a; Tsolaki, *et al.*, 1998]. Of

18 specimens collected from patients with PcP, 4 groups were identified and it was noted that more than one mtLSUrRNA type could be present per patient specimen [Latouche, *et al.*, 1997a; Latouche, *et al.*, 1997b; Tsolaki, *et al.*, 1998]. The most informative locus displaying the highest degree of polymorphisms is the ITS, making it the most sensitive DNA region for detecting intraspecies biodiversity [Latouche, *et al.*, 1997a; Latouche, *et al.*, 1997b, Tsolaki, *et al.*, 1998]. High degrees of strain diversity have been detected in many studies. Strain diversity, calculated as the number of different strains detected as a percent of the number of specimens analysed, has been reported to be between 27 % (4 strains / 15 specimens) and 82 % (14 strains / 17 specimens) [Lu, *et al.*, 1994; Tsolaki, *et al.*, 1996; Latouche, *et al.*, 1997a; Latouche, *et al.*, 1997b, Lee, *et al.*, 1998; Miller, *et al.*, 1999; Atzori, *et al.*, 2001; Nevez, *et al.*, 2001a; Volpe, *et al.*, 2001; Matos, *et al.*, 2003; Nevez, *et al.*, 2003].

Typing, employing ITS nucleotide sequence variations, has become an important tool for epidemiological studies of PcP. Various authors have used different methods for naming ITS types, but Lee *et al*. constructively established a consensus ITS1 sequence of 161 bp and ITS2 of 192 bp. Fifteen ITS1 sequevars were named with capitals A through O and fourteen ITS2 sequevars were named in lowercase a through n [Lee, *et al.*, 1998].

Since ITS strain typing has been applied to *P. jirovecii*, globally prevalent types have been identified, along with investigations into geographical variations, distribution in infected lungs, recurrent infections, and strain transmissibility. ITS type Eg (23 – 86% prevalence) and Ne (11.6 – 56% prevalence) have been found to be the most prevalent types with a worldwide distribution [Tsolaki, *et al.*, 1996, Lee, *et al.*, 1998, Miller, *et al.*, 1999, Tsolaki, *et al.*, 1999; Helweg-Larsen, *et al.*, 2001; Nimri, *et al.*, 2002; Matos, *et al.,* 2003]. Types Be, Hh, Nb and Nc have only been detected in the USA [Lee, *et al.*, 1998]. Types Ne and Na have been reported to be the most frequent types obtained from second and subsequent episodes of PcP and it was suggested that they could represent more transmissible or persistent strains [Miller, *et al.*, 1999; Tsolaki, *et al.*, 1999]. Uncertainty exists as to the genetic stability of the

ITS locus and whether presentation of PcP is due to reactivation of latent strains or *de novo* acquisition from the environment. Studies have reported the persistence of a specific type over short periods during the same episode of PcP, as well as persistence of the same type during subsequent episodes, suggesting either failure to clear the fungus or reacquisition of the same strain from the environment [Latouche, *et al.*, 1997b; Tsolaki, *et al.,* 1999]. These observations have been used to suggest that genotypes are stable *in vivo* and validate the reproducibility of the technique [Latouche, *et al.*, 1997b]. However, apparent genotype switching (the detection of different genotypes at different time points during one episode of PcP) has been observed frequently [Tsolaki, *et al.*, 1996; Helweg-Larsen, *et al.*, 2001; Matos, *et al.*, 2003]. Helweg-Larsen *et al*. reported genotype switching in 6 / 10 patients with recurrent PcP and in 10 / 19 patients who had repeat bronchoscopy investigations within the same episode of PcP. It was noted that such genotype changes were found only in samples obtained after an interval of >6 days in the latter group [Helweg-Larsen, *et al.*, 2001a]. Keely *et al*., on sequencing two distinct genes, the ITS regions encoded by nuclear chromosomes and the mtLSUrRNA gene located in the mitochondrion, reported that changes in the ITS types correlated to changes seen in the mtLSUrRNA. They concluded that the coincidence of changes in the sequence of these genes, one nuclear and the other mitochondrial encoded, excluded mutation as the cause of the genetic differences between *P. jiroveci* strains isolated during different episodes of PcP and supported the hypotheses that recurrent PcP is caused by re-infection rather than by reactivation of latent organisms [Keely & Stringer, 1997]. Co-infection with more than one ITS genotype per PcP episode has been demonstrated in up to 60% of specimens, 17% have been reported to harbour three genotypes, 3% four genotypes and one study found 2 / 207 specimens containing 6 genotypes. [Lu, *et al.*, 1994; Lee, *et al.*, 1998, Tsolaki, *et al.*, 1998; Miller, *et al.*, 1999; Tsolaki, *et al.*, 1999; Nevez, *et al.*, 2001a]. There seems to be a geographic tendency to co-infection frequency as 75% patients from Atlanta were seen to harbour significantly more strain co-infections than San Francisco (6.7%) and Seattle (8.3%) [Nimri, *et al.*, 2002]. Finding multiple strains per PcP episode has been supported by genotyping employing different genes / techniques. Genotyping with mtLSUrRNA has also shown co-infection with different strains

[Latouche, *et al.*, 1997a; Latouche, *et al.*, 1997b, Tsolaki, *et al.*, 1998]. In support of DNA sequence variation data as described above, Nahimana *et al*. and Hauser *et al*. using single strand conformational polymorphism (SSCP) of 4 genomic regions reported up to 65% of patients co-infected with different strain types [Hauser, *et al.*, 1997; Nahimana, *et al.*, 2000]. Discrepancies have been detected on comparison of either ITS or mtLSUrRNA types obtained from post-mortem lung biopsies and other specimens [Atzori, *et al.*, 2001]. In addition, compartmentalisation of mtLSUrRNA and ITS types were observed in discrete sections of lung, suggesting that it cannot be assumed *a priori* that from one specimen strain types are fully representative of the whole organ [Ambrose, *et al.*, 2001; Atzori, *et al.*, 2001; Helweg-Larsen, *et al.*, 2001a]. Based on such observations it was suggested that *P. jiroveci* infection is not a clonal infection, and that repeated *de novo* acquisition of the opportunistic pathogen is likely to occur [Tsolaki, *et al.*, 1996].

1.3.2 Coalescent statistical parsimony network analysis

1.3.2.1 *P. jirovecii* ribosomal DNA

The nuclear rRNA is encoded by an operon consisting of the 18S, 5.8S, and 26S rRNA genes occurring in tandem. Between the 18S and 5.8S and between the 5.8S and 26S rRNA are the internal transcribed spacers 1 and 2 (ITS1 and ITS2), respectively. The 18S and 26S rRNA genes are flanked by the 5' external transcribed spacer (5' ETS) and 3' external transcribed spacer (3' ETS), respectively (Figure 1.1). The operon is referred to as ribosomal DNA (rDNA). During transcription by RNA polymerase I, a 35S pre-rRNA transcript is created that contains the above mentioned elements. During maturation of the pre-rRNA, the transcribed spacers are removed in a series of processing steps carried out by endo- and exonucleases [Venema, *et al.*, 1995]. The pre-rRNA folds into a cruciform-like structure which directs maturation by providing targets for RNA binding, and bringing cleavage sites into closer proximity. Initial cleavage occurs within ITS1 followed by maturation of the 3' 18S termini and 5' 5.8S termini. Subsequent cleavage reactions yield the matured 18S, 5.8S and 26S rRNA's without transcribed spacers [Good, *et al.*, 1997]. Analysis

of ITS1 has suggested that it has always functioned as a spacer between the prokaryotic unlinked 16S and 23S rRNA genes. ITS2 however, is considered to have evolved from a former expansion segment (ES), elements that cause length variations in eukaryotic 25/28S rRNA, which at the base of the eukaryotic tree separated the 5' part from the rest of the eukaryotic analogue to the 23S rRNA of *Escherichia coli* [Torres, *et al.*, 1990].

Figure 1.1 *P. jirovecii* ribosomal DNA structure
Redrawn from Edman *et al*. [Edman, *et al.*, 1989]

1.3.2.2 ITS based phylogenetic reconstruction

The ITS gene sequences have been used for phylogenetic reconstruction in many orders and genera, particularly plants and lower eukaryotes [Torres, *et al.*, 1990; O'Donnel & Cigelnik, 1997; Hijri, *et al.*, 1999; Pélandakis, *et al.*, 2000; Marcilla, *et al.*, 2001; Marcilla, *et al.,* 2002; Rodriguez-Lanetty, 2003]. Ribosomal DNA, and particularly ITS sequences, have been reported to be useful markers for resolving supra specific, specific, subspecific, strain and population-level relationships in organisms [Marcilla, *et al.*, 2001; Marcilla, *et al.*, 2002]. Since spacer regions evolve under fewer constraints than protein coding genes, the rate of spacer evolution is very fast. This makes inferences based on spacer phylogenies particularly useful for studying divergences within the last 50 million years [Hillis & Dixon, 1991]. Ribosomal DNA spacer regions are not free from constraints as are pseudogenes, as they have to maintain a certain degree of secondary structure, the maintenance of which requires compensatory mutations for stabilising stem-loop structures [Hillis & Dixon,

1991]. Most eukaryotic organisms contain many, up to thousands, of copies of rDNA paralogues, homogenisation of the sequences occurs by concerted evolution. It is mainly intergenic recombination, unequal crossing over, and gene conversion that homogenises the sequences of the paralogous rDNA repeats [Buckler, *et al.*, 1997]. Guintoli *et al*. has shown that *Pneumocystis* obtained from rats have at most 2 copies of rRNA [Guintoli, *et al.*, 1994], reminiscent of *Tetrahymena* that contains exceptionally few rDNA copies [Yao, *et al.*, 1974]. Despite the fact that ITS sequences have been successful for reconstructing phylogenies among taxa and species, discordant or unresolved results have been reported, these have been attributed mainly to xenology and homoplasy [O'Donnel, *et al.*, 1997; Hijri, *et al.*, 1999]. Despite the widespread use of ITS sequence typing for epidemiological studies of *P. jirovecii*, no phylogenetic or population genetic analyses have been forthcoming. Helweg-Larsen reported that phylogenetic attempts employing Neighbour-Joining failed to resolve, as bootstrap values were all < 0.5 [Helweg-Larsen, *et al.*, 2001b]. In addition Hsueh *et al*. published a Maximum Likelihood tree of ITS sequences obtained from *Pneumocystis* isolated from *Maccaca mulata*, however, resolution was poor and no useful inferences could be made [Hsueh, *et al.*, 2001].

1.3.2.3 Intraspecific gene genealogies

Classical phylogenetic heuristic methods were designed to infer relationships between genes sampled from individuals belonging to different species (phylogeny *sensu strictu*) [Posada & Crandall, 2001]. The different species are assumed to be hierarchical and the result of reproductive isolation and population fissions, leading to fixation of mutations and non-overlapping gene pools. The evolution of such systems is assumed to be tree like with dichotomous branching patterns since it infers speciation events [Posada & Crandall, 2001; Rosenberg & Nordborg, 2002]. The direction of evolution, although it is not always known if the tree is unrooted, is assumed to be linear [Allaby & Brown, 2001]. Tokogeny, however, is not hierarchical but the result of sexual reproduction or recombination, with resultant relatively recent mutations not yet fixed in the population [Posada & Crandall, 2001]. The genealogical outcomes of intraspecific evolution, such as recombination and homoplasies

generates reticulate relationships for which classical phylogenetic algorithms such as Maximum Likelihood, Maximum Parsimony or Minimum Evolution, do not compensate for, and their assumptions are violated [Posada & Crandall, 2001]. The results of a recent evolutionary timescale means that ancestral states still exist and multiple apomorphies are being fixed or lost from the population, resulting in a multifurcating rather than a dichotomous branching pattern [Allaby & Brown, 2001]. Population genetic methods that allow persistent ancestral nodes, multifurcations and reticulations are necessary to account for intraspecific population dynamics. The stochastic process known as the coalescent is an extension of classical population genetic models. The underlying theme is that in the absence of selection, sampled lineages can be viewed as randomly picking their parents as one goes back in time. Whenever two lineages pick the same parent they are said to coalesce. Eventually all lineages coalesce into the most recent common ancestor (MRCA) of the sample [Rosenberg & Nordborg, 2002]. Modelling population structure as a network does not assume reproductive isolation and enables multiple ancestry, apomorphy and extant ancestral states to be portrayed, and is a much more realistic description than tree building for intraspecific analyses [Allaby & Brown, 2001].

1.4 TREATMENT AND RESISTANCE DEVELOPMENT

1.4.1 Standard treatment and prophylaxis

The first report that trimethoprim sulphamethoxazole (TMP-SMX) (cotrimoxazole) may be of clinical use in treating PcP was by Hughes *et al*. who in 1974 demonstrated the efficacy of cotrimoxazole in a murine PcP model [Hughes, *et al.*, 1974]. A follow up clinical study demonstrated the efficacy of TMP-SMX at 20/100 mg/kg per day, administered for 14 days for treating PcP in humans [Hughes, *et al.*, 1975]. With the advent of the AIDS pandemic it was realised that the outcome of AIDS and non-AIDS associated PcP was similar with cotrimoxazole treatment, however, AIDS patients required longer duration of therapy to attain the same results. In addition, it was established that intravenous vs. oral cotrimoxazole

presented no statistically significant advantage to treatment outcome [PCP Therapy Group, 1984]. Later studies comparing aerosolised pentamidine to cotrimoxazole reported a greater risk (3.25 X) for recurrence of PcP if aerosolised pentamidine treatment was administered [Hardy, *et al.*, 1992]. Many treatment failures or failure of prophylaxis using high dose cotrimoxazole were attributed to non-compliance due to side effects. This prompted further comparisons of cotrimoxazole, aerosolised pentamidine and dapsone. Ioannidis *et al*. reported cotrimoxazole to be superior and lower doses (one double strength tablet three times per week instead of daily) increased tolerance to this agent without losing efficacy. Low doses of dapsone had less toxic effects, but this was at the expense of efficacy. It was observed that although high doses of aerosolised pentamidine improved efficacy it failed to prevent toxoplasmosis, and it was concluded that the lower dose cotrimoxazole was the superior regimen for preventing PcP in patients with AIDS [Ioannidis, *et al.*, 1996].

Highly active antiretroviral therapy (HAART) remains the most effective approach to preventing opportunistic infections in HIV-infected persons; however, some patients are unable to take HAART, are not ready to start therapy or have failed therapy. In addition to HAART, specific prophylactic regimens provide survival benefits, even for those receiving HAART. Primary prophylaxis for opportunistic infections in adults and adolescents (including pregnant woman and patients receiving HAART) is recommended to be initiated when $CD4^+$ cell counts reach <200/μl, if the person has a history of oropharyngeal candidiasis, if the $CD4^+$ ratio drops below 14%, or if the person had a history of an AIDS-defining illness. Recommended chemoprophylaxis is cotrimoxazole, 1 double strength tablet per day or 1 single strength tablet per day. Alternatively, 1 double strength tablet three times a week can be used. Alternative regimens include dapsone, dapsone plus pyrimethamine plus leucovorin, or aerosolized pentamidine. It is considered safe to discontinue primary prophylaxis in patients responding to HAART with a 3-6 month sustained rise in $CD4^+$ cells from <200/μl to >200/μl. Patients experiencing a recurring episode should be administered secondary prophylaxis (long term maintenance chemoprophylaxis) with the above mentioned regimens. Evidence suggests it safe to suspend secondary prophylaxis

when a patient's $CD4^+$ cell count exceeds 200/μl for ≥3 months as a consequence of HAART [USPHS/IDSA Prevention of Opportunistic Infections Working Group, 1999].
In children indications for initiating primary prophylaxis are: HIV-infected or HIV-indeterminate infants aged 1-12 months; HIV-infected children 1-5 years with CD4+ cells <500/μl or $CD4^+$ ratio < 15%; HIV-infected children 6-12 years with $CD4^+$ <200/μl or $CD4^+$ ratio of <15%. The following is recommended as primary prophylaxis in these groups: cotrimoxazole at 150/750 mg/m^2/day in 2 two divided doses three times a week on consecutive days, or single dose three times a week on consecutive days, or 2 divided doses three times a week on alternate days. Alternative regimens include dapsone, aerosolised pentamidine or atovaquone. If PcP recurs after treatment of an acute infection, prophylaxis is recommended for life. The regimen is as above for primary prophylaxis [USPHS/IDSA Prevention of Opportunistic Infections Working Group, 1999].

The drug of choice for treatment of PcP in adults and paediatric patients is cotrimoxazole at TMP 15 mg/kg/day, SMX 75 mg/kg/day, orally or intravenously in 3 or 4 divided doses for 14–21 days. Alternatively, pentamidine, trimetrexate, atovaquone or primaquine may be used. [Walzer, *et al.*, 2000].

1.4.2 Mechanism of action of cotrimoxazole

Folates play an essential role in metabolism where they act as cofactors. Folates are synthesised *de novo* in prokaryotes and lower eukaryotes, but higher organisms need preformed folates to be absorbed via the diet. In the biosynthesis of folates (Fig 1.2) *p*-aminobenzoic acid (*p*ABA) condenses with 2-amino-4-hydroxy-6-hydroxymethyl-7,8-dihydropteridine pyrophosphate (DHPPP), a reaction catalysed by dihydropteroate synthase (DHPS) [E.C. 2.5.1.15], to form dihydropteroate (DHP). Dihydrofolate synthase (DHFS) [E.C. 6.3.2.12] then catalyzes the addition of glutamate to DHP to produce dihydrofolate (DHF). DHF is then reduced by dihydrofolate reductase (DHFR) [E.C. 1.5.1.3] to produce tetrahydrofolic acid, i.e.

folate, in a form available as cofactor for many essential reactions of metabolism [Ouellete, *et al.*, 1998].

Trimethoprim and sulfamethoxazole are substrate analogues in folate metabolism in prokaryotes and lower eukaryotes and act by competitive inhibition. Sulphamethoxazole (SMX) targets the enzyme DHPS, which catalyzes the condensation of *p*ABA, to which SMX is an analogue, with dihydropteroate pyrophosphokinase (DHPPP). In addition to sulphonamides competitive inhibition of DHPS resulting in inhibition of folate synthesis, sulpha drugs are metabolised to the sulpha-containing folate analogue sulpha-dihydropteroate (sulpha-DHP) that in itself inhibits metabolism [Patel, *et al.*, 2003]. Trimethoprim (TMP) acts on DHFR, which catalyses the reduction of DHF to tetrahydrofolate. Sulphonamides and trimethoprim act in tandem to inhibit folate biosynthesis [Ouellette, *et al.*, 1998].

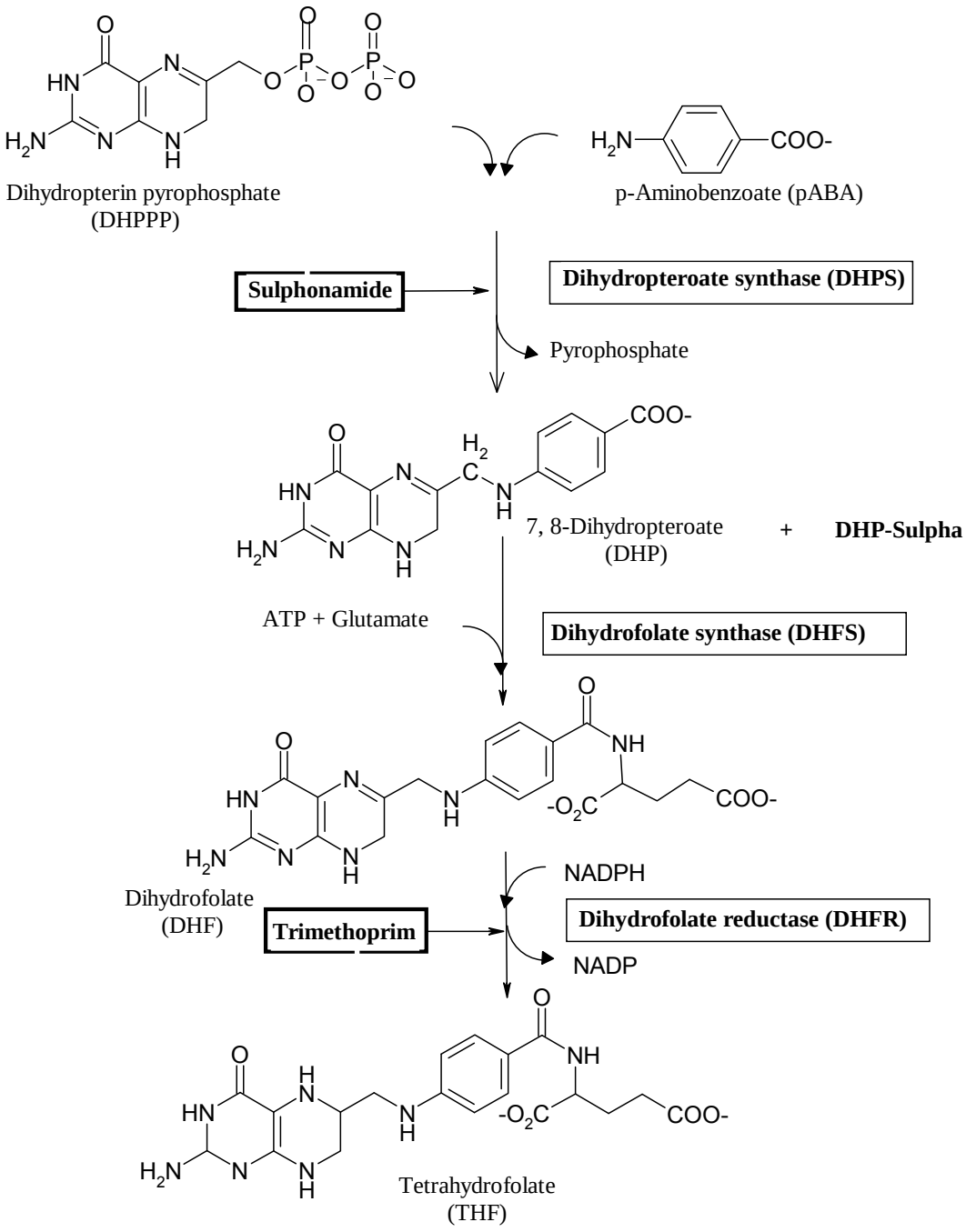


Figure 1.2 Schematic diagram of the folate synthesis pathway

Key enzymes and inhibitors are boxed. Based on Ouellette, *et al.*, 1998.

1.4.3 Development of resistance to cotrimoxazole

P. carinii DHPS was characterised in 1992 as being part of a trifunctional folic acid synthase (fas) gene encoding dihydroneopterin aldolase, hydroxymethyldihydropterin

pyrophosphokinase (DHPPK) and DHPS [Volpe, *et al.*, 1992; Volpe, *et al.*, 1993]. Lane *et al*. compared the DHPS gene of *Pneumocystis* from various hosts and observed polymorphisms suggestive of different evolutionary pressures. The ratio of nonsynonymous:synonymous amino acid changes in the human (100%) was greater than that from the rodent isolates (36%) and the transversion:transition ratio was higher in human (0.54) than rodent isolates (0.07). These observations imply that human isolate's DHPS genes have been under selective pressure, possibly due to longterm exposure to sulphonamides [Lane, *et al.*, 1997]. Polymorphisms reported in *P. jirovecii* DHPS genes are at codon positions 23, 55, 57, 60, 111, and 248. Three of these, at positions 55, 57, and 60 were similar to DHPS "hotspots" of other organisms known to be involved in sulphonamide resistance development [Lane, *et al.*, 1997]. The detection frequency of *P. jirovecii* DHPS genes with mutations at codons 55 and 57 from Europe and the USA, have been reported to be between 20.4% - 69.5% [Mei, *et al.*, 1998; Helweg-Larsen, *et al.*, 1999; Santos, *et al.*, 1999; Visconti, *et al.*, 1999; Beard, *et al.*, 2000; Huang, *et al.*, 2000; Takahashi, *et al.*, 2000; Nahimana, *et al.*, 2003; Kazanjian, *et al.*, 2004; Montes-Cano, *et al.*, 2004].

The first association of DHPS mutations and prophylaxis / sulphonamide exposure was made by Kazanjian *et al*. who in 1998 observed that mutations at positions 55 and 57 were more common in patients who received and failed sulpha or sulphone prophylaxis (5/7) than those who did not receive (2/13) prophylaxis [Kazanjian, *et al.*, 1998]. Subsequently more reports appeared that associated sulphone / sulpha exposure with mutations at codon 55 and 57, suggesting development of mutations during treatment / prophylaxis [Ma, *et al.*, 1999; Nahimana, *et al.*, 2003a]. DHPS mutations have been reported in 62% - 80.3% of patients exposed to sulpha / sulphone agents compared to 23% - 53.8% in patients not receiving these drugs [Helweg-Larsen, *et al.*, 1999; Santos, *et al.*, 1999; Huang, *et al.*, 2000; Kazanjian, *et al.*, 2000; Nahimana, *et al.*, 2003a]. Mutation frequency has been reported to be increasing; in patients receiving sulpha prophylaxis from 46% in 1994-1995 to 88% in 2000-2001 and in patients not exposed from 38% in 1996-1997 to 54% in 2000-2001 [Kazanjian, *et al.*, 2004].

Geographic location has been shown to be an independent predictor for harbouring a mutated strain [Beard, *et al.*, 2000; Huang, *et al.*, 2000]. Specific geographic areas have been associated with a markedly increased risk of mutations occurring. The organisms from the West coast of the USA, including San Francisco and Seattle, have been shown to have a significantly higher incidence of mutations than those in Atlanta [Beard, *et al.*, 2000; Huang, *et al.*, 2000]. On a finer scale, hospitalisation in a specific hospital has been associated with resistance gene detection [Nahimana, *et al.*, 2003a]. These studies have suggested that acquisition of mutated strains may occur *de novo* from the environment or spread from person-to-person. [Beard, *et al.*, 2000; Huang, *et al.*, 2000; Nahimana, *et al.*, 2003a].

The role of DHPS mutations in clinical outcome is still controversial. DHPS mutations have been associated with failure of sulphonamide prophylaxis [Kazanjian, *et al.*, 1998; Mei, *et al.*, 1998; Kazanjian, *et al.* 2000; Visconti, *et al.*, 2001]. However, little evidence exists that treatment failure is associated with DHPS mutations in that a) it has been reported that the majority of patients with DHPS mutations respond to standard cotrimoxazole therapy [Kazanjian, *et al.*, 2000], b) both DHPS wild type and mutation types have similar clinical responses to standard cotrimoxazole therapy [Visconti, *et al.*, 2001], and c) pathophysiological factors appear to have a greater degree of association with treatment failure than DHPS mutations [Kazanjian, *et al.*, 1998]. A few reports have suggested an association of DHPS mutations with sulphonamide therapy failure, Kazanjian *et al*. reported 7% of patients with DHPS wild type genes failed therapy, while 28% of patients harbouring DHPS mutations failed therapy [Kazanjian, *et al.*, 2000]. What has become evident is that sulphonamide exposure and length thereof, increases the risk of acquiring DHPS mutations [Ma, *et al.*, 1999; Huang, *et al.*, 20000; Kazanjian, *et al.*, 2000; Nahimana, *et al.*, 2003b]. Despite the lack of clinical data on treatment failure, *in vitro* site-directed mutagenesis of the orthologous folic acid synthase gene of *Saccharomyces cerevisiae* have indicated that DHPS mutations at codon 55 and 57 may confer resistance to sulpha / sulphones. However, results indicated that a single mutation at codon 55 renders the enzyme resistant to sulphanilamide and a single mutation at codon 57 renders the enzyme resistant to sulphanilamide and sulphadoxine. The

paradox however, is that the double mutation 55 + 57 renders the organism hypersensitive to sulphamethoxazole and dapsone. In addition, the double mutant enzyme has a reduced affinity for the substrate (*p*ABA) – and growth requires the addition of *p*ABA [Iliades, *et al.*, 2004; Meneau, *et al.*, 2004].

It is well known that other pathogens, notably *Escherichia coli, Streptococcus pneumoniae, Neisseria gonorrhoea* and *Plasmodium falciparum* develop stepwise resistance to these agents by point mutations in the gene coding for the sulphonamide target DHPS and trimethoprim target DHFR [Lopez, *et al.*, 1987; Swedberg, *et al.*, 1993; Brooks, *et al.*, 1994; Wang, *et al.*, 1997; Qvarnaström & Swedberg, 2000]. The region at position 55 - 57 comprises Thr-Arg-Pro corresponding to the *E. coli* DHPS gene positions 62 - 64, a region known to be an active site involved in binding both substrate and sulphonamide. It has been found to be a highly conserved region in all DHPS sequences identified to date. In the *E. coli* DHPS gene, Thr_{62} is involved in binding the substrate (pterin) with two hydrogen bonds, one of which is lost if Thr is replaced by Ala. Thr_{62} in *E. coli* is homologous to Thr_{55} in *P. jirovecii*. The adjacent amino acid in *E. coli* is Arg_{63}, its homologue in *P. jirovecii* is Arg_{56}, which is involved in binding both pterin substrate and sulphonamides. Thus, the substitution of Ala_{55} for Thr_{55} in the *P. jirovecii* enzyme is likely to affect Arg_{56} and its ability to bind sulphonamides. In addition to this, the observed substitution of Ser_{57} for Pro_{57}, which is also adjacent to Arg_{56}, could affect binding to both substrate and drug [Kazanjian, *et al.*, 1998].

Studies have indicated that, in contrast to the high prevalence of DHPS mutations, DHFR polymorphisms are rare [Ma, *et al.*, 1999]. A lower selective pressure is thought to be exerted by trimethoprim on DHFR, since it has been shown from *in vitro* studies and animal models that trimethoprim contributed only marginally to the efficacy of cotrimoxazole [Edman, *et al.*, 1989; Ma, *et al.*, 1999]. On comparing percentages of isolates harbouring DHPS (43%) and DHFR (0%) mutations, it was suggested that DHPS gene mutations were not random, but indicative of selective pressure on DHPS and less so on DHFR [Ma, *et al.*, 1999]. DHFR gene polymorphism was reported for the first time in 2002 from Japan, where DHFR genes

with synonymous and nonsynonymous nucleotide substitutions were observed. The two synonymous substitutions T540C and T312C and two amino acid substitutions Ala67Val and Cys116Tyr did not correlate with cotrimoxazole treatment failure. The authors suggested that Cys116Tyr may have been selected during therapy [Takahashi, *et al.*, 2002]. It was reported that DHPS but not DHFR polymorphisms contributed to cotrimoxazole PcP treatment failures [Takahashi, *et al.*, 2002].

In South Africa, Ruffini and Madhi found 28% of HIV-1 infected children that developed PcP, had a history of being on cotrimoxazole prophylaxis at the time of their illness, raising concern to the possible emergence of sulphonamide resistant strains in South Africa [Ruffini & Madhi, 2002].

1.5 OBJECTIVES

1. To compare a) different PCR primers directed at various gene targets, and b) PCR techniques with IF microscopy for the detection of *P. jirovecii* in clinical specimens.

2. To determine the extent of co-infection associated with PcP in South Africa.

3. To determine local *P. jirovecii* ITS1-5.8S-ITS2 rDNA strain types in Cape Town, and model lineage evolution employing a coalescent-theory based statistical parsimony network analysis.

4. To investigate the possible emergence of cotrimoxazole-resistant strains of *P. jirovecii* in South Africa.

CHAPTER 2

MATERIALS AND METHODS

2.1 CLINICAL SPECIMENS

2.1.1 Clinical specimens for PCR performance evaluation

For PCR performance evaluation, 78 sequential clinical specimens submitted to the Pathology Laboratories at Tygerberg Hospital, South Africa, for identification of *P. jirovecii* (June 2001 to April 2002) were collected and stored at -80°C. Specimens comprised: sputum (14), tracheal aspirates (TASP) (50), bronchoalveolar lavage fluids (BAL) (9) and fresh lung biopsy tissues (5). In addition, archived formalin-fixed, paraffin-wax embedded lung histology sections (12) previously confirmed (1981 - 1999) as PcP-positive by methanamine silver staining, were obtained from the Department of Anatomical Pathology (1981 to 1999) and included in the study.

2.1.2 Sputum specimens for co-infection investigations

Microbiology and virology laboratory data obtained from the 78 sequential specimens employed in section 2.1.1 were investigated for indications of co-infections. In addition to routine records, HIV data were available for 40 of the 78 patients. *M. tuberculosis* culture-confirmed sputum specimens (663) were obtained from the Medical Research Council (South Africa) Unit for Tuberculosis Operational and Policy Research. The specimens were collected from patients residing in five provinces in South Africa *viz.* KwaZulu Natal (192), Western Cape (72), Eastern Cape (148), North West (177) and Mpumalanga (74). HIV status for 648 of 663 patients was known.

2.1.3 Specimens for ITS genotyping

Clinical specimens (20) were obtained from 19 patients diagnosed with PcP, attending the Tygerberg Hospital. Included were three specimens from twin babies both of whom were HIV-positive. The babies presented with PcP simultaneously and were admitted together to the paediatric intensive care unit. From twin 1 two specimens were analysed: a tracheal aspirate and lung biopsy (collected six days after the tracheal aspirate), and from twin 2 a lung biopsy was obtained concurrently with the lung biopsy of twin 1.

2.2 DIAGNOSTIC DATA: IMMUNOFLUORESCENCE AND CO-INFECTION

2.2.1 Indirect immunofluorescence

Indirect IF was performed as the standard diagnostic test by medical technologists at Tygerberg Hospital. The DETECT IF *Pneumocystis carinii* indirect immunofluorescence kit (Shield Diagnostics Ltd. Dundee, UK) was used to detect *P. jirovecii* cysts in clinical samples according to the manufacturer's instructions.

2.2.2 Co-infection

Microbiology and virology laboratory records of the patients diagnosed with PcP by indirect immunofluorescence were investigated retrospectively for reports of other pathogens detected during the episode of PcP. HIV status data were obtained concurrently where available.

TB culture confirmed specimens (2000 – 2003) were obtained from the Medical Research Council, Unit for Tuberculosis Operational and Policy Research. HIV data of the patients from whom specimens were taken was available for 648 of 663 patients. Specimens were supplied as accession numbers and no patient names were made available. The only demographic data provided were provinces of residence of the patients.

2.3 PCR INVESTIGATIONS

2.3.1 Sample treatments prior to DNA extraction

2.3.1.1 BAL's, tracheal aspirates and sputum

Mucoid specimens of 1 to 5 ml were treated with an equal volume of 0.1 M 1,4-dithiothreitol (DTT) (Roche Molecular Biochemicals, Mannheim, Germany), vortexed and incubated at 37°C for 30 min until liquefied. The suspension was centrifuged at 3 000x**g** for 15 min and the supernatant discarded. The pellet was resuspended in 500 µl sterile deionised water (dH_2O). DNA extraction was performed using the High Pure Template Preparation Kit (Roche) as for isolation of nucleic acids from whole blood, buffy coat, or cultured cells according to the manufacturer's instructions with a few modifications. Briefly, Tissue Lysis Buffer (4 M urea, 200 mM Tris, 20 mM NaCl, 200 mM EDTA, pH 7.4) and 20 µl Proteinase K (20 mg/ml) was added to 200 µl sample, vortexed and incubated overnight until a clear homogeneous liquid was obtained. DNA was then extracted employing the Roche system as described in section 2.3.2.1.

2.3.1.2 Fresh biopsy tissue

Extraction from fresh biopsy tissue was performed using the High Pure Template Preparation Kit (Roche) as for isolation of nucleic acids from mammalian tissue. Briefly, 25 – 50 mg of tissue was mechanically disrupted with a sterile pestle. Tissue Lysis Buffer (4 M urea, 200 mM Tris, 20 mM NaCl, 200 mM EDTA, pH 7.4) and 40 µl Proteinase K (20 mg/ml) was added to the sample, vortexed and incubated at 37°C overnight until liquefied. After the overnight incubation an additional 40 µl Proteinase K (20 mg/ml) was added, and incubated at 56°C for 2 h. Remaining insoluble tissue was carefully removed by drawing up with a 200 µl pipette tip. DNA was then extracted from the solution employing the Roche system as described in section 2.3.2.1.

2.3.1.3 Formalin-fixed paraffin wax-embedded tissue

Extraction from formalin-fixed paraffin wax-embedded tissue was performed using the High Pure Template Preparation Kit (Roche) as for isolation of nucleic acids from formalin-fixed paraffin wax-embedded tissue sections, according to the manufacturer's instructions. To deparaffinize tissue sections 3 – 4 microtome slices were soaked in xylene (Sigma-Aldrich, Missouri, USA) for 30 min. The sample was centrifuged at 6 000x**g** for 10 sec and the supernatant discarded. The sections were then incubated for 10 sec in the following series of ethanol concentrations to rehydrate the tissue: 100 % ethanol, 80 %, 60 %, 40 % and lastly in dH_2O. The sample was centrifuged at 6 000x**g** for 10 sec and the supernatant was discarded. Of the remaining tissue, 25 – 50 mg was used and processed as follows: Tissue Lysis Buffer (4 M urea, 200 mM Tris, 20 mM NaCl, 200 mM EDTA, pH 7.4) and 40 µl Proteinase K (20 mg/ml) was added to the sample, vortexed and incubated overnight at 37°C until liquefied. After the overnight incubation an additional 40 µl Proteinase K (20 mg/ml) was added and incubated at 56°C for 2 h. Remaining insoluble tissue was removed by carefully drawing up with a 200 µl pipette tip. DNA was then extracted from the solution employing the Roche system as described in section 2.3.2.1.

2.3.1.4 TB-positive sputum specimens (663 specimens, section 2.1.2)

Specimens (200 µl) were treated with an equal volume of 1N NaOH, 0.1N sodium citrate and N-acetyl-L-cysteine. The suspension was vortexed and incubated at 37°C for 30 min until liquefied. After centrifugation at 3 000x**g** for 15 min the supernatant was discarded and DNA extracted from the pellet employing the Promega system as described in section 2.3.2.2.

2.3.2 DNA extraction

2.3.2.1 DNA extraction: Roche system

To 200 µl treated specimen an equal volume binding Buffer (6 M guanidine-HCl, 10 mM urea, 10 mM Tris-HCl, 20% Triton X-100 (v/v), pH 4.4) was added and the solution was incubated at 72°C for 10 min. Isopropanol (Sigma) was added and mixed. The mix was then transferred to the upper reservoir of a combined High Pure filter tube-collection tube assembly and centrifuged at 6 000x**g** for 1 min. The elute and collection tube was discarded and the filter combined with a new collection tube. Inhibitor Removal Buffer (ethanol, 5 M guanidine HCl and 20 mM Tris-HCl at pH 6.6) was added and the assembly centrifuged at 6 000x**g** for 1 min. The elute and collection tube was discarded. The filter was then combined with a new collection tube; Wash buffer (ethanol, 20 mM NaCl and 2 mM Tris-HCl, at pH 7.5) was added and centrifuged at 6 000x**g** for 1 min. The elute and collection tube was discarded and the filter combined with a new collection tube. This step was repeated once. The assembly was then centrifuged at maximum speed (16 000x**g**) for 10 sec to remove any residual Wash Buffer. The collection tube was discarded and the filter combined with a 1.5 ml sterile reaction tube. Prewarmed (70ºC) Elution Buffer (10 mM Tris at pH 8.5) was added and the assembly centrifuged at 6 000x**g** for 1 min. Aliquots of the extracted DNA were stored at – 80ºC and a working aliquot kept at 4 – 8ºC.

2.3.2.2 DNA extraction: Promega system

DNA extraction was performed employing the Wizard® SV Genomic DNA Purification System (Promega, Madison, USA) as for purification of genomic DNA from mouse-tail clippings or animal tissue using a microcentrifuge. To the pellet obtained in section 2.3.1.4 200 μl Nuclei Lysis Buffer, 450 μl 0.5M EDTA ph 8.0, 20 μl Proteinase K (20 mg/ml), and 5 μl Rnase A Solution (4 mg/ml) was added and incubated at 55°C overnight. Wizard® SV Lysis Buffer (250 μl) was added and vortexed. The lysate was transferred to a minicolumn assembly and centrifuged at 13 000x**g** for 3 min. The elute was discarded and the sample subjected to washing: 650 μl Wizard® Wash solution was added and centrifuged at 13 000x**g** for 1 min after which the elute was discarded. The washing step was repeated for a total of 4 washes. The elute was discarded and the minicolumn was reassembled and centrifuged at 13 000x**g** for 2 min to dry the binding matrix. Nuclease free water (250 μl) was added and incubated at room temperature for 2 min. The assembly was centrifuged at 13 000x**g** for 2 min. Aliquots of the eluted purified DNA were stored at – 80ºC and a working aliquot kept at 4 – 8ºC.

2.3.2.3 Gel electrophoresis for determining DNA integrity and PCR product detection and sizing

Electrophoresis was performed on a 0.5% for DNA integrity and 1.4% UltraPure (GibcoBRL®) agarose gel for PCR product detection. Gels were run for 45 min at 80 V using Tris-acetate EDTA buffer. Ethidium bromide was used to stain DNA bands and captured by UV transillumination. The 100 bp DNA molecular weight marker XIV (Roche) was used to estimate expected PCR products.

2.3.3 PCR PROCEDURES

A summary of primers employed and resultant PCR product sizes are given in Table 2.1. Details of PCR mixtures and cycling conditions (Applied Biosystems GeneAmp® PCR System 9700, PE Biosystems, Ca, USA) are described in the following sections (2.3.3.1 – 2.3.3.7).

TABLE 1 PCR primers employed

Primers		Sequences 5′ - 3′	Size (bp)	Reference
ITS				
First	1724F	AAGTTGATCAAATTTGGTC	550	Lu, *et al.*, 1995
	ITS2R	CTCGGACGAGGATCCTCGCC		
Nested	ITS1F	CGTAGGTGAACCTGCGGAAGGATC		
	ITS2R1	GTTCAGCGGGTGATCCTGCCTG		
DHPS				
First	F1	CCTGGTATTAAACCAGTTTTGCC	300	Lane, *et al.*, 1997
	B45	CAATTTAATAAATTTCTTTCCAAATAGCATC		
Nested	A HUM	GCGCCTACACATATTATGGCCATTTTAAATC		
	BN	GGAACTTTCAACTTGGCAACCAC		
DHPS	A HUM	GCGCCTACACATATTATGGCCATTTTAAATC	750	Takahashi, *et al.*, 2000
Single	B HUM	CATAAACATCATGAACCCG		
DHFR				
First	FR208	GCAGAAAGTAGGTACATTATTACGAGA	798	Ma, *et al.*, 1999
	FR1038	AACCAGTTACCTAATCAAACTATATTGC		
Nested	FR242	GTTTGGAATAGATTATGTTCATGGTGTACG		
	FR1018	GCTTCAAACCTTGTGTAACGCG		
MSG				
	JKK14	GAATGCAAATCCTTACAGACAACAG	249	Huang, *et al.*, 1999
Heminested	JKK15	GAATGCAAATCTTTACAGACAACAG		
	JKK17	AAATCATGAACGAAATAACCATTGC		
MtLSUrRNA				
Single	pAZ102-E	GATGGCTGTTTCCAAGCCCA	346	Wakefield, *et al.*, 1996
	pAZ102-H	GTGTACGTTGCAAAGTACTC		
Nested	pAZ102-X	GTGAAATACAAATCGGACTAGG	260	
	PAZ102-Y	TCACTTAATATTAATTGGGGAGC		
18S rRNA				
One-tube - nested	Pc1	CCAGATTAGCTTTTGCTGATCGCGGG	265	Mathis, *et al.*, 1997
	Pc2	TTTACTTCCTCTAAATGACCAAATTTGATC		
	Pc5	CAGAGCCAGCAAGTTCATTTC		
	Pc6	CCAAATTTGATCAACTTTCCAG		
Real-time 5S rRNA	5S F	AGTTACGGCCATACCTCAGA	Tm = 82.5°C +/- 2.0°C	Larsen, *et al.*, 2002
	5S R	AAAGCTACAGCACGTCGTAT		
Single	5S F	AGTTACGGCCATACCTCAGA	120	Kitada, *et al.*, 1991
	5S R	AAAGCTACAGCACGTCGTAT		

2.3.3.1 Internal transcribed spacer (ITS)

Nested PCR. Nested PCR of the internal transcribed spacer (ITS) region was performed according to the method of Lu *et al.* [Lu*, et al.*, 1995]. For the first PCR the forward primer 1724F: 5′ – AAGTTGATCAAATTTGGTC and reverse primer ITS2R: 5′ – CTCGGACGAGGATCCTCGCC was used. The primers for the nested (second round amplification) consisted of the forward ITS1F: 5′ – CGTAGGTGAACCTGCGGAAGGATC and reverse ITS2R1: 5′ – GTTCAGCGGGTGATCCTGCCTG. Amplification was performed in a total volume of 25 µl. The reaction mix comprised: 2 µl of DNA template, 2 mM $MgCl_2$, 0.2 µM of each primer, 0.2 mM of each dNTP, 0.625 units *Taq* DNA polymerase in a PCR buffer (20 mM Tris-HCl pH 8.0, 0.1 mM EDTA, 1 mM DTT, 50% (v/v) glycerol and stabilizers) (Invitrogen, UK). For the second round of amplification 1 µl of the first-round product was used as template and the concentration of each primer was 0.4 µM.

Temperature cycling: A three step initial phase comprising 94°C for 10 min followed by 47°C for 5 min (while *Taq* DNA polymerase was added as a hot start) and 72°C for 5 min. Amplification: 35 cycles comprising 94°C for 1 min; 47°C for 1 min; and 72°C for 2 min followed by a final extension period of 10 min at 72°C. For the second round of amplification an initial 94°C for 10 min was followed by 35 cycles comprising 94°C for 1 min; 47°C for 1 min; and 72°C for 2 min followed by a final extension period of 10 min at 72°C.

2.3.3.2 Dihydropteroate synthase (DHPS)

Nested PCR. Nested PCR of the DHPS gene was performed as previously described [Lane*, et al.*, 1997]. It consisted of a first round PCR with forward primer DHPS F1: 5′ – CCTGGTATTAAACCAGTTTTGCC and reverse primer DHPS B_{45}: 5′ – CAATTTAATAAATTTCTTTCCAAATAGCATC. The primers for the second round PCR consisted of the forward primer DHPS A_{HUM}: 5′ – GCGCCTACACATATTATGGCCATTTTAAATC and reverse DHPS BN: 5′ – GGAACTTTCAACTTGGCAACCAC. Amplification was performed in a total volume of 25 µl. The reaction mix comprised: 2 µl of DNA template, 2 mM $MgCl_2$, 0.4 µM of

each primer, 0.2 mM of each dNTP, 0.625 units *Taq* DNA polymerase in a PCR buffer (20 mM Tris-HCl pH 8.0, 0.1 mM EDTA, 1 mM DTT, 50% (v/v) glycerol and stabilizers) (Invitrogen). For the second round of amplification 1 µl of the first-round product was used as template. Temperature cycling: A three step initial phase comprising 94°C for 5 min followed by 52°C for 5 min (while *Taq* DNA polymerase was added as a hot start) and 72°C for 5 min. Amplification: 35 cycles comprising 92°C for 30 sec; 52°C for 30 sec; and 72°C for 1 min followed by a final extension period of 5 min at 72°C. For the second round of amplification an initial 94°C for 5 min was followed by 35 cycles comprising 92°C for 30 sec; 55°C for 30 sec; and 72°C for 1 min followed by a final extension period of 5 min at 72°C.

2.3.3.3 Dihydropteroate synthase single

A single step PCR was performed as previously published [Takahashi*, et al.*, 2000] with the forward primer DHPS A_{HUM}: 5′ - GCGCCTACACATATTATGGCCATTTTAAATC and reverse primer DHPS B_{HUM}: 5′ – CATAAACATCATGAACCCG. Amplification was performed in a total volume of 25 µl. The reaction mix comprised: 2 µl of DNA template, 2 mM $MgCl_2$, 0.4 µM of each primer, 0.2 mM of each dNTP, 0.625 units *Taq* DNA polymerase in a PCR buffer (20 mM Tris-HCl pH 8.0, 0.1 mM EDTA, 1 mM DTT, 50% (v/v) glycerol and stabilizers) (Invitrogen). Temperature cycling: A three step initial phase comprising 94°C for 5 min followed by 45°C for 5 min (while *Taq* DNA polymerase was added as a hot start) and 72°C for 5 min. Amplification: 35 cycles comprising 94°C for 1 min; 45°C for 1 min; and 72°C for 2 min followed by a final extension period of 10 min at 72°C.

2.3.3.4 Dihydrofolate reductase (DHFR)

Nested PCR. A nested PCR was performed as described by Ma *et al*. [Ma*, et al.*, 1999]. For the first round of amplification the forward primer FR208: 5′ – GCAGAAAGTAGGTACATTATTACGAGA and the reverse primer FR1038: 5′ – AACCAGTTACCTAATCAAACTATATTGC was used. For the second round PCR the forward primer FR242: 5′- GTTTGGAATAGATTATGTTCATGGTGTACG and reverse primer FR1018: 5′ – GCTTCAAACCTTGTGTAACGCG was used. Amplification was performed in a total volume of 25 µl. The reaction mix comprised: 2 µl of DNA

template, 2 mM $MgCl_2$, 0.25 µM of each primer, 0.2 mM of each dNTP, 0.625 units *Taq* DNA polymerase in a PCR buffer (20 mM Tris-HCl pH 8.0, 0.1 mM EDTA, 1 mM DTT, 50% (v/v) glycerol and stabilizers) (Invitrogen). For the second round of amplification 1 µl of the first-round product was used as template. Temperature cycling: A three step initial phase comprising 95°C for 5 min followed by 60°C for 5 min (while *Taq* DNA polymerase was added as a hot start) and 72°C for 5 min. Amplification: 35 cycles comprising 94°C for 1 min; 60°C for 2 min; and 72°C for 3 min followed by a final extension period of 10 min at 72°C. For the second round of amplification an initial 94°C for 5 min was followed by 35 cycles comprising 94°C for 1 min; 60°C for 2 min; and 72°C for 3 min followed by a final extension period of 10 min at 72°C.

2.3.3.5 Major surface glycoprotein (MSG)

Hemi-nested PCR. The MSG PCR was performed according to the method of Huang *et al*. [Huang*, et al.*, 1999] with the forward primer JKK14: 5′ – GAATGCAAATCCTTACAGACAACAG and a combination of the two reverse primers JKK15: 5′ – GAATGCAAATCTTTACAGACAACAG and JKK17: 5′ – AAATCATGAACGAAATAACCATTGC. Amplification was performed in a total volume of 25 µl. The reaction mix comprised: 2 µl of DNA template, 3 mM $MgCl_2$, 1 µM final concentration of the 3 primers (in eqimolar ratio), 0.4 mM of each dNTP, 0.625 units *Taq* DNA polymerase in a PCR buffer (20 mM Tris-HCl pH 8.0, 0.1 mM EDTA, 1 mM DTT, 50% (v/v) glycerol and stabilizers) (Invitrogen). Temperature cycling: A three step initial phase comprising 96°C for 1 min followed by 65°C for 5 min (while *Taq* DNA polymerase was added as a hot start) and 72°C for 5 min. Amplification: 43 cycles comprising 95°C for 1 min; 65°C for 1 min; and 72°C for 1 min followed by a final extension period of 10 min at 72°C.

2.3.3.6 Mitochondrial large subunit rRNA (mtlsurRNA)

Nested PCR. The nested PCR was performed according to Wakefield *et al.* [Wakefield*, et al.*, 1996]. It consisted of the forward primer pAZ102-E: 5′ – GATGGCTGTTTCCAAGCCCA and reverse primer pAZ102-H: 5′ – GTGTACGTTGCAAAGTACTC. For the nested reaction the forward primer pAZ102-

X: 5′ – GTGAAATACAAATCGGACTAGG and reverse primer pAZ102-Y: 5′ - TCACTTAATATTAATTGGGGAGC was used. Amplification was performed in a total volume of 25 µl. The reaction mix comprised: 2 µl of DNA template, 3 mM $MgCl_2$, 1 µM of each primer, 0.4 mM of each dNTP, 0.625 units *Taq* DNA polymerase in a PCR buffer (20 mM Tris-HCl pH 8.0, 0.1 mM EDTA, 1 mM DTT, 50% (v/v) glycerol and stabilizers) (Invitrogen). For the second round of amplification 1 µl of the first-round product was used as template. Temperature cycling: A three step initial phase comprising 94°C for 2 min followed by 50°C for 5 min (while *Taq* DNA polymerase was added as a hot start) and 72°C for 5 min. Amplification: 40 cycles comprising 94°C for 1.5 min; 50°C for 1.5 min; and 72°C for 2 min followed by a final extension period of 10 min at 72°C. The nested second round PCR was performed on specimens that did not amplify after the first round PCR and consisted of 94°C for 1.5 min and amplification of 40 cycles comprising 94°C for 1.5 min; 50°C for 1.5 min; and 72°C for 2 min followed by a final extension period of 10 min at 72°C.

2.3.3.7 Small subunit ribosomal RNA (18s rRNA)

One-tube nested PCR: The nested PCR was performed according to Mathis *et al*. [Mathis*, et al.*, 1997]. It consisted of the forward outer primer PC1: 5′ – CCAGATTAGCTTTTGCTGATCGCGGG and reverse outer primer PC2: 5′ – TTTACTTCCTCTAAATGACCAAATTTGATC. The inner forward primer PC5: 5′ – CAGAGCCAGCAAGTTCATTTC and reverse primer PC6: 5′ - CCAAATTTGATCAACTTTCCAG was used. Amplification was performed in a total volume of 25 µl. The reaction mix comprised: 2 µl of DNA template, 4 mM $MgCl_2$, 3 nM of each of the outer primers (PC1 and PC2), 1.5 µM of each of the inner primers (PC5 and PC6), 0.2 mM of each dNTP and 0.625 units *Taq* DNA polymerase in a PCR buffer (20 mM Tris-HCl pH 8.0, 0.1 mM EDTA, 1 mM DTT, 50% (v/v) glycerol and stabilizers) (Invitrogen). Temperature cycling: A three step initial phase comprising 94°C for 5 min followed by 50°C for 5 min (while *Taq* DNA polymerase was added as a hot start) and 72°C for 5 min. Amplification: 20 cycles comprising 94°C for 30 sec; 72°C for 30 sec; and 30 cycles comprising 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec followed by a final extension period of 5 min at 72°C.

2.3.4 REAL-TIME 5S RIBOSOMAL RNA PCR

2.3.4.1 Real-time amplification

PCR was performed according to Palladino *et al*. [Palladino*, et al.*, 2001]. It consisted of the forward primer 5S sense: 5′ – AGTTACGGCCATACCTCAGA and reverse primer 5S antisense: 5′ – AAAGCTACAGCACGTCGTAT. Amplification was performed in a total volume of 20 µl. The reaction mix comprised 10 µl of DNA template, 1.5 mM $MgCl_2$, 0.5 µM of each primer and 2 µl FastStart SYBR Green I in LightCycler glass capillary tubes (Roche). Temperature cycling: the real-time PCR was performed in a LightCycler I System (Roche). Amplification: denaturation and FastStart *Taq* activation at 95°C for 10 min followed by 38 cycles comprising heating at 20°C/sec to 95°C with a 15 sec hold; cooling at 20°C/sec to 55°C with a 10 sec hold and heating at 20°C/sec to 72°C with a 10 sec hold. This was immediately followed by a melting curve analysis to determine the melting temperature (Tm) of the dsDNA product. This consisted of heating at 20°C/sec to 95°C with no hold, cooling at 20°C/sec to 40°C with a 15 sec hold, and then heating at 0.2°C/sec to 95°C during which fluorescence (F1) was measured at 530 nm. A Tm of 82.5°C +/- 2.0°C was indicative of *P. jirovecii* [Palladino, 2001].

2.3.4.2 Standard curve

For creating a standard curve for absolute quantitative analysis a conventional PCR was performed according to Kitada *et al.* [Kitada*, et al.*, 1991], employing the same primers as for the real-time PCR reaction with a positive control specimen. It consisted of a forward primer 5S sense: 5′ – AGTTACGGCCATACCTCAGA and reverse primer 5S antisense: 5′ – AAAGCTACAGCACGTCGTAT. Amplification was performed in a total volume of 50 µl. The reaction mix comprised 5 µl of DNA template, 1.5 mM $MgCl_2$, 0.5 µM of each primers, 0.2 mM of each dNTP and 1.25 units *Taq* DNA polymerase in a PCR buffer (20 mM Tris-HCl pH 8.0, 0.1 mM EDTA, 1 mM DTT, 50% (v/v) glycerol and stabilizers) (Invitrogen). Temperature cycling: the PCR was performed in a thermocycler (Applied Biosystems GeneAmp® PCR System 9700) with a three step initial phase comprising 95°C for 9 min followed by 50°C for 5

min (while *Taq* DNA polymerase was added as a hot start) and 72°C for 5 min. Amplification: 35 cycles comprising 94°C for 30 sec; 55°C for 60 sec; and 72°C for 60 sec followed by a final extension period of 5 min at 72°C.

PCR products were separated by electrophoresis in a 1.4% UltraPure (GibcoBRL®) agarose gel for 45 min at 80V using Tris-acetate EDTA buffer. Ethidium bromide was used to stain the DNA products and was examined and captured by UV transillumination. The 100 bp DNA molecular weight marker XIV (Roche) was used to estimate the expected 120 bp PCR product. The PCR product was purified (Wizard® SV gel and PCR clean-up system, Promega) and amplicon concentration was obtained by measuring absorbance at 260 nm from which tenfold dilutions were prepared. In addition, a known positive clinical specimen was serially diluted and incorporated in the standard curve estimation. The final standard curve contained: 1 X 10^{-1}; 1 X 10^{1} and 1 X 10^{3}; 1 X 10^{6} and 1 X 10^{12} copies/μl. All standard curve reactions were performed in duplicate. The standard curve was calculated employing the second derivative maximum analysis method incorporated in the LightCycler quantification software package (Roche).

2.4 GENOTYPING AND COALESCENT PHYLOGENETIC ANALYSIS

2.4.1 Specimens

Clinical specimens (20) submitted to the Pathology Laboratories at Tygerberg Hospital, South Africa, for identification of *P. jirovecii* were investigated (section 2.1.1.).

2.4.2 ITS gene amplification

Sample preparation and DNA extraction was performed as described in sections 2.3.1 and 2.3.2. Amplification of the ITS gene regions was performed as per section

2.3.3.1. In addition, high fidelity nested PCR of the internal transcribed spacer (ITS) region was performed as described by Lu *et al*. [Lu*, et al.*, 1995] employing *Pwo* DNA polymerase (Roche) according to the manufacturer's instructions. The high fidelity PCR was included to confirm *Taq* generated genotype sequences. *Pwo* is a highly processive 5'–3' DNA polymerase and possesses a 3'–5' exonuclease activity (proofreading). Employing this polymerase increases fidelity to >10-fold that of *Taq* DNA polymerase. The same primers as for the *Taq* polymerase reactions (Table 2.1) were employed. Amplification was performed in a total volume of 50 µl. The reaction mix comprised 4 µl of DNA template, 2 mM $MgSO_4$, 0.2 µM of each primer, 0.2 mM of each dNTP, 1.25 units *Pwo* DNA polymerase in a PCR buffer (20 mM Tris-HCl pH 7.5, 100 mM KCl, 1 mM DTT, 0.1 mM EDTA, 0.5% Nonidet P40 (v/v), 0.5% Tween 20 (v/v), and 50% (v/v) glycerol) (Roche). For the second round of amplification 2 µl of the first-round product was used as template and the concentration of each primer was 0.4 µM. Temperature cycling: the PCR was performed in a thermocycler (Applied Biosystems GeneAmp® PCR System 9700). Initial denaturation of 94°C for 2 min. Amplification: 10 cycles comprising 94°C for 15 sec; 55°C for 30 sec; and 72°C for 45 sec followed by 15 cycles comprising 94°C for 15 sec; 55°C for 30 sec; 72°C for 45 sec (+ 5 sec for each cycle); and a final extension at 72°C for 7 min. For the second round of amplification cycling conditions were the same. PCR products of expected size (550 bp) were separated by electrophoresis as described in section 2.3.2.3.

2.4.3 Subcloning

Subcloning of purified ITS PCR products was undertaken employing pGEM® -T Vector System (Promega) according to the manufacturer's instructions. The pGEM® -T vector contains 3′ terminal thymidine at both ends that assist ligation of PCR products into plasmid by preventing recircularisation of the vector, and providing compatible T overhangs for PCR products generated by *Taq* DNA polymerases. *Taq* DNA polymerases add a single deoxyadenosine, in a template-independent fashion, to the 3′ -ends of the amplified fragment. The vector contains a multiple cloning

region within the α - peptide coding region of β-galactosidase. Insertional inactivation of the α-peptide allows recombinant clones to be directly identified by colour screening on indicator plates.

2.4.3.1 PCR product preparation

Purified PCR product (from section 2.4.2) concentration was estimated by comparison to the DNA molecular weight marker XIV (Roche) and diluted to 10 ng/μl with nuclease free water. PCR products generated by *Taq* DNA polymerase contains A overhangs (required for cloning), however, PCR products generated by *Pwo* DNA polymerase are blunt ended and require a A-tailing procedure. Briefly: to five microliters *Pwo* PCR product 1 μl *Taq* DNA polymerase 10X reaction buffer with $MgCl_2$ (Promega), dATP to a final concentration of 0.2 mM, and 5 units *Taq* DNA polymerase (Invitrogen) was added to a final reaction volume of 10 μl, and incubated at 70°C for 20 min.

2.4.3.2 Ligation

Ligation was performed according to the manufacturer's instructions. Briefly: The pGEM® -T vector and control insert DNA was centrifuged at 4 000x**g** for 30 sec to collect the contents at the bottom of the tubes. The ligation reaction was set up as in Table 2.2 in 0.5 ml tubes with low DNA-binding capacity (Promega).

2.4.3.3 Transformation

The ligated products from section 2.4.3.2 were introduced into high efficiency (1 X 10^8cfu/μg DNA) competent JM 109 cells (Promega Corporation, Madison, WI, USA). The genotype of JM 109 is *rec*A1, *end*A1, *gyr*A96, *thi*, *hsd*R17 (r_K-,m_K+), *rel*A1, *sup*E44, Δ(*lac-pro*AB), [F′, *tra*D36, *pro*AB, *lac*I^qZΔM15].

TABLE 2.2 Ligation reactions

	Standard Reaction Volume (μl)	Positive Control* Volume (μl)
2X rapid Ligation Buffer, T4 DNA Ligase	5	5
pGEM®-T Easy vector (50 ng/μl)	1	1
PCR Product	1	-
Control insert DNA	-	2
T4 DNA Ligase (3 Weiss units/μl)	1	1
Deionised water	2	1
Final volume	10	10

* Positive control was run with every batch

The reactions were mixed by pipetting and incubated at 4°C overnight.

Transformation was performed according to the manufacturer's instructions. Briefly: The ligation reaction tubes were centrifuged at 4 000x**g** for 20 sec. Two microliters of the ligation solution was transferred to a new reaction tube on ice. JM 109 cells (Promega) were thawed for 5 min in an ice bath, and mixed by gentle flicking. To the ligation solution 50 μl of cells was added and mixed by gentle flicking. The tubes were left on ice for 20 min and subsequently heat-shocked at 42°C for 45 – 50 sec in a water bath and returned to ice for 2 min. Room temperature SOC medium (950 μl) was added to the tubes containing cells transformed with the ligation reaction and incubated for 1.5 h in a shaking (150 rpm) water bath at 37°C. The cells were subsequently pelleted by centrifugation at 1 000x**g** for 10 min, and the pellet resuspended in 200 μl SOC medium. Two Luria-Bertanie (LB) medium agar plates containing 100 μg/ml ampicillin, 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and 80 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (LB/ampicillin/IPTG/X-Gal plates) were equilibrated to room temperature for each reaction and 100 μl of the SOC reaction mixture was plated on each plate. The plates were then incubated at 37°C overnight.

2.4.3.4 Selecting transformants

From two LB/ampicillin/IPTG/X-Gal plates, ten white colonies, labelled clones a – j, were picked and streaked onto LB/ampicillin plates and incubated at 37°C overnight. From the resulting growth, half of a single, well isolated colony from each of five clones (a – e) was suspended in 50 µl nuclease free water and subjected to PCR as described below (2.4.3.5). The rest of the confluent growth of the 10 clones (a – j) were resuspended in Mueller-Hinton broth with 10% glycerol and stored at - 80°C.

2.4.3.5 Insert amplification

Amplification was performed employing vector specific forward primer M13F (5' – GTTTTCCCAGTCACGAC – 3') and reverse primer M13R (5' – CAGGAAACAGCTATGAC –3') in a total volume of 25 µl. The reaction mix comprised 2 µl of DNA template, 2 mM $MgCl_2$, 0.5 µM of each primer, 0.2 mM of each dNTP, 0.625 units *Taq* DNA polymerase (Invitrogen) in a PCR buffer (20 mM Tris-HCl pH 8.0, 0.1 mM EDTA, 1 mM DTT, 50% (v/v) glycerol and stabilizers) (Invitrogen). The cycling conditions were 95°C for 2 min followed by 25 cycles of 95°C for 30s; 45°C for 30s and 72°C for 30s, with a final extension at 72°C for 5 min in a thermocycler (Applied Biosystems GeneAmp® PCR System 9700). PCR products were separated by electrophoresis (section 2.3.2.3). A PCR product of the expected size (806 bp) confirmed the presence of the correct insert and was prepared for direct sequencing using ExoSAP-IT (USB Corporation, Cleveland, USA). Sequencing was performed employing vector specific primers M13F and M13R, according to the manufacturer's instructions as described in section 2.4.4.

2.4.4 Sequencing

The PCR product concentrations were estimated by comparison to the DNA molecular weight marker XIV (Roche) concentration standards incorporated in the molecular weight markers. The PCR products were diluted to 10 ng/µl with nuclease-free water. PCR products were prepared for direct sequencing using the ExoSAP-IT

(USB Corporation) enzyme digestion. The enzyme combination of shrimp alkaline phosphatase and exonuclease 1 hydrolyses any remaining dNTP's and degrades any residual single-stranded primers or extraneous single-stranded DNA, preparing the PCR product for dideoxy cycle sequencing. The procedure was performed according to the manufacturer's instructions. Briefly, 2 µl of ExoSAP-IT was added to 5 µl diluted PCR product. The mix was incubated, using a thermocycler (Applied Biosystems GeneAmp® PCR System 9700) at 37°C for 15 min and then at 80°C for 15 min to irreversibly inactivate the enzymes. A final concentration of 5 ng/µl DNA was used for sequencing.

Sequencing reactions were performed using the ABI Prism® BigDye™ Terminator Ready Reaction v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions. Briefly, half reactions were performed as follows: 4 µl ready Reaction Premix, 2 µl BigDye Sequencing Buffer, 3.2 pmol primer (M13 F and/or M13 R), 3 – 10 ng template DNA, and nuclease free water was added for a final volume of 20 µl.

Temperature cycling: the PCR was performed in a thermocycler (Applied Biosystems GeneAmp® PCR System 9700) with an initial denaturation at 96°C for 1 min, followed by 25 cycles comprising 96°C for 10 sec; 50°C for 5 sec; and 60°C for 4 min. Capillary electrophoresis was performed on an ABI Prism® 3100 Genetic Analyser (Applied Biosystems) according to the manufacturer's instructions, by the Central Analytical Facility, University of Stellenbosch.

2.4.5 Coalescent statistical parsimony network analysis

ITS1 and ITS2 sequences were aligned with all previously published ITS sequences employing Clustal W, incorporated in the BioEdit software package (version 5.06; Tom Hall, Department of Microbiology North Carolina State University [http://www.mbio.ncsu.edu/BioEdit/page2.html]), with subsequent visual inspection and optimisation. ITS genotypes were assigned and named according to Lee *et al*.

[Lee*, et al.*, 1998]. An area within the ITS1 contains a homopolymeric tract (located between positions 62 – 71 that has 10 T bases in tandem) that has been shown to yield variable nucleotide numbers when sequenced repeatedly and was omitted from genotyping [Lee*, et al.*, 1998]. New types were only assigned if found in more than one patient specimen.

Statistical parsimony was applied using the computer program TCS (version 1.13; Clement, M., J. Derington, and D. Posada. Brigham Young University. [http://inbio.byu.edu/Faculty/kac/crandall_lab/] [Clement*, et al.*, 2000]. ITS1 and ITS2 sequences were concatenated. Gaps were treated as missing characters, but subsequent to coalescence network analysis, included to reveal haplotype subdivisions within the obtained haplotypes. Unresolved loops were optimised by applying coalescence theory to obtain the most parsimonous network [Rosenberg*, et al.*, 2002].

2.5 DHPS AND DHFR GENE SCREENING AND SEQUENCING

2.5.1 Specimens

One hundred and seventy eight sequential specimens, that included the 78 described in section 2.1.1, submitted to the Pathology Laboratories at Tygerberg Hospital, South Africa, for identification of *P. jirovecii* (June 2001 to February 2003) were collected and stored at -80°C. In addition, 23 of 663 TB-positive specimens that produced an amplicon by mtLSUrRNA nested PCR (section 4.2.2) were included.

2.5.2 Amplification and sequencing

Sample preparation (section 2.3.1) and DNA extraction (section 2.3.2) was undertaken and amplification of DHPS and DHFR genes were performed as described in sections 2.3.3.2 and 2.3.3.4, respectively. PCR products were

sequenced as described in section 2.4.4 employing DHPS forward and reverse primers Ahum and Bnest, and DHFR forward and reverse primers FR242 and FR1018.

2.5.3 DHPS and DHFR sequence alignment

DNA sequences were analysed using the computer program Chromas Version 1.45 [C. McCarthy, Griffith University, Australia]. The deduced DNA sequences were converted to FASTA format and compared to published wild type sequences, deposited in the GenBank repository, using the multiple sequence alignment computer program BioEdit (version 5.06; Tom Hall, Department of Microbiology, North Carolina State University [http://www.mbio.ncsu.edu/BioEdit/page2.html]). The *P. jirovecii* sequences obtained were compared to the *P. jirovecii* wild type genes with the following GenBank accession numbers: DHPS U66279 [Lane*, et al.*, 1997] and DHFR AF090368.1 [Ma, 1999]. Sequences producing double peaks indicative of mixed strain types were submitted to subcloning as described in section 2.4.3, and resequenced.

2.5.4 Genome walking

The Universal GenomeWalker™ kit (BD Bioscience Clontech, Palo Alto, CA) was employed for exploring upstream sequences of currently employed DHPS primer annealing sites according to the manufacturer's recommendations. Genome walking is performed by construction of a pool of adaptor-ligated genomic DNA fragments (libraries). Genomic DNA is digested with various restriction enzymes to create blunt-ended genomic fragments and is ligated to adapter sequences. A primary PCR is performed using an outer adaptor primer (AP1) and an outer gene specific primer (GSP1). A nested PCR is performed on the product of the primary PCR employing a nested adaptor primer (AP2) and a nested gene specific primer (GSP2). Each of the DNA fragments – which begin in a known sequence at the 5´ end of GSP2 and

extend into unknown adjacent genomic DNA – can then be cloned and further analysed.

2.5.4.1 Library construction

An open-lung biopsy confirmed by methanamine silver staining and PCR for the presence of *P. jirovecii*, as well as a BAL specimen confirmed by IF and PCR for the presence of *P. jirovecii* were investigated. DNA extraction was performed as described in section 2.3.1 and 2.3.2. The quality of genomic DNA was evaluated by running 1 μl of extracted DNA on 0.5% agarose gel. In addition, the purity of the extracted DNA was evaluated by subjecting the DNA to digestion by *Dra* I. The digestion reaction was performed as follows: Briefly, 5 μl DNA was digested with 16 U *Dra* I in *Dra* I restriction buffer (BD Bioscience Clontech) in a total volume of 20 μl. Digestion occurred at 37°C overnight. The digested DNA was evaluated on 0.5% agarose gel by electrophoresis.

For each library construction three blunt end digestions were performed, a positive human genomic DNA control was included with each library construction. Each reaction consisted of 25 μl genomic DNA, 8 μl restriction enzyme (*Dra* I, *Eco*R V, *Pvu* II), 10 μl restriction enzyme buffer, and dH_2O to a final volume of 100 μl. The reactions were incubated at 37°C for 2 h, vortexed at slow speed and returned to 37°C for overnight digestion. From each reaction 5 μl was subjected to 0.5% agarose gel electrophoresis.

Digested DNA was purified by phenol / chloroform ethanol precipitation. Briefly, 95 μl phenol (Sigma) was added to each tube, vortexed at slow speed, and centrifuged at 6 000x**g** for 30 sec. The aqueous phase was aspirated, to which 95 μl chloroform (Sigma) was added, vortexed at slow speed and centrifuged at 6 000x**g** for 30 sec. The aqueous phase was aspirated to which 190 μl ice cold 95% ethanol (Sigma), 9.5 μl 3 M NaOAc (pH 4.5), and 20 μg glycogen (Fermentas, Vilnius, Lithuania) was added and vortexed at slow speed. The solution was then centrifuged at 14 000x**g** for 10 min and the pellet washed in 100 μl ice-cold 80% ethanol (Sigma). After

centrifugation at 14 000x**g** for 15 min the pellet was air dried and resuspended in 20 μl TE (10/01, pH 7.5) (Sigma). One microliter of digest solutions were electrophoresed on 0.5% agarose gels and DNA quantified by visual inspection.

Four microliters of digested DNA (50 pg/μl) was ligated to adaptors by the addition of 1.9 μl GenomeWalker adaptor, 1.6 μl 10X ligation buffer, and 0.5 μl T4 DNA ligase. The reaction was incubated at 4°C overnight in an Applied Biosystems 9700 Thermalcycler. The ligation reaction was terminated after overnight incubation by heat inactivation at 70°C for 5 min. To the reaction 72 μl TE (10/1, pH 7.4) (Sigma) was added and vortexed at slow speed.

2.5.4.2 DNA walking

A PCR was undertaken employing the BD Advantage™ II PCR enzyme system (BD Bioscience Clontech) with primers GSP1 (DHPS B_{45} – see Table 2.1) and AP1. Amplification was performed in a total volume of 50 µl. The reaction mix comprised:
1 µl of DNA template, 2.5 mM $Mg(Oac)_2$, 10 μM of each primer, 0.2 mM of each dNTP, Advantage II Genomic Polymerase buffer and 1μl Advantage II polymerase mix. Temperature cycling consisted of a two step cycle: 7 cycles comprising 94°C for 2 sec and 72°C 3 min, followed by 32 cycles of 94°C, 2 sec and 67°C, 3 min. Final extension was at 67°C for 4 min.

A nested reaction was performed with primers GSP2 (DHPS Bn, see table 2.1) and AP2. Amplification was performed in a total volume of 50 µl. The reaction mix comprised: 1 µl of primary PCR product, 2.5 mM $Mg(OAc)_2$, 10 μM of each primer, 0.2 mM of each dNTP, Advantage II Genomic Polymerase buffer and 1μl Advantage II polymerase mix. Temperature cycling consisted of a two step cycle: 5 cycles comprising 94°C for 2 sec and 72°C for 3 min, followed by 20 cycles of 94°C for 2 sec and 67°C for 3 min. Final extension was at 67°C for 4 min. PCR products (5 μl) were analysed on 1.5% agarose gels by electrophoresis. Products were subjected to DNA sequencing as described in section 2.4.4 employing GSP2.

2.5.5 Extended DHPS and DHFR primer investigations

2.5.5.1 Specimens

Specimens employed consisted of two categories: four specimens that had yielded a DHPS or DHFR PCR product using previously employed primers (section 2.3.3), and five specimens that displayed discordant DHPS/DHFR-negative *vs*. mtLSUrRNA nested PCR-positive results.

2.5.5.2 DHPS primer design

New primers were designed employing the computer program GeneFisher [Geigerich*, et al.*, 1996] according to software recommendations. For DHPS primer searches the published *P. jirovecii* hydroxymethyl-dihydropterin pyrophosphokinase / dihydropteroate synthase (Genbank AF139132) [Ma*, et al.*, 1999] was employed as the input sequence. The following parameters were specified: Primer size 18 –31; primer GC content 45% – 65%; melting temperature (Tm) 55°C - 65°C; PCR distance 500 – 1000 bp; 3´ clamp GC content 45% – 55%; Maximum GC clamp size 4. Primers PSGF1 and PSGF2 derived. One set of primers was empirically designed in order to include all previously employed primer annealing sites and assigned as primers LR1 and LR2. In addition, the previously published primer B45 was modified subsequent to detection of errors of the published primer sequence [Lane*, et al.*, 1997] and redesigned as B45b.

Reaction mixture and cycling parameters as described in section 2.3.3.2 were used but with appropriate annealing temperatures. Forward primer PSGF1 5´ TTTATAAATGCAGGGGCGACGA and reverse primer PSGF2 5´ CCCGTATAATATCACAGCCTCCTA. Annealing temperature 52ºC, PCR product size 628 bp. Forward primer LR1 5´ CAAATTAGCGTATCGAATGACCT and reverse primer LR2 5´ TGCAAAATTACAATCAATCAACCAAAG. Annealing temperature 62ºC, PCR product size 1030 bp. Forward primer F1b 5´ GTTAATCCTGGTATTAAACAGTTTTGCCAT and reverse primer B45b 5´ CAATTCAATAAACTCCTTTCCAAATAGCATC. Annealing temperature 52ºC, PCR product size 911 bp.

2.5.5.3 DHPS primer combinations

Reaction mixture and cycling parameters as described in section 2.3.3.2 were used but with an appropriate annealing temperature. Forward primer DHPS F1: 5′ – CCTGGTATTAAACCAGTTTTGCC and reverse primer DHPS B_{HUM}: 5′ – CATAAACATCATGAACCCG. Annealing temperature 52ºC, PCR product size 838 bp.

2.5.5.4 DHFR primer design

New primers were designed employing the computer program GeneFisher [Geigerich*, et al.*, 1996] according to software recommendations. For DHFR primer searches the published *P. jirovecii* dihydrofolate reductase gene (Genbank AF090368) [Ma*, et al.*, 1999] was employed as the input sequence. The following parameters were specified: Primer size 18 –31; primer GC content 45% – 65%; melting temperature (Tm) 55°C - 65°C; PCR distance 500 – 1200 bp; 3´ clamp GC content 45% – 55%; Maximum GC clamp size 4. Primer pair FRGF1 and FRGF2 was derived.

Reaction mixture and cycling parameters as described in section 2.3.3.4 were used but with an appropriate annealing temperature and extension time. Forward primer FRGF1 5´ AGATTCCTGTACTGTGGCTTCTGA and reverse primer FRGF2 5´ ACCCTGAGGAACTTTACTTCCA. Annealing temperature 65ºC, extension time 1 min. PCR product size 1167 bp.

2.5.5.5 Gene sequencing

PCR products obtained in 2.5.5.2 – 2.5.5.4 were subjected to DNA sequencing as described in section 2.4.4 employing primers used for amplification.

2.6 ETHICAL APPROVAL

Approval to perform this study was obtained from the Stellenbosch University Division of Research Development and Support, Subcommittee C (Protocol Numbers 2001/C081 and 2002/C085). Work on *M. tuberculosis* sputum specimens obtained from the Unit for Tuberculosis Operational and Policy Research had received approval from the Medical Research Council Bioethics committee as part of the TB surveillance protocol.

CHAPTER 3

PCR DETECTION

3.1 INTRODUCTION

Culture of *P. jirovecii* is still elusive and diagnostic investigations have in the past relied on the microscopic detection of cysts or trophic forms of the fungus in respiratory fluids obtained by BAL, TASP, induced sputum or from tissues obtained from transbronchial or open lung biopsies. The problems are that a single stain cannot detect all the *P. jirovecii* life cycle stages, variable observer skill and interpretational differences. Early diagnosis of PcP in immunocompromised patients, a life threatening complication if not treated correctly, may lower the morbidity and mortality associated with it. The PCR has been reported to be superior to staining techniques for the detection, as it is more sensitive and specific [Leign*, et al.*, 1993]. The major advantages of PCR are the ability to detect low numbers of organisms (sensitivity) and the very specific nature of PCR methods. The first report of PCR for clinical detection of *P. jirovecii* in BAL was by Wakefield *et al*., employing primers directed at the mtLSUrRNA [Wakefield*, et al.*, 1990a]. Subsequently a number of

reports have been published on PCR for the detection of *P. jirovecii* in clinical samples employing various chromosomal loci [Wakefield*, et al.*, 1990a; Kitada*, et al.*, 1991; Tamburrini*, et al.*, 1993; Honda*, et al.*, 1994; Lee*, et al.*, 1994; Chouaid*, et al.*, 1995; De Luca*, et al.*, 1995; Moonens*, et al.*, 1995; Lu*, et al.*, 1995; Skøt*, et al.*, 1995; Lane*, et al.*, 1997; Mathis*, et al.*, 1997; Rabodonirina*, et al.*, 1997; Ribes*, et al.*, 1997; Huang*, et al.*, 1999; Sandhu*, et al.*, 1999; Sing*, et al.*, 2000; Torres*, et al.*, 2000; Helweg-Larsen*, et al.*, 2002; Kasolo*, et al.*, 2002; Lishimpi*, et al.*, 2002]. The sensitivity and specificity of PCR methods using these different targets varies [De Luca*, et al.*, 1995; Lu*, et al.*, 1995]. More recently real-time PCR has been described as being a sensitive and specific technique with the potential for quantitative analysis to differentiate colonisation from infection [Kaiser*, et al.*, 2001; Palladino*, et al.*, 2001; Larsen*, et al.*, 2002a; Larsen*, et al.*, 2002b; Larsen*, et al.*, 2004]. Discrepant results have been reported when comparing IF staining techniques with PCR. Factors that could lead to discordant results include the observation that PCR can rapidly convert from positive to negative following initiation of specific treatment for PcP, whereas cyst wall specific stains can detect empty cysts subsequent to successful treatment / clearance [Armbruster*, et al.*, 1995].

The perception by clinicians at Tygerberg Hospital that the *P. jirovecii* IF method appears to produce both false-positive and false-negative results prompted evaluation- and comparison of IF and recent molecular detection techniques. However, primer selection plays a significant role in the diagnosis of PcP by PCR and various primers and PCR techniques should be evaluated for local strains / conditions [De Luca*, et al.*, 1995].

3.2 RESULTS

3.2.1 PCR evaluation

A study to evaluate the detection of *P. jirovecii* from various clinical specimens by nine PCR assays was undertaken. PCR amplification was performed with

mtLSUrRNA nested, mtLSUrRNA single-round, DHPS nested, MSG hemi-nested, ITS nested, 18S one-tube nested, DHPS single, and DHFR nested primers. Amplification products are shown in Figures 3.1 – 3.8. 5S Real-time PCR products detected by SYBR Green fluorescence are shown in Figure 3.9. Real-time PCR products, characterised as melting at 82.5ºC, were confirmed as 120 bp (Figure 3.10).

Of 90 specimens processed, depending on the primers employed, an amplification product indicative of *P. jirovecii* was obtained in 9 to 62 specimens (Table 3.1). The highest detection rate was observed for the mtLSUrRNA nested (69%), followed by real-time 5S (54%), mtLSUrRNA single (47%), DHPS nested (42%), MSG heminested (27%), ITS nested (24%), 18S nested (20%), DHPS single (10%), and DHFR nested (10%). Concordances between the PCR reactions varied from 41.9% to 94.9% (Table 3.2). Least concordance was seen for DHPS single vs. mtLSUrRNA nested PCR (41.9%). The highest degree of concordance among the PCR methods was mtLSUrRNA nested vs. real-time 5S (88.9%), DHPS single vs. 18S (90.5%), and 18S vs. DHPS nested PCR (94.9%). The mtLSUrRNA single and nested reactions (same primers employed for first round) displayed a concordance of 64.9% with the nested reaction detecting 20 additional specimens to the single-round PCR. DHPS nested and DHPS single PCR (different primers employed) displayed a concordance of 82.4%. The highest detection rate was obtained with mtLSUrRNA nested and 5S real-time PCR for which discordant results are shown and further analysed in Table 3.3. By considering a positive PCR targeting another gene, other than mtLSUrRNA and / or staining as confirmation, results suggested two false-negative mtLSUrRNA nested PCR results (specimens 4 and 5, Table 3.3), and 12 false-negative real-time 5S rRNA PCR results (specimens 6 – 17, Table 3.3).

P. jirovecii strain typing which involved sequencing of ITS PCR products was performed as described in Chapter 5. It is of interest that of six specimens, which were ITS PCR positive, the products on sequencing contained the amplified homologous region of *Candida albicans* in five specimens and *Cryptococcus magnus* in one specimen. For two of these specimens all other PCR products were negative.

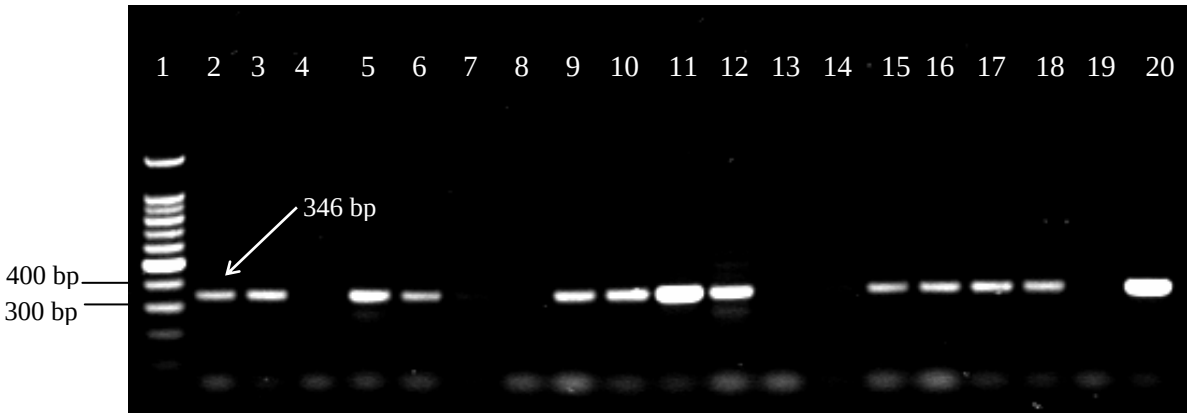


Figure 3.1 ***P. jirovecii*** **detection employing mtLSUrRNA single-round PCR**

Positive PCR fragment: 346 bp. Lane 1: 100 bp DNA ladder; Lane 19: Negative control; Lane 20: Positive control.

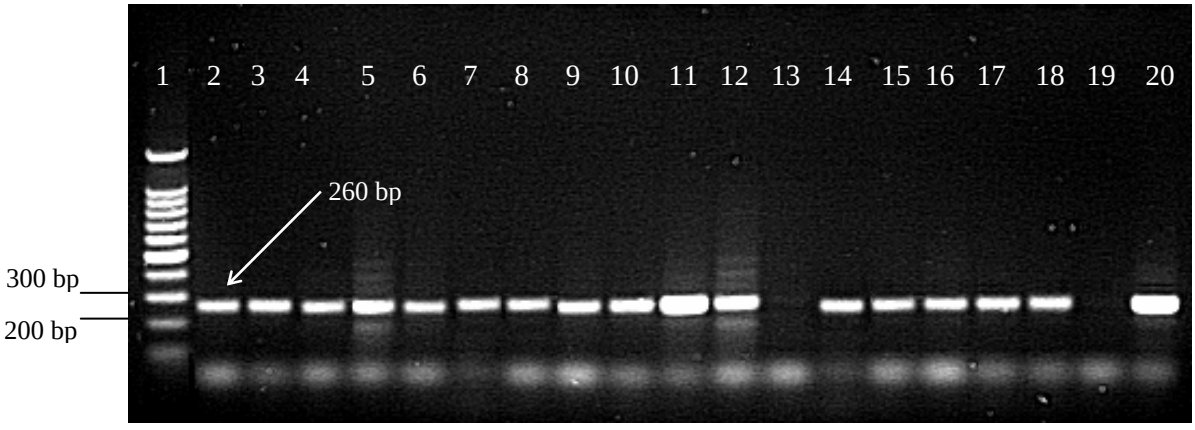


Figure 3.2 ***P. jirovecii*** **detection employing mtLSUrRNA nested PCR**

Positive PCR fragment: 260 bp. Lane 1: 100 bp DNA ladder; Lane 19: Negative control; Lane 20: Positive control.

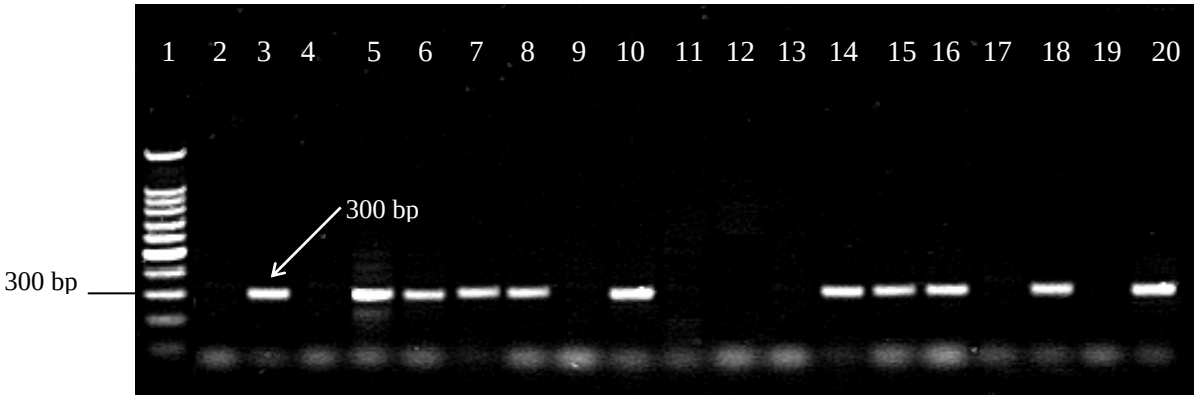


Figure 3.3 ***P. jirovecii*** **detection employing DHPS nested PCR**

Positive PCR fragment: 300 bp. Lane 1: 100 bp DNA ladder; Lane 19: Negative control; Lane 20: Positive control.

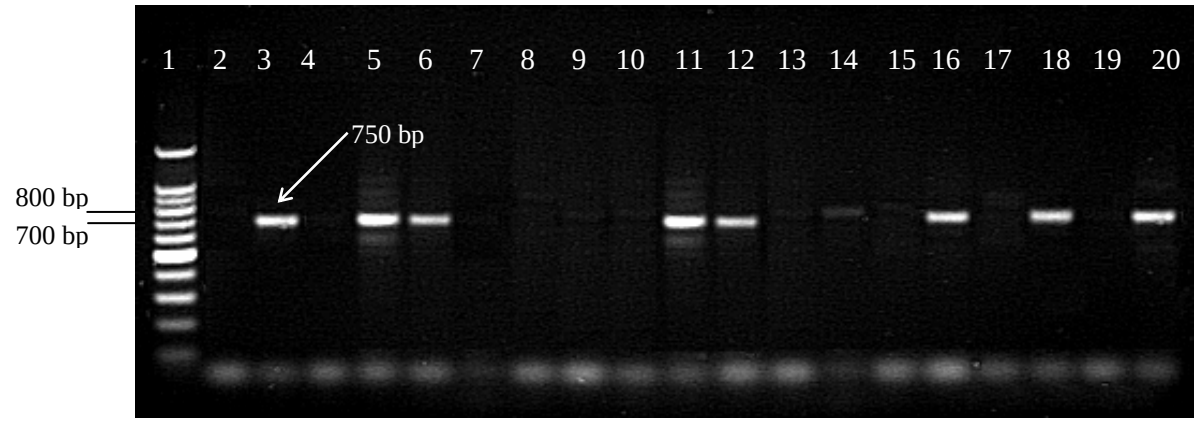


Figure 3.4 ***P. jirovecii*** **detection employing DHPS single PCR**

Positive PCR fragment: 750 bp. Lane 1: 100 bp DNA ladder; Lane 19: Negative control; Lane 20: Positive control.

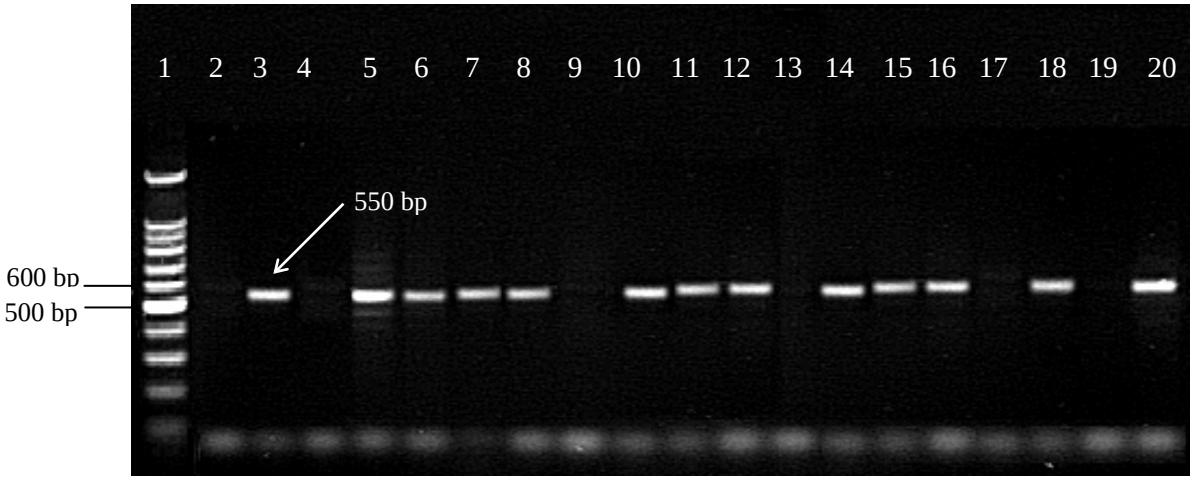


Figure 3.5 ***P. jirovecii*** **detection employing ITS nested PCR**

Positive PCR fragment: 550 bp. Lane 1: 100 bp DNA ladder; Lane 19: Negative control; Lane 20: Positive control.

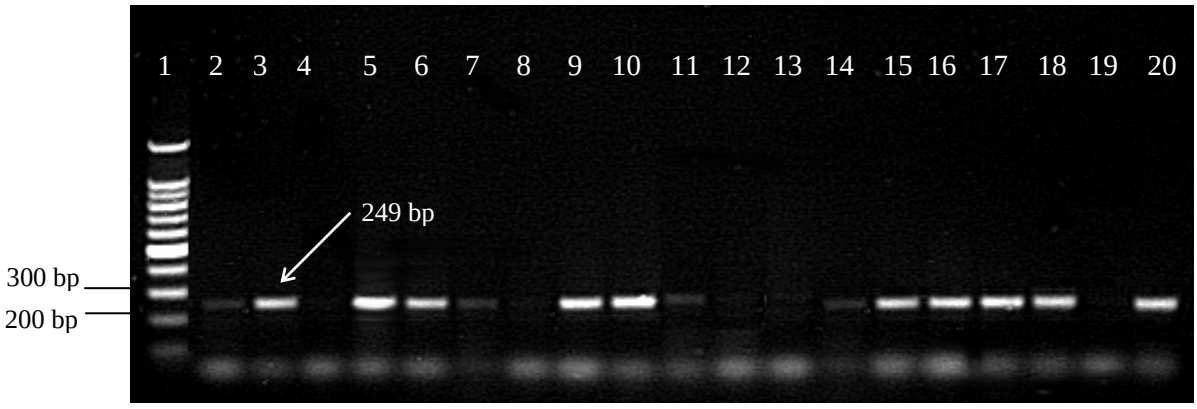


Figure 3.6 ***P. jirovecii*** **detection employing MSG hemi-nested PCR**

Positive PCR fragment: 249 bp. Lane 1: 100 bp DNA ladder; Lane 19: Negative control; Lane 20: Positive control.

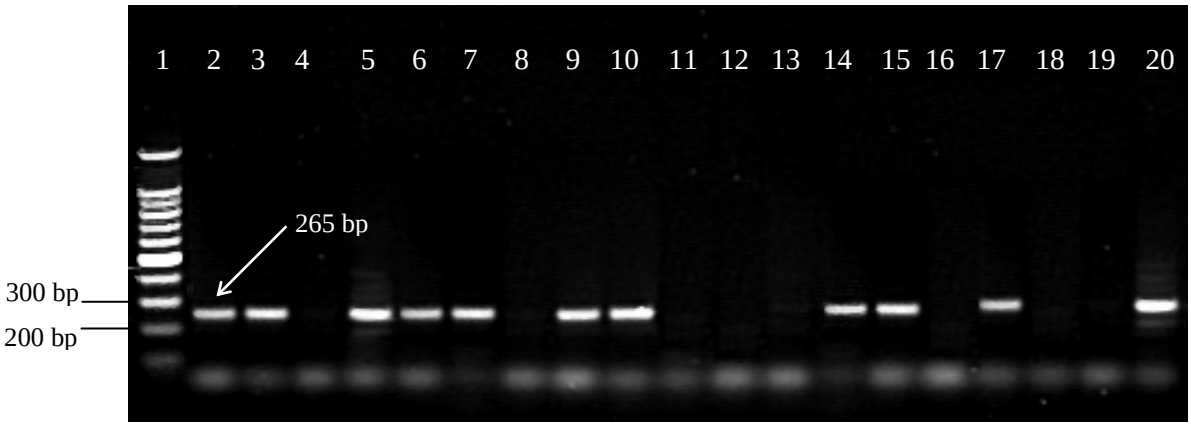


Figure 3.7 ***P. jirovecii*** **detection employing 18S one-tube nested PCR**

Positive PCR fragment: 265 bp. Lane 1: 100 bp DNA ladder; Lane 19: Negative control; Lane 20: Positive control.

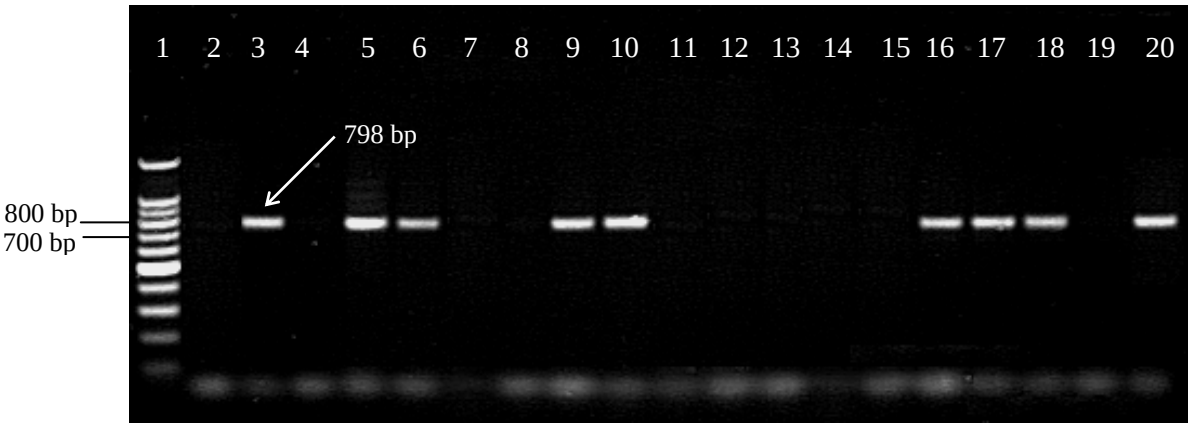


Figure 3.8 ***P. jirovecii*** **detection employing DHFR nested PCR**

Positive PCR fragment: 798 bp. Lane 1: 100 bp DNA ladder; Lane 19: Negative control; Lane 20: Positive control.

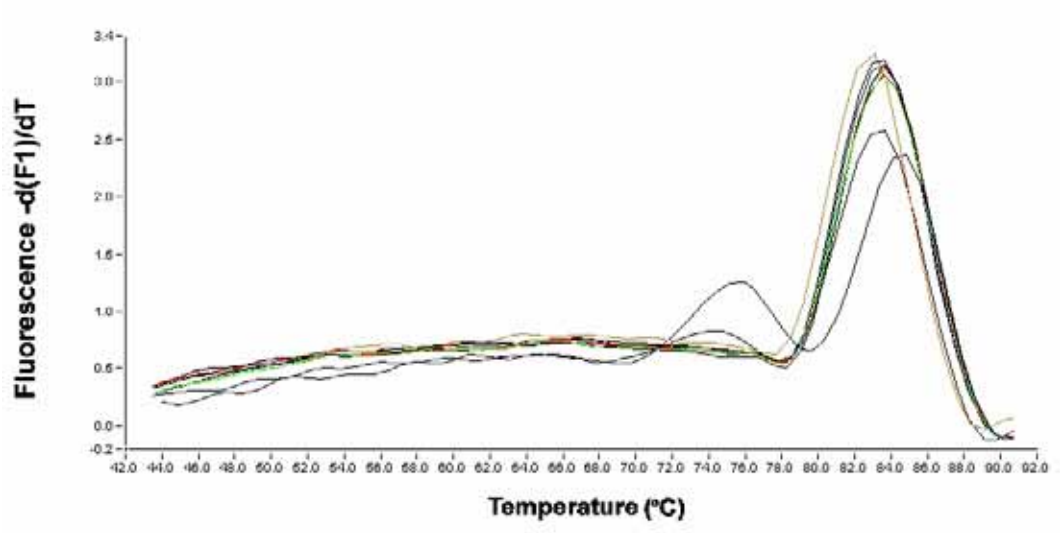


Figure 3.9 Real-time 5S rRNA PCR melting curve analysis

Positive signal at Tm 82.5°C +/- 2.0°C.

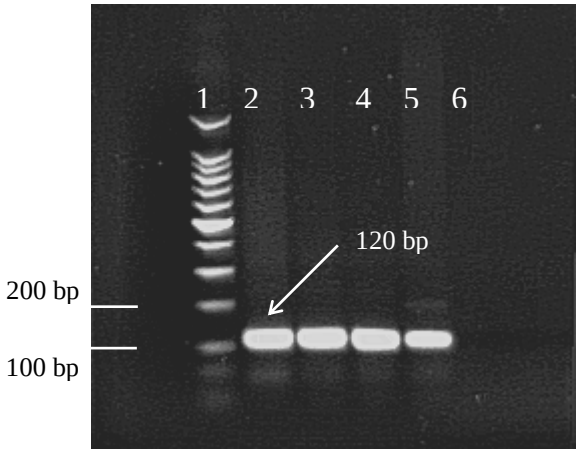


Figure 3.10 Real-time 5S PCR products

Lane 1: 100 bp DNA ladder; Lane 6: Negative Control

Table 3.1 PCR detection results

	BAL	TASP	Sputum	Bx	PET	Total (%)
	n = 9	*n* = 50	*n* = 14	*n* = 5	*n* = 12	*n* = 90
mtLSUrRNA nested	6	31	8	5	12	**62 (69)**
Real-time 5S	2	26	6	4	11	**49 (54)**
mtLSUrRNA1	4	27	3	3	5	**42 (47)**
DHPS nested	2	23	5	3	5	**38 (42)**
MSG heminested	0	9	3	3	9	**24 (27)**
ITS nested	1	15	2	2	2	**22 (24)**
18S one-tube nested	0	8	3	4	3	**18 (20)**
DHPS single	1	4	2	2	0	**9 (10)**
DHFR nested	0	3	0	3	3	**9 (10)**

BAL = Bronchoalveolar lavage fluid; TASP = Tracheal aspirate fluid; Bx = fresh lung biopsy; PET = Paraffin wax-embedded lung tissue

Table 3.2 Concordance (%) among PCR techniques evaluated

	Real-time 5S	Mt LSU rRNAn	Mt LSU rRNA1	DHPSn	MSGn	ITSn	18S	DHPSs	DHFRn
Real-time 5S	-								
Mt LSU rRNAn	88.9	-							
Mt LSU rRNA1	56.6	64.9	-						
DHPSn	52.2	56.1	54.1	-					
MSGn	73.3	69.1	51.1	72.3	-				
ITSn	62.2	60.2	58.2	74.5	74.5	-			
18S	61.1	62.2	54.1	94.9	87.2	87.2	-		
DHPSs	52.2	41.9	54.0	82.4	77.0	78.4	90.5	-	
DHFRn	56.5	49.3	61.6	83.6	79.5	78.1	87.7	87.7	-

Percent concordance between PCR methods = (concordant positives + concordant negatives) / all tests X 100. n = nested; s = single round; hn = heminested; 1-tn = one-tube nested

Table 3.3 Real-time and mtLSUrRNA nested discordant PCR results

(Following page)

a DETECT IF *Pneumocystis carinii* indirect immunofluorescense kit (Shield Diagnostics)

b Tracheal aspirate

c Paraffin wax-embedded lung biopsy

d Methanamine silver histological staining

e Bronchoalveolar lavage

Dark grey shading = false mtLSUrRNA nested – negative results

Light grey shading = false 5S real-time PCR results

Table 3.3 Real-time and mtLSUrRNA nested discordant PCR results

No.	Specimen type	mtLSUrRNA nested	Real-time	Other PCR positive	Supporting tests
1	Sputum	-	+	None	IF[a] -
2	TASP[b]	-	+	None	IF -
3	TASP	-	+	None	None
4	TASP	-	+	DHPS nested	IF -
5	TASP	-	+	MSG	None
6	TASP	+	-	MtLSUrRNAs, DHPS nested	IF +
7	TASP	+	-	MtLSUrRNAs, DHPS nested	IF +
8	TASP	+	-	MtLSUrRNAs, DHPS nested	IF +
9	TASP	+	-	ITS nested, DHPS nested	IF +
10	TASP	+	-	MtLSUrRNAs, DHPS nested	IF -
11	TASP	+	-	MtLSUrRNAs, DHPS nested	IF -
12	TASP	+	-	mtLSUrRNAs, DHPS nested	IF -
13	Sputum	+	-	mtLSUrRNAs, DHPS nested	IF -
14	PET lung[c]	+	-	MtLSUrRNAs	MS[d] +
15	Biopsy	+	-	MtLSUrRNAs	IF +
16	BAL[e]	+	-	MtLSUrRNAs	IF +
17	TASP	+	-	MtLSUrRNAs	IF +
18	BAL	+	-	MtLSUrRNAs	IF -
19	BAL	+	-	MtLSUrRNAs	Not done

3.2.2 Real-time quantitative analysis

Quantitative analysis was performed on discordant 5S real-time, mtLSUrRNA nested and DHPS nested PCR specimens to determine whether a correlation existed between organism concentration and method performance. The standard curve employed for quantitative analysis (Figure 3.11) had the following characteristics (employing the second derivative maximum analysis method incorporated in the LightCycler software suite): Slope = -0.658, Intercept = 33.5, Error = 0.155, and r = -0.96. The quantitative data obtained on the discordant results are shown in Table 3.4. No correlation was observed between target copy number and performance of a PCR method.

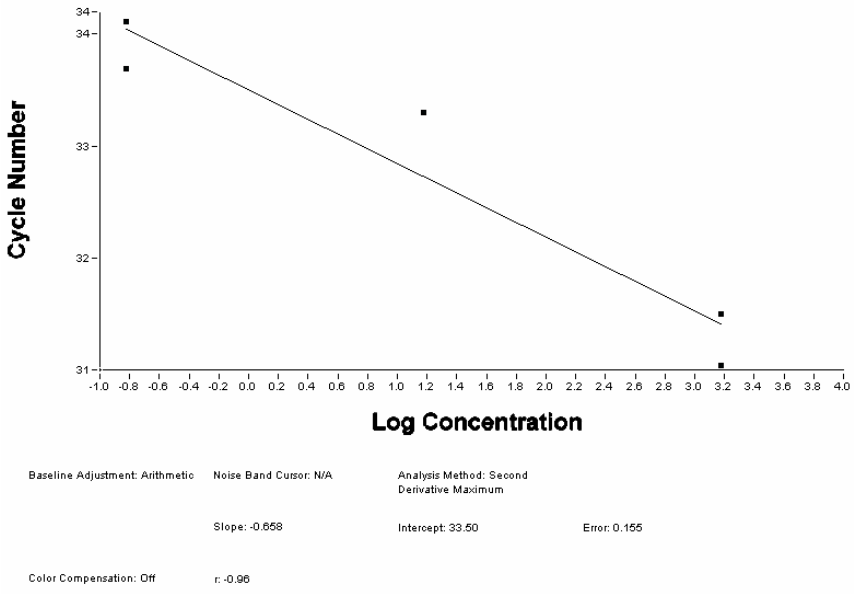


Figure 3.11 Standard curve obtained for quantitative real-time 5S rRNA PCR

Table 3.4 Quantitative analysis of discordant mtLSUrRNA, DHPS and 5S real-time PCR results

Specimen No.	PCR Profile	Target copy number (targets/μl)[#]
1	mtLSUrRNA n - DHPS n -	1.06×10^{-1}
2	mtLSUrRNA n - DHPS n -	2.43×10^{7}
3	mtLSUrRNA n - DHPS n -	7.37×10^{1}
4	mtLSUrRNA n + DHPS n +	2.96×10^{8}
5	mtLSUrRNA n + DHPS n -	1.82×10^{-1}
6	mtLSUrRNA n + DHPS n -	1.44×10^{3}
7	mtLSUrRNA n + DHPS n -	4.56×10^{5}
8	mtLSUrRNA n + DHPS n -	7.09×10^{4}
9	mtLSUrRNA n + DHPS n -	4.94×10^{3}
10	mtLSUrRNA s + mtLSUrRNA n + DHPS n -	1.05×10^{3}
11	mtLSUrRNA s + mtLSUrRNA n + DHPS n -	3.96×10^{10}
12	mtLSUrRNA s + mtLSUrRNA n + DHPS n -	4.59×10^{-1}
13	mtLSUrRNA s + mtLSUrRNA n + DHPS n -	2.85×10^{-1}
14	mtLSUrRNA s + mtLSUrRNA n + DHPS n -	3.13×10^{-1}

[#] As determined by quantitative PCR employing 5S rRNA real-time PCR with 10 μl DNA template.

n = nested; s = single-round

Shading depicts the four PCR profile groups

3.2.3 PCR vs. standard staining techniques

PCR results were compared to IF and histological staining (methenamine silver) by specimen type (Table 3.5). Concordances were calculated among the results obtained (Table 3.6). Agreement of PCR and IF on TASP ranged from 16% - 48%, for BAL from 22% - 44%, and for sputum from 36% - 57%. PCR agreement with fresh biopsies ranged from 40% - 100% and that on formalin-fixed paraffin wax-embedded lung biopsy specimens from 0% - 100% (Table 3.6). It is of note that, compared to histological techniques (methanamine silver staining), PCR on respiratory fluid specimens subjected to IF displayed remarkable discordances. The two most sensitive PCR assays (mtLSUrRNA nested and 5S real-time PCR) produced concordance to IF on TASP of 46 – 48%, BAL of 44%, and sputum of 43% - 50%, whilst compared to methanamine silver histology (often regarded as gold standard) concordance of 80 – 100% was obtained. This alludes to questioning the reliability of IF on respiratory fluids. When compared to IF mtLSUrRNA nested PCR appeared the most concordant PCR method (48%), followed by 5S real-time PCR (45%). Similarly, on methanamine silver stained histology sections, mtLSUrRNA nested PCR was most sensitive (100%), followed by 5S real-time PCR (88%).

3.2.3.1 Comparison of mtLSUrRNA PCR to IF

Since the mtLSUrRNA nested PCR demonstrated the highest detection rate among the primer combinations (Table 3.1) and the highest degree of concordance to staining (Table 3.6), 74 mtLSUrRNA nested PCR results were evaluated against IF (Table 3.7). MtLSUrRNA PCR detected *P. jirovecii* in 42 / 74 whilst IF reported a positive signal from 56 / 74 specimens (Tables 3.7 and 3.8). Direct comparison of mtLSUrRNA nested PCR to IF yielded a sensitivity and specificity of 55.4% and 38.9%, respectively (Table 3.7), whilst comparison of IF to mtLSUrRNA yielded a sensitivity of 73.8% and specificity of 21.9% (Table 3.8).

Table 3.5 PCR technique results vs. IF and histology staining by specimen type in 2X2 format

		Staining Results									
		IF						Histology			
		TASP		BAL		Sputum		PET		Biopsy	
		Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
Real-time 5S *n* = 90	**Positive**	19	6	2	0	3	3	11	0	4	0
	Negative	21	4	5	2	5	3	1	0	1	0
Mt LSU rRNAn *n* = 90	**Positive**	18	4	3	1	3	2	12	0	5	0
	Negative	22	6	4	1	5	4	0	0	0	0
Mt LSU rRNAs *n* = 90	**Positive**	19	8	3	1	1	2	5	0	3	0
	Negative	21	2	4	1	7	4	7	0	2	0
DHPSn *n* = 90	**Positive**	17	6	1	1	3	2	5	0	3	0
	Negative	23	4	6	1	5	4	7	0	2	0
MSGn *n* = 86	**Positive**	9	0	0	0	2	1	9	0	3	0
	Negative	28	9	7	2	6	5	3	0	2	0
ITSn *n* = 90	**Positive**	12	3	1	0	2	0	2	0	2	0
	Negative	28	7	6	2	6	6	10	0	3	0
18S *n* = 90	**Positive**	7	1	0	0	2	1	3	0	4	0
	Negative	33	9	7	2	6	5	9	0	1	0
DHPSs *n* = 69	**Positive**	3	1	1	0	2	0	0	0	2	0
	Negative	26	2	6	2	5	4	12	0	3	0
DHFRn *n* = 68	**Positive**	3	0	0	0	0	0	3	0	3	0
	Negative	27	2	6	2	7	4	9	0	2	0

Table 3.6 Concordance (%) of PCR techniques to staining by specimen type [#]

	IF				Histology		
	TASP	**BAL**	**Sputum**	**Total**	**Biopsy**	**PET***	**Total**
	n = 50	*n* = 9	*n* = 14	*n* = 73	*n* = 5	*n* = 12	*n* = 17
Real-time 5S	46	44	43	**45**	80	92	**88**
MtLSUrRNA n	48	44	50	**48**	100	100	**100**
mtLSUrRNA s	42	44	36	**41**	60	42	**47**
DHPS nested	42	22	50	**41**	60	42	**47**
MSG hn	39	22	50	**39**	60	75	**71**
ITS n	38	33	57	**41**	40	17	**24**
18S n	32	22	50	**34**	80	25	**41**
DHPS s	16	33	55	**27**	40	0	**12**
DHFR n	16	25	36	**22**	60	25	**35**

[#] Calculated from Table 3.6.

* Formalin-fixed paraffin-wax embedded lung biopsy specimens

Table 3.7 Sensitivity and specificity of mtLSUrRNA PCR vs IF

		IF		
		Positive	Negative	*Total*
mtLSUrRNA PCR	Positive	31 [a]	11 [c]	*42*
	Negative	25 [b]	7 [d]	*32*
Total		*56* [a + b]	*18* [c + d]	*74*

Sensitivity: 55.4%

Specificity: 38.9%

Sensitivity = a / (a + b) x 100; Specificity = d / (c + d) x 100

Table 3.8 Sensitivity and specificity of IF vs mtLSUrRNA

		MtLSUrRNA nested		
		Positive	Negative	*Total*
IF	Positive	31	25	*56*
	Negative	11	7	*18*
Total		*42*	*32*	*74*

Sensitivity: 73.8%

Specificity: 21.9%

3.2.4 Analysis of discrepant PCR and IF results

Discrepancies between results obtained from mtLSUrRNA nested PCR and IF were seen with 36 / 74 specimens (Tables 3.7 and 3.8). This high degree of discrepancy prompted investigations of clinical data by review of hospital records and histology and / or cytology results. The findings are summarised in sections 3.2.4.1 and 3.2.4.2.

3.2.4.1 PCR-negative IF-positive

PCR-negative IF-positive discrepant results were seen in 25 specimens. Following review of hospital records, histology and/or cytology results and DHPS nested PCR (confirmed by sequencing), specimen results were as follows: (Summarised in Table 3.9a.)

False IF positive (7 / 25 specimens)

- One specimen, clinically the patient was considered not to have PcP and the histology result was negative.
- Three specimens, clinically the patients were considered not to have PcP, one of these was negative by cytology and the other two patients recovered without specific PcP treatment.
- Two patients were negative by cytology and/or histology.
- One patient was diagnosed and successfully treated for *M. tuberculosis*.

False mtLSUrRNA PCR negative (9 / 25 specimens)

- Eight specimens produced a DHPS nested PCR amplicon that was confirmed by sequencing.
- One specimen on re-amplification was mtLSUrRNA nested PCR positive.

Unresolved (9 / 25 specimens)

- On six specimens no other laboratory tests were performed to exclude PcP, and patients received cotrimoxazole.

- Three specimens failed to produce an amplification product with a template preparation control employing a universal bacterial PCR (Template quality suspected).

Table 3.9a Analysis of discrepant mtLSUrRNA PCR-negative IF-positive results by incorporating clinical records and cytology/histology findings and additional PCR results where available

Discrepancy	Number of specimens	Clinical records and cytology, histology and DHPS PCR results where available	Consideration
PCR – IF +	1	Clinically not considered PcP and histology negative	7 PCR negative, thus false IF-positive
	3	Clinically not considered PcP, one patient cytology negative, and two patients improved without treatment	
	2	Cytology and/or histology negative	
	1	Diagnosed with TB and successfully treated for TB	
	6	No other laboratory tests performed to exclude PcP, received cotrimoxazole.	9 Unresolved
	3	Suspect template DNA, possible PCR-negative	
	8	DHPS PCR positive	9 False PCR negative
	1	Repeat PCR-positive	

3.2.4.2 PCR-positive IF-negative

PCR-positive IF-negative discrepant results were seen in 11 specimens. The discrepant results are summarised in Table 3.9b. Following review of hospital records, histology and/or cytology results and DHPS nested PCR (confirmed by sequencing) results, specimen results were as follows:

False IF negative (1 / 11 specimens)

- One patient on repeat sampling was IF positive.

Unresolved (10 / 11 specimens)

- Four patients were clinically considered to have PcP and received cotrimoxazole.
- Of six patients insufficient clinical data was recorded.

Table 3.9b Analysis of discrepant mtLSUrRNA PCR-positive IF-negative results by incorporating clinical records and cytology/histology findings and additional PCR results where available

Discrepancy	Number of specimens	Clinical records and cytology, histology and DHPS PCR results where available	Consideration
PCR + IF -	1	Repeat IF-positive	False IF-negative
	4	Clinically considered PcP and received cotrimoxazole	10 Unresolved
	6	No clinical records available	

Of the 36 specimens that produced discrepant results, 19 could not be resolved due to insufficient data although for 10 specimens false negative IF results are indicated (Tables 3.9a and 3.9b). The unresolved cases included: a) template DNA suspect (3 specimens); b) No laboratory investigations or clinical records that supported or excluded PcP (10 specimens); c) insufficient clinical evidence to exclude PcP (6 specimens).

Including the review of hospital records, histology and/or cytology results and DHPS nested PCR (confirmed by sequencing), mtLSUrRNA yielded a sensitivity and specificity of 77.5% and 93.3%, respectively. The resultant positive and negative predictive values were 96.9% and 60.9% (Table 3.10). Comparing IF to reviewed results the sensitivity and specificity was 96.9% and 60.9%, and the positive and negative predicted values were 77.5% and 93.3%, respectively (Table 3.11). From Table 3.10 and 3.11 the derived prevalence of *P. jirovecii* in the sampled population was 69.9% based on mtLSUrRNA nested PCR results, and 74% based on IF results.

Table 3.10 Sensitivity and specificity of mtLSUrRNA PCR vs IF with clinical review

		Reviewed data		
		Positive	Negative	*Total*
mtLSUrRNA PCR {	Positive	31 [a]	1 [c]	*32* [a + c]
	Negative	9 [b]	14 [d]	*23* [b + d]
Total		*40*	*15*	*55*

Sensitivity: 77.5%

Specificity: 93.3%

Positive predictive value: 96.9%

Negative predictive value: 60.9%

Prevalence: 69.9%

Positive predictive value = a / (a + c); Negative predictive value = d / (b + d);

Prevalence = a + b / (a + b + c)

Table 3.11 Sensitivity and specificity of IF vs mtLSUrRNA PCR with clinical review

			Reviewed data		
			Positive	Negative	*Total*
IF	{	Positive	31	9	*40*
		Negative	1	14	*15*
	Total		*32*	*23*	*55*

Sensitivity: 96.9%

Specificity: 60.9%

Positive predictive value: 77.5%

Negative predictive value: 93.3%

Prevalence: 74%

3.3 DISCUSSION

The perception by clinicians at Tygerberg Hospital that the *P. jirovecii* IF method appears to produce both false-positive and false-negative results prompted evaluation and comparison of IF and PCR techniques.

3.3.1 PCR evaluation

The mtLSUrRNA nested PCR employed against the South African clinical specimens yielded the highest level of sensitivity, followed by 5S rRNA real-time PCR. MtLSUrRNA is a nested reaction targeting a multicopy gene and lends itself to the detection of very low numbers of organisms, as reported in other studies [Rabodonirina*, et al.*, 1997; Ribes*, et al.*, 1997; Sing*, et al.*, 2000; Torres*, et al.*, 2000]. MtLSUrRNA nested PCR detected considerably more *P. jirovecii* than the single-round PCR, 62 vs. 42 positive specimens. 5S Real-time PCR performed sub-optimally, since utilisation of a sensitive amplicon detection technique (real-time

fluorescence detection) targeting a multicopy gene present at > 10^3 copies per haploid genome [Kitada*, et al.*, 1991] one would expect a higher detection rate than conventional mtLSUrRNA nested PCR. However, 5S real-time PCR detection was similar to mtLSUrRNA single-round PCR. The detection limit for conventional mtLSUrRNA single PCR and 5S PCR have been suggested to be 10 nuclei / μl and 160 nuclei / μl (as tested on rat isolates), respectively [De Luca*, et al.*, 1995]. This implies that mtLSUrRNA should be more sensitive than conventional 5S PCR. The concordance of conventional 5S and mtLSUrRNA single PCR has been reported to be 93.6% [De Luca*, et al.*, 1995]. However, in the current study, the use of real-time PCR has shifted the concordance of conventional mtLSUrRNA single and 5S real-time PCR to 56.6%. The employment of fluorescent detection chemistry has improved the sensitivity of the 5S PCR technique, which was more sensitive in the current study than conventional mtLSUrRNA single-round PCR. Both 5S and mtLSUrRNA single PCR primers have been tested in previous studies for specificity: mtLSUrRNA single primers have been tested against *Aspergillus nidulans, A. fumigatus, Cryptococcus neoformans, Candida albicans* and non-*albicans strains*, *Mycobacterium tuberculosis*, *Saccharomyces cerevisiae, and Histoplasma capsulatum* [Wakefield*, et al.*, 1990b; Lu*, et al.*, 1995], and 5S primers against *Corynebacterium kutshceri*, *Pasteurella pneumotropica*, *C*. *albicans, C*. *glabrata*, *Aspergillus fumigatus*, *C*. *neoformans* and *M*. *tuberculosis* [Kitada*, et al.*, 1991]. Although many clinically more relevant organisms could have been included for testing specificity, 5S real-time PCR has the advantage of melting curve analysis to confirm the sequence identity of the amplicon, and mtLSUrRNA products are verified by employing nested primers. It is widely assumed that real-time PCR is more sensitive than conventional amplification systems. Careful analysis of published reports reveals that real-time PCR has equal sensitivity to conventional amplification formats [Palladino*, et al.*, 2001; Flori*, et al.*, 2004]. In the current study analysis of discordant mtLSUrRNA nested and 5S real-time PCR indicates that mtLSUrRNA nested PCR produced two false-negative results, whilst 5S real-time PCR produced 12 false-negative reports. This reinforces the value of conventional mtLSUrRNA nested PCR for the epidemiological detection of *P. jirovecii* from clinical specimens.

The inability to amplify the DHPS gene from all *P. jirovecii* PCR-positive specimens (38 vs. 62 for mtLSUrRNA nested) has been reported by other investigators in the USA [Beard*, et al.*, 2000; Huang*, et al.*, 2000]. A plausible explanation as to why DHPS fails to amplify all mtLSUrRNA positive specimens is that DHPS is a single nuclear encoded gene whereas mtLSUrRNA is a mitochondrial encoded gene and many mitochondria may be present at any one time within the cytoplasm. This may also be the reason for the poor performance of the DHPS single-round PCR as one would expect a lower yield than with the nested reaction.

DHFR detected only 9 specimens, compared to 62 by mtLSUrRNA nested PCR. These findings are similar to those reported by Lu *et al*. (Indiana, USA) who obtained detection rates of 23% employing DHFR [Lu*, et al.*, 1995]. However, in contrast, De Luca *et al.* reported similar sensitivities on comparing the two techniques (mtLSUrRNA: 72.7% and DHFR: 71%) [De Luca*, et al.*, 1995]. Similar to the reported DHFR 5S concordance of 56.8% by De Luca *et al*. [De Luca*, et al.*, 1995], the current study yielded a concordance of 61.6%. In support of similar sensitivities obtained, a detection limit of 160 nuclei / μl was reported for rat isolates on DHFR and 5S PCR [De Luca*, et al.*, 1995].

The poor performances of the MSG (24 MSG positive vs. 42 mtLSUrRNA single positive) seen in this study is in contrast to that reported by Huang *et al*. Working on specimens obtained from patients in the USA in 1999, they found that MSG PCR sensitivity exceeded that of mtLSUrRNA single PCR, and that MSG PCR yielded a positive signal from as little as 16 fg total DNA as compared to 1.6 pg by mtLSUrRNA single PCR [Huang*, et al.*, 1999]. A reason for the observed low sensitivity and concordance is unclear, but speculation as to why MSG primers were seen not to be as efficient as the mtLSUrRNA primers on these recent specimens from South Africa is that the MSG gene (involved in antigenicity) is known to undergo constant rearrangement and such events may have resulted in modified primer annealing site [Garbe & Stringer, 1994; Kutty*, et al.*, 2001].

The lack of sensitivity of the ITS PCR (24% detection vs. 47% detection by mtLSUrRNA single-round PCR) is in contrast to previous studies reported in the literature. ITS PCR has been shown to be more sensitive (100% sensitivity) than mtLSUrRNA single PCR (87% sensitive), with an apparent detection limit of 3 organisms per reaction [Lu*, et al.*, 1995]. It is of importance to note that while Lu *et al*. reported that ITS PCR did not amplify DNA from various other organisms including *C. albicans* and *C. neoformans* [Lu*, et al.*, 1995], in the present study, on subcloning and sequencing of the *P. jirovecii* indicative 550 bp amplicons, the homologous region of *C. albicans* (five specimens) and *Cryptococcus magnus* (one specimen) was demonstrated. For two of these specimens all other PCR reactions were negative.

Previous reports of the high sensitivity of 18S rRNA nested PCR (100% detection compared to 87% detection by mtLSUrRNA single PCR) [Lu, 1995], is in contradiction to what was obtained from the current study (20% 18S detection vs. 47% mtLSUrRNA single PCR detection). In addition, Mathis *et al*. reported that by omitting the nested reaction of 18S PCR a detection rate similar to that of single-round mtLSUrRNA PCR is obtained, but when performing a nested reaction, up to a 100 fold increase in sensitivity was obtained compared to a single step 18S and mtLSUrRNA PCR. A detection limit of 3 cysts per reaction was reported for the nested 18S PCR [Mathis*, et al.*, 1997].

3.3.2 Real-time quantitative analysis

Quantitative analysis of discordant 5S real-time, mtLSUrRNA nested and DHPS nested PCR was performed to determine if a correlation exists between target copy number and PCR method performance. From the data obtained no correlation to target copy number could be observed, indicating that factors other than organism concentration play a role in the variable efficacy of these three PCR methods.

3.3.3 Analysis of PCR vs. IF

It should always be borne in mind, that when comparing techniques sampling can influence result outcomes e.g. one PCR-negative specimen, on re-amplification was seen to be positive, and on repeat staining of an IF-negative specimen cysts were subsequently demonstrated. Direct comparison of mtLSUrRNA to IF yielded a poor sensitivity and specificity, 55.4% and 41.2%, respectively. When IF was compared to mtLSUrRNA nested PCR results a sensitivity- and specificity of 73.8% and 21.9% was obtained. The apparent lack of sensitivity of PCR compared to IF was due to IF reporting a positive signal from 56 specimens and PCR from 42 (∆ 14 specimens). Where discrepancies (36 specimens) were observed, clinical information, cytology/histology and additional DHPS PCR (sequence confirmed) findings were taken into consideration. Seven false IF-positive results were uncovered that could be substantiated by good clinical and laboratory findings to exclude PcP. False IF signals could be due to the inability of fluorescence microscopy to differentiate true positives from non-specific background fluorescence as morphology is not weighted to the same extent as in conventional non-fluorescent stains. In addition, empty cysts, which will be detected by IF but not by PCR, have been shown to persist in lung tissue for 4 – 6 weeks after successful treatment [Armbruster*, et al.*, 1995]. One specimen was considered a false IF-negative (true PCR-positive). This could be due to the inability of IF to accurately detect trophic forms of *P. jirovecii* that can often outnumber cysts in clinical specimens [El-Sadr & Gidhu, 1986]. Nine specimens failed to produce an amplification product on mtLSUrRNA PCR. These were considered false negative results due to amplification (and sequence confirmation) by DHPS nested primers. The reasons for these results are unclear as it defies the target copy number hypothesis, and alternative factors should be considered. It is of interest that when compared to the gold standard of methenamine silver histology-staining, mtLSUrRNA, and in fact most other PCR assays, performed much better than when compared to IF.

Subsequent to the review of clinical records and additional staining and PCR results, the IF method was seen to exhibit high sensitivity (96.9%) but low specificity (60.8%), evidence suggests false-positive (9/23) results. MtLSUrRNA PCR displayed a sensitivity of 77.5% and specificity of 93.3%. The positive predictive value obtained by PCR (96.9%) exceeds that of IF (77.5%), indicating that with a population of low prevalence of disease PCR is most effective. However, the negative predictive value of IF (93.3%) exceeds that of PCR (60.9%), and would be a more appropriate choice of test for populations with a high prevalence of disease. Prevalence seen in the population studied (specimens submitted specifically on suspicion of PcP, or performed to exclude PcP) was 69.9% - 74%. Exact conclusions as to the most suitable diagnostic method for this population are confounded by the high number of unresolved clinical cases (19 / 74 = 25.7%).

Similar studies employing mtLSUrRNA primers found a higher sensitivity of PCR compared to staining techniques and a reduced specificity, owing to the detection of very low numbers of organisms [Moonens*, et al.*, 1995; Ribes*, et al.*, 1997]. This is frequently encountered when a nested PCR is employed and has been suggested to indicate colonisation or subclinical infection [Rabodonirina*, et al.*, 1997; Ribes*, et al.*, 1997; Sing*, et al.*, 2000; Torres*, et al.*, 2000]. However, comparable sensitivities and specificities of PCR to IF have been reported [Moonens*, et al.*, 1995; Mathis*, et al.*, 1997]. After review of clinical data Mathis *et al*. reported sensitivities and specificities of 18S PCR and IF to be 94.8% and 99.1%, and 93.8% and 100%, respectively. However, it was noted that PCR seemed more sensitive on sputum samples and IF on BAL samples [Mathis*, et al.*, 1997]. This could be due to the higher incidence of background non-specific fluorescence occurring in sputum as opposed to BAL.

CHAPTER 4

CO-INFECTION

4.1 INTRODUCTION

Worldwide, PcP and pulmonary tuberculosis (TB), caused by *Mycobacterium tuberculosis*, are the primary lung infections associated with HIV. Their respective prevalence, however, varies geographically. *P. jirovecii* is the most frequent cause of opportunistic pneumonia in the USA and Europe, while *M. tuberculosis* seems to be the main pulmonary pathogen in African HIV-positive patients [Orlovic*, et al.*, 2001]. In the developing world *M. tuberculosis* appears to be the most commonly encountered co-infecting pathogen associated with PcP, occurring in up to 66% of PcP cases [MacLeod*, et al.*, 1989; Abouya*, et al.*, 1992; Machiels*, et al.*, 1992; Atzori*, et al.*, 1993; Weinberg & Duarte, 1993; Malin*, et al.*, 1995; Mahomed*, et al.*, 1999]. Pathogens, other than *M. tuberculosis*, commonly encountered include *S. pneumoniae*, *S. aureus* and CMV [MacLeod*, et al.*, 1989; Hsiao*, et al.*, 1997]. Rates of co-infection associated with PcP and any other pathogen, range from 20% - 70% [Fisk*, et al.*, 2003]. Paediatric patients with PcP seem particularly at risk for co-infection with CMV as rates of up to 68% among African patients [Jeena*, et al.*, 1996;

Graham*, et al.*, 2000], and 58% among Thai patients have been reported [Bhoopat*, et al.*, 1994].

It has been suggested that a surprisingly high frequency of *P. jirovecii* and *M. tuberculosis* co-infections may occur in TB hyperendemic areas [Orlovic*, et al.*, 2001]. Orlovic *et al.* reported the co-existence of PcP and TB in 9.9% of induced sputum specimens obtained from HIV-positive patients [Orlovic*, et al.*, 2001]. Similarly Aderaye *et al*. reported the detection of *P. jirovecii* in Ethiopian patients with proven TB in 13.5% of HIV-positive and 4.3% of HIV-negative patients respectively. These investigators reported a prevalence of PcP in 30.3% of HIV-positive patients with suspected TB [Aderaye*, et al.*, 2003].

To investigate the rate of co-infections associated with PcP a retrospective review of laboratory data was undertaken to identify pulmonary pathogens commonly encountered in association with PcP. In addition, to investigate the possible contribution that *P. jirovecii* may have on patients in South Africa with pulmonary tuberculosis, *P. jirovecii* directed PCR was performed on a sputum bank created from a TB surveillance study.

4.1 RESULTS

4.2.1 Retrospective laboratory investigation for co-infections

Of 78 sequential specimens submitted to the microbiology laboratory at Tygerberg Hospital for routine diagnosis of PcP, 62 produced an amplicon indicative of the presence of *P. jirovecii* by mtLSUrRNA nested PCR (section 3.2.1, Table 3.1). Twenty (32.2%) of these specimens were collected from patients with co-existing pulmonary infections (Table 4.1). The pathogenic organisms were: Gram-negative bacilli (*Klebsiella pneumoniae*, *Acinetobacter* spp., and *Pseudomonas aeruginosa*) in 9 / 62 (14.5%) patients, CMV in four patients (6.5%), *M. tuberculosis* in three (4.8%), and respiratory syncytial virus (RSV) in two (3.2%) patients. The *Acinetobacter* spp. and *Pseudomonas aeruginosa* might not have been significant isolates. Co-infection with three pathogens was not uncommon. *P. jirovecii*, *M. tuberculosis* and *Candida*

albicans were observed in two patients, *P. jirovecii*, *C. albicans* and *S. pneumoniae* in one patient, and *P. jirovecii* with two different Gram-negative bacilli in one patient. The significance of the *C. albicans* was not known. Co-infections occurred with higher frequency in HIV-positive patients (6 / 18) than HIV-negative patients (2 / 18), however, 10 / 18 patients with co-infections had an unknown HIV status. Of the patients with laboratory demonstrated *P. jirovecii* infection, as detected with mtLSUrRNA nested PCR (section 3.1), and record of HIV-status (40 patients), 45% were HIV-negative, 45% HIV-positive, and 10% were immunocompromised due to cytotoxic chemotherapy or systemic lupus erythaematosis.

Table 4.1 PcP patients with pulmonary co-infections

Co-infecting pathogen	Total	Immuno-suppressed*	HIV status		
			Positive	Negative	Unknown
Cytomegalovirus	4	1	2	0	1
Respiratory cyncytial virus	2	0	1	0	1
Gram negative bacteria[#]	9	0	1	2	6
M. tuberculosis	2	0	1	0	1
M. tuberculosis and *C. albicans*	1	1	0	0	0
S. pneumoniae and *C. albicans*	1	0	1	0	0
Two different Gram negative bacteria	1	0	0	0	1
Total	**20**	**2**	**6**	**2**	**10**

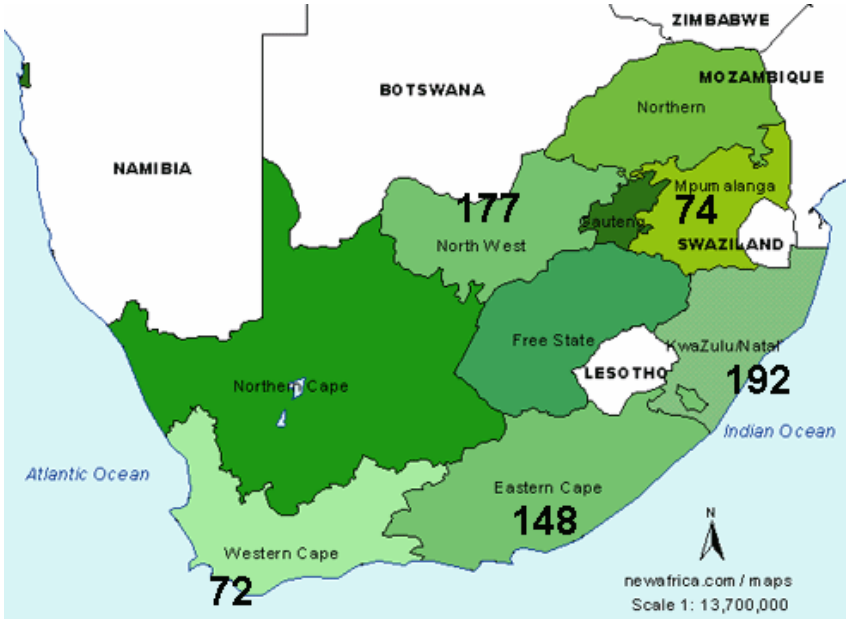
* Organ transplant recipients and systemic lupus erythaematosis

[#] *Klebsiella pneumoniae, Acinetobacter spp, Pseudomonas aeruginosa.*

4.2.2 *P. jirovecii M. tuberculosis* co-infections

Sputum specimens, collected from patients residing in five provinces of South Africa, for a TB surveillance study, were screened by PCR for the presence of *P. jirovecii* (Figure 4.1). MtLSUrRNA nested PCR detected *P. jirovecii* DNA in 23 / 663 *M. tuberculosis* culture-positive sputum specimens (Table 4.2). Of HIV-positive patients with confirmed TB 4.3% had *P. jirovecii* DNA detectable in sputum. Of HIV-negative patients with confirmed TB 2.5% had *P. jirovecii* DNA detectable in sputum. Detection was highest in patients from the Western Cape (3 / 72) (4.2%), followed by those from North West Province (7 / 177) (4.0%), KwaZulu Natal (7 / 192) (3.7%), Eastern Cape (5 / 148) (3.4%), with the lowest detection in Mpumalanga (1 / 74) (1.4%). The prevalence of HIV in the sampled population was 45.4% and the prevalence of *P. jirovecii* 3.5%. Of the TB patients co-infected/colonised with *P. jirovecii* 13 / 23 were HIV-positive, 9 / 23 were HIV-negative and one patient had an unknown HIV status. *P. jirovecii* was detected more frequently in HIV-positive vs. HIV-negative patients residing in KwaZulu Natal (6 / 7 vs. 1 / 7), Western Cape (3 / 3 vs. 0 / 3) and North West (5 / 7 vs. 2 / 7); however, in the Eastern Cape *P. jirovecii* was detected more frequently in HIV-negative patients (5 / 5 vs. 0 / 0). The single case of *P. jirovecii* detected from Mpumalanga was from an individual whose HIV status was unknown.

Figure 4.1 Provinces from which sputum specimens were obtained for screened co-existing *P. jirovecii* and *M. tuberculosis*[#]



[#] Numbers of specimens screened are indicated.

Table 4.2 Detection of *P. jirovecii* by mtLSUrRNA nested PCR in sputum from *M. tuberculosis* culture-positive specimens

	n	HIV-positive (%)	PcP detection (%)	PcP detection by HIV status		
				Positive	Negative	Unknown
KwaZulu Natal	192	55.7	7 (3.7)	6	1	0
Western Cape	72	30.6	3 (4.2)	2	1	0
Eastern Cape	148	23.0	5 (3.4)	0	5	0
North West	177	52.0	7 (4.0)	5	2	0
Mpumalanga	74	62.2	1 (1.4)	0	0	1
5 Provinces Total	**663**	**45.4**	**23 (3.5)**	**13**	**9**	**1**

4.3 DISCUSSION

Of 78 sequential specimens submitted to Tygerberg Hospital for routine PcP diagnosis 62 were positive for *P. jirovecii* by mtLSUrRNA nested PCR. Of these patients who were initially suspected of having PcP on clinical grounds, 32.2% harboured co-infecting pathogens in the respiratory tract. The most frequent co-infections seen in the patient population tested were Gram-negative bacilli (14.5%), followed by CMV (6.2%). The significance of the Gram-negative bacilli is unknown. Previous reports have indicated high co-infection rates of *P. jirovecii* and CMV, particularly in the paediatric population where rates between 5% and 100% in HIV-positive patients have been reported [Michalany*, et al.*, 1987; Weinberg*, et al.*, 1993; Bhoopat*, et al.*, 1994; Graham*, et al.*, 1995; Jeena*, et al.*, 1996; Mootsikapun*, et al.*, 1996; Hsiao*, et al.*, 1997]. In South Africa Jeena *et al*. reported 68% co-infection of CMV and *P. jirovecii* in HIV-positive paediatric cases [Jeena*, et al.*, 1996]. In contrast to a study in Zimbabwe, in which *S. pneumoniae* was the co-infecting pathogen in up to 50% of HIV-positive adults [McLeod*, et al.*, 1989], our data suggest a low co-infection rate (1 / 62) with *S. pneumoniae.*

The high rate of detection of *P. jirovecii* by molecular techniques in immunocompetent patients (45% of specimens submitted for routine PcP diagnosis) is worrisome. Sing *et al*. reported detecting *P. jirovecii* in 17% - 20% of immunocompetent patients not presenting with PcP [Sing*, et al.*, 2000]. In our study population one cannot assume a high false positive rate, as argued by Sing *et al*. since the obtained positive predicted value for mtLSUrRNA nested PCR is 96.9% (section 3.3.4.2, Table 3.10). This implies that a real problem exists in that PcP in South Africa may be occurring in immunocompetent patients more frequently than expected.

The detection of *P. jirovecii* in 3.5% of sputum samples from patients with culture confirmed pulmonary TB is of concern. The diagnostic yield of sputum samples for detection of *P. jirovecii* is very low [Masur*, et al.*, 1988], suggesting that this figure is possibly an underestimate. Orlovic *et al*. reported the detection of *M. tuberculosis* in 9.9% of South African patients who were on treatment for PcP [Orlovic*, et al.*, 2001]. Higher prevalence of co-infection has been reported throughout the developing

world. In Brazil and Tanzania induced sputum specimens stained with Toluidine-blue have diagnosed co-infection in 25% and 66% of HIV-positive patients respectively [Atzori*, et al.*, 1993; Pitchenik*, et al.*, 1983]. Studies employing BAL and transbronchial biopsy specimens with standard staining have shown co-infection in HIV-positive patients ranging from 5% to 40% [McLeod*, et al.*, 1989; Abouya*, et al.*, 1992; Machiels & Urban, 1992; Bhoopat*, et al.*, 1994; Malin*, et al.*, 1995; Mahomed*, et al.*, 1999]. Geographic variations in the frequency of co-infecting pathogens have been noted. From Mexico reports of *M. tuberculosis P. jirovecii* co-infections in HIV-positive patients have reached 24%, whereas lower rates have been reported in the USA (6%) and Italy (5%) [Mohar*, et al.*, 1992].

The clinical and radiographic presentation of TB can mimic that of PcP, particularly in immunocompromised individuals. Orlovic *et al*. reported that the most common symptoms in patients co-infected with *P. jirovecii* and *M. tuberculosis* was a productive or dry cough, night sweats, dyspnoea, and fever. In addition, radiographic presentation of bilateral patchy infiltrates or reticulonodular patterns, focal consolidation, hilar / paratracheal lymphadenopathy, pleural effusion, and necrotizing or cavitation were seen in co-infected patients, exemplifying the difficulties in differentiating co-existence of the two pathogens [Orlovic*, et al.*, 2001]. Aderaye *et al*. reported that 13.5% of patients diagnosed with TB on chest radiography tested positive for *P. jirovecii* employing nested PCR, with no microbiological evidence of TB [Aderaye*, et al.*, 2003].

In the current study, 3.5% of patients diagnosed initially as having TB were co-infected / colonised with *P. jirovecii*. In addition, of patients diagnosed initially as having PcP 4.8% were co-infected with *M. tuberculosis*. Although the data sets were vastly different in terms of statistical power, it would seem evident that when encountering TB or PcP, clinicians in South Africa can expect co-infection in 3.5% - 4.8% of patients.

Although the clinical outcome and relevance of *P. jirovecii* co-infection with *M. tuberculosis* is unknown from our study design, Orlovic *et al*. reported from South Africa that clinical response was only seen once the second infection was adequately

treated [Orlovic*, et al.*, 2001]. In patients with dual infection, *M. tuberculosis* appears to exert a more dominant role in the disease process [Orlovic*, et al.*, 2001]. *P. jirovecii* is generally regarded as a pathogen of low virulence, which in HIV-positive patients emerges later during the progressive loss of cell mediated immunity, and appears initially as a more subtle disease with symptoms absent for weeks to months prior to the requirement for hospitalisation [Lucas*, et al.*, 1990; Orlovic*, et al.*, 2001]. In many cases more virulent pathogens such as *M. tuberculosis* may trigger an acute disease, with detection of subclinical *P. jirovecii*. However, ignoring laboratory detection of *P. jirovecii* could lead to unresponsiveness to TB treatment regimes, or the infection may remain subclinical for a latent period, but emerge as a serious pneumonia when cellular immunity has diminished.

On analysis of the value of nested PCR for different immunological groups of patients Sing *et al*. reported that nested PCR and IF displayed good correlation in the HIV-positive group. In the other patient groups (transplant recipients, malignancy, otherwise immunosuppressed, immunocompetent) nested PCR and IF yielded a poor correlation, mainly with BAL but also with sputum and TASP [Sing*, et al.*, 2000]. The poor concordance of mtLSUrRNA nested PCR and IF from the present study (Chapter 3) may be due to the high representation of HIV-negative, non-immunocompromised individuals diagnosed with PcP (45%). This suggests that other immunosuppressive factors such as nutrition or co-infections may predispose our study population to the development of PcP. In support of this suggestion, nutrition was the first condition recognised as predisposing to PcP in children [Burke & Good, 1973]. The detection of *P. jirovecii* in HIV-positive patients by nested PCR should alert clinicians to possible early developmental stages of disease as colonisation rarely occurs in HIV-infected patients without leading to overt PcP [Sing*, et al.*, 2000].

CHAPTER 5

GENOTYPING AND COALESCENT PHYLOGENETIC ANALYSIS

5.1 INTRODUCTION

Pneumocystis pneumonia (PcP) is a major contributor to morbidity and mortality in immunocompromised individuals [Rimland, *et al.*, 2002; Ruffini, *et al.*, 2002]. Many molecular epidemiological techniques are not applicable for typing of *Pneumocystis,* as it cannot readily be propagated *in vitro*. Regions that have been investigated for designing a typing method include the mitochondrial large subunit rRNA (mtLSUrRNA), mitochondrial small subunit rRNA (mtSSUrRNA), *arom* locus, and internal transcribed spacer regions (ITS) [Tsolaki, *et al.*, 1998]. Sequence diversity of *P. jirovecii* ITS1 and ITS2 regions made these regions suitable for typing of *P. jirovecii* [Lu, *et al.*, 1994]. Ribosomal DNA of *P. jirovecii* is present as a single copy in the cell and is transcribed as a single transcript, with 18S rRNA, 5.8S rRNA and 26S rRNA occurring in tandem [Guintoli, *et al.*, 1994]. The rRNA genes are separated by

ITS1 between 18S rRNA and 5.8S rRNA, and ITS2 between 5.8S rRNA and 26S rRNA [Edman, *et al.*, 1989].

Globally the most frequently encountered genotypes are Eg and Ne [Tsolaki, *et al.*, 1996; Lee, *et al.*, 1998; Tsolaki, *et al.*, 1998]. Latouche *et al*. proposed that as a specific type was seen to persist during the same episode of PcP, genotype switching did not occur [Latouche, *et al.,* 1997]. However, in a study conducted on 19 patients, within the same episode of PcP, genotype changes were observed in 53% of the patients [Helweg-Larsen, *et al.*, 2001]. In addition co-infection with more than one genotype has been reported in a high proportion of PcP episodes [Lu, *et al.*, 1994; Tsolaki, *et al.*, 1996; Tsolaki, *et al.*, 1998; Tsolaki, *et al.*, 1999; Nahimana, *et al.*, 2000; Nimri, *et al.*, 2002]. The current understanding is that *P. jirovecii* infection is not clonal, and that repeated *de novo* acquisition of ITS types is likely to occur [Tsolaki, *et al.*, 1996; Latouche, *et al.*, 1997].

Conventional molecular phylogenetic analysis of tree building is based on homologous characters between species that are assumed reproductively isolated with ascendance based on linear, dichotomous speciation events [Rosenberg, *et al.*, 2002]. These assumptions do not hold when analysing intraspecific nucleotide evolution at the population level. Focus is shifted to a recent evolutionary timescale, which implies that ancestral states may still exist, multiple apomorphies may be present, sexual reproduction may take place and recombination may be involved [Rosenberg, *et al.*, 2002]. Coalescent theory addresses these issues as it models genealogical processes of selectively neutral genes from a population, looking backward in time whereby all lineages will eventually coalesce into a single lineage termed the most recent common ancestor of the sample [Rosenberg, *et al.*, 2002].

5.2 RESULTS

5.2.1 ITS1 and ITS2 genotypes

From the 20 clinical specimens (19 patients) 83 clones were sequenced and ITS types assigned. Twelve new ITS1 sequences (Roman numerals Fig. 5.1) and 13 new ITS2 sequences (Arabic numerals Fig. 5.2) were found. A novel ITS2 type was identified in three patients and designated u (Table 5.1; Fig.5.2). Four new combinations of previously reported ITS1 and ITS2 sequences were demonstrated: Eo, Je, Ge and No.

PCR employing DNA polymerase *Pwo* demonstrated a reduced specificity when compared to *Taq* DNA polymerase reactions. Although *Pwo* polymerase produced amplicons of the expected size, upon cloning and sequencing 35 clones, most PCR products were shown to be human DNA and/or the homologues ITS regions of *Candida albicans.* However, successful amplification of four new types employing *Pwo* confirmed the sequences of Eu, Xg, E12 and XI11 (Table 5.1 and Figs. 5.1 and 5.2). A single ITS type was detected in 3 specimens; 5 specimens contained 2 types; 8 specimens 3 types; 2 specimens 4 types; 1 specimen 5 types and 1 specimen 6 types. Sequencing of two specimens, a tracheal aspirate and a biopsy, collected from one patient yielded nine different types (specimens 2 and 3 Table 5.1). The most frequent type detected in the population studies was Eg (14/19 patients, 33/83 clones), followed by Gg (4/19, 7/83), Eu (3/19, 5/83) and Gh (2/19, 2/83) (Table 5.1).

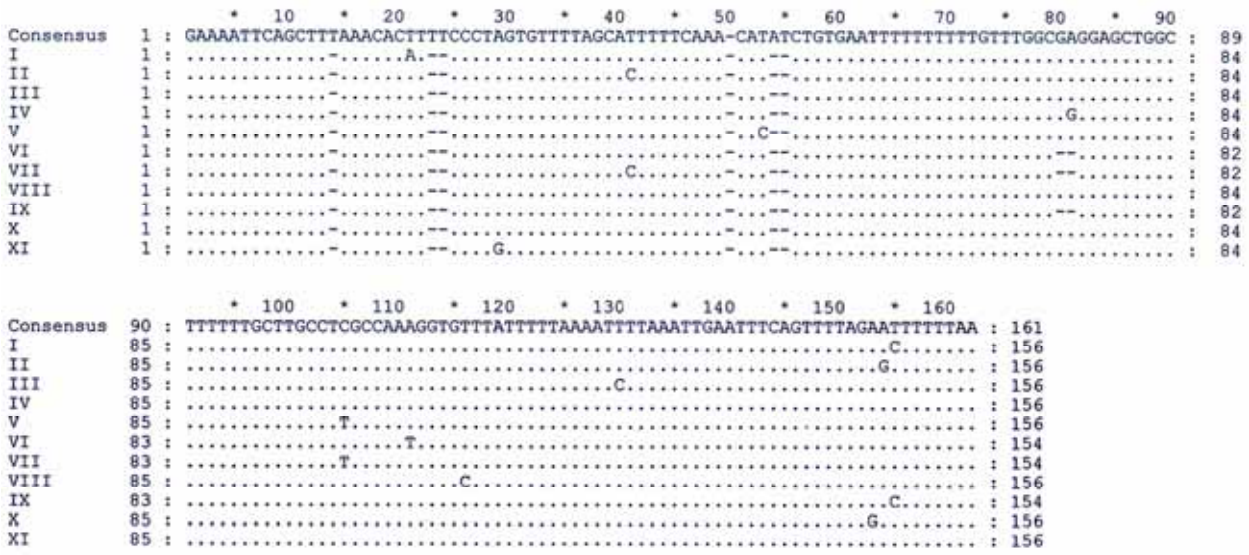


Figure 5.1 Alignment of new ITS1 sequences

GenBank accession numbers I - XI: AY328043 – AY328053

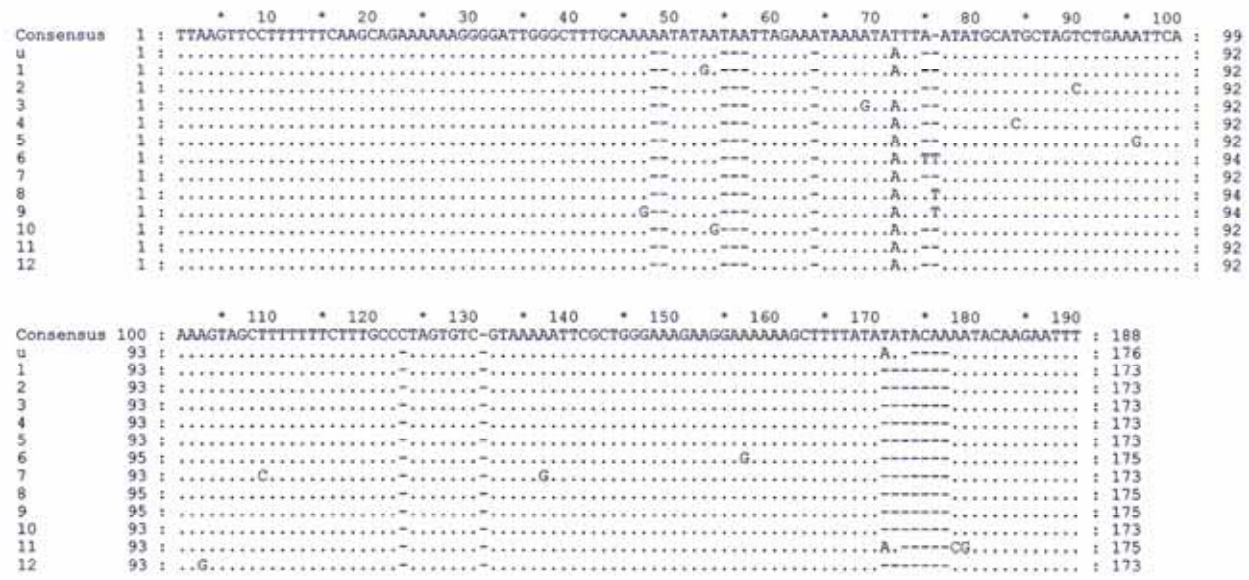


Figure 5.2 Alignment of new ITS2 sequences

GenBank accession numbers u: AY328054 and 1 –12: AY328055 – AY328066

Table 5.1 ITS types demonstrated from 20 specimens (83 clones)

Specimen	ITS types obtained from each specimen					
1	E g	E u				
2*#	J e	N e	I g	E o	E g	II g
3*#	E g	III g	IV 1	N g		
4#	E g	G e	N o			
5	G b	E b	E g			
6	E 2	E g				
7	V 3	E g	E 4			
8	G g	G h	VI g			
9	E 5	E g				
10	VII 6	E 7	G h	G 6		
11	E g					
12	E 8	E g	E 9	G 8		
13	E g	G g				
14	E g					
15	VIII 10	G g	IX g			
16	E g	E u	X g			
17	E a					
18	E u					
19	E g	XI 11	E 12			
20	E g	G g				

* Specimen 2 and 3 from same patient – tracheal aspirate and biopsy, respectively.

Specimens from twins – twin 1 specimens 2 & 3, twin 2 specimen 4.

Novel ITS2 type u designated as it was demonstrated from three patients.

New ITS1 and ITS2 sequences that were only detected once are indicated by Roman and Arabic numerals respectively.

The 95% parsimony distance matrix and resultant statistical parsimony network are shown in Table 5.2 and Figure 5.3. Coalescence suggests the major ancestral haplotype is Eg that radiates microevolutionary minor haplotypes. Unresolved loops are indicated between types Eo, Ge, Ne and No, as well as for Eo, No and Ng, and Ng, Ig and IXE. There appears to be several haplotypes that can be considered as "missing" from the sampling performed. Of major interest was specimens received from baby twins who presented with PcP simultaneously (Table 5.1). The tracheal aspirate of twin 1 revealed ITS types Je, Ne, Eg, Eo, Ig and IIg, and the biopsy types Eg, Ne, IIIg and IV1. In total, twin 1 presented with nine types obtained from 14 clones with only type Eg common to both specimens. From the biopsy of twin 2, 3

types Eg, Ge and No were identified from six clones. The only type common to both twins was Eg (Fig. 5.4).

Table 5.2 Real (observed among sequences) distance matrix analysis on the 33 different ITS1 - ITS2 sequences obtained

		Real distances																								
Haplotype	**#**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**
Eg	**1**	--	4	3	2	1	2	2	2	1	2	1	2	3	1	1	1	1	3	2	1	1	2	1	1	3	1
Je	**2**	4	--	3	6	3	6	2	4	5	6	5	4	7	5	3	5	5	6	6	4	5	6	5	5	6	5
Ne	**3**	3	3	--	3	2	5	1	1	4	5	2	3	6	4	2	4	4	5	5	3	4	5	4	4	5	4
I g	**4**	2	6	3	--	3	4	4	2	3	4	1	4	5	3	3	3	3	5	4	3	3	4	1	3	5	3
Eo	**5**	1	3	2	3	--	3	1	1	2	3	2	3	4	2	2	2	2	3	3	1	2	3	2	2	3	2
II g	**6**	2	6	5	4	3	--	4	4	3	4	3	4	5	3	3	3	3	3	4	3	3	4	3	3	5	3
Ge	**7**	2	2	1	4	1	4	--	2	3	3	3	2	5	3	1	3	3	4	4	2	3	4	3	3	4	3
No	**8**	2	4	1	2	1	4	2	--	3	4	1	4	5	3	3	3	3	4	4	2	3	4	3	3	4	3
III g	**9**	1	5	4	3	2	3	3	3	--	3	2	3	4	2	2	2	2	4	3	2	2	3	2	2	4	2
IV 1	**10**	2	6	5	4	3	4	3	4	3	--	3	4	5	3	2	2	3	4	4	2	3	4	2	3	5	3
Ng	**11**	1	5	2	1	2	3	3	1	2	3	--	3	4	2	2	2	2	4	3	2	2	3	2	2	4	2
E 2	**12**	2	4	3	4	3	4	2	4	3	4	3	--	5	3	1	3	3	5	4	3	3	4	3	3	5	3
V 3	**13**	3	7	6	5	4	5	5	5	4	5	4	5	--	4	4	4	4	4	5	4	4	5	4	4	6	4
E 4	**14**	1	5	4	3	2	3	3	3	2	3	2	3	4	--	2	2	2	4	3	2	2	3	2	2	4	2
Gh	**15**	1	3	2	3	2	3	1	3	2	2	2	1	4	2	--	2	2	4	3	2	2	3	2	2	4	2
VI g	**16**	1	5	4	3	2	3	3	3	2	2	2	3	4	2	2	--	2	4	3	2	2	3	2	2	4	2
E 5	**17**	1	5	4	3	2	3	3	3	2	3	2	3	4	2	2	2	--	4	3	2	2	3	2	2	4	2
VII 6	**18**	3	6	5	5	3	3	4	4	4	4	4	5	4	4	4	4	4	--	5	2	5	5	4	4	5	4
E 7	**19**	2	6	5	4	3	4	4	4	3	4	3	4	5	3	3	3	3	5	--	3	3	4	3	3	5	3
G 6	**20**	1	4	3	3	1	3	2	2	2	2	2	3	4	2	2	2	2	2	3	--	3	3	2	2	3	2
E 9	**21**	1	5	4	3	2	3	3	3	2	3	2	3	4	2	2	2	2	5	3	3	--	3	2	2	4	2
VIII 10	**22**	2	6	5	4	3	4	4	4	3	4	3	4	5	3	3	3	3	5	4	3	3	--	3	3	5	3
IX g	**23**	1	5	4	1	2	3	3	3	2	2	2	3	4	2	2	2	2	4	3	2	2	3	--	2	4	2
X g	**24**	1	5	4	3	2	3	3	3	2	3	2	3	4	2	2	2	2	4	3	2	2	3	2	--	4	2
XI 11	**25**	3	6	5	5	3	5	4	4	4	5	4	5	6	4	4	4	4	5	5	3	4	5	4	4	--	4
E 12	**26**	1	5	4	3	2	3	3	3	2	3	2	3	4	2	2	2	2	4	3	2	2	3	2	2	4	--

Produced with program TCS (version 1.13; Clement, M., J. Derington, and D. Posada. Brigham Young University). ITS1 and ITS2 sequences concatenated. Gaps treated as missing data. Parsimony limit: 95%.

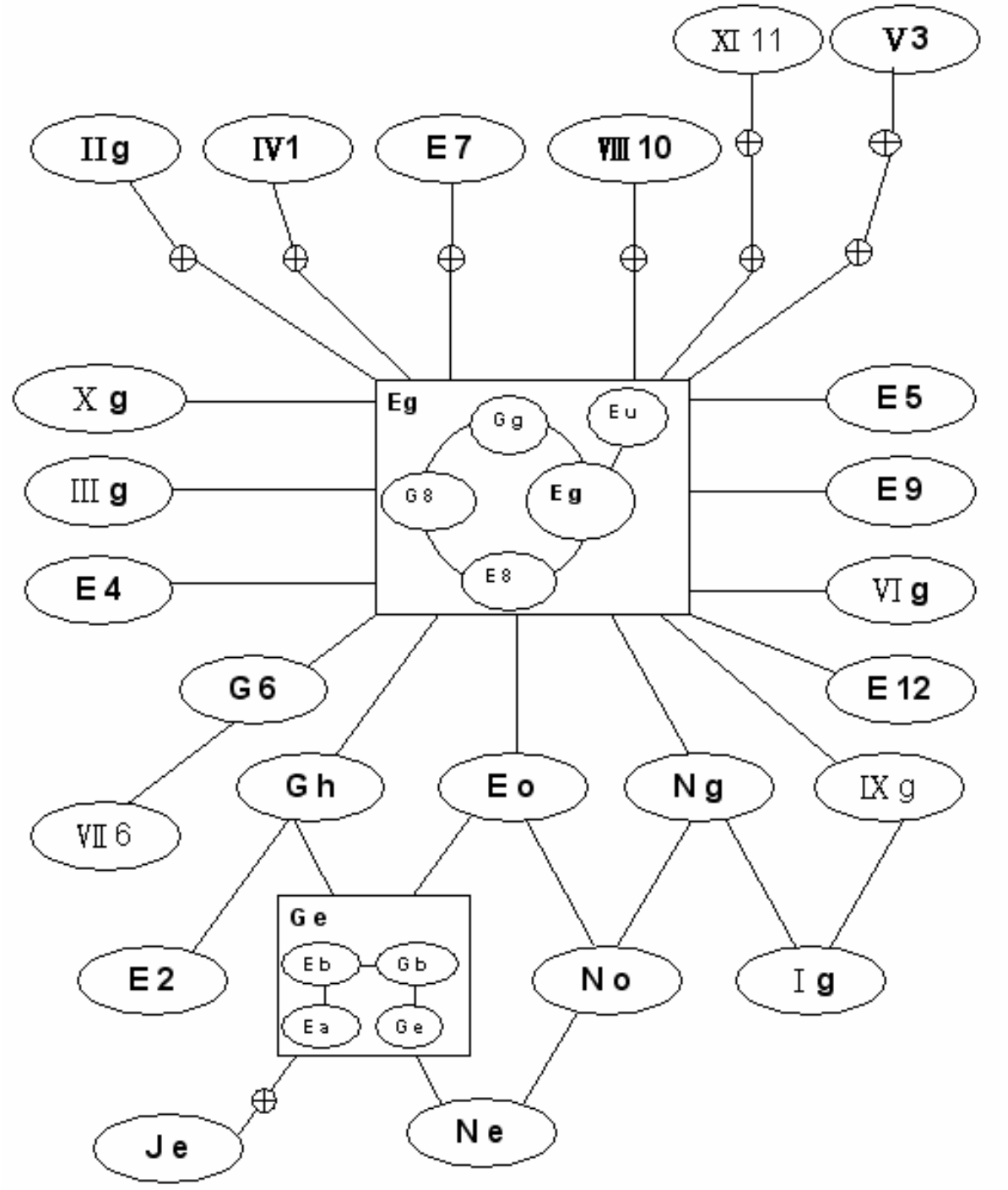


Figure 5.3 ITS haplotype network

Derived from Table 5.2 employing coalescent theory [Posada & Crandall, 2001]. Lines connecting haplotypes are equivalent to one mutational difference with empty nodes representing haplotypes not found in the population. Haplotype subdivisions incorporating indel information (one indel depicted by a connecting line) are shown in the boxed haplotypes Eg and Ge.

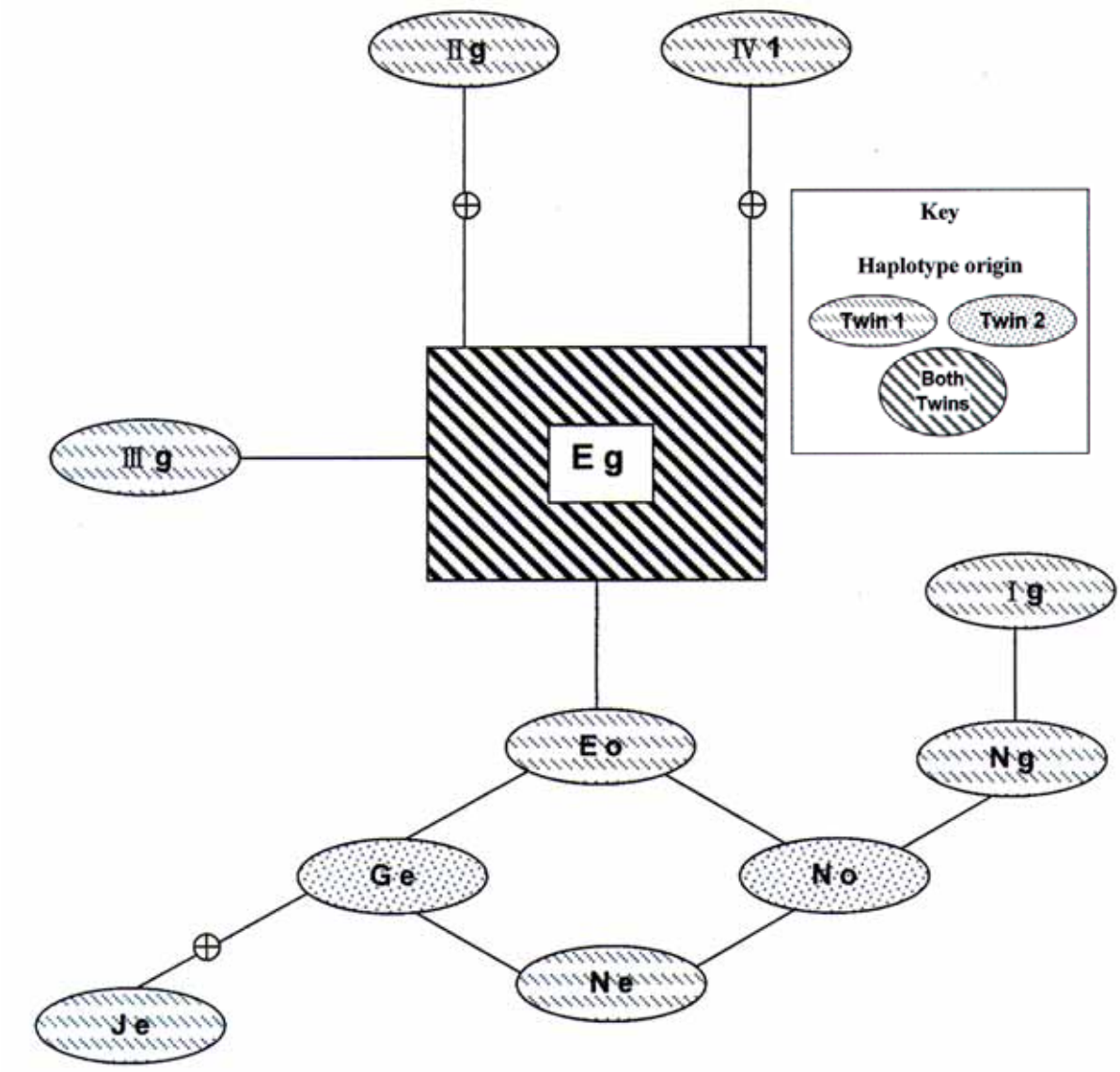


Figure 5.4 ITS haplotype network focusing on twins that presented concurrently with PcP.

Lines connecting haplotypes are equivalent to one mutational difference with empty nodes representing haplotypes not found in the population.

5.2.2 5.8S rRNA genotypes

From the 83 clones analysed thirteen different 5.8S rDNA sequences were detected (Fig. 5.5). Eighty-two clones contained a CG at position 115 – 116. Of the 20 clinical specimens examined, 13 contained a single 5.8S rDNA type; three contained 2 types; three contained 3 types and 1 contained 4 different types. From the twins, the tracheal aspirate of twin 1 possessed four different 5.8S rRNA types and the biopsy 2 types, with only one type common to both the biopsy and the tracheal aspirate. Twin 2 harboured two 5.8S rDNA types. One type associated with ITS types Eg and Ge was also present in both tracheal aspirate and biopsy specimens from twin 1, but the second type exhibited a genotype that was demonstrated in twin 2 only (linked to ITS type No, Fig. 5.4). No linkage association could be demonstrated between the ITS and 5.8S rDNA types obtained.

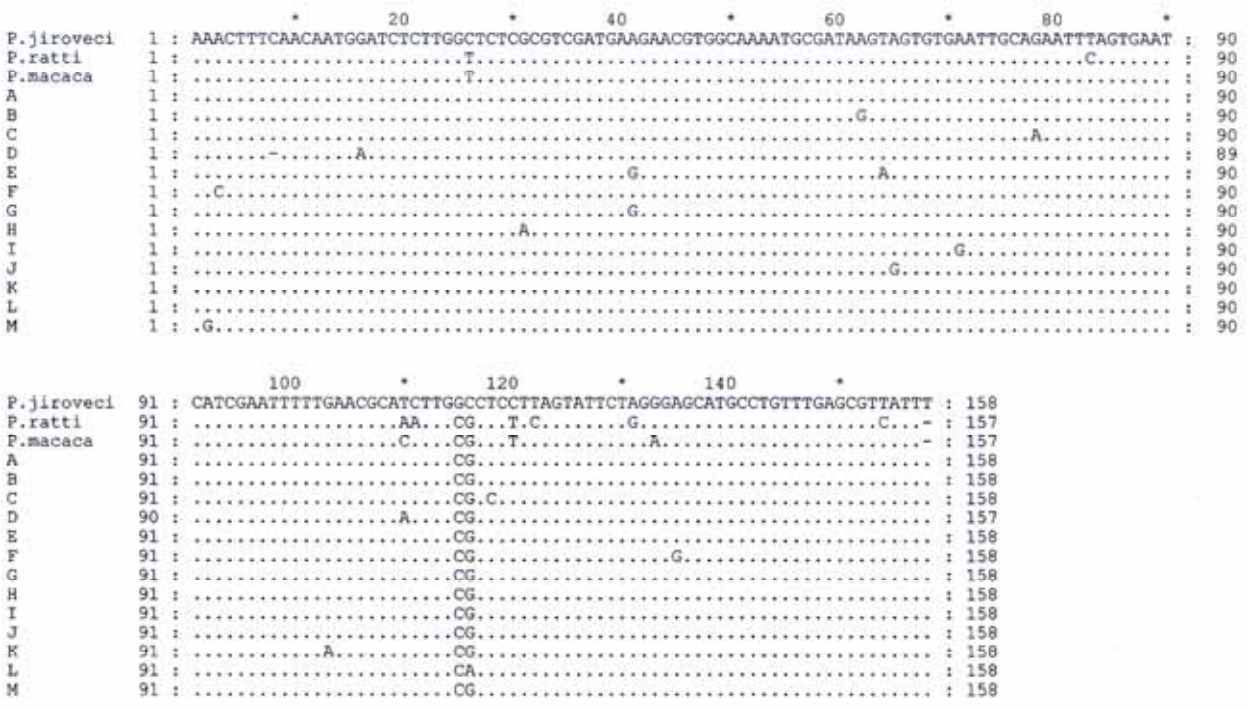


Figure 5.5 Alignment of new 5.8S rDNA sequences

GenBank accession numbers A – M: AY328067 – AY328078 and AY330724. *P. ratti*: L27658; *P. macaca*: AF 288848; *P. jirovecii* (*P. carinii* f. sp . *hominis*): AF013954

5.3 DISCUSSION

The most prevalent ITS1 and ITS2 types demonstrated were Eg (14/19 patients), similar to studies from other continents [Tsolaki, *et al.*, 1996; Tsolaki, *et al.*, 1998; Totet, *et al.*, 2003], followed by Gg (4/19), Eu (3/19) and Gh (2/19). Coalescent theory proposes that haplotypes of low frequency occupy tips and those of high frequency internal nodes [Golding, *et al.*, 1987; Excoffier, *et al.*, 1989]. Older alleles have a greater probability of having produced mutated derivatives, and therefore a greater probability of becoming interior haplotypes than do younger alleles, with the result that haplotypes of greater antiquity occur at internal nodes and haplotypes of recent evolutionary origin occur preferentially at the tips of a cladogram [Crandall & Templeton, 1993]. Since Eg is the most frequently isolated local and global allele and as an internal haplotype positioned at the centre of the cladogram, it can be postulated that Eg is the major ancestral haplotype with radiated recent evolutionary lineages occurring at the tips.

The ITS type combinations Eo, Je, Ge and No were unique to the South African samples and in addition 12 new ITS1 and 13 new ITS2 sequences were demonstrated. A novel ITS2 type that was detected in three different temporally separated patients was designated u (GenBank accession number AY328054). It was observed that all the new ITS1 and ITS2 sequences (singletons) detected in South Africa were connected to the cladogram network with 0.95 confidence (95% parsimony limit), a finding in accordance with the prediction that in a geographically subdivided population with limited gene flow, a singleton is more likely to be connected to an allele in the same population than an allele in a different population [Watterson, 1985].

Homoplasy and / or recombination was evident from unresolved loops in the cladogram. An internal ambiguity was seen within the major haplotype Eg. Eg, Gg, G8 and E8 were interconnected in the cladogram forming a closed loop that could be broken at any connection point. Since the subdivision within Eg was created by the incorporation of indel occurrences, and since indels are mutations evolving in a

completely parsimonous fashion [Crandall & Templeton, 1993], recombination is strongly supported by the analysis. External ambiguities, with tip halpotypes having alternative connections to a number of haplotypes, seem to have occurred frequently. This is indicative of a homoplasious fashion of evolution [Crandall & Templeton, 1993]. The occurrence of an internal ambiguity observed in the haplotype network from the twin could be the result of either recombination or homoplasies. Since the connections are due to nucleotide mutations and not indels, and nucleotide mutations do not necessarily evolve in a completely parsimonous fashion, one cannot exclude either explanation.

Co-infection with different *P. jirovecii* strain types appeared common in the patient population investigated, with 15/19 patients (79%) harbouring more than one genotype. Two specimens from one patient, taken only six days apart revealed the presence of nine different genotypes. In a study conducted by Lee *et al*. it was suggested that samples containing six or more genotypes resulted from cross contamination during processing [Lee, *et al.*, 1998]. However, in the present study cross contamination could be excluded as types Je, Ne, Ig, Eo and IIg found in one specimen were not detected in any other specimens analysed. Other studies, on sequencing up to 5 clones/specimen have reported the presence of 3 – 5 types from a specimen [Lu, *et al.*, 1994; Helweg-Larsen, *et al.*, 2001; Nimri, *et al.*, 2002; Nevez, *et al.*, 2003].

ITS analysis of *P. macacae* employing classical phylogenetic approaches, as performed by Hsueh *et al*. [Hsueh, *et al.*, 2001], does not appear to provide for adequate intraspecies resolution. If *Pneumocystis* ITS regions undergo recombination or if there is a high rate of homoplasies, classic analysis would in effect be unresolved due to a saturation effect. On applying coalescence based statistical parsimony analysis on ITS regions from *P. jirovecii*, the parsimony network clearly shows linkage loops between haplotypes, indicative of homoplasies or recombination. Morphological investigations on *Pneumocystis* conducted in 1984 certainly support recombination in that synaptonemal complexes, indicative of meiosis in the “early precyst” stage, were reported [Matsumoto, *et al.*, 1984].

Based on sequence information and haplotype frequency data, genotype Eg was identified as the outgroup and most probable major ancestral haplotype within the population group. In support of the coalescence approach adopted, genotyping conducted worldwide has shown that the most frequently encountered ITS type is Eg [Tsolaki, *et al.*, 1996; Latouche, *et al.*, 1997; Lee, *et al.*, 1998; Tsolaki, *et al.*, 1998; Nimri, *et al.*, 2002; Nevez, *et al.*, 2003; Totet, *et al.*, 2003]. As certain genotypes, most notably type Eg are over represented, dissemination of specific ITS types, appears to be the major mode of propagation.

Linkage of 5.8S rDNA types with ITS types was not evident, indicating that different parental strains may harbour very similar ITS types or recombination may occur. Although the relevance of 5.8S rDNA sequence polymorphisms necessitates further investigations, as an adjunct to ITS regions they may assist in distinguishing strain types within populations with similar ITS genotypes. When considering the number of different ITS1 and ITS2 genotype combinations reported, recombinational events could well contribute to the degree of heterogeneity observed worldwide.

CHAPTER SIX

COTRIMOXAZOLE RESISTANCE

6.1 INTRODUCTION

The long-term use of trimethoprim and sulphonamides, for prophylaxis and treatment of PcP, has prompted investigations into the possibility that resistance could be emerging and result in breakthrough cases and treatment failures [Helweg-Larsen, *et al.*, 1999; Huang, *et al.*, 2000; Kazanjian, *et al.*, 1998; Visconti, *et al.*, 2001]. Point mutations in the *P. jirovecii* DHPS gene have been reported and have been associated with prophylactic failures [Kazanjian, *et al.*, 1998; Mei, *et al.*, 1998; Kazanjian, *et al.,* 2000; Visconti, *et al.*, 2001]. It is well known that other pathogens, notably *Escherichia coli, Streptococcus pneumoniae, Neisseria gonorrhoea* and *Plasmodium falciparum* develop resistance to sulphonamides by point mutations in DHPS genes [Lopez, *et al.*, 1987; Swedberg, *et al.*, 1993; Brooks, *et al.*, 1994; Wang, 1997; Qvarnaström & Swedberg, 2000].

Studies have shown that mutations detected in *P. jirovecii* DHPS arise during therapy, suggesting selection for resistance [Ma, *et al.*, 1999; Huang, *et al.*, 20000;

Kazanjian, *et al.*, 2000; Nahimana, *et al.*, 2003b]. DHPS mutations have been reported in up to 68.5% of specimens screened in the USA [Huang, *et al.*, 2000], 40% in Paris [Santos, *et al.*, 1999], 35.5% in Spain [Montes-Cano, *et al.*, 2004], 35% in Italy [Visconti, *et al.*, 1999], 25% in Japan [Takahashi, *et al.*, 2000], 20.4% in Denmark [Helweg-Larsen, *et al.*, 1999], and 7% in China [Kazanjian, *et al.*, 2004]. A significantly higher proportion of mutations have been detected from patients recently exposed to sulphonamides [Kazanjian, *et al.*, 1998]. In the USA up to 80.3% of mutations in *P. jirovecii* DHPS have been detected in patients with recent exposure to sulphonamides, whereas up to 47.5% have been recovered from patients without sulphonamide exposure [Huang, *et al.*, 2000]. Similarly, in Denmark 62% of mutations have been detected in patients with exposure vs. 10.5% without [Helweg-Larsen, *et al.*, 1999]. *De novo* acquisition of mutated strains in patients without prior exposure to cotrimoxazole has also been demonstrated in certain geographic areas, notably San Francisco and Seattle in the USA [Beard, *et al.*, 2000; Huang, *et al.*, 2000], and Lyon [Nahimana, *et al.*, 2003a] as well as Paris [Latouche, *et al.*, 2003] in France, suggesting geographic area as an independent predictor for harbouring *P. jirovecii* containing a DHPS mutation. However, there is little supportive clinical evidence that DHPS gene mutations effect strain responses to standard sulphonamide therapy [Kazanjian, *et al.*, 2000].

Dihydrofolate reductase (DHFR), the target of trimethoprim, has been investigated for mutations that could play an additional role in resistance to cotrimoxazole. However, animal models have suggested a minimal role of trimethoprim in the action of cotrimoxazole [Edman, *et al.*, 1989]. Polymorphisms in the DHFR gene have been detected, but no mutation has been associated with known DHFR enzymatic active sites or with treatment / prophylaxis failure [Takahashi, *et al.*, 2002].

A major limitation to the screening of DHPS and DHFR gene mutations is the inability of currently recommended primers to amplify DHPS and DHFR genes [Lu, 1995; Beard, *et al.*, 2000; Huang, *et al.*, 2000]. In Chapter 3 we report the lack of sensitivity of DHPS nested PCR vs. mtLSUrRNA nested PCR, in that only 38 / 81 mtLSUrRNA-positive specimens produced a DHPS amplicon. Even more problematic were DHFR

primers, with only 9 / 81 mtLSUrRNA-positive specimens giving DHFR gene products.

In order to investigate this apparent lack of sensitivity and ensure primer annealing sites were fully compatible both genome walking as well as different primer combinations and newly designed primers were employed (Fig. 6.1). In addition, on comparing the sequence of the DHPS primer B45, published by Lane *et al*. [Lane, *et al.*, 1997] with the DHPS sequence (GenBank AF139132), primer – target mismatches of three nucleotides were evident (Figure 6.2).

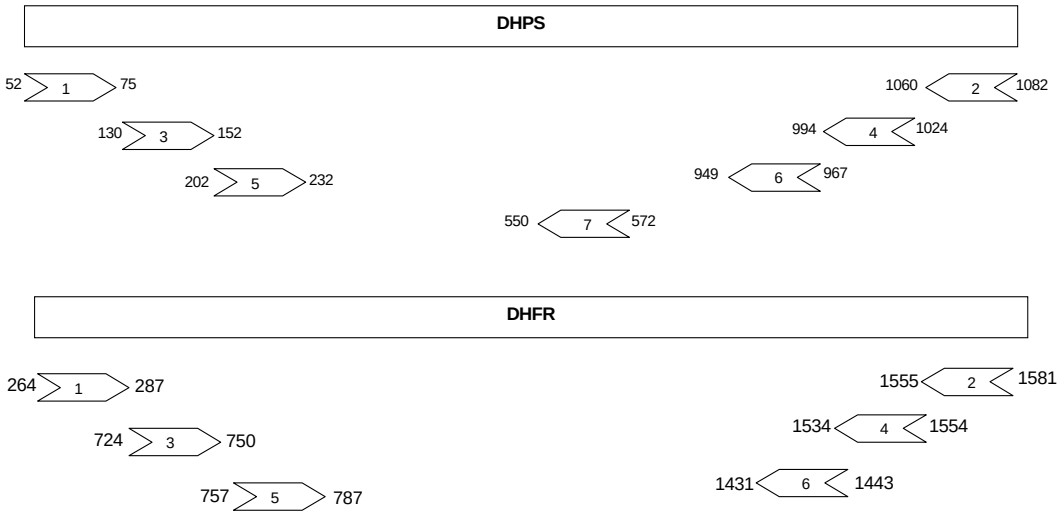


Figure 6.1PCR primer annealing sites to DHPS and DHFR genes

DHPS nucleotide positions according to GenBank AF139132.

DHPS primers: 1, LR1; 2, LR2; 3, F1; 4, B45; 5, Ahum; 6, Bhum; 7, Bnest

DHFR nucleotide positions according to GenBank AF090368

DHFR primers: 1, FRGF1; 2, DHFR1038; 3, DHFR208; 4, DHFR1018; 5, DHFR242; 6, FRGF2

Genomic target[#] : 5´ GATGCTATTTGGAAAGGAGTTTATTGAATTG

DHPS B45 [*] : 5´ GATGCTATTTGGAAAGAAATTTATTAAATTG

Corrected [*] : 5´ GATGCTATTTGGAAAGGAGTTTATTGAATTG

Figure 6.2DHPS Primer mismatch

[#] DHPS GenBank AF139132

* Sequence of reverse complement to reverse primer

6.2 RESULTS

6.2.1 DHPS gene investigations

6.2.1.1 Genome walking

DNA extracted from the *P. jirovecii*-positive lung biopsy and BAL specimens was successfully digested employing *Dra* I, *Eco*R V, *Pvu* II (Figure 6.3). Genomic libraries were constructed by ligating digests to adapters. Primary and nested PCR yielded PCR products (Figure 6.4). However, subsequent DNA sequence analysis indicated that PCR products obtained were of human chromosomal origin.

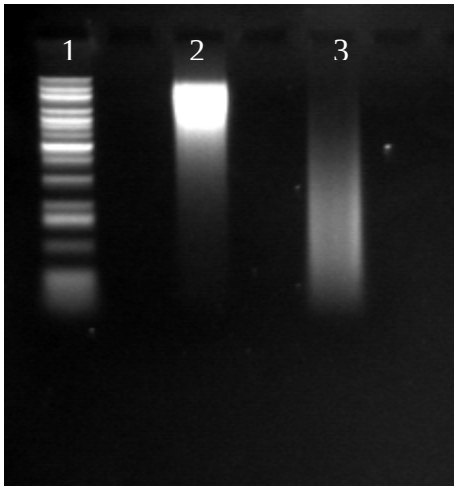


Figure 6.3 *DraI* digested genomic DNA

Lane 1, 10Kb Molecular weight marker; Lane 2, Clinical specimen 1;
Lane 3, Clinical specimen 2. Clinical specimen 2 was subsequently employed.

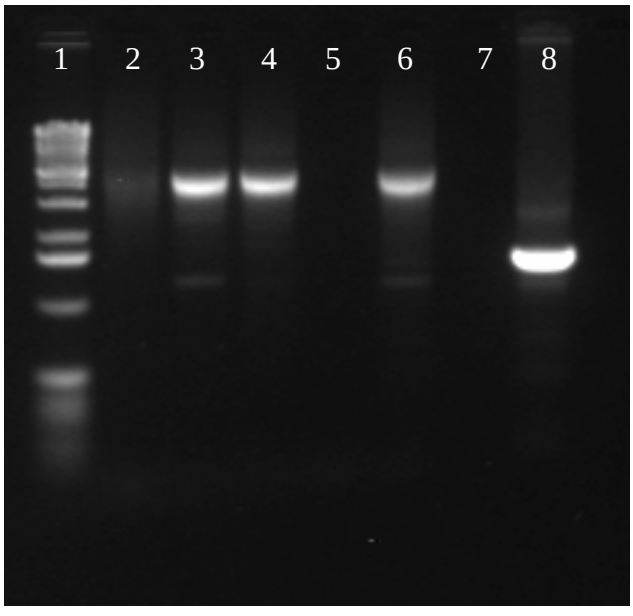


Figure 6.4 Genome walking nested PCR products

Lane 1, 1Kb molecular weight marker; Lane 2 – 6, nested PCR products; Lane 7, negative control; Lane 8, positive (kit) control

6.2.1.2 Optimisation of DHPS amplification

The corrected B45 primer (Figure 6.2) was employed on eight mtLSUrRNA-positive specimens that failed to produce an amplification product with the original B45 reaction. No additional DHPS genes were demonstrated on using the corrected primer sequence.

Optimisation of the new primer combination LR1 and LR2 was performed. The concentration of $MgCl_2$ that produced the strongest PCR product (1030 bp) was 2.5 mM. Due to the large LR1 + LR2 PCR product size, dNTP concentrations and PCR extension time were also assessed. To investigate the possible role that DNA template concentration may have on amplification attempts, template preparation dilutions (1:10 and 1:50) were incorporated. No increase in sensitivity was noted.
A proofreading enzyme with a chemical hotstart modification (Proofstart®) was evaluated. A range of $MgCl_2$ concentrations (1.5 mM, 2 mM, 2.5 mM) was attempted as well as the addition of Q-solution® which enables amplification of targets with high GC content or stable secondary structure. No increase in sensitivity was noted.

Amplification employing LR1 and LR2 with optimised conditions was successful in detecting 3 / 28 discordant specimens previously amplified by mtLSUrRNA PCR but not by previously reported DHPS PCR primers (Table 6.1). Similarly, of the 28 discordant specimens DHPS genes were amplified with the new primer combination F1 and Bhum from the same three samples (Table 6.1).

The DHPS PCR products obtained with primers LR1 and LR2 (1030 bp) from specimens failing to produce amplification products with previously published primers (three specimens) were sequenced. On comparison to the wild type DHPS gene, no mutations were detected.

Table 6.1 DHPS PCR amplification analysis

Primer strategy	No. Specimens	
	Positive	Negative
MtLSUrRNA	28	0
Ahum + Bhum	0	28
LR1 + LR2	3	25
F1 + Bhum	3	25

6.2.2 DHFR gene investigations

The four FRGF1 and FRGF2 PCR products (1189 bp) were sequenced in order to determine the possible existence of mutations located at primer annealing sites. No mutations in the obtained sequences were identified when compared to the DHFR wild type sequence.

6.2.2.1 Optimisation of DHFR amplification

The new primer combination FRGF1 and FRGF2 successfully amplified four specimens that were DHFR positive with previously employed primers. However, on applying FRGF1 and FRGF2 primers to 23 specimens previously amplified by mtLSUrRNA but negative by previous DHFR primers (discordant), no further DHFR gene products were demonstrated.

6.2.3 DHPS mutations

DHPS nucleotide positions according to Ma *et al*. [Ma, *et al.*, 1999]. Of 56 DHPS genes amplified and sequenced, 54 contained the wild type sequence. Strains from one patient, obtained from a tracheal aspirate and post-mortem lung biopsy specimens, contained DHPS genes with the double mutation A346G and C352T (Thr55Ala; Pro57Ser). The tracheal aspirate was collected one day after initiation of cotrimoxazole therapy. Review of clinical records indicated that the patient had no previous exposure to sulphonamides in the recent past. The lung biopsy specimen was obtained 18 days after the tracheal aspirate. The mutation was detected in 1 / 5 clones from the tracheal aspirate, while only wild type sequences were detected in five clones from the post-mortem lung biopsy. All viral, bacterial and fungal cultures performed on the specimens were negative. The post-mortem finding attributed the cause of death to ventilation damage. Sequencing of ITS gene regions (5 clones from each specimen) revealed only type Eg - present in both tracheal aspirate and post-mortem lung biopsy specimens. The effect of DHPS mutations on the enzyme active site is depicted in Figure 6.5. A specimen collected from a second patient yielded DHPS with a synonymous substitution: T432C.

6.2.4 DHFR mutations

DHFR nucleotide positions referred to are according to Takahashi *et al*. [Takahashi, 2002]. Of 27 DHFR genes amplified and sequenced 3 genotypes were detected (Figure 6.6). The wild type gene sequence (genotype 1) was detected in 23 specimens. Three specimens contained a C1046T (nucleotide positions according to) transition mutation (amino acid substitution Ala67Val) (genotype 2). A further specimen contained two nucleotide substitutions: a transversion C1022G (amino acid substitution Arg59Gly) and a nucleotide transition C1126T, located in the intron region of DHFR (genotype 3). Genotypes 2 and 3 were obtained from patients within 3 days of initiation of cotrimoxazole therapy. None of the patients had prior exposure to cotrimoxazole. Sequencing the ITS gene regions from 5 clones / specimen

demonstrated that both patients' *P. jirovecii* harbouring genotype 2 contained ITS genotypes Eg and Eu.

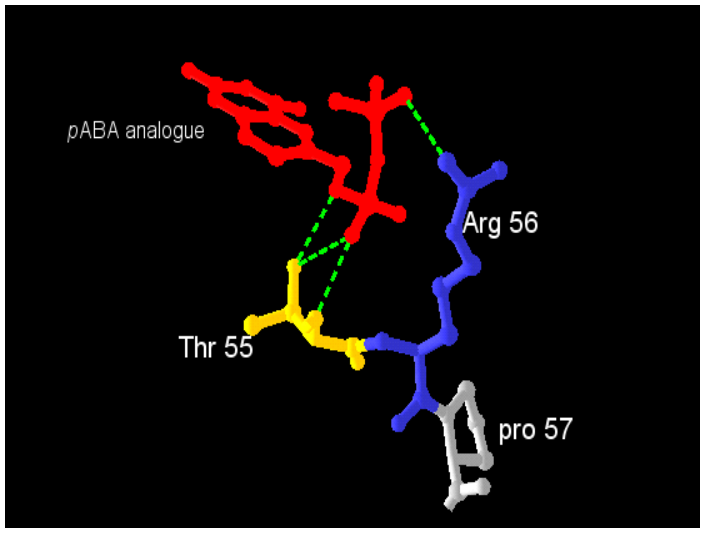


Figure 6.5a Wild type DHPS active site

Wild type: $Thr_{55}Arg_{56}Pro_{57}$
Hydrogen bonds to substrate (red) are shown in green
Produced with the program Deep View from the
published *P. carnii* DHPS amino acid sequence (GenBank M86602) [Volpe, 1992]

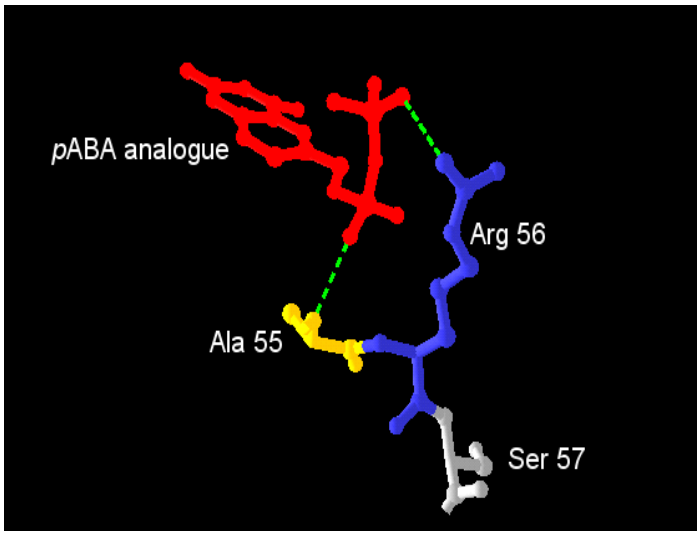


Figure 6.5b Mutation modified DHPS active site

Double mutation: Thr55Ala, Arg56, Pro57Ser
Hydrogen bonds to substrate (red) are shown in green
Produced with the program Deep View from the
published *P. carnii* DHPS amino acid sequence (GenBank M86602) [Volpe, 1992]

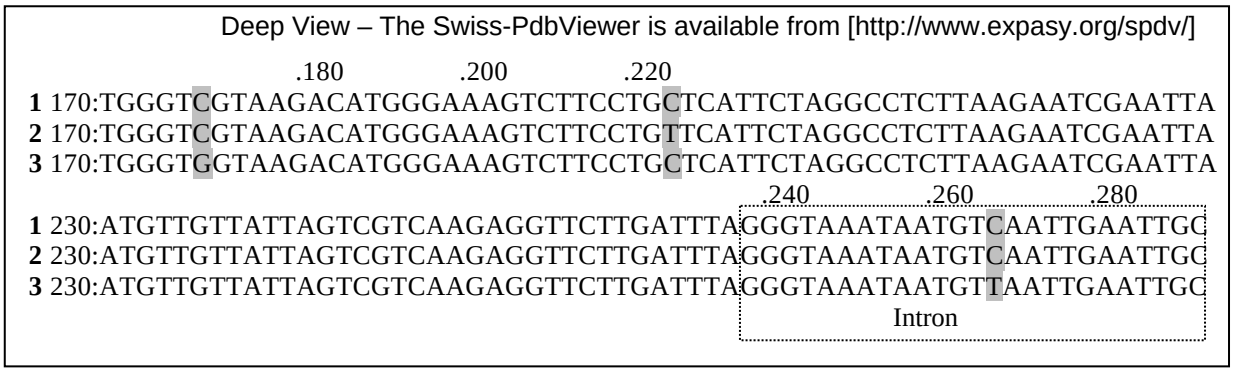


Figure 6.6 DHFR genotypes identified

1: wild type genotype 1; 2: genotype 2; 3: genotype 3.

Polymorphic sites: 175, 200, 278

Nucleotide positions according to Takahashi *et al*. [Takahashi, 2002].

6.3 DISCUSSION

Currently the greatest impediment to genetic research on *P. jirovecii* is the lack of an *in vitro* culture system. The inability to obtain isolated *Pneumocystis* genetic material, free from human (host) DNA contamination, in any significant amount, is a serious obstacle as was evident from the failure of genome walking attempts.

A serious limitation to screening DHPS and DHFR genes for mutations is the inability to obtain amplification products from specimens that had amplified by employing other primers, especially mtLSUrRNA. The corrected DHPS B45 primer did not resolve DHPS detection. The DHPS primer combination of F1 and Bhum and new primers LR1 and LR2 amplified 3 / 28 previously discordant specimens. Investigation into the possible existence of mutations at primer annealing sites indicated 1) that for the three previously discordant DHPS specimens analysed and 2) for four 1189 bp fragments of the DHFR gene, annealing mismatches were not present. These data suggest that factors other than primer sequence mismatches, such as internal secondary structure or dimerisation could occur and prevent the F1 B45 primer combination from amplifying target sequences optimally. The observation that the addition of Q-solution did not improve amplification suggests that target secondary structure or high CG content does not appear to be responsible. The only other

explanation is that both DHPS and DHFR genes are single copy and thus provide fewer target copies for PCR amplification when compared to multicopy genes such as mtLSUrRNA or MSG. An additional point of note is that LR1 + LR2; and F1 + Bhum primers did amplify three further DHPS genes, perhaps emphasising that to decrease annealing temperatures and primer base numbers could be detrimental to product recovery.

Despite the application of new primers and primer combinations, a serious lack of sensitivity was still evident for amplifying DHPS and DHFR genes. Many studies have reported on the lack of sensitivity of DHPS primers: Totet *et al*. and Beard *et al*. reported the amplification of DHPS genes by nested PCR from 73.3% and 85.7% of specimens producing amplicons by mtLSUrRNA single round PCR [Beard, *et al.*, 2000; Totet, *et al.*, 2004]. On reporting amplification of specimens obtained from patients clinically diagnosed with PcP, DHPS nested PCR amplified between 43.5% [Lane, *et al.*, 1997] and 71.9% - 94% of specimens [Huang, *et al.*, 2000; Helweg-Larsen, *et al.*, 1999; Kazanjian, *et al.*, 2000; Kazanjian, *et al.*, 1998]. Although few studies have investigated DHFR gene polymorphisms, discrepant reports have emerged on the amplification success. De Luca *et al*. and Takahashi *et al*. have reported a 71% - 100% success rate employing single round PCR whereas Lu *et al*, in comparison to five other PCR techniques reported successful amplification of DHFR genes by single round PCR in 23% of specimens detected by 18S and ITS nested PCR reactions [Lu, *et al.*, 1995]. Perhaps when more studies emerge on investigations of DHFR gene polymorphisms discrepancies will become apparent.

On sequencing 56 DHPS genes one patient from the Western Cape (2001) was found to harbour *P. jirovecii* that contained DHPS genes with the double mutation Thr55Ala; Pro57Ser. This suggests a DHPS mutation prevalence of <2% in the Western Cape population sampled in South Africa. The double mutation is thought to signify the emergence of resistance to sulphonamides, as the mutations occur in a region of the DHPS gene involved in binding both substrate and sulphonamides [Kazanjian, *et al.*, 1998]. Although the mutations do not appear to be associated with therapy, they were recently reported as the first description of indicative

sulphonamide resistance development in *P. jirovecii* from South Africa [Robberts, *et al.*, 2004]. Studies from other countries have shown the presence of DHPS mutations in specimens obtained both from patients with (up to 80.3%) and without (up to 53.8%), prior exposure to sulphonamides [Helweg-Larsen, *et al.*, 1999; Santos, *et al.*, 1999; Huang, *et al.*, 2000; Kazanjian, *et al.*, 2000; Nahimana, *et al.*, 2003a]. It has been suggested that the geographic area of residence is an independent predictor for harbouring *P. jirovecii* with DHPS mutations, possibly related to local sulphonamide prescribing practices [Beard, *et al.*, 2000]. This was evident in a recent study contrasting the high prevalence of mutations in the USA with a very low prevalence in China, where cotrimoxazole is not used as extensively [Kazanjian, *et al.*, 2004]. Mechanisms other than point mutations in the DHPS active site may be responsible for resistance to sulphonamides as has been suggested to be the case for *S. cerevisiae* and *P. falciparum*. Bayly *et al*. reported the selection of *S. cerevisiae* strains with decreased sensitivities to sulphamethoxazole in the presence of *p*ABA or folic acid, and have proposed that a physiological response involving gene or transported activation may be responsible [Bayly, *et al.*, 2001]. In addition, Wang *et al*. have demonstrated that for *P. falciparum* an exogenous source of folate may have a major role in determining resistance levels, and that this characteristic seems to be linked to DHFR alleles [Wang, *et al.*, 1997b]. An alternative hypothesis is that sulpha drug uptake may simply be inhibited by competitive transport of folic acid into cells [Bayly, *et al.*, 2001].

The DHFR mutation Ala67Val has recently been reported from Japan [Takahashi, *et al.*, 2002]. The occurrence of the same mutation in the Western Cape could either be indicative of spread of a particular strain, or more significantly may indicate selective pressure on the enzyme by the widespread use of trimethoprim. It was, however suggested that DHFR Ala67Val from *P. jirovecii* should not influence patient management as the mutation is not located in conserved gene regions and is not close to the active site [Takahashi, *et al.*, 2002]. The detection of the novel DHFR mutation Arg59Gly located in the structural region of the enzyme may support the suggestion that the enzyme appears to be under selective pressure; however, the substitution is not located at a highly conserved position. Although the mutation

detected in the intron region of DHFR is not expected to have an influence on the enzymatic functions of DHFR, it may be of use in enhancing multilocus sequence typing.

CHAPTER 7

CONCLUSIONS

Laboratory diagnosis of PcP is difficult as there is currently no technique available for culturing the organism. In South Africa IF is presently the standard laboratory technique for diagnosis of PcP. However, there is a perception that the *P. jirovecii* IF method produces both false-positive and false-negative results. In this study molecular detection employing the PCR was evaluated and compared to IF. PCR primer selection has been shown to play a significant role in the diagnosis of PcP and therefore various primers and PCR techniques were evaluated for local strains / conditions [De Luca, *et al.*, 1995]. Results obtained confirmed major discrepancies exist between primer / target combinations. Concordance between the nine PCR techniques evaluated ranged from 41.2% to 94.4%. Detection rates of the techniques differed substantially. Of the standard PCR techniques compared, detection was highest employing primers directed at the mtLSUrRNA gene in a nested reaction. The mtLSUrRNA gene target, on employing single-round PCR, remained the most readily detectable even in comparison to nested reactions directed at other genes.

Real-time PCR, with its sophisticated chemistry and detection platforms has been reported as having superior sensitivity and specificity when compared to standard techniques. However, the present study showed that mtLSUrRNA nested PCR detected *P. jirovecii* in 15% more clinical specimens than 5S real-time PCR, suggesting that real-time PCR may not be superior universally. While the practical advantages of shorter cycling times and the automated detection of PCR products do assist in rapid diagnosis, when employing nuclear 5S rRNA as gene target, it is at the expense of sensitivity.

On analysis of quantitative data obtained with real-time PCR, no apparent factor related to organism / target concentration per specimen, explained the discrepant standard PCR (DHPS nested and mtLSUrRNA nested) and real-time (5S rRNA) results. This suggests that factors other than target copy number play a role in the detection limit of genes as targets in PCR techniques. The mtLSUrRNA nested PCR employed against local strains was the most sensitive technique, followed by 5S rRNA real-time PCR. The testing of known positive lung biopsies, although few in number, confirmed the value of mtLSUrRNA primers in the South African setting. The study emphasises the requirements for correct primer selection for the detection of *P. jirovecii*.

As mtLSUrRNA nested PCR demonstrated the highest level of detection, results were compared to those obtained from routine IF staining. The correlation obtained was poor and prompted review of clinical records for discordant results. Both PCR and IF have the potential of producing false-positive and –negative results. It is therefore recommended they be performed in parallel and discrepant results assessed following investigation of further specimens together with the clinical presentation.

Due to the perceived increase in incidence of PcP infections in Africa, and the recent provision of antiretroviral therapy, *P. jirovecii* should be monitored nationally employing complementary sensitive techniques that are suitable to South African strains.

Contrary to reports that identified *S. pneumoniae*, *M. tuberculosis* and CMV as the most prevalent co-infections associated with PcP, a higher than expected proportion of local patients were co-colonised with Gram-negative bacilli in the respiratory tract. The clinical significance of some of the gram-negative bacilli isolated was not known.

With the high burden of TB in South Africa, and particularly the Western Cape, clinicians should be alerted to the possibility of *M. tuberculosis* and *P. jiro*vecii co-infection. Patients presenting with diffuse pneumonia should be investigated for both infections. In addition, when encountering TB patients unresponsive to therapy clinicians should be aware of the possibility of concurrent PcP, and it is recommended that both infections be treated when *P. jirovecii* can be demonstrated in the laboratory. If *P. jirovecii* is left untreated fulminant PcP may occur later as cell mediated immunity wanes in HIV-infected patients.

Local patient populations may be predisposed to PcP due to factors other than HIV as it was seen that 45% of patients diagnosed with PcP by PCR were HIV-negative. Similarly, of TB patients with detectable *P. jirovecii* 9 / 23 were HIV-negative. The high prevalence of PcP in HIV-negative non-immunosuppressed patients in the Western Cape region is of concern. It warrants further investigation into physiological and/or co-infection states that may predispose to *P. jirovecii* infection. It is clear that against a backdrop of the high prevalence of HIV and under-nourished communities, South Africa has not placed sufficient emphasis on detecting *P. jirovecii*, a situation that should be addressed with provision of funding for the necessary investigations.

Based on sequence information and haplotype frequency data, genotype Eg was identified as the outgroup and most probable major ancestral haplotype and MRCA within the population group. In support of the coalescence approach adopted, genotyping conducted worldwide has shown that the most frequently encountered ITS type is Eg. As certain genotypes, most notably type Eg are over represented, dissemination of specific ITS types, appears to be the major mode of propagation.

Linkage of 5.8S rDNA types with ITS types was not evident, indicating that different parental strains may harbour very similar ITS types or recombination may occur. Although the relevance of 5.8S rDNA sequence polymorphisms necessitates further investigations, as an adjunct to ITS regions they may assist in distinguishing strain types within populations with similar ITS genotypes. When considering the number of different ITS1 and ITS2 genotype combinations reported, recombination could well contribute to the degree of heterogeneity observed worldwide.

Investigation into pathogen population dynamics should consider employing a coalescent approach to resolve molecular epidemiological questions as it provides a more realistic approach to intraspecies relatedness.

A patient in the Western Cape was found to be infected with a strain of *P.jirovecii* with a double mutation Thr55Ala; Pro57Ser mutation in the DHPS gene. The double mutation is thought to signify the emergence of resistance to sulphonamides, as the mutations occur in a region of the DHPS gene involved in binding both substrate and sulphonamides. Although the mutations do not appear to be associated with therapy, they were recently reported as the first description of indicative sulphonamide resistance development in *P. jirovecii* from South Africa [Robberts, 2004a; Robberts, In Press]. Subsequently Zar *et al*. have reported similar mutations and frequencies in the DHPS gene of *P. jirovecii* from the Western Cape [Zar, *et al.*, 2004]. The low rate of mutations in the Western Cape suggests that selective pressure on local strains may not as yet have reached levels as found in the USA or Europe, but rather resemble those of China [Kazanjian, *et al.*, 2004]. To date there remains little evidence that such mutated strains impair clinical response to standard sulphonamide therapy. The detection of DHFR Ala67Val should not influence patient management as the mutation is not located in conserved gene regions and is not close to the active site.

Interestingly ITS type Eu, which to date has only been identified in South Africa [Robberts, *et al.*, 2004b] was associated with the DHFR mutation Ala67Val. Furthermore, the DHFR genotype Arg59Gly and C278T (located in the intron) has not been previously described. The occurrence of these DHFR genotypes could have potential in the development of multilocus sequence typing of *P. jirovecii*. It is inappropriate when investigating clinical specimens, to link DHPS/DHFR types with ITS genotypes since more than one ITS genotype can be obtained from a patient with a single DHPS/DHFR type.

Due to the perceived increase in incidence of PcP infections in Africa, and the recent provision of antiretroviral therapy, *P. jirovecii* should be monitored nationally for prevalence and susceptibility to co-trimoxazole employing appropriate techniques that are suitable to the South African strains.

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LIST OF PUBLICATIONS

Congress presentations (Poster)

1. Robberts, F. J. L., L. J. Chalkley, and L. D. Liebowitz. *Pneumocystis carinii* – Clinically problematic, diagnostically perplexing. 46th Academic Year Day, Stellenbosch University, Tygerberg. August 2002. Abstract No. 181.

2. Robberts, F. J. L., L. J. Chalkley, and L. D. Liebowitz. *Pneumocystis carinii* – Clinically problematic, diagnostically perplexing. Pathogenomics: 42nd Annual congress of the Federation of South African Societies of Pathology. Bloemfontein. July 2002. Abstract No. P46.

Congress presentations (Platform)

1. Robberts, F. J. L., L. D. Liebowitz, and L. J. Chalkey. Sequence variation of the ITS1-5.8S-ITS2 rDNA gene region of *Pneumocystis jiroveci* and typing of South African strains. 47th Academic Year Day, Stellenbosch University, Tygerberg. August 2003. Abstract No. 173.

2. Robberts, F. J. J., L. J. Chalkley, and L. D. Liebowitz. Evaluation of molecular diagnostic techniques and cotrimoxazole resistance surveillance in Cape Town. Antimicrobial resistance congress. Durban. October 2003. Invited speaker.

3. Robberts, F. J. L., L. D. Liebowitz, and L. J. Chalkey. Sequence variation of the ITS1-5.8S-ITS2 rDNA gene region of *Pneumocystis jiroveci* and typing of South African strains. 8th International workshop on opportunistic protists. Hilo, Hawaii. July 2003. Abstract No. A10.

4. Robberts, F. J. L., L. D. Liebowitz, and L. J. Chalkley. Genotyping and coalescent phylogenetic analysis of *Pneumocystis jiroveci* from South Africa. Understanding life: Microbes to Man, SASM/SAGS congress. Stellenbosch. April 2004. Abstract No. 41.

5. Robberts, F. F. L., L. J. Chalkley, K. Weyer, P. Goussard, and L. D. Liebowitz. Detection and emergence of cotrimoxazole-resistant strains of *Pneumocystis jiroveci* in South Africa. 44th Annual conference of the Federation of the South African Societies of Pathology: Pathsplash, Stellenbosch. July 2004. Abstract No. MP6. Invited speaker.

6. Robberts, F. J. L., L. D. Liebowitz, and L. J. Chalkey. Molecular epidemiology of Pneumocystis jirovecii in South Africa. PcP in South Africa conference, Sandringham. October 2004. Invited speaker.

Publications

1. Robberts, F. J. L., L. J. Chalkley, and L. D. Liebowitz. Pneumocystis Pneumonia (PCP). South African Dental Journal 2002;57(11):451-453.

2. Robberts, F. J. L., L. D. Liebowitz, and L. J. Chalkley. Typing and coalescent phylogenetic analysis of *Pneumocystis jiroveci* from South Africa. Journal of Clinical Microbiology 2004;42(4):1505-1510.

3. Robberts, F. J. L., L. J. Chalkley, K. Weyer, P. Goussard and L. D. Liebowitz. Novel dihydrofolate reductase gene mutations in South African strains of *Pneumocystis jirovecii*. Journal of Clinical Microbiology. 2005;43(3):1443-1444.