Resolution of mixed dihydropteroate synthase (DHPS) genotypes in respiratory specimens from patients with *Pneumocystis jirovecii* pneumonia from Gauteng, South Africa

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“An achievement is a bondage. It obliges one to a higher achievement.”

- Albert Camus

DECLARATION

I, Bhavani Poonsamy, declare that this dissertation is a result of my own work, unless otherwise stated. It is being submitted for the Degree of Master of Science in Medicine in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

(Signature of candidate)

04 October 2011

(Date)

ABSTRACT

Pneumocystis pneumonia (PCP) is one of the most prevalent diseases in HIV-positive and other immunosuppressed patients. It is caused by the opportunistic fungal pathogen *Pneumocystis jirovecii*. Dihydropteroate synthase (DHPS) mutations in *P. jirovecii* have been linked to resistance to trimethoprim-sulphamethoxazole (cotrimoxazole), the main treatment and prophylaxis used for PCP. DHPS mutations have been identified globally, predominantly in developed countries. This study investigated the *P. jirovecii* DHPS genotypes in PCP-positive patients from Chris Hani Baragwanath Hospital in Gauteng Province, South Africa.

During the period March 2005 through June 2009, 266 patients were enrolled in the study and 306 specimens were collected. *P. jirovecii* was identified in 67% (205/306) of these specimens with quantitative real-time PCR (qPCR). The qPCR had a sensitivity of 98% and a specificity of 70%, compared with the immunofluorescence assay (IFA). Using sequencing and cloning techniques, 64% (110/173) of the nested PCR-positive specimens contained *P. jirovecii* with mutant DHPS genotypes. There was no association between patients harbouring *P. jirovecii* with mutant DHPS genotypes and in-hospital patient outcome (p-value = 0.19). As part of this project the Roche MagNA Pure Compact (RMPC) instrument and technology was validated for use as a new DNA extraction method. The RMPC was quick and easy to use compared to the Qiagen manual extraction method.

The specificity of the qPCR was compromised by the high number of apparent false positive results obtained by the assay. However, as the IFA is an imperfect gold standard,

these are probably true cases of infection or colonisation. This study found a higher proportion of *P. jirovecii* with DHPS mutant genotypes than wild type in PCP patients, which is unusual. The most probable reason for this is the widespread use of sulfa drugs, which are thought to select for these mutations. While this study did not find an association between DHPS mutations and adverse patient outcome, there have been contradictory findings. If further investigations reveal that DHPS mutations affect patient treatment or outcome, it will have major implications for the management of PCP in the country.

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NOMENCLATURE

β	Beta
A	Adenine
AFB	Acid-fast bacilli
AIDS	Acquired immunodeficiency syndrome
Ala	Alanine
ARV	Antiretroviral
BAL	Broncho-alveolar lavage
BDG	β-D-glucan
bp	Base pairs
C	Cytosine
CA	California
cART	Combined antiretroviral therapy
CD4	Cluster of differentiation 4
CHB	Chris Hani Baragwanath
Ct	Cycle threshold
DDT	Dithiothreitol
DFA	Direct immunofluorescence assay
DHPS	Dihydropteroate synthase
DMP	Diagnostic media products
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide phosphate
EDTA	Ethylenediaminetetra-acetic acid
et al.	*et alia* (and others)
f. sp.	forma specialis
fas gene	Folic acid synthesis gene
FITC	Fluorescein isothiocyanate
G	Guanine
HAART	Highly active antiretroviral therapy
HDF	Hidiformamide
HIV	Human immunodeficiency virus
IFA	Immunofluorescence assay
IPC	Internal positive control
LB	Luria broth
LDH	Lactate dehydrogenase

M1	Mutation 1; mutation at position 165
M2	Mutation 2; mutation at position 171
M3	Mutation 3; mutations at positions 165 and 171
MA	Massachusetts
$MgCl_2$	Magnesium chloride
MGP	Magnetic glass particles
n/a	Not applicable
NA	Nucleic acid
NaCl	Sodium chloride
NHLS	National Health Laboratory Service
NICD	National Institute for Communicable Diseases
nPCR	Nested PCR
NTC	No target template control
PABA	Para-amino benzoic acid
PBS	Phosphate buffered saline
PCP	*Pneumocystis* pneumonia
PCR	Polymerase chain reaction
P. jirovecii	*Pneumocystis jirovecii*
Pro	Proline
PRU	Parasitology Reference Unit
qPCR	Quantitative PCR
R2	Coefficient of determination
RCF	Relative centrifugal force
RFLP	Restriction fragment length polymorphism
RMPC	Roche MagNA Pure Compact
RNase P	Ribonuclease P
rpm	Revolutions per minute
SA	South Africa
SAM	S-adenosylmethionine
Ser	Serine
SOC	Super optimal broth with catabolite repression
SSCP	Single strand conformation polymorphism
T	Thymine
TAE	Tris/acetic acid/EDTA
TB	Tuberculosis
Thr	Threonine
TMP-SMX	Trimethoprim-sulphamethoxazole, cotrimoxazole
U	Units
USA	United States of America
UV	Ultraviolet
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

CHAPTER 1 - INTRODUCTION

1.1 *Pneumocystis jirovecii*

1.1.1 History

In 1909, *Pneumocystis* was first identified by Charlos Chagas in Brazil. He was examining the lungs of guinea pigs that were inoculated with trypanosome-positive blood. He believed that he was looking at a new life stage of the protozoan flagellate *Trypanosoma cruzi* and therefore suggested the name of *Schizotrypanum*. Antonio Carini, in 1910, found the same organism in rat lungs also infected with trypanosomes and thought that it was a new type of trypanosome. In 1912, P. Delanoë and M. Delanoë found the same organisms in the lungs of sewer rats uninfected with trypanosomes and concluded that this was a different species and they proposed the name *Pneumocystis carinii* [1-4].

For many years after it was discovered, it was believed that *P. carinii* was a protozoan because it displayed morphological features typical of protozoa and because it responded to anti-protozoan treatment. However, in the 1970s, there were suggestions that *P. carinii* may be a fungus. This was confirmed in 1988, after sequencing of the small ribosomal RNA subunit [1;2;4;5].

Further study showed that there were different forms of *Pneumocystis* specific for different hosts [2]. *Pneumocystis* has been found in humans, rats, mice, pigs and horses, amongst others. A trinomial naming system was used to differentiate the species, e.g. *Pneumocystis carinii* f. sp. *carinii* referred to the species of *Pneumocystis* that infected rats and *Pneumocystis carinii* f. sp. *hominis* referred to the species that infected humans [6]. As

sufficient taxonomic information became available, nomenclature was revised. The form that infects humans was named *Pneumocystis jirovecii* after Otto Jirovec, a pathologist who initially reported *Pneumocystis* in humans, and the form that infects rats was named *P. carinii* [2;6]. PCP was originally the abbreviation for *Pneumocystis carinii* pneumonia. After the organism's name was changed to *P. jirovecii*, the abbreviation was kept and subsequently stands for *Pneumocystis* pneumonia.

1.1.2 Classification

Pneumocystis species are currently classified in the Phylum Ascomycota, Subphylum Taphrinomycotina, Class Pneumocystidomycetes, Order Pneumocystidales, and Family Pneumocystidaceae [2;7].

1.1.3 Morphology and life cycle

To date, *Pneumocystis* organisms have not been successfully cultured, which is the core reason why so much remains unknown about the morphology and life cycle of the organism [8;9]. Nonetheless, hypothesised life cycles have been established and are periodically revised, based largely on microscopic observations. The two main stages of the organism that were identified initially, were the trophozoite stage measuring 2 - 5 µm (Figure 1.1) and the cyst stage measuring 6 - 7 µm (Figure 1.2) [2;9]. This partially explains why *Pneumocystis* was initially believed to be a parasite, as these are the two typical life stages of protozoan parasites.

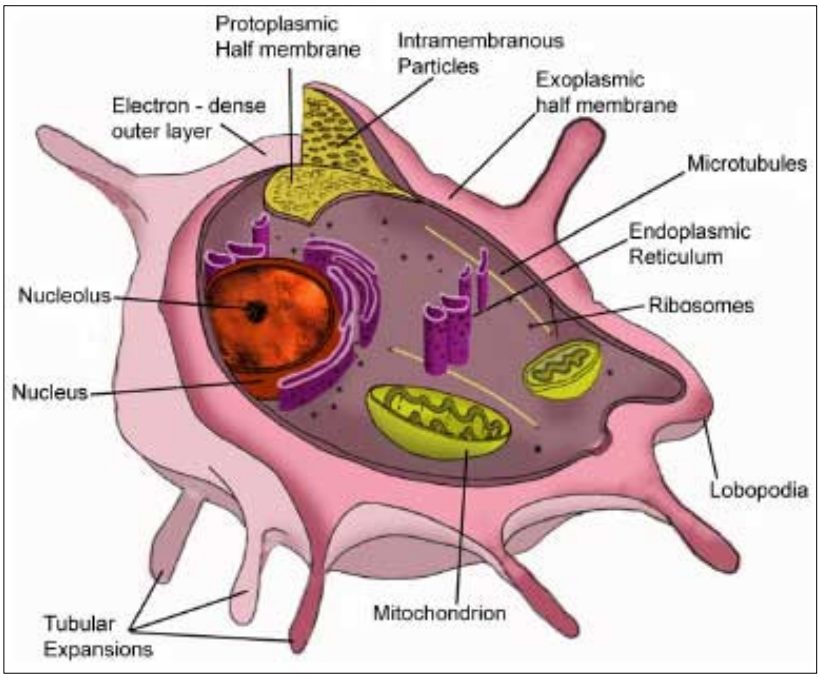


Figure 1.1 Schematic diagram of the trophozoite form of *P. jirovecii* [9]

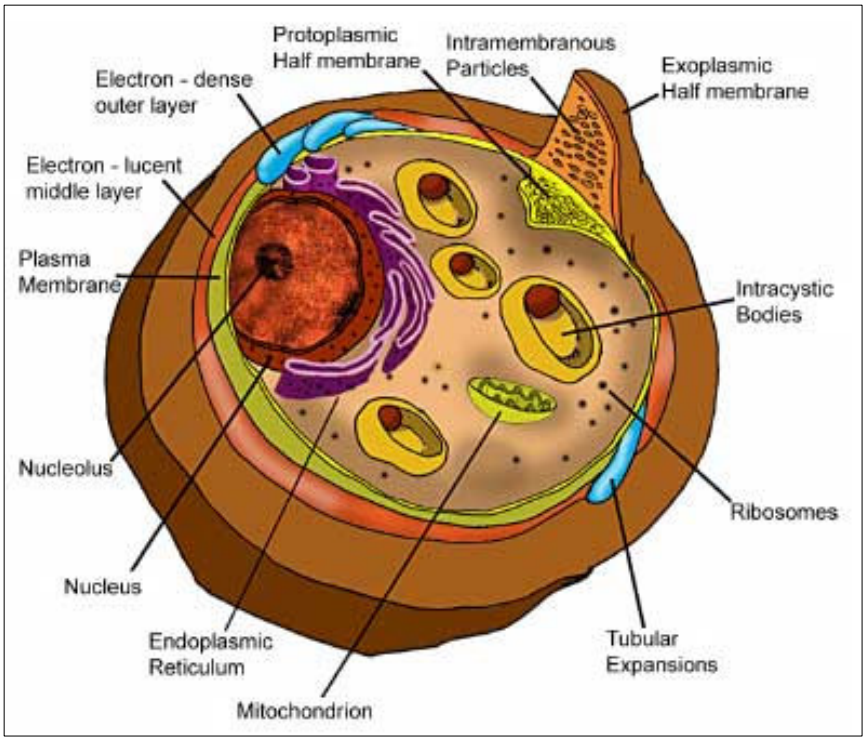
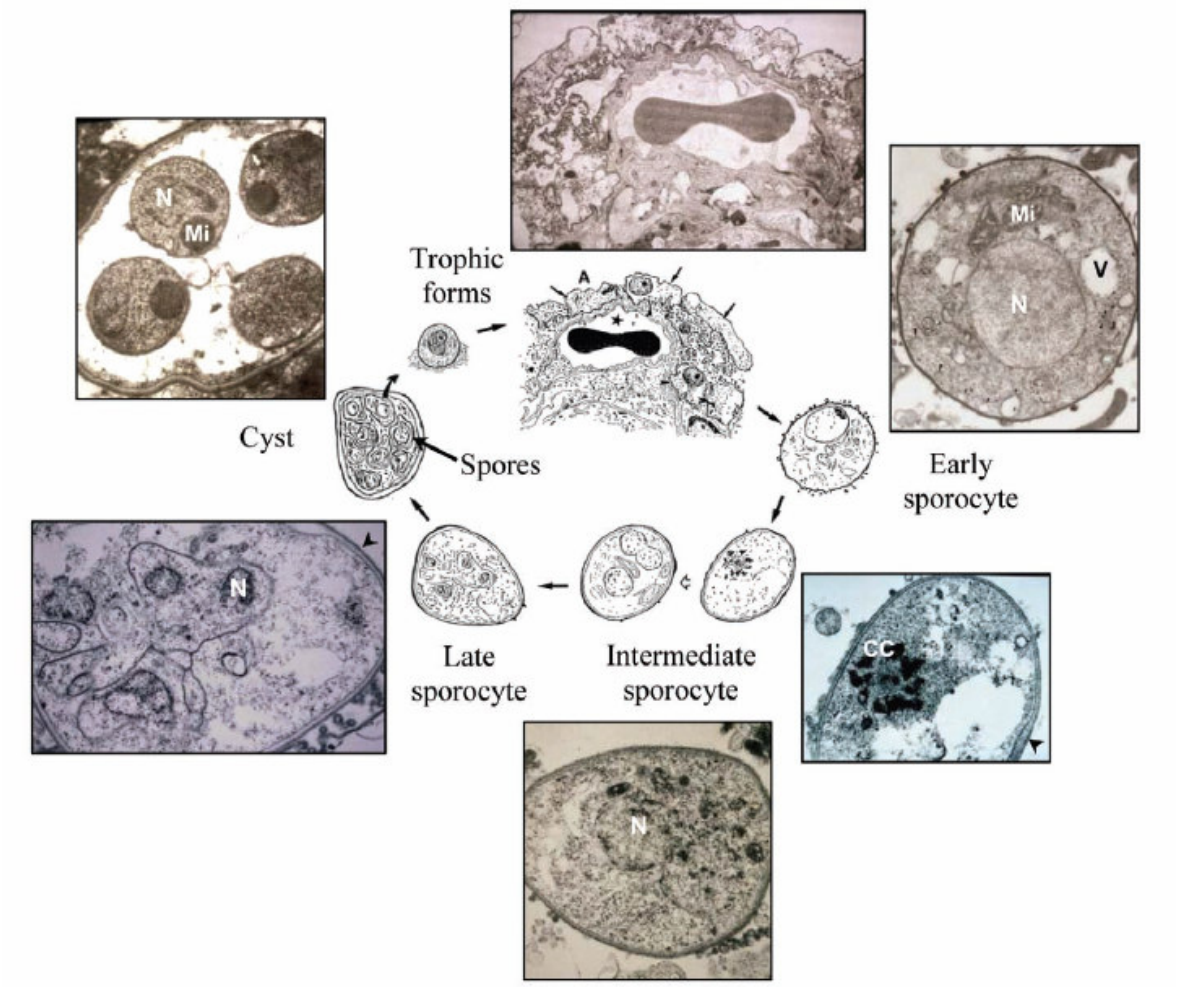


Figure 1.2 Schematic diagram of the cyst form of *P. jirovecii* [9]

According to one of the most recent proposed life cycles, *Pneumocystis* is thought to involve three main stages, namely the trophozoite, the sporocyte and the cyst [10]. The trophozoite stage conjugates to form a sporocyte, which undergoes meiosis and then mitosis to give rise to the cyst form. The cyst form contains eight spores which, when fully developed, are released as eight trophic forms (Figure 1.3).



A hypothetical *Pneumocystis* life cycle illustrated by transmission electron micrographs and corresponding interpretation drawings of organisms developing in mammalian lungs. Mononuclear thin-walled trophic forms (small arrows) are attached to type 1 epithelial alveolar cell that is close to a capillary vessel (star). Following conjugation, trophic forms would evolve into thin-walled round early sporocyte. While electron-lucent layer (arrowhead) develops in intermediate sporocytes, meiotic nuclear division proceeds. An additional mitotic replication leads to a thick-walled late sporocyte (arrowhead) containing eight nuclei. In the mature cyst, the eight spores are fully delineated. These forms are able to leave the cyst and subsequently attach to type I alveolar cells. A: alveolar space; CC: condensed chromosomes and spindle microtubules; N: nucleus; Mi: mitochondrion; V: vacuole. Arrowheads indicate thick cell wall.

Figure 1.3 Proposed *Pneumocystis* life cycle. Adapted from Aliouat-Denis *et al.* [4]

1.1.4 Infection and transmission

While the exact transmission route of *Pneumocystis* is still largely unknown, there are two key theories. The first is that PCP infection arises from the reactivation of a latent infection and the second is that PCP infection is a result of a new exposure or recent transmission.

As most healthy children are seropositive in the first few years of life [11-13] and *P. jirovecii* is a known opportunistic pathogen that causes infection in people who are immunosuppressed, it is believed by some that this is due to the reactivation of a latent infection [14]. However, this theory has been challenged by the fact that *Pneumocystis* organisms have not been found in many immunocompetent people on follow-up, which suggests that the organism does not persist long after infection and produces a rather transient carrier state [15;16].

The other possibility, namely that patients who are immunosuppressed acquire a new infection of *Pneumocystis* appears to be gaining support. The source of this infection is not indisputably known. The reservoir for the organism could be healthy or ill individuals colonised with the fungus (i.e. carriers), patients with current PCP infection or even the environment [11;15;17]. The possible routes of transmission include person-to-person and airborne.

Healthy individuals have been shown to harbour the organism without infection [16;18-20]. These findings prove that asymptomatic infection or colonisation occurs. Colonisation has been defined as the isolation of *P. jirovecii* DNA in respiratory samples from patients who show no signs and symptoms of PCP. Colonisation is not known to lead to PCP [18;19]. In the USA, a large proportion (22/32, 69%) of HIV-positive patients without

PCP, were found to be colonised with *P. jirovecii* [21]. In contrast, a very low prevalence (0.3%, 1/384) of colonisation was found in Tanzania among HIV-positive and negative patients suspected of having TB. However, investigators used oral-wash specimens, which is not an optimal specimen type [22]. Colonised people may also play a large role in the transmission of PCP.

Air samples from hospitals have been tested and found to contain *P. jirovecii* DNA, supporting the theory of airborne transmission [23;24]. This leads to the strong possibility of nosocomial spread of the organism and has lead to suggestions that PCP patients should be isolated from immunosuppressed patients [20;23;25-29]. An animal study exposed rats to sterile and unsterile surroundings, food, water, as well as PCP-infected food. Only the immunosuppressed rats housed in an unsterile environment acquired PCP; therefore it was concluded that airborne transmission was very likely [30].

Many studies have suggested that person-to-person spread is the most probable route of transmission [20;29]. Outbreaks of PCP among transplant patients support this theory [27;31]. On the contrary, it has been suggested that person-to-person transmission and transmission from PCP patients to susceptible hosts may occur but may not be the main route of transmission [32;33].

Most of the recent studies agree with the recent transmission theory rather than the reactivation of a latent infection theory [34;35]. It is suggested by some researchers that infection arises from recent transmission, because their results show that *P. jirovecii* dihydropteroate synthase (DHPS) mutations change over time [35;36]. These authors state that if reactivation of a latent infection were occurring then the genotypes would remain

the same. To dispute this, Nahimana *et al*. [14] suggested that reactivation may occur and that the DHPS mutations may be selected for within the patients, under the pressure of the sulfa drugs. Investigations on the genotypes of *P. jirovecii* in association with place of birth versus place of diagnosis were done. Allelic variation patterns in *P. jirovecii* were not associated with patients' place of birth, but rather with place of diagnosis [37]. This finding strengthens the argument that patients acquire new infections.

1.2 *Pneumocystis* pneumonia (PCP)

Pneumocystis pneumonia is one of the most prevalent diseases in HIV-positive patients [2;18] but also affects certain non-HIV-infected groups. These are mainly patients on immunosuppressant medications such as glucocorticosteroids [2;14;38]. Mortality rates for PCP patients in developed countries range from 7% to 21% [39-41]. In-hospital mortality in children was found to be between 39% and 47% in South Africa (SA) [42;43]. PCP incidence may be affected by seasonality and climatic factors, particularly mean temperature [44-46].

1.2.1 Clinical manifestations

The signs and symptoms of PCP in immunocompromised patients are very much the same as those for other atypical pneumonias [3]. The main symptoms of PCP are a dry, non-productive cough and shortness of breath [3;47-49]. Fever and hypoxia are also common in PCP patients [48;50-52]. Patients presenting with the symptoms listed above should be further examined or tested to determine if they may have PCP.

Symptoms in HIV-infected patients are usually seen for longer periods of time but are milder compared to HIV uninfected patients, despite the higher fungal loads present in HIV-patients [48;53-55]. In addition the mortality rate is higher in non-HIV patients [48;55;56].

1.2.2 Diagnosis

PCP is a difficult disease to diagnose due to its non-specific signs and symptoms; in addition, infected immunosuppressed patients commonly have other opportunistic infections [57]. PCP is often the HIV/AIDS indicator disease and should be suspected in all HIV-positive patients with respiratory symptoms [42].

1.2.2.1 Clinical and radiological diagnosis

A chest x-ray of a patient with early presentation of PCP may show a perihilar haze, and late presentation generally shows bilateral interstitial infiltration [3;51-53]. This can progress to dense, diffuse alveolar infiltrates if the patient is not treated or is treated unsuccessfully. PCP is often diagnosed clinically, especially in SA, but the value of this is debatable [47;50;51]. A low CD4 cell count, specifically less than 200 cells/mm^3, is a risk factor for PCP, but infection can occur in patients with CD4 cell counts greater than 200 cells/mm^3 [14;38;47;50-52;58]. The CD4 count is less valuable in HIV-negative patients with PCP, who can have counts from 42 to 900 cells/mm^3 [59].

1.2.2.2 Laboratory diagnosis

Laboratory diagnosis is important because of the non-specific signs and symptoms of PCP [51]. A number of different methods have been explored for the diagnosis of PCP because

the organism cannot be maintained in culture. In the past, various stains were used to identify *Pneumocystis* organisms. These included Grocott's methenamine silver, toluidine blue O, cresyl violet and Giemsa stains [50;53;60].

Identification methods have naturally evolved over time. Electron microscopy has successfully shown the trophozoite, sporocyte and cyst stages of *Pneumocystis* in respiratory specimens, but because of its precise and lengthy processing steps, it cannot be used to routinely diagnose PCP. In recent times, the most commonly used diagnostic methods for PCP are indirect immunofluorescence assay (IFA) and polymerase chain reaction (PCR). For IFA diagnosis, broncho-alveolar lavage (BAL) is recommended in order to obtain a reliable result [42;51]. However, an induced sputum, which has been shown to have a good yield, may also be used [61]. The invasive procedures required to obtain these specimens, puts the already weak patient under further stress so measures must be taken to monitor the patient during the procedure.

Other respiratory specimens may be used for IFA but the sensitivity of the test will decrease. Due to the nature of the disease, patients are not often able to expectorate sputum samples. The sputum induction procedure can only be carried out by a trained nurse. For this, the patient must inhale a mist of hypertonic saline created by a nebulizer. As arterial blood desaturation may result, the patient's arterial blood saturation should be measured with a pulse oximeter during sputum induction [53]. This technique was used to collect the samples for this study.

PCR assays are used, but as they are very sensitive [especially quantitative real-time PCR (qPCR)], they identify colonisation in addition to infection which may make the results

less valuable [51]. DNA copy number cut-offs to differentiate colonisation and infection, for qPCR assays have been suggested but need to be standardised for routine use [51;62-64]. Colonisation studies should make use of qPCR assays, or results may be questionable. An example is a study that found a low rate of colonisation in HIV-infected adults and their children [65], but this may be because they used a nested PCR instead of a qPCR, which is more sensitive. Due to the sensitivity of PCR, a variety of specimen types have been successfully used to identify the presence of *P. jirovecii*, such as induced sputum, broncho-alveolar lavage, oral wash and tracheal aspirate specimens [66-68]. Whichever method is used to identify *P. jirovecii*, the results should be coupled with the clinical presentation of the patient, especially because of colonisation [69].

Molecular methods have also been employed to determine drug resistance levels, because in-vitro susceptibility tests cannot be done due to the lack of a culture system. With respect to PCR, it must be remembered that this method and other related molecular methods do not identify the whole organism, as most histologic methods do [70;71].

DNA extraction is a method that is carried out prior to DNA molecular analysis [72]. It is important that the DNA is isolated carefully to ensure that it is not fragmented. The typical extraction procedure involves cell disruption to free the DNA, removal of contaminants namely RNA and proteins, and precipitation of the DNA. This is done in the presence of a chelating agent which binds to the magnesium ions, which are needed by the DNA degrading enzymes (DNases). DNA extraction methods have developed over time; some methods involve the use of magnetic beads and others use spin columns [73;74]. Several of these methods have been incorporated into automated systems thereby creating an easy-to-use procedure.

Some serum markers have been found to be associated with PCP but the diagnostic significance is unclear. The first is lactate dehydrogenase (LDH) which has been shown to increase in patients with PCP, but it has also been noted that this increase is probably due to the lung inflammation and injury associated with PCP rather than the disease itself [42;47;52]. The second marker is (1->3) β-D-glucan (BDG), which forms part of the cell wall of most fungi and has been used as a serum marker for other fungal diseases; therefore again this marker is not specific to PCP. However, many studies have shown that BDG can be a useful marker for PCP, especially if coupled with the clinical presentation of the patient [14;38;75;76]. Testing for these serum markers to diagnose PCP is an option but due to the non-specific nature of the tests it may not be practical [57;77].

The essential metabolite S-adenosylmethionine (SAM) cannot be synthesised by *Pneumocystis*, as it is by other organisms. For this reason, determining the level of SAM in people infected with *P. jirovecii* was investigated as another promising test for PCP. It was successfully shown that SAM levels became depleted during PCP infection and after treatment, levels of SAM increased [78]. On the contrary, other investigators found that SAM was not a reliable marker for PCP [79].

1.2.3 Prophylaxis and treatment

Patients with severe immunodeficiency should receive PCP prophylaxis [47;58]. In SA guidelines stipulate that all patients with CD4 cell counts ≤200 cells/mm^3 should be given PCP prophylaxis [80]. This is usually in the form of the sulfa-containing drug trimethoprim-sulphamethoxale (TMP-SMX or cotrimoxazole). This drug also provides protection against another opportunistic pathogen – *Toxoplasma gondii*, as well as many bacterial infections. The most common side effect of this drug is a maculopapular rash that

develops more frequently in HIV-positive patients. An antihistamine can be prescribed, to reduce this reaction or the patient can be desensitised. However, in the case of other more serious side effects such as fever, hepatitis and mucous membrane lesions, an alternative drug should be used. Dapsone is an alternative to TMP-SMX.

Treatment drugs for PCP are very similar to the PCP prophylactic drugs. Cotrimoxazole is the first choice [47;49;68]. Dapsone plus trimethoprim or clindamycin plus primaquine can be used in cases of adverse reactions to TMP-SMX. Atovaquone and pentamidine are other choices. Pentamidine has been linked to worse outcome compared to TMP-SMX and clindamycin plus primaquine [81]. Adjunctive steroids such as prednisone should be given to reduce the host inflammatory response.

PCP prophylaxis or treatment is not always effective for reasons including side effects such as toxicity, treatment failure and non-adherence [18;82;83]. Heffelfinger *et al*. [82] conducted a large study in America to determine the extent and reasons for non-adherence. They found that 18% of patients were non-adherent and the most common factors associated with non-adherence were drug abuse and mental health problems. In SA, non-adherence is also a problem, but for reasons far different. The main reason is limited or no access to medication.

Some studies have shown that cotrimoxazole prophylaxis decreases the incidence of PCP in HIV-patients [84;85]. A South African study that showed that children on PCP prophylaxis were less likely to develop PCP compared to those not on these drugs [42]. However, another study in SA proved that this is not always the case. In 2002, Madhi *et al*. [86] carried out a study on South African children with pneumonia. They found that

children who received TMP-SMX prophylaxis did not have a significantly reduced number of PCP episodes compared to children who did not receive prophylaxis. However, they did find that PCP patients who were on PCP prophylaxis had a better outcome than those who were not.

1.2.4 Epidemiology

1.2.4.1 Global PCP

PCP was the most common opportunistic infection in HIV/AIDS patients in developed countries such as the United States of America (USA) [40;87]. For this reason, when the HIV burden was at its highest, the number of PCP cases increased. However, due to effective public health interventions, namely PCP prophylaxis and HAART, PCP has decreased in most developed countries [40;46;87;88]. A study in the USA analysed national data over a 20-year period from 1986 to 2005, and showed that hospitalisation and hospital mortality for AIDS-associated PCP has decreased over this period. This period included the introduction of chemoprophylaxis and potent combination antiretroviral therapy [40]. PCP continues to be a major health problem among people who are unaware of their HIV status, have limited or no access to treatment, and do not adhere to antiretroviral therapy or PCP prophylaxis [18;47;51].

It was initially believed that PCP in HIV-positive patients, was not as prevalent in Africa and the rest of the developing world, compared to the developed world. However, more recently, studies have proven otherwise and reasons for the earlier findings, such as small study groups, have been proposed [42;47;58;89-91]. Studies carried out in Zimbabwe (1992-1993), Kenya (1999-2000), Uganda (1999-2000) and Ethiopia (2004-2005) on HIV-

positive adult patients who were sputum smear-negative for AFB, documented PCP prevalence rates of 33% (31/64), 37% (19/51), 39% (32/83) and 30% (39/131), respectively [58;90;92;93].

1.2.4.2 PCP in South Africa

PCP is of great concern in SA because of the burden of HIV/AIDS [89]. There are a limited number of published articles regarding PCP in SA, especially in adults. Wood *et al.* [100], found 22% of HIV-positive clinic attendees in the Western Cape had PCP, the second highest HIV-related disease after TB [91;94]. A post-mortem study done on the bodies of HIV/AIDS patients in the Eastern Cape found a 9% PCP prevalence [95]. Ramogale *et al.* [96] reported that pneumonia (PCP and bacterial) was the second highest cause of maternal deaths in a hospital in KwaZulu-Natal, SA. Ten percent of acute pneumonia cases in HIV-positive children were due to PCP in 1998 [42], and a similar study carried out in 2006 to 2008 found a 21% prevalence [43]. From these data, we can conclude that PCP is a major health problem in our country.

1.3 Resistance

1.3.1 Mechanism of resistance

As with many organisms, drug resistance has become an important problem. *Pneumocystis jirovecii* has been shown to have potential resistance to sulfa drugs. If confirmed, resistance will reduce the efficacy of these widely-used drugs [97].

Sulphonamide drugs have been used for over 60 years and are effective because they target the enzymes utilised in the folate synthesis pathway [98]. Many microorganisms, including *P. jirovecii,* need to produce their own folate as they are not able to transfer it into their cells. The multifunctional *fas* (folic acid synthesis) gene codes for the enzymes dihydroneopterin aldolase, hydroxymethyldihydropterin pyrophosphokinase and DHPS [99], which are required for folate synthesis. Sulfa drugs inhibit these enzymes and since humans do not have these enzymes, they are not adversely affected. Mutations in the *fas* gene are thought to alter the enzyme's structure and therefore sulfa drugs are not as effective, as their active binding site has been modified [84]. Figure 1.4 illustrates the possible mechanism of resistance caused by mutations in the DHPS region of the *fas* gene, which will further be referred to as the DHPS gene for simplicity.

Two DHPS mutations in *P. jirovecii* that are thought to be associated with sulfa resistance are non-synonymous point mutations at nucleotide positions 165 and 171 [37;100;101]. A mutation at position 165 (from adenine to guanine) causes an amino acid change from threonine to alanine at codon 55 and a mutation at position 171 (from cytosine to thymine) causes a change from proline to serine at a codon 57. Combinations of both mutations have also been seen. These DHPS mutations have been linked to sulfa drug resistance in other organisms such as *Plasmodium falciparum, Neisseria meningitidis, Streptococcus pneumoniae* and *Streptococcus pyogenes,* which shows that this highly-conserved region is a common target for mutations that can cause drug resistance [102-105].

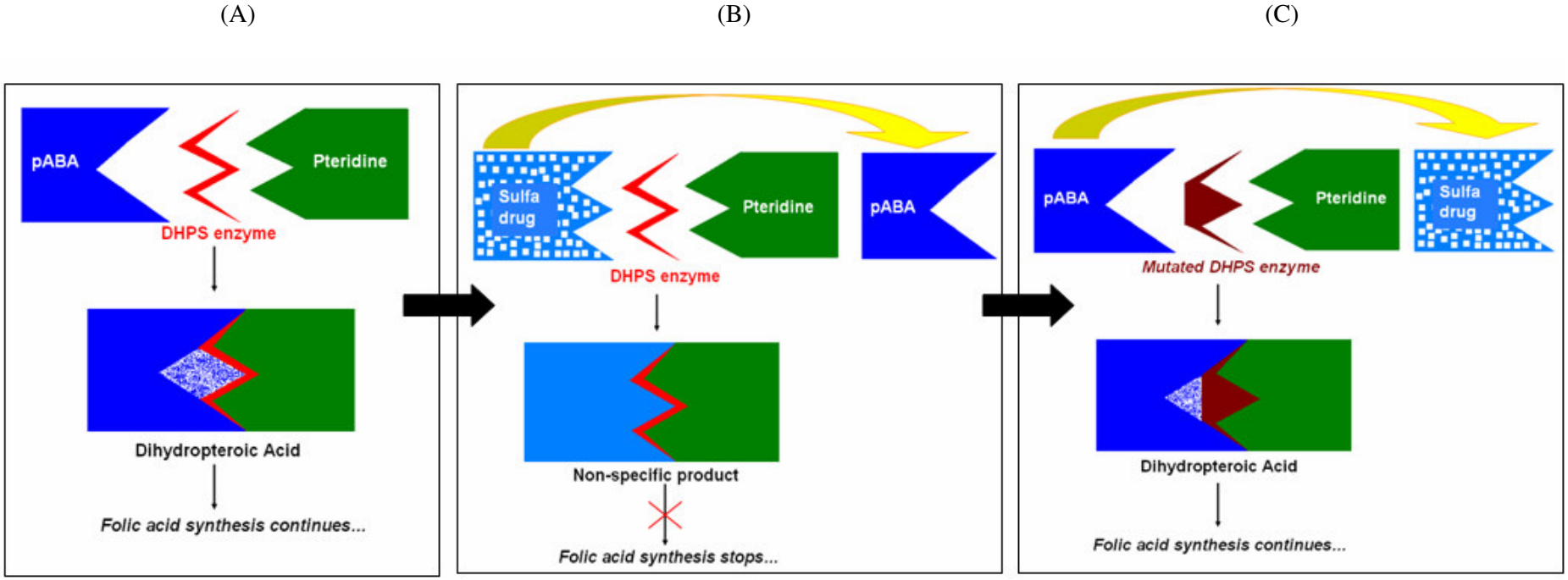


Figure 1.4 Mechanism of *P. jirovecii* resistance to sulfa drugs caused by mutations in the DHPS region of the *fas* gene (Graphic by B. Poonsamy)

(A): **Normal scenario - DHPS enzyme** catalyses the condensation of **pABA** and **pteridine** to form dihydropteroic acid. Further steps in this pathway lead to folic acid synthesis and eventually DNA synthesis, hence the organism continues to survive. (B): **Treatment with sulfa drugs - Sulfa drugs** are chemical analogues of **pABA** and bind to **DHPS**, thus competitively inhibiting the enzyme and folate synthesis resulting in the death of the organism. (C) **DHPS mutations -** DHPS gene mutations are thought to cause structural changes in the enzyme's substrate binding site, which interferes with sulfa binding and possibly causes sulfa resistance, thereby allowing folic acid synthesis to continue as normal.

1.3.2 Global *P. jirovecii* DHPS mutation patterns

Published data show that DHPS mutations in *P. jirovecii* are more prevalent in developed countries, with prevalence rates ranging between 0 - 81% [14;34-37;67;100;101;106-121]. In comparison, mutation rates in developing countries range from 0 - 12% [34;35;122-128]. This correlates with the findings on PCP infection prevalence in developing countries and is probably due to the limited use of sulfa drugs. It may also be related to the limited number of studies and the small sample sizes used. The mean sample size in the studies from developed countries was 100 (range, 6 - 394), compared to a mean sample size of 24 (range, 6 - 57) in the developing countries' studies. Two studies in South Africa used larger sample sizes (79 and 151) and found substantially higher DHPS mutation rates (38% and 56% respectively) [68;129].

The highest prevalence of DHPS mutations has been found in North America, particularly the USA. Studies in Europe show varying prevalence, and studies in South America are limited. Except for the two studies from our group [68;129], the prevalences of mutations in Africa, Asia and Australia are low (Figure 1.5).

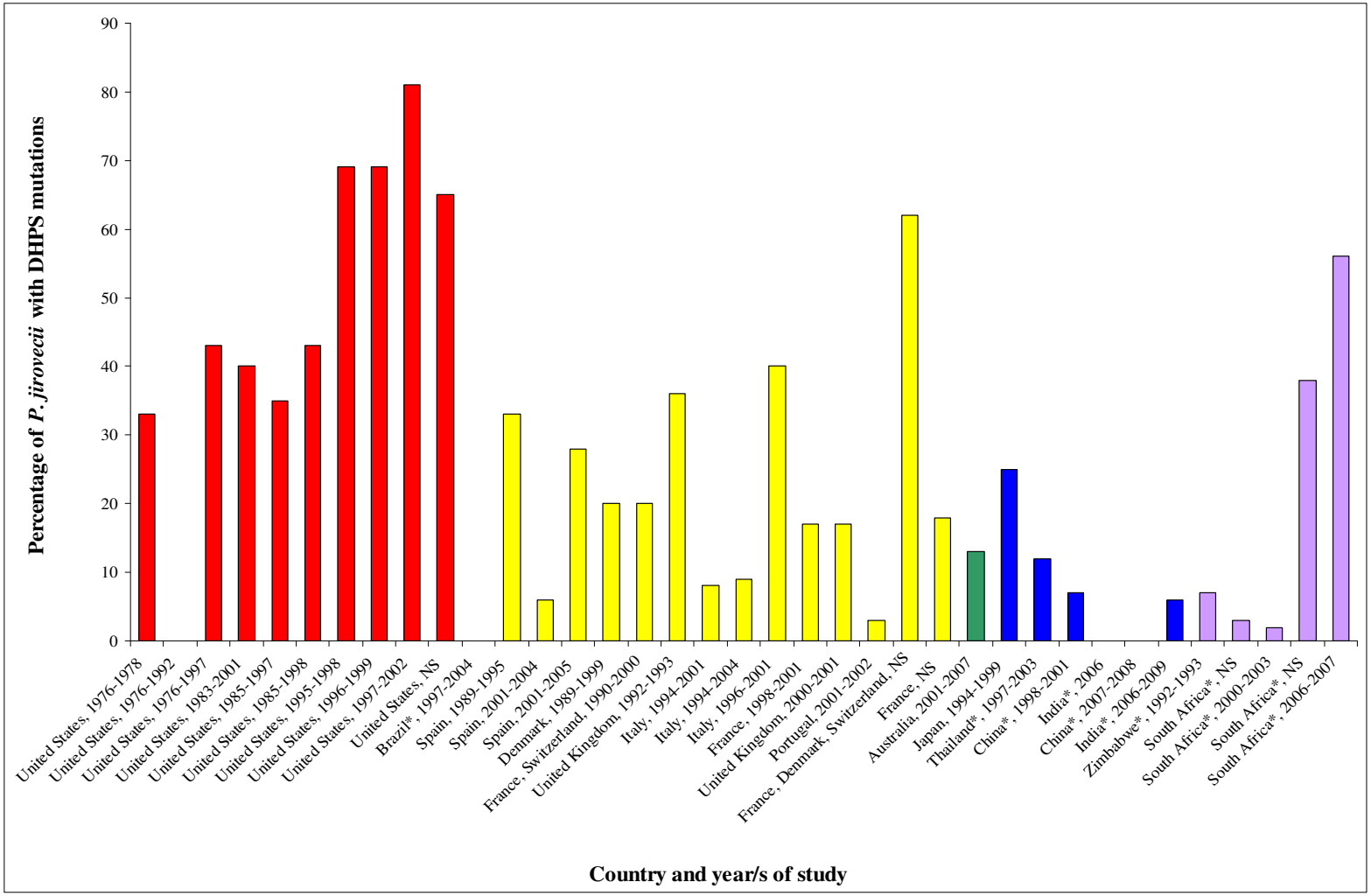


Figure 1.5 Comparison of the prevalence of DHPS mutations in *P. jirovecii* studies carried out on different continents** [14;34-37;67;68;100;101;106-112;114-129]

**Key: Red bars = North America, Yellow bars = Europe, Green bar = Oceania, Blue bars = Asia, and purple bars = Africa. NS = not stated, * = developing countries.

1.3.3 DHPS mutations, sulfa prophylaxis and patient outcome

Many studies have shown that DHPS mutations are more likely to occur as a result of sulfa drug prophylaxis failure or more generally, in patients that have been previously exposed to sulfa drugs [35;67;101;106;113;116;118;119;130]. The theory of these mutations arising from selective pressure of sulfa drugs has been supported by other studies [34;35]. A study in Spain found a higher rate of DHPS mutations in the pre-combined antiretroviral therapy (pre-cART) period compared to the cART period, and these mutations were associated with previous sulfa drug prophylaxis [36]. However, DHPS mutations have also been found in patients who have not been exposed to sulfa drugs [110], which again suggests person-to-person transmission of *P. jirovecii*.

In developed countries prophylactic drugs are more commonly administered and this is the most plausible reason for the high rates of DHPS mutations in these countries. Kazanjian *et al*. [34] showed that in the USA where sulfa drugs are widely used, the prevalence of DHPS mutations was higher (40%) compared to China (7%) where using prophylactic sulfa drugs for PCP is not common. This strongly suggests that the drugs select for the mutations.

Studies in SA have found a range of prevalence of DHPS mutations (Table 1.1). The two more recent studies [68;129] showed that a high prevalence of *P. jirovecii* DHPS mutations in patients from SA exists. It is possible that in SA we have an emerging sulfa drug resistance problem, as sulfa drugs are now being more widely used.

Table 1.1 *P. jirovecii* DHPS mutation studies conducted in South Africa [68;124;125;129]

Year of publication	Province/s of study	Year/s of study	Study population	Samples with DHPS mutant genotypes* (number)
2004	Western Cape	Not stated	HIV-positive children	3% (1/30)
2005	KwaZulu-Natal, Western Cape, Eastern Cape, North West and Mpumalanga	2000-2003	Not stated	2% (1/53)
2006	Gauteng	2004	HIV-positive adult inpatients	38% (30/79)
2010	Gauteng (69%); Eastern Cape, Mpumalanga, Free State, Limpopo, Northern Cape	2006-2007	Adults and children (HIV status unknown)	56% (85/151)

*Includes only mutations at nucleotide positions 165 and 171.

The important question of whether these mutations have an impact on patient outcome remains unanswered. Published data to date provide conflicting evidence. Reasons for this include inconsistent study definitions (e.g., for treatment outcome or previous exposure) and small data sets [84;113;131]. These reasons also explain why much else is unclear with regard to PCP. Several studies have found that patients harbouring *P. jirovecii* with mutant DHPS genotypes present with more severe disease and have a worse outcome [108;112;113;116], while others found that mutations were not associated with adverse patient outcome [36;114]. Valerio *et al*. [110] showed that DHPS mutations were associated with possible sulfa drug treatment failure.

1.3.4 Identification of DHPS mutations

To identify the DHPS mutations, a number of molecular processing options are available, such as sequencing, restriction fragment length polymorphism (RFLP) and single strand

conformation polymorphism (SSCP) [101;111;132]. However, all these methods have the limitation of not being able to resolve the complex mixed DHPS genotypes. These mixed genotypes can be resolved if their PCR products are cloned into an appropriate vector, amplified by PCR and sequenced.

Many studies that examined DHPS genotypes found that some patients harboured these complex mixed *P. jirovecii* DHPS genotypes [68;106;109;110;129]; therefore there is a need to resolve these genotypes. By resolving these mixed genotypes, we can re-group PCP patients according to their specific DHPS genotype and look for a possible link between DHPS genotype and patient outcome. We can possibly determine if patients with one *P. jirovecii* strain containing double mutants have a different outcome compared to patients with two *P. jirovecii* strains containing single mutants. It is possible that *P. jirovecii* strains with double mutations have increased resistance compared to strains with single mutations, as has been found with *P. falciparum* [84].

1.4 Study aim and objectives

Aim: To resolve mixed DHPS genotypes in *Pneumocystis jirovecii* in induced sputum specimens collected from patients suspected of having *Pneumocystis* pneumonia.

Objectives:

1. To validate the Roche MagNA Pure Compact automated extraction system.

2. To determine the fungal load present in each patient specimen with a quantitative real-time PCR assay, in order to identify the patients infected with *P. jirovecii*.

3. To sequence a fragment of the DHPS region of the *P. jirovecii fas* gene in these patients, thereby determining the DHPS genotypes and identifying patients harbouring complex mixed DHPS genotypes.

4. To resolve the complex mixed DHPS genotypes by cloning and resequencing the DHPS gene.

5. To determine if there is any association between DHPS genotype and in-hospital patient outcome.

CHAPTER 2 - MATERIALS AND METHODS

2.1 Patient recruitment, sample and clinical data collection

This project forms part of a larger study entitled "Survey and management of drug-resistant *Pneumocystis jirovecii* in South Africa". Patient recruitment started in March 2005 and concluded in June 2009. Adult patients admitted to the Respiratory Unit at the Chris Hani Baragwanath (CHB) Hospital, who were clinically suspected of having *Pneumocystis* pneumonia, were invited to enrol in the study. Once informed consent was obtained (Appendix A), an induced sputum sample was taken by a qualified study nurse. Repeat specimens were taken on request from the clinician. The enrolled patients were monitored and clinical data were collected (Appendix B) until the patient was discharged or died. All clinical data were analysed for the larger study; for this project, only in-hospital patient outcome information was analysed.

2.2 *Pneumocystis jirovecii* diagnosis

2.2.1 *P. jirovecii* diagnosis by IFA

P. jirovecii diagnosis was done by the Parasitology Reference Unit (PRU) of the National Institute for Communicable Diseases (NICD). This accredited national reference laboratory is the largest *P. jirovecii* testing laboratory in the country and hence was well placed to carry out testing. Specimens were pre-digested by adding equal volumes of a 1.4-dithiothreitol (DTT) (Roche Diagnostics GmbH, Mannheim, Germany) working solution (Appendix C) to the specimen. Specimens were vortexed well for at least 30 seconds and incubated at 37°C for 15 minutes. This allowed the DTT to digest the sputum by breaking down any mucus present in the specimen. After incubation, specimens were vortexed again

and if clumps of mucus were still present, 1 ml of DTT stock (65 mM) (Appendix C) was added and the specimen was incubated at 37°C for a further 15 minutes.

To concentrate the specimen, the digested sputum was transferred to a 15 ml centrifuge tube and topped up to 14 ml with PBS pH 7.2 (Diagnostic Media Products, NHLS, SA). This was centrifuged at 2 000 rpm for 5 minutes. The supernatant was decanted and the pellet was resuspended in the remaining liquid. This suspension was applied thinly to a well of a clean slide with a pipette. Slides were air dried for 20 minutes and thereafter fixed with methanol for 2 minutes. After the methanol evaporated, 40 µl of stain from the Light Diagnostics Direct Immunofluorescence Assay (DFA) kit for the detection of *P. jirovecii* (Millipore Corporation, Billerica, MA 01821) was pipetted onto the well and carefully spread over the entire well surface. Slides were incubated in a humid chamber at 37°C for 30 minutes. The stain contained anti-*P. jirovecii* monoclonal antibodies that were labelled with fluorescein isothiocyanate (FITC). The antibodies bound to cysts and trophozoites of *P. jirovecii*. After incubation, excess stain was removed and the slide was washed twice in wash buffer (supplied in the Light Diagnostics DFA Kit). One drop of mounting medium (supplied in the Light Diagnostics DFA Kit) was added to the well and a coverslip was placed on the well.

The entire well was examined with an ultraviolet (UV)-equipped microscope under 400x magnification for any fluorescing antibody-antigen complexes. The presence of fluorescent green cysts and/or clusters of cysts on a red-stained background confirmed the presence of *P. jirovecii* (i.e. a positive result). The typical clusters of cysts have a honeycomb appearance (Figure 2.1). The parasite load was quantified into the following categories for the positive specimens:

- \+ : 2 cysts/ clusters of cysts,
- ++ : >2-10 cysts/ clusters of cysts, and
- +++ : >10 cysts/ clusters of cysts.

If only one cyst or cluster of cysts was observed, a possibly positive result was reported. If no cysts or clusters were seen and there was sufficient background staining, a negative result was reported.

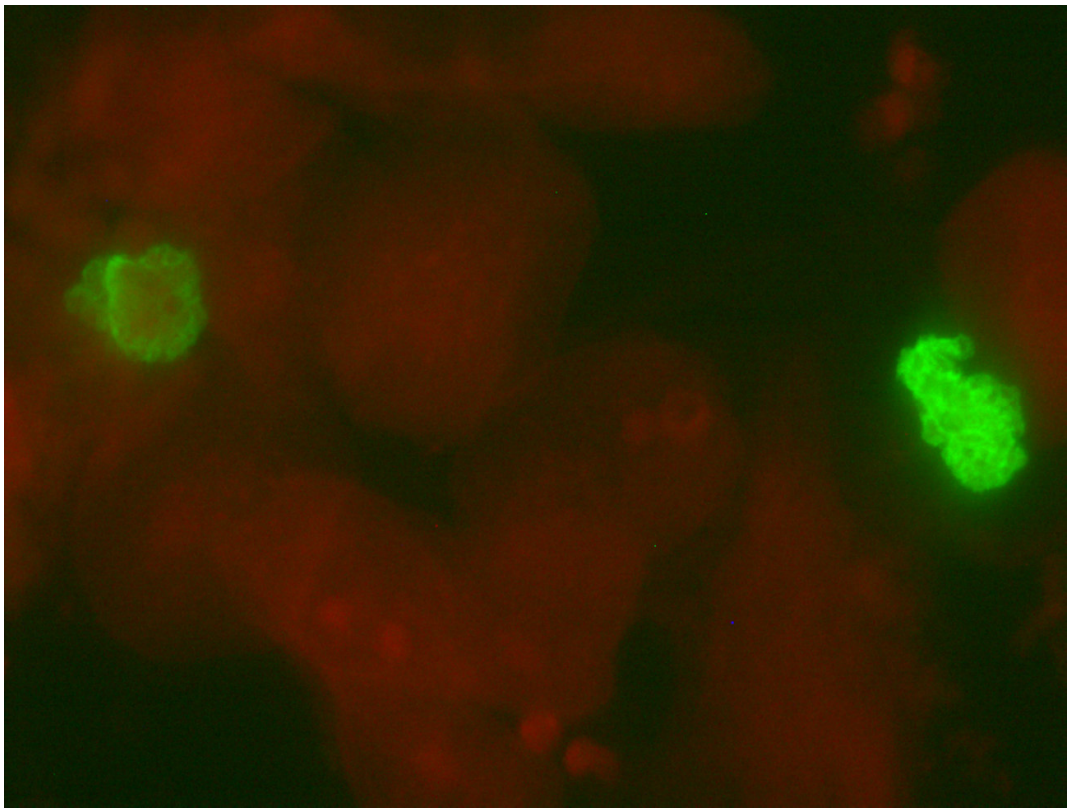


Figure 2.1 Micrograph showing two fluorescent green clusters of *P. jirovecii* cysts on a red-stained background, 400x magnification

For quality control, positive and negative control slides were stained with every new kit used. In addition a positive control slide was stained every week. Tests were repeated if the controls failed, the background was insufficient or the slide appeared cloudy under magnification.

Sample washing

To prepare samples for molecular analysis, samples were washed to remove reagents such as DTT from the sample. This was done by adding 10 ml of 10x PBS (Diagnostic Media Products) to the digested, concentrated samples, vortexing and centrifuging at 3 000 rpm for 5 minutes. The supernatants were decanted; the pellet was resuspended in the remaining liquid (about 200 µl) and the suspension was transferred to a 1.5 ml labelled tube, and then stored at -20°C.

2.2.2 Validation of the Roche MagNA Pure Compact

An automated DNA extraction instrument, the Roche MagNA Pure Compact (RMPC) (Roche Diagnostics), was purchased for this study and for other routine work of the PRU. In keeping with good laboratory practice, the new piece of equipment was validated. The validation formed part of this project. A series of experiments (Experiments 1 – 7) was conducted to assess the RMPC for contamination, repeatability and efficiency, in comparison with the previously used manual extraction method with the QIAamp DNA Mini Kit (Qiagen Inc, Valencia, CA 91355).

The automated RMPC method makes use of preloaded reagent cartridges and robotic transfer of the sample from one well to the next. This method uses magnetic glass particles (MGP), which extract the DNA (Figure 2.2). The Qiagen (manual) method makes use of spin columns. First the organism is lysed to release the DNA, the DNA is then bound to the QIAamp silica membrane and washed twice to remove any residual contaminants, thereby improving the purity of the DNA. Lastly, the purified DNA is eluted from the QIAamp spin column in a concentrated form (Figure 2.3).

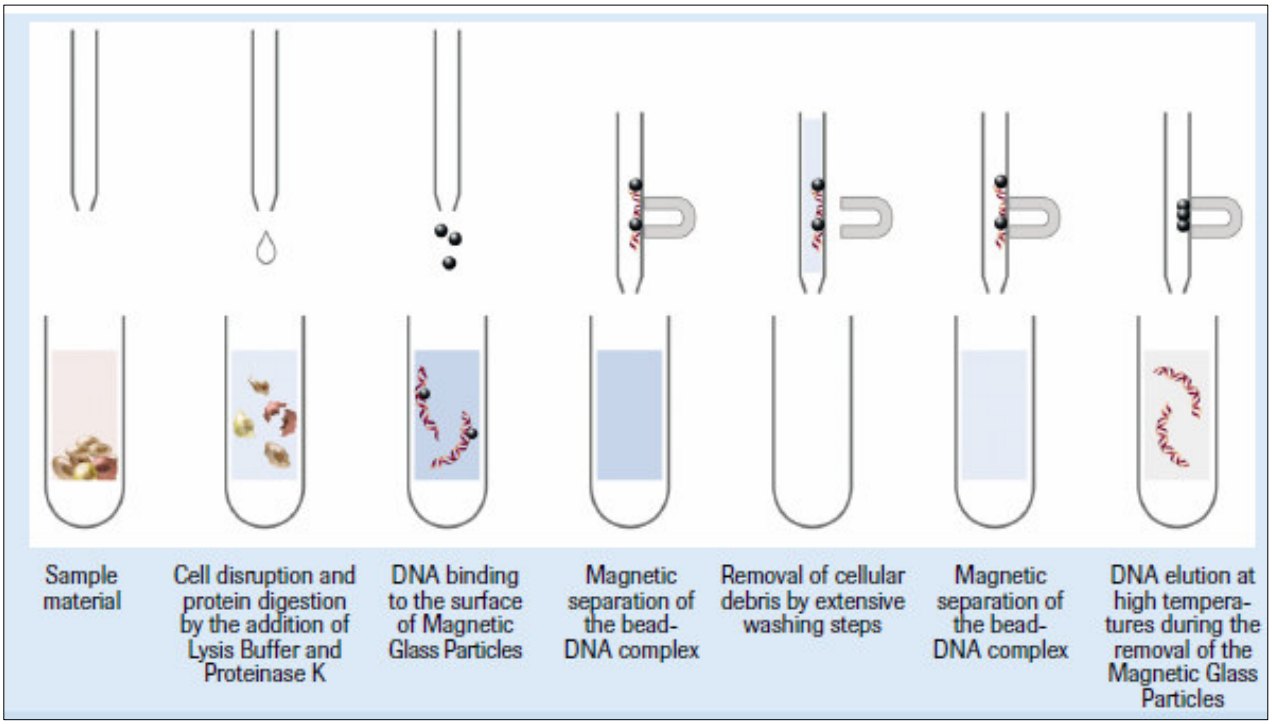


Figure 2.2 Principle of nucleic acid extraction in the Roche MagNA Pure Compact [133]

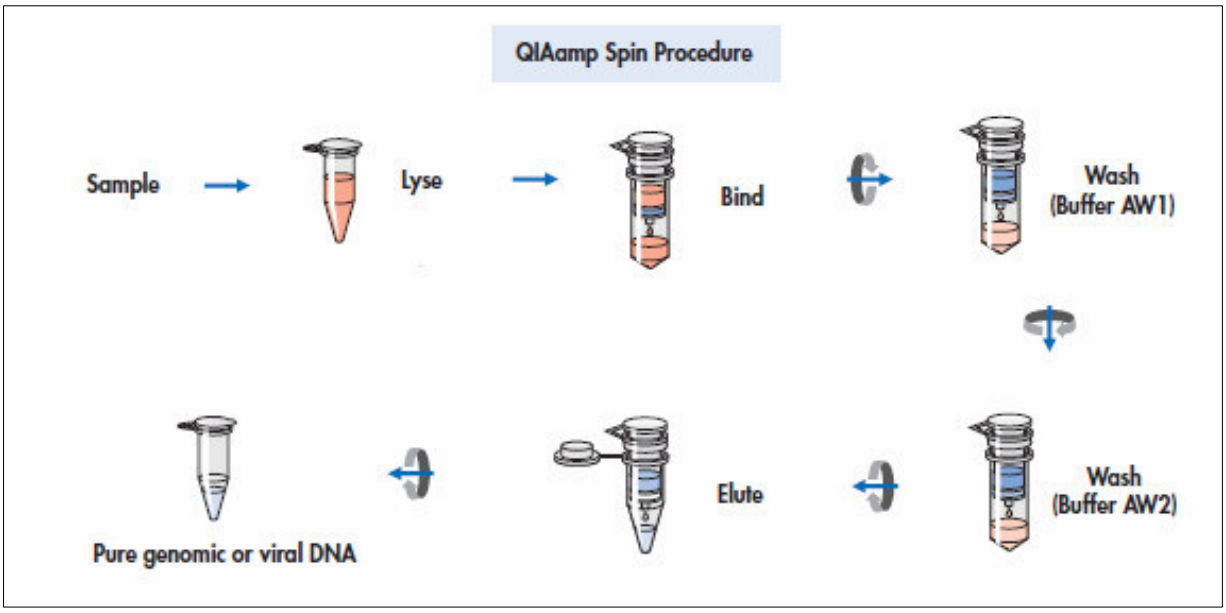


Figure 2.3 Principle of nucleic acid extraction with the Qiagen DNA Mini Kit. Adapted from the Qiagen DNA Mini Kit handbook [134].

Cross-contamination in the RMPC (Experiment 1)

When extracting DNA with the Qiagen method, specimens were handled individually, to prevent contamination. However, in the RMPC all specimens (up to eight) were open and processed simultaneously; this led to the question of possible cross-contamination due to aerosols. To check for cross-contamination in the RMPC, four highly positive specimens and four negative samples (sterile water) were placed in alternating positions in the RMPC and processed. The method used is detailed in section 2.2.3.1. A qPCR (as described in section 2.2.3.2) was performed on all eight extracted DNA specimens to determine the copy number of any *P. jirovecii* DNA present.

Comparison of the automated RMPC versus the manual Qiagen method (Experiment 2)

To compare these automated and manual extraction methods, 12 IFA-positive and 12 IFA-negative specimens were extracted with both methods. The manual extraction method used is detailed in Appendix D. All extracted DNA were processed further by a qPCR assay.

Technical optimisation of the use of the RMPC reagents (Experiment 3)

When performing automated DNA extractions, it was noticed that the well containing the magnetic beads in the reagent cartridge (Figure 2.4) had varying amounts of beads at the bottom of the well. In some cartridges the beads had adhered to the sides of the well. As the magnetic beads form a crucial part of this automated extraction method, this experiment was designed to compare the effect of shaking the cartridges to ensure all the magnetic beads were at the bottom of the cartridges before use, as opposed to not shaking the cartridges.

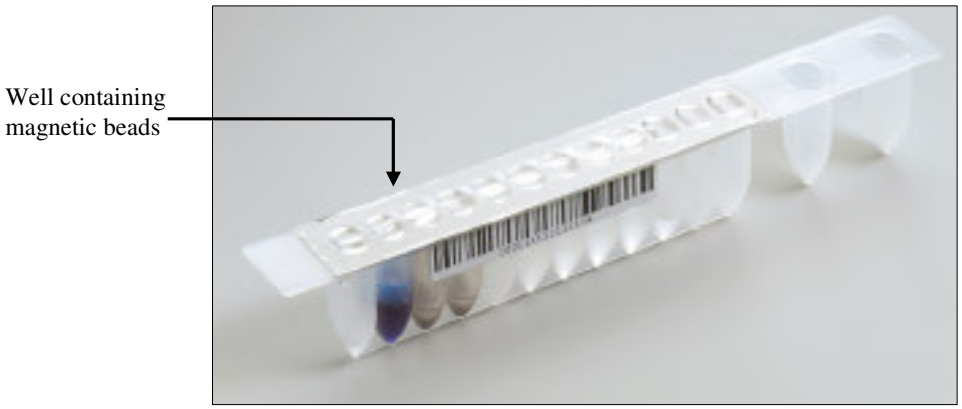


Figure 2.4 Roche MagNA Pure reagent cartridge needed for DNA extraction with the Roche MagNA Pure Compact [133]

The same 12 positive specimens from Experiment 2 were extracted again but in this experiment each cartridge was shaken well before being loaded into the RMPC. The extracted DNA from the 12 positives from Experiment 2 (in which the cartridges were not shaken) and the newly extracted DNA (cartridges shaken) were amplified by qPCR.

Repeat of the comparison of the automated RMPC versus the manual Qiagen method experiment (Experiment 4)

Following the previous experiment, the results of the comparison experiment may have been influenced by the cartridges not being shaken. Therefore, the qPCR was repeated on the DNA extracted manually and the DNA extracted by the RMPC with the cartridges shaken.

Robustness (Experiment 5)

The starting volume which is the volume of washed sputum that is digested, and the loading volume, which is the volume of digested material that is loaded into the RMPC, can be altered. In order to determine the optimal starting and loading volumes, the same positive specimen was processed at starting volumes of 20 µl and 50 µl and loading

volumes of 100 µl and 200 µl. A qPCR assay was performed on the extracted DNA to determine any differences in DNA copy number.

Repeatability (Experiment 6)

To test for repeatability three specimens with a low, medium and high copy number of DNA respectively were processed in triplicate with both the RMPC and Qiagen extraction methods. A qPCR assay was performed on the extracted DNA.

Varying instrument protocols (Experiment 7)

Roche Diagnostics suggested using a different protocol on the RMPC called the bacterial protocol [previously the total nucleic acid (NA) protocol was used], which they loaded onto the RMPC. The results of the two protocols on the RMPC were compared with the manual extraction method. Three specimens with low, medium and high copy numbers were processed in triplicate with the three methods; a qPCR assay was performed on the extracted DNA.

2.2.3 *P. jirovecii* diagnosis by real-time PCR

2.2.3.1 DNA extraction

Following the validation, all specimens were extracted with the automated RMPC. Respiratory specimens were first digested by adding 150 µl of bacterial lysis buffer (Roche Diagnostics) and 20 µl of proteinase K (Roche Diagnostics) to 50 µl of the washed sample. This was incubated for 2 hours at 56°C. DNA was extracted with the RMPC and the MagNA Pure Compact Nucleic Acid isolation kit (Roche Diagnostics), as per the manufacturer's instructions. Samples were loaded into the instrument and the bacterial

lysis protocol was selected. The 100 µl of extracted DNA were frozen at -70°C. For quality control, positive and negative controls were included in each extraction run.

2.2.3.2 Quantitative real-time PCR

Fungal load was determined with a qPCR assay [129] targeting a region coding for the mitochondrial large subunit (MtLSU) rRNA with an Applied Biosystems 7500 real-time PCR instrument (Applied Biosystems, Foster City, CA 94404).

In each assay, the final 25 µl reaction mixture consisted of 12.5 µl of 1x TaqMan universal master mix (Applied Biosystems), 0.25 µl of primer/probe mix at 20 µM each (1 part forward primer: 1 part reverse primer: 1 part MGB probe: 2 parts water) (Table 2.1), 2.5 µl of 10x Exo IPC (internal positive control) Mix, 0.5 µl of 50x Exo IPC DNA, 4.25 µl of water and 5 µl of DNA. The master mix was pipetted into 96 well MicroAmp plates (Applied Biosystems). Each plate contained standards (Appendix C) which were performed in triplicate, and included 10^{-10} (17 copies), 10^{-9} (170 copies), 10^{-8} (1 700 copies), 10^{-7} (17 000 copies) and 10^{-6} (170 000 copies) dilutions. Plates also contained negative or NTC (no target template control) wells and an IPC block well. Plates were covered with an adhesive optic film (Applied Biosystems) and centrifuged at 2 800 rpm for 5 minutes. The plate was loaded into the 7500 PCR instrument and an absolute quantification (standard curve) assay was setup on the Applied Biosystems SDS v1.3.1 program. Detectors FAM and VIC and the passive reference ROX were selected. The PCR assay parameters were as follows: 2 minutes at 50°C, 10 minutes at 95°C, followed by 45 cycles of 15 seconds at 95°C and 1 minute at 60°C. Quantification was based on extrapolation to standard curves generated by amplification of the standards. The standards were serial dilutions of a linearised plasmid (pCR2.1) which contained the *P. jirovecii*

DNA fragment defined by primers LSU1 and LSU2 (Table 2.1). The result analysis was done with the 7500 System Software. Real-time PCR results were expressed as target sequence copy number per 5 µl, further referred to as DNA copy number. For quality control, IPC controls, PCR positive controls and positive and negative DNA extraction controls were included in each real-time assay. An RNAse P PCR to detect inhibition (Appendix G) was done on all IFA-positive specimens that were qPCR negative.

Table 2.1 Primers used for *P. jirovecii* PCR assays [129]

PCR	Primer/probe name	Oligonucleotide sequence
Real-time qPCR	Primer LSU1 (forward)	5´-AAA TAA ATA ATC AGA CTA TGT GCG ATA AGG-3´
Real-time qPCR	Primer LSU2 (reverse)	5´-GGG AGC TTT AAT TAC TGT TCT GGG-3´
Real-time qPCR	Probe LSUP1	FAM 5´-AGA TAG TCC AAA GGG AAA C-3´TAMRA (Applied Biosystems)
Nested DHPS PCR – primary	Primer SMIF1 (forward)	5' CAA ATT AGC GTA TCG AAT GAC C 3'
Nested DHPS PCR – primary	Primer SMIB2 (reverse)	5' GCA AAA TTA CAA TCA ACC AAA GTA 3'
Nested DHPS PCR – secondary and Cycle sequencing	Primer SMIF6 (forward)	5' AGC GCC TAC ACA TAT TAT GG 3'
Nested DHPS PCR – secondary	Primer SMIB7 (reverse)	5' GTT CTG CAA CCT CAG AAC G 3'
Cycle sequencing*	Primer Mini-B7 (reverse)	5' CTG CAA CCT CAG AAC G 3'

* Unpublished

2.3 Primary sequencing

2.3.1 Nested PCR

All real-time PCR positive specimens (i.e. DNA copy number >0) were selected for further processing. The DHPS gene was amplified by a nested PCR (nPCR) protocol [129] in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany).

In each primary PCR assay, the final 25 µl reaction mixture consisted of 10.75 µl of water, 2.5 µl of 10x buffer, 2.5 µl of 1.25 mM dNTPs, 3 µl of 25 mM $MgCl_2$, 0.5 µl of 50 µM primer SMIF1 (Table 2.1), 0.5 µl of 50 µM primer SMIB2 (Table 2.1), 0.25 µl (5U/µl) Supertherm *Taq* DNA polymerase and 5 µl of DNA.

PCR tubes were loaded into the Eppendorf Mastercycler. The cycling parameters were as follows: an initial extended denaturation step at 94°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds for denaturation, 55°C for 1 minute for annealing, 72°C for 2 minutes for extension and a final extended extension cycle of 72°C for 7 minutes. Primary PCR products were stored at 4°C short term until the secondary PCR was performed. For long term storage the products were stored at -20°C.

In each secondary PCR assay, the final 25 µl reaction mixture consisted of 14.75 µl of water, 2.5 µl of 10x buffer, 2.5 µl of 1.25 mM dNTPs, 3 µl of 25 mM $MgCl_2$, 0.5 µl of 50 µM primer SMIF6 (Table 2.1), 0.5 µl of 50 µM primer SMIB7 (Table 2.1), 0.25 µl (5U/µl) Supertherm *Taq* DNA polymerase and 1 µl of DNA.

PCR tubes were loaded into the Eppendorf Mastercycler. The cycling parameters were as follows: an initial extended denaturation step at 94°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds for denaturation, 52°C for 30 seconds for annealing, 72°C for 1 minute for extension and a final extended extension cycle of 72°C for 7 minutes. Secondary PCR products were stored at 4°C short term until gel electrophoresis was performed. For long term storage the products were stored at -20°C.

For quality control, negative controls were included in each primary and secondary PCR to identify any possible contamination. Positive controls were not required as the specimens were known positives.

2.3.2 Gel electrophoresis

PCR products were resolved on a 1.5% agarose gel stained with ethidium bromide. The gel was made by adding 0.45 g of agarose to 30 ml 1x TAE buffer (Appendix C). This was dissolved in a microwave by heating for 1 minute. After cooling, 1.5 µl of 10 mg/ml ethidium bromide was added; the solution was poured into the gel-setting chamber, and left to set for 45 - 60 minutes. Five microlitres of PCR product, 1 µl of 6x DNA loading dye (Fermentas, Burlington, Ontario, L7N 3N4) and 4 µl of 1x TAE buffer were mixed and loaded into the wells. Four microlitres of a Generuler 100 bp plus DNA ladder (Fermentas) was added to the first well of the gel. Samples were run at 110 volts for at least 35 minutes. The gel was viewed with an ultraviolet transilluminator (GelDoc, Vacutec, Johannesburg, South Africa) to observe if the 278 bp amplicon was present.

2.3.3 Sequencing of PCR products

Following amplification, nPCR products were purified with the QIAquick PCR purification kit (Qiagen Inc). Five volumes (100 µl) of Buffer PB were added to 1 volume (20 µl) of the PCR sample. This was vortexed and briefly centrifuged. To bind the DNA, the sample was applied to the QIAquick column (in 2 ml collection tube) and centrifuged for 60 seconds. The flow-through was discarded and the collection tube was blotted to remove excess fluid. The QIAquick column was placed back into the same tube and washed by adding 750 µl of Buffer PE to the column and centrifuging for 60 seconds. This

step was repeated and the tube was centrifuged for an additional 1 minute to remove any residual ethanol from Buffer PE. The QIAquick column was placed in a clean labelled 1.5 ml microcentrifuge tube, and the collection tube was discarded. To elute DNA, 25 µl of Buffer EB was added to the centre of the QIAquick membrane. The column was left to stand for 1 minute, and then centrifuged for 1 minute. The column was discarded and the purified product was stored at 4°C for a short while, if the sequencing reaction was not done immediately.

Sequencing was done in both directions with the BigDye v3.1 Terminator Cycle Sequencing Kit (Applied Biosystems). Each cycle sequencing reaction had a final volume of 10 µl which consisted of 6 µl water, 1 µl of 5x sequencing buffer, 2 µl Big Dye terminator v3.1, 0.5 µl of 1 µM primer (SMIF6 or Mini-B7) and 0.5 µl of cleaned PCR product. Cycling parameters consisted of 25 cycles of denaturation at 96°C for 20 seconds, annealing at 50°C for 15 seconds and extension at 60°C for 4 minutes. Samples were stored at 4°C for short term or at -20°C for long term.

Products were purified with the DyeEx 2.0 Spin Kit (Qiagen). The Qiagen spin column was vortexed well to remove bubbles from the gel and centrifuged at 2 700 rpm for 3 minutes to remove the liquid. The spin column was placed in a clean labelled 1.5 ml microcentrifuge tube, and the collection tube was discarded. The sequencing product was carefully pipetted into the middle of the gel and the tubes were centrifuged at 2 700 rpm for 3 minutes. The columns were discarded and the cleaned product was vacuum dried in an Eppendorf Concentrator (Eppendorf) for 20 minutes at room temperature. Samples were stored at 4°C for short term or at -20°C for long term.

Twelve microlitres of Hi-Di Formamide (HDF) was added to the dried purified sample. This was vortexed and incubated at 95°C for 2 minutes. Samples were then placed on ice for 1 minute, pipetted into a 96 well optical plate which was centrifuged briefly. Electrophoresis was performed in an Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems), with the 'Rapidseq36_POP7_3.1' programme and the '3130POP7_BDTv3' analysis protocol. The 'Sequence analysis software v5.2' was used to obtain the results and the 'Bioedit Software' was used to analyse the sequences.

This initial sequencing was referred to as primary sequencing, and the mutations were classed and named according to Table 2.2 for simplicity. The genotypes labelled 'basic genotypes' and 'resolvable mixed genotypes' are the DHPS genotypes which could be determined from the primary sequencing. The 'irresolvable mixed genotypes' are the genotypes which were cloned and resequenced in order to discern their DHPS genotypes.

Table 2.2 Different possible primary sequencing DHPS genotype results with their corresponding bases at nucleotide positions 165 and 171 and the resulting amino acids at codons 55 and 57, respectively

Name	Description	Base/s at nucleotide position 165, and resulting amino acid at codon 55	Base/s at nucleotide position 171, and resulting amino acid at codon 57
Basic genotypes			
WT	Wild type, no mutations	A, Thr	C, Pro
M1	Single mutation at position 165	G, Ala	C, Pro
M2	Single mutation at position 171	A, Thr	T, Ser
M3	Double mutation	G, Ala	T, Ser
Resolvable mixed genotypes			
WT + M1	Mix of wild type and M1	A, Thr/ G, Ala	C, Pro
WT + M2	Mix of wild type and M2	A, Thr	C, Pro/ T, Ser
M3 + M1	Mix of M3 and M1	G, Ala	T, Ser/ C, Pro
M3 + M2	Mix of M3 and M2	G, Ala/ A, Thr	T, Ser
Irresolvable mixed genotypes			
Unknown mix		A, Thr/ G, Ala	C, Pro/ T, Ser

2.4 Cloning

Specimens with irresolvable mixed DHPS genotypes were cloned with the TOPO TA Cloning® Kit (Invitrogen, Carlsbud, California 92008). To ligate the PCR product into the vector, a 6 µl reaction mixture containing 3 µl of water, 1 µl of salt solution (1.2 M NaCl; 0.06 M $MgCl_2$), 1 µl of pCR®2.1-TOPO® vector (Figure 2.5) (kept on ice) and 1 µl of PCR product was prepared. This was incubated at room temperature for 30 minutes.

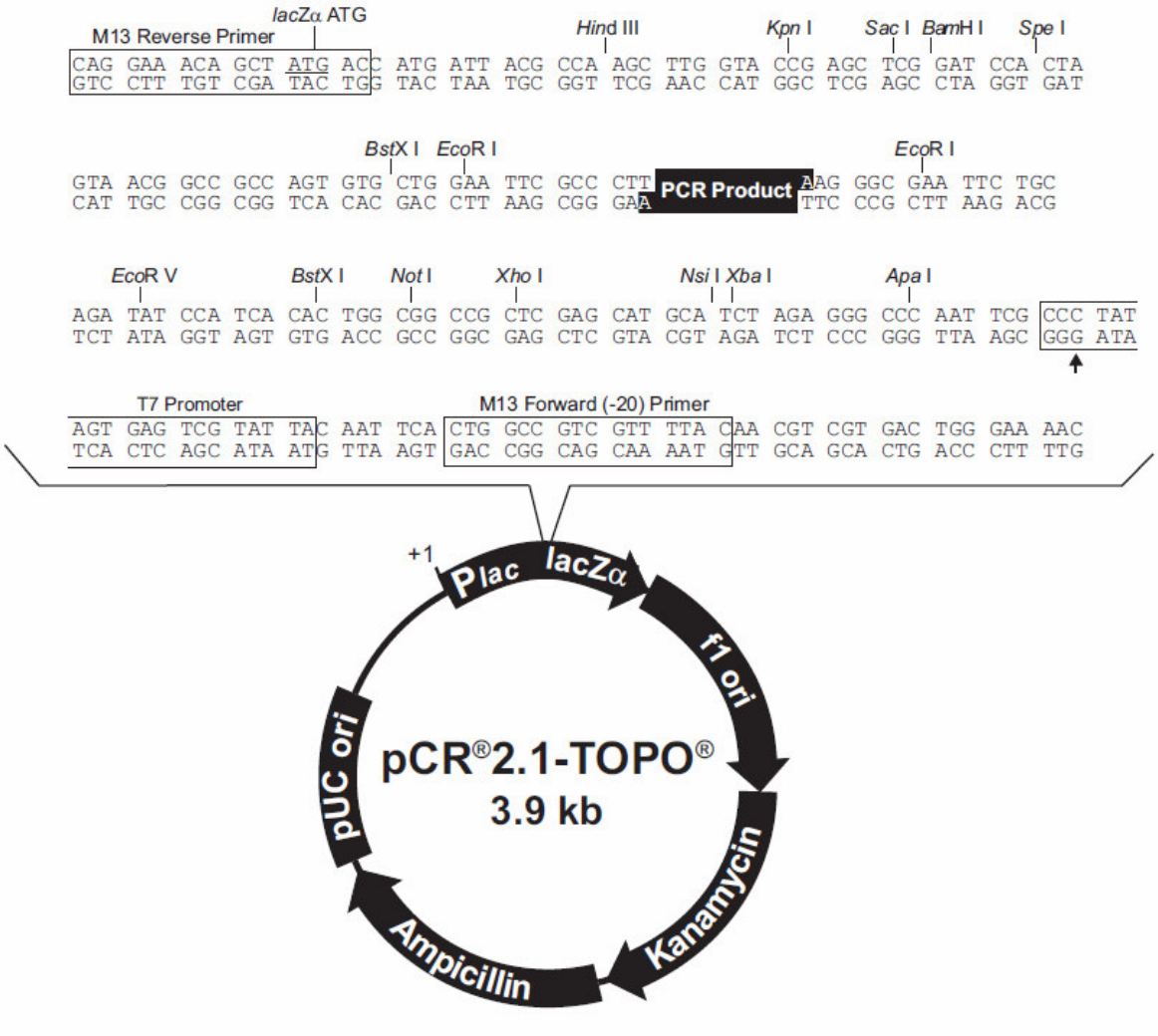


Figure 2.5 pCR 2.1-TOPO® vector, showing restriction sites, used to clone the *P. jirovecii* DHPS gene

For the transformation procedure, 3 µl of the ligation mix was added to 80 µl of One Shot® TOP10 chemically competent cells (*E. coli*). This was incubated on ice for 30 minutes. The mixture was heat shocked at 42°C for 30 seconds, which enabled the cells to momentarily take in the vectors. To maximise the transformation efficiency of competent cells, 250 µl of SOC medium was added and this was incubated for 1,5 hours at 37°C in a shaking incubator (200 rpm). Forty microlitres of X-gal (40 mg/ml) (Appendix C) was streaked on LB plates and kept at 37°C until use. Fifty microlitres of transformed cells were streaked onto the plate and incubated at 37°C overnight, preferably for 18-20 hours. For long term storage, 3 ml of Luria broth with ampicillin (Appendix C) and 50 µl of the transformed cells were incubated at 37°C in a shaking incubator overnight to increase cell count.

A 20 µl master mix was prepared by adding 12.2 µl of water, 2 µl of 10x buffer, 1.6 µl of 10 mM dNTPs, 2.4 µl $MgCl_2$, 0.8 µl of 20 µM M13 forward primer (M13 primers included in TOPO TA Cloning® Kit), 0.8 µl of 20 µM M13 reverse primer and 0.2 µl Taq polymerase. A pipette tip was used to pick off white colonies (i.e. transformed cells) and inoculate the master mix. The tip was also used to inoculate a patch plate for short term storage of the colonies, and 2 ml of Luria broth (clone culture) for long term storage. Eight to ten white colonies and one blue colony (which served as a control) from each sample was used. Recombinant clones were screened by PCR. The cycling parameters were as follows: an initial extended denaturation step at 94°C for 10 minutes, 25 cycles of denaturation at 94°C for 30 seconds, annealing at 45°C for 30 seconds, extension at 72°C for 1 minute and an extended final extension step at 72°C for 7 minutes. The PCR products were run on a 1.5% agarose gel, as previously described. The expected product size was 480 bp (plasmid size of 202 bp + PCR insert size of 278 bp). Eight to ten random clones were selected from the successful clones. These were sent to Inqaba Biotec (Pretoria,

South Africa) for sequencing to establish their individual DHPS genotypes. Sequencing was done with the M13 forward primer. To differentiate from the initial (primary) sequencing, this subsequent sequencing was referred to as secondary sequencing.

The transformed cells (800 µl) that were left overnight in the shaking incubator were added to 200 µl of sterile glycerol, mixed well and frozen at -70°C. The plates used for recombinant selection were sealed with parafilm and stored at 4°C. LB patch plates were incubated overnight at 37°C. After incubation, LB patch plates were sealed with parafilm and stored at 4°C. The clone culture (850 µl) was added to 150 µl sterile glycerol, mixed well and stored at -70°C. The plates were kept for short term (1-2 weeks) in case they were needed again, after which they were discarded.

2.5 Data management and analysis

Sequences were analysed with the BioEdit sequence alignment editor and compared against one of the DHPS gene sequences obtained from Genbank (Accession number AF139132). Data were analysed with InStat software; the two-tailed Fisher's exact test was used to determine associations. A p-value less than 0.05 was considered significant.

2.6 Ethical clearance

All required ethical clearances were obtained from the Committee for Research on Human Subjects of the University of Witwatersrand (protocol number M040612, 28/06/2004 – Appendix E). Informed written consent was obtained from all patients enrolled in the study. Permission to conduct the study was obtained from the Superintendent of CHB Hospital.

CHAPTER 3 – RESULTS

3.1 Patient recruitment, sample and clinical data collection

From March 2005 through June 2009, 280 patients were recruited from the CHB Hospital, and 320 induced sputum samples were obtained from these patients. Informed consent forms were not signed by 14 patients and therefore they were excluded from the study, resulting in 306 specimens for analysis.

There were 195 females and 71 males in the study group and the mean patient age was 35 years (range, 19 – 60). All 259 patients with a known HIV status were HIV positive. The in-hospital outcomes of the patients were recorded as follows; 74% (197/266) were discharged, 18% (48/266) died and 1% (2/266) refused hospital treatment. No data were available for 7% (19/266).

3.2 *Pneumocystis jirovecii* diagnosis

3.2.1 *P. jirovecii* diagnosis by IFA

Of the 306 specimens tested for *P. jirovecii* by the IFA detection method, 51% (156/306) were IFA positive, 4% (11/306) were IFA possibly positive and 45% (138/306) were IFA negative (Figure 3.1). The IFA test could not be completed for one specimen due to a laboratory error that occurred during processing; however this specimen was included in all further analysis as the DNA extraction was successful.

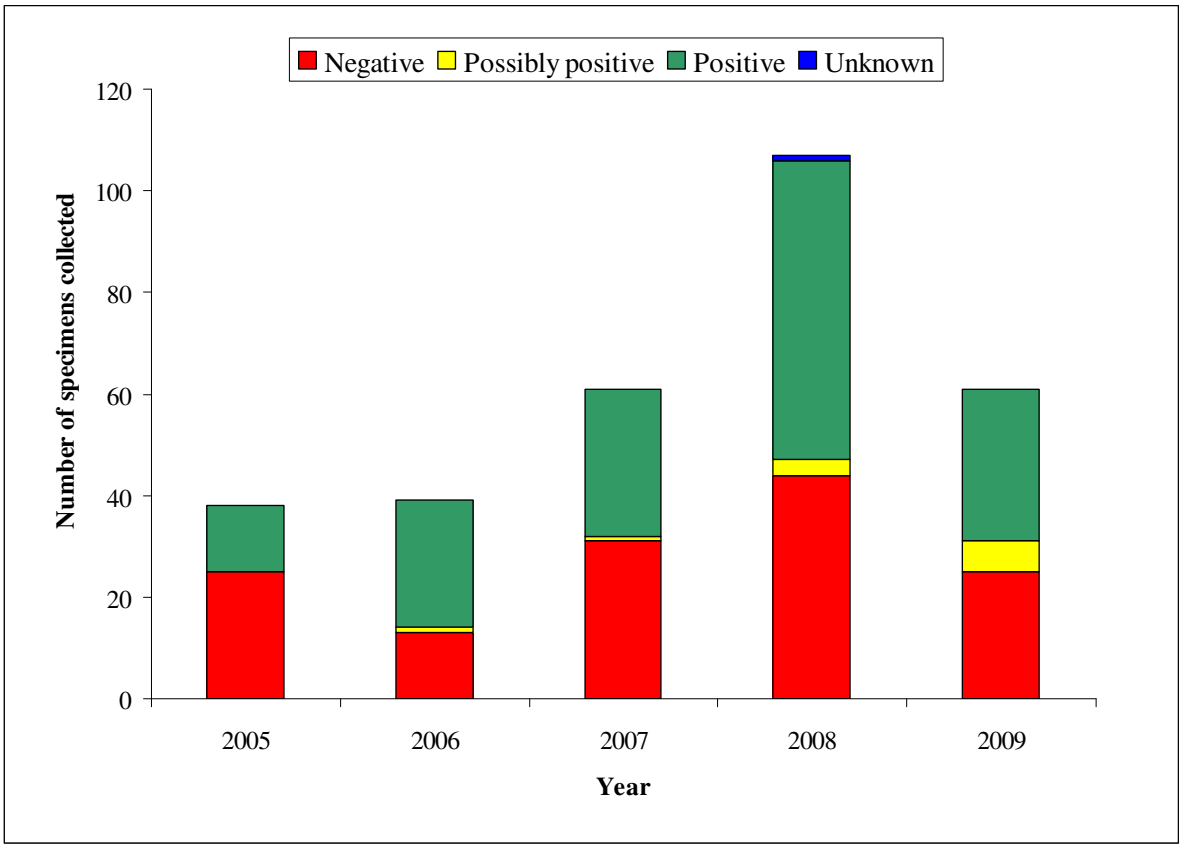


Figure 3.1 Number of specimens collected and immunofluorescence assay results for *P. jirovecii* by year, 2005-2009, Gauteng, South Africa*

*2005: 10 month period (Mar – Dec), 2009: 6 month period (Jan – Jun)

3.2.2 Validation of Roche MagNA Pure Compact

All results for the validation experiments are shown in Appendix F.

Cross-contamination in the RMPC (Experiment 1)

All four positive specimens yielded high copy numbers between 1 100 000 and 11 600 000. None of the four negative specimens produced a positive qPCR result.

Comparison of the automated RMPC versus the manual Qiagen method (Experiment 2)

From the 12 IFA-negative specimens, 12 were negative by the automated method and 11 by the manual method. Fourteen copies of DNA were present in one specimen extracted by the manual method. From the 12 IFA-positive specimens, all were positive by both methods. Overall, the manual method resulted in a significantly higher yield of DNA (p-value = 0.01, Wilcoxon matched-pairs signed-rank test).

Technical optimisation of the use of the RMPC reagents (Experiment 3)

All 12 IFA-positive specimens resulted in higher copy numbers of DNA, when the cartridges were shaken compared to when they were not (p-value = <0.05, Wilcoxon matched-pairs signed-rank test). Following this experiment, cartridges were shaken as a routine step for all subsequent automated extractions.

Repeat of the comparison of the automated RMPC versus the manual Qiagen method experiment (Experiment 4)

Of the 12 IFA-positive specimens, eight produced a higher copy number when extracted manually and four when extracted with the robot (cartridges shaken). However, there was no significant difference between the two methods (p-value = 0.20, Wilcoxon matched-pairs signed-rank test).

Robustness (Experiment 5)

Samples with the higher starting volume and higher loading volume yielded higher copy numbers of DNA.

Repeatability (Experiment 6)

The consistency of the copy numbers for each repeated specimen was similar for both methods (p-value = 0.09, paired *t*-test).

Varying instrument protocols (Experiment 7)

The new bacterial protocol resulted in a better yield of DNA compared to the total NA protocol. There was no significant difference between the two RMPC protocols (p-value = >0.05, Dunn's multiple comparisons test), and the RMPC bacterial protocol and the Qiagen method (p-value = >0.05), but there was between the RMPC total NA protocol and the Qiagen method (p-value = <0.05). Following this experiment, the bacterial protocol was used in all subsequent automated extractions.

A comparison between the two extraction methods is shown in Table 3.1.

Table 3.1 Comparison between the Qiagen and Roche MagNA Pure Compact extraction methods

Criteria	Qiagen	Roche MagNA Pure Compact
Length	1 - 2 hours excluding pre-digestion time of 2 hours	< 30 minutes excluding pre-digestion time of 2 hours
Number of specimens	Any number of specimens can be extracted at one time	Restricted to 8 specimens per run (6 excluding controls)
Labour	Labour intensive and greater chance of human error	Less labour intensive and more user-friendly
Cost	±R40 per sample	±R50 per sample
Maintenance of instrument	N/A	Minimal maintenance of RMPC required

3.2.3 *P. jirovecii* diagnosis by real-time PCR

Quantitative real-time PCR was conducted on DNA extracts from all 306 specimens. Sixty-seven percent (205/306) of the specimens were positive for *P. jirovecii* DNA (i.e. they had a DNA copy number greater than 0) and 33% (101/306) were negative for

P. jirovecii (Table 3.2). Using the IFA method as the gold standard, there were four false negatives qPCR results (three IFA positive and one IFA possibly positive). The extracted DNA from the three IFA-positive specimens was diluted and the qPCR repeated to eliminate the possibility of PCR inhibition due to excess amounts of DNA; the qPCR results remained the same. Two of the three specimens were positive for inhibition, when DNA was amplified with the RNAse P PCR assay. The IFA slide for the last specimen that was stored at 4°C was also reviewed and the green fluorescent objects did not correspond with the typical morphology of *P. jirovecii*.

Table 3.2 Comparison of the *P. jirovecii* immunofluorescence assay (IFA) and the quantitative real-time PCR (qPCR) assay results, 2005-2009, Gauteng, South Africa (N = 306)

		IFA Result		
		Positive	**Possibly positive**	**Negative**
qPCR result	Positive*	153	10	41
	Negative	3	1	97

*The sample without an IFA result (explained previously) was qPCR positive and is not included in the table.

To determine the statistical measures of performance of the qPCR assay, the IFA-possibly positive results were combined with the IFA-positive results. Using the IFA test as the gold standard, the sensitivity and specificity of the qPCR assay were calculated to be 98% and 70%, respectively. The positive and negative predictive values were 80% and 96%, respectively.

The median copy numbers of DNA correlated with the IFA results (Table 3.3). There were three outliers in the 41 IFA-negative, qPCR-positive results (25 799, 33 825 and 147 500 copies of DNA) that were excluded from the range given in Table 3.3.

Table 3.3 Median copy number of *P. jirovecii* DNA for each immunofluorescence assay (IFA) result category, for all quantitative real-time PCR (qPCR) positive specimens, Gauteng, South Africa, 2005-2009

IFA result category	Median copy number of DNA in qPCR positive specimens (range)
IFA negative	**45** (2 – 15 377)
IFA possibly positive	**493** (60 – 2 566)
IFA positive	**12 817** (7 – 704 257)
IFA +	195 (7 – 48 923)
IFA ++	1 779 (93 – 56 260)
IFA +++	23 643 (137 – 704 257)

To determine if there was a seasonal trend in PCP cases, the qPCR-positive specimens were plotted according to month of collection (Figure 3.2).

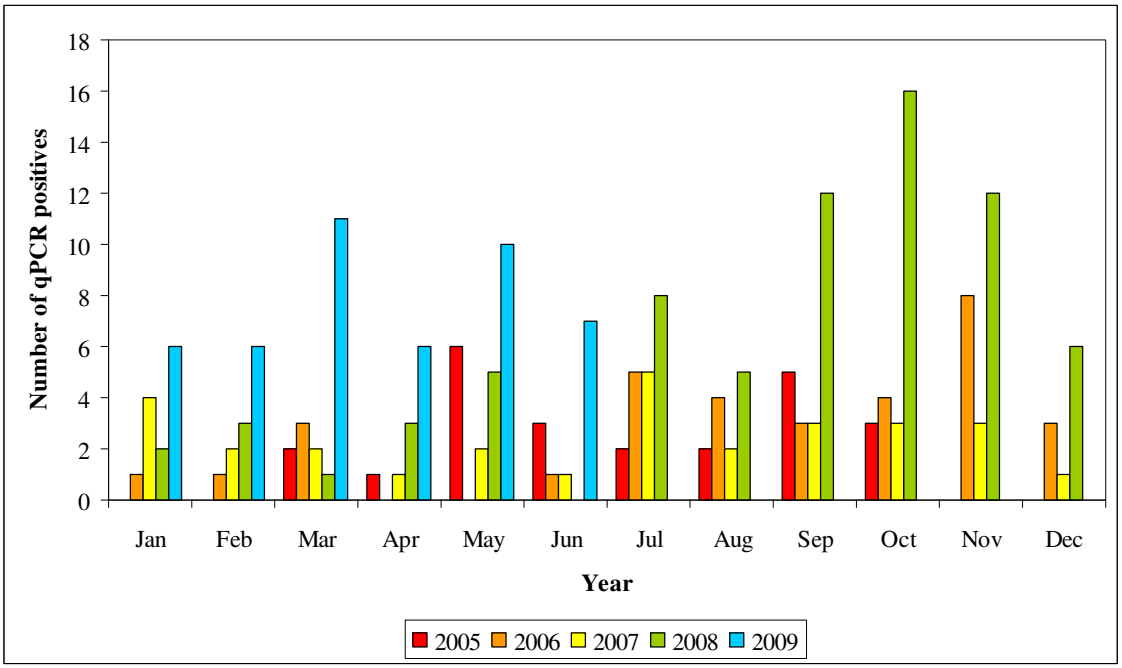


Figure 3.2 Number of specimens with positive *P. jirovecii* quantitative real-time PCR (qPCR) results by month and year of collection, 2005-2009, Gauteng, South Africa*

*2005: 10 month period (Mar – Dec), 2009: 6 month period (Jan – Jun)

3.3 Primary sequencing

3.3.1 Nested PCR and gel electrophoresis

The DHPS gene of *P. jirovecii* was amplified by nPCR for all specimens with a qPCR-positive result, and the products were resolved by agarose gel electrophoresis. The product size was 278 base pairs (Figure 3.3). The results showed that 84% (173/205) of the qPCR positives were positive, and 16% (33/205) negative, by the nPCR assay (Table 3.4).

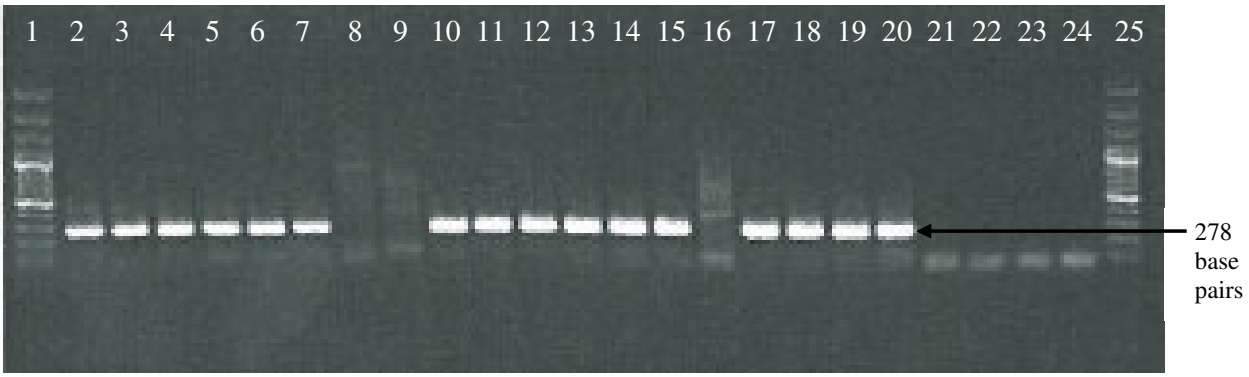


Figure 3.3 Agarose gel showing PCR-amplified 278 base pair product of the *P. jirovecii* DHPS gene*

*Lanes 1 and 25: 100 bp molecular weight marker, lanes 2-7, 10-15, 17-20: positive results, lanes 8-9, 16: negative results, and lanes 21-24: primary and secondary PCR negative controls.

Table 3.4 Comparison of the *P. jirovecii* immunofluorescence assay (IFA) and the nested PCR (nPCR) assay results, 2005-2009, Gauteng, South Africa (N = 206)

		IFA Result		
		Positive	**Possibly positive**	**Negative**
nPCR result	Positive*	145	8	19
	Negative	8	2	23

*The sample without an IFA result (explained previously) was nPCR positive and is not included in the table.

To determine the statistical measures of performance of the nPCR assay, the IFA-possibly positive results were combined with the IFA-positive results. Using the IFA test as the gold standard, the sensitivity and specificity of the nPCR assay were calculated to be 94% and 55% respectively. The positive and negative predictive values were 89% and 70% respectively. The nPCR tended to be positive for specimens with high copy numbers (14 - 704 257) and negative for specimens with low copy numbers (2 – 369) (Table 3.5). As previously discussed, the three outliers in the 19 IFA-negative, nPCR-positive results were excluded from the range given in Table 3.5.

Table 3.5 Median copy number of *P. jirovecii* DNA in the positive and negative nested PCR (nPCR) specimens, 2005-2009, Gauteng, South Africa

IFA result category	Median copy number of DNA in qPCR positive specimens (range)	
	nPCR positive	**nPCR negative**
IFA positive	**14 498** (57 - 704 257)	**98** (7 - 369)
IFA possibly positive	**729** (134 - 2 566)	**131** (60 - 202)
IFA negative	**823** (14 – 15 377)	**12** (2 - 64)

3.3.2 Sequencing of PCR products

Figures 3.4, 3.5 and 3.6 illustrate examples of electropherograms of a basic genotype, resolvable mixed genotype and irresolvable mixed genotype, respectively. The maroon rectangles in the electropherograms identify nucleotide positions 165 and 171, respectively. Primary sequencing results of the nPCR products showed the presence of seven of the nine possible DHPS genotypes, with the wild type genotype being most prevalent (Table 3.6).

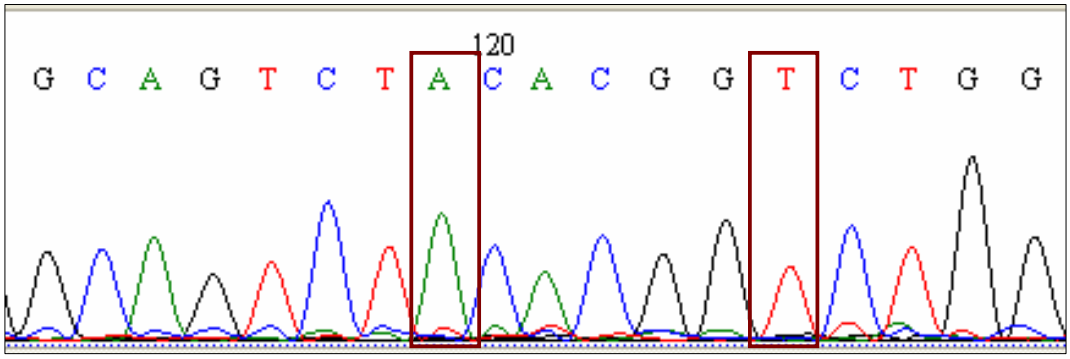


Figure 3.4 Electropherogram showing a *P. jirovecii* basic DHPS genotype (M2) result

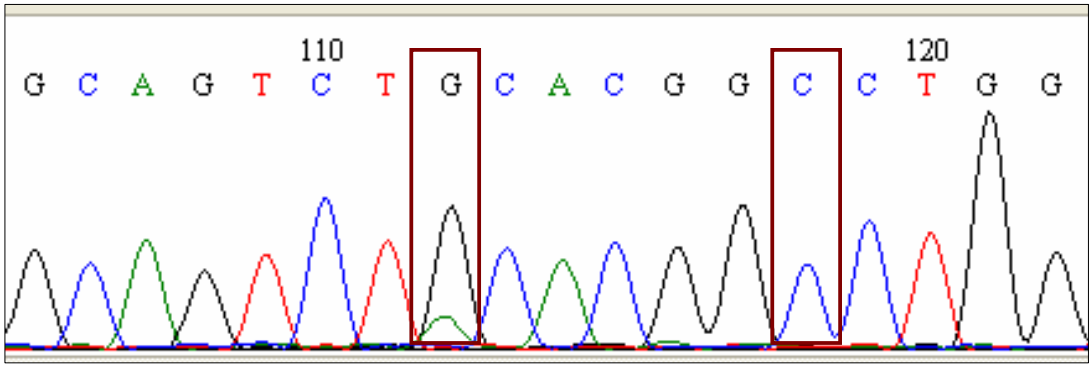


Figure 3.5 Electropherogram showing a *P. jirovecii* resolvable mixed DHPS genotype (WT + M1) result

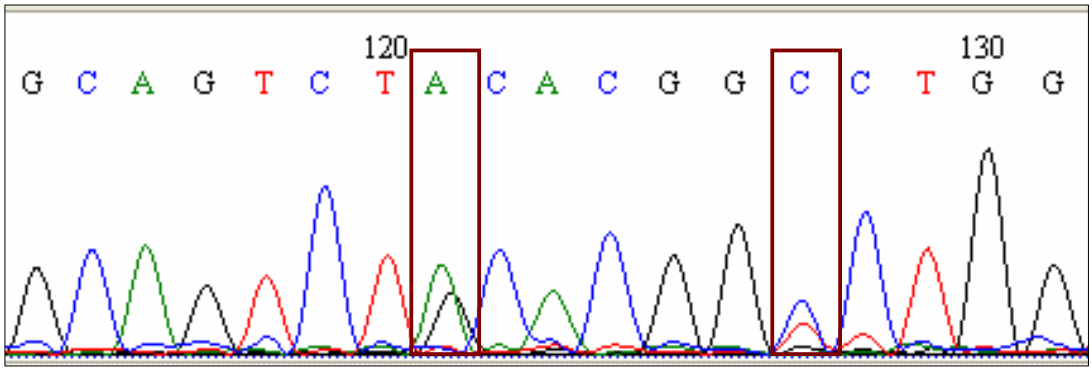


Figure 3.6 Electropherogram showing a *P. jirovecii* irresolvable mixed DHPS genotype result

Table 3.6 Results of primary sequencing of the *P. jirovecii* DHPS gene, 2005-2009, Gauteng, South Africa (N = 173)

Genotype		Number (%)
WT (Wild type)		**61 (35%)**
Single and double mutations	M1 (Mutation 1)	0 (0%)
	M2 (Mutation 2)	11 (6%)
	M3 (Mutation 3)	12 (7%)
Resolvable mix	WT + M1	11 (6%)
	WT + M2	35 (20%)
	M1 + M3	0 (0%)
	M2 + M3	2 (1%)
Irresolvable mix		41 (24%)

A large proportion (24%) of the primary sequencing results showed irresolvable mixed genotypes (Figure 3.7), which could not be resolved by the initial sequencing. The *P. jirovecii* DHPS gene from these 41 specimens were cloned and resequenced.

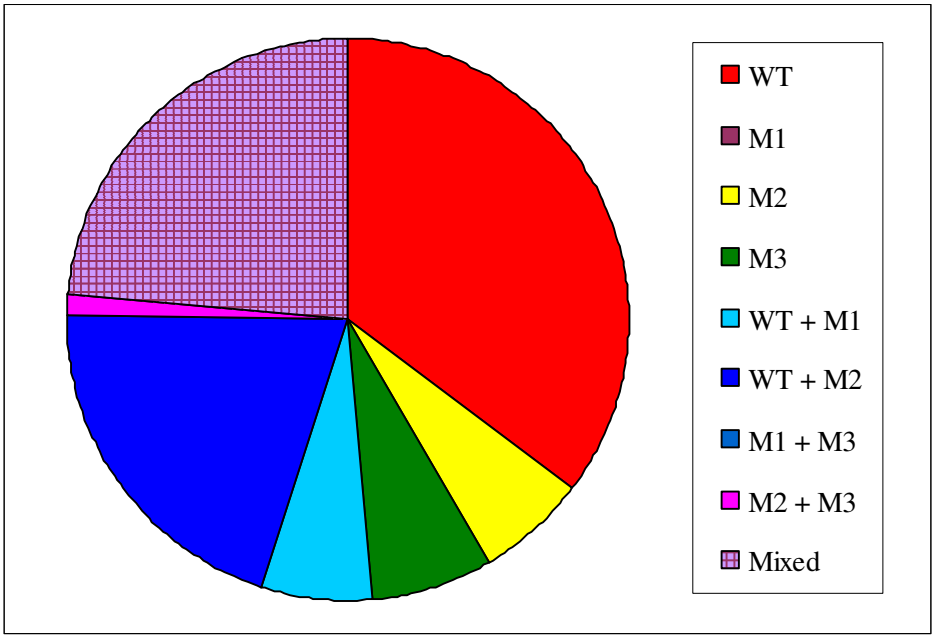


Figure 3.7 Primary sequencing results of the *P. jirovecii* DHPS gene, 2005-2009, Gauteng, South Africa*

*Key: WT = wild type, M1 = mutation 1 (mutation at position 165), M2 = mutation 2 (mutation at position 171), M3 = mutation 3 (mutations at positions 165 and 171), Mixed = irresolvable mixed genotypes

3.4 Resolution of mixed DHPS genotypes

Thirteen different genotypes were identified with the secondary sequencing, with the wild type + M3 genotype being most prevalent (Table 3.7). Figure 3.8 highlights the predominant genotypes from both the primary and secondary sequencing. Fifteen percent (6/41) and 24% (10/41) of the secondary sequencing were basic genotypes and resolvable mixed genotypes respectively.

Table 3.7 Secondary sequencing results of the DHPS gene in *P. jirovecii* isolated from patients with PCP, 2005-2009, Gauteng, South Africa (N = 41)

Genotype		Number (%)
WT (Wild type)		2 (5%)
Single and double mutations	M1 (Mutation 1)	0 (0%)
	M2 (Mutation 2)	1 (2%)
	M3 (Mutation 3)	3 (7%)
Resolvable mixed genotypes	WT + M1	5 (12%)
	WT + M2	1 (2%)
	M1 + M3	1 (2%)
	M2 + M3	3 (7%)
Mixed genotypes	WT + M3	**11 (27%)**
	WT + M1 + M2	3 (7%)
	WT + M1 + M3	1 (2%)
	WT + M2 + M3	7 (17%)
	M1 + M2 + M3	2 (5%)
	WT + M2 + M1 + M3	1 (2%)

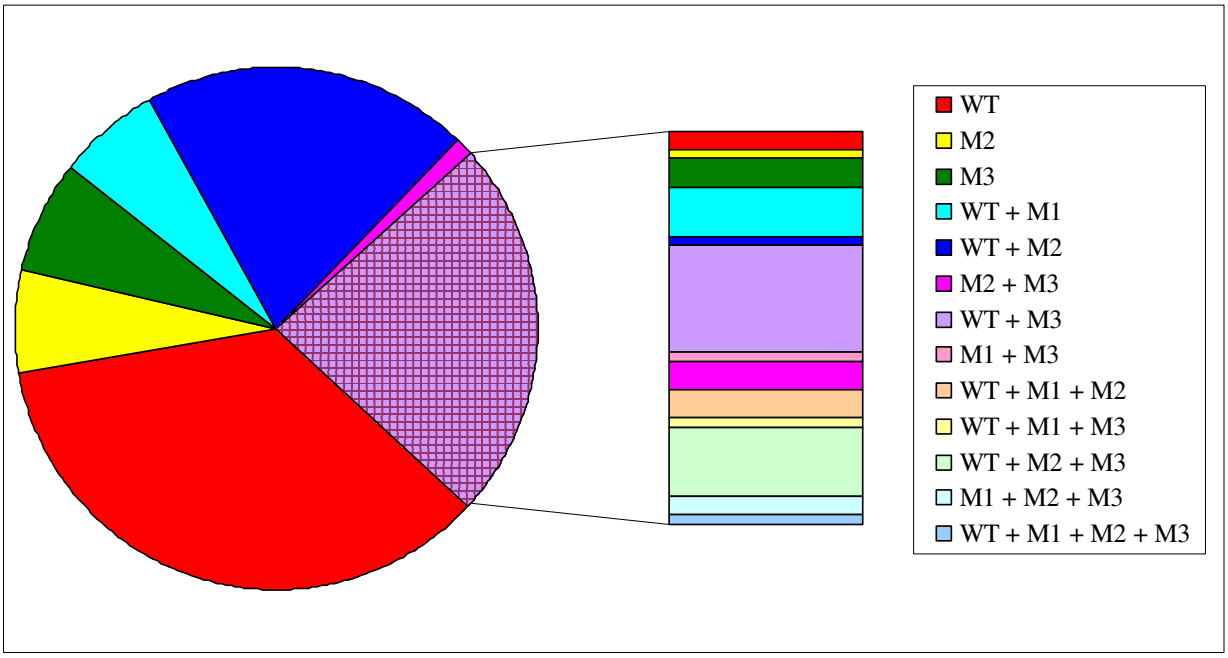


Figure 3.8 Secondary sequencing results (represented by the bar) of all irresolvable mixed genotypes (hatched purple) identified by primary sequencing (represented by the pie) of the *P. jirovecii* DHPS gene, 2005-2009, Gauteng, South Africa*

*Key: WT = wild type, M1 = mutation 1 (mutation at position 165), M2 = mutation 2 (mutation at position 171), M3 = mutation 3 (mutations at positions 165 and 171)

When the results of primary and secondary sequencing were combined, the two predominant genotypes were wild type (36%), and the wild type + M2 genotype (21%) (Table 3.8 and Figure 3.9).

Table 3.8 Combined final sequencing results of the *P. jirovecii* DHPS gene, 2005-2009, Gauteng, South Africa (N = 173)

Genotype		Number (%)
WT (Wild type)		**63 (36%)**
Single and double mutations	M1 (Mutation 1)	0 (0%)
	M2 (Mutation 2)	12 (7%)
	M3 (Mutation 3)	15 (9%)
Mixed genotypes	WT + M1	16 (9%)
	WT + M2	**36 (21%)**
	M1 + M3	1 (1%)
	M2 + M3	5 (3%)
	WT + M3	11 (6%)
	WT + M1 + M2	3 (2%)
	WT + M1 + M3	1 (1%)
	WT + M2 + M3	7 (4%)
	M1 + M2 + M3	2 (1%)
	WT + M1 + M2 + M3	1 (1%)

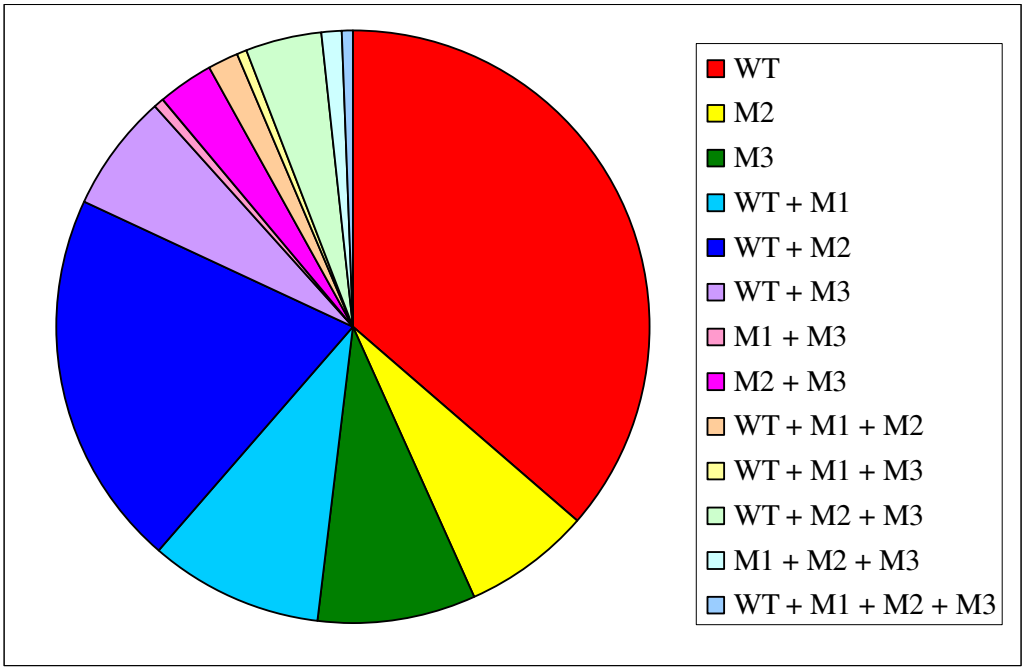


Figure 3.9 Final sequencing results of the *P. jirovecii* DHPS gene, 2005-2009, Gauteng, South Africa*

*Key: WT = wild type, M1 = mutation 1 (mutation at position 165), M2 = mutation 2 (mutation at position 171), M3 = mutation 3 (mutations at positions 165 and 171)

Forty patients had a repeat specimen taken; the results for these patients are shown in Appendix H. The majority (53%, 21/40) of patients had negative results for both specimens, 25% (10/40) of patients had positive qPCR results for both specimens, 15% (6/40) patients had an initial negative result and a subsequent positive result and 8% (3/40) had an initial positive result and a subsequent negative result. Of the ten patients who had positive results for both specimens, the *P. jirovecii* DHPS genotypes were different in five patients.

3.5 DHPS genotypes and in-hospital patient outcome

There was no significant difference between age and DHPS genotype (p-value = 0.09; Student's *t*-test). There was no association between in-hospital outcome and DHPS genotype (p-value = 0.19; Fisher's exact test), but there was between gender and DHPS genotype (p-value = 0.02; Fisher's exact test) (Table 3.9).

Table 3.9 Comparison of age, gender and in-hospital patient outcome with *P. jirovecii* DHPS genotypes, 2005-2009, Gauteng, South Africa

	Wild type	Mutations	p-value
Age, median	33	36	0.09
Gender			0.02
Male	23	20	
Female	43	87	
In-hospital patient outcome			0.19
Died	19	44	
Discharged	44	65	

When each mutant DHPS genotype was compared to the wild type genotype, only the M2 genotype was associated (p-value = 0.02, Fisher's exact test) with patient in-hospital mortality (Table 3.10).

Table 3.10 Comparison of each *P. jirovecii* DHPS genotype and in-hospital patient outcome, 2005-2009, Gauteng, South Africa (N = 162)*

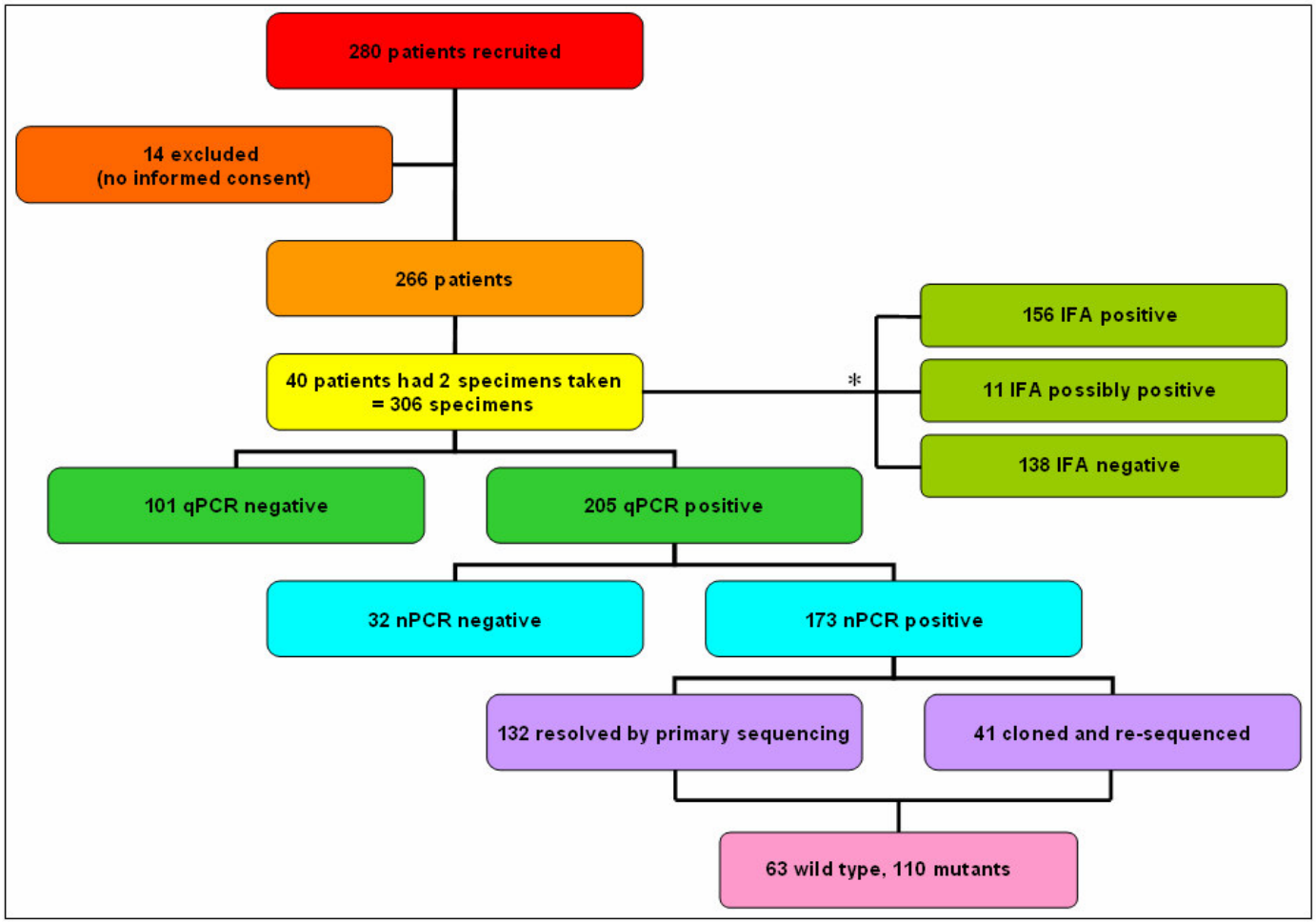
	Died	Discharged	p-value
Wild type (WT)	18	40	n/a
Mutations	42	62	0.31
M2 (Mutation 2)	8	3	**0.02**
M3 (Mutation 3)	8	7	0.14
WT + M1 (M1 = Mutation 1)	8	7	0.14
WT + M2	13	23	0.66
WT + M3	2	8	0.71
M1 + M3	0	1	1.00**
M2 + M3	1	3	1.00**
WT + M1 + M2	1	1	0.54**
WT + M1 + M3	0	1	1.00**
WT + M2 + M3	1	5	0.66**
M1 + M2 + M3	0	2	1.00**
WT + M1 + M2 + M3	0	1	1.00**

*The first result of the 10 patients who had repeat specimens taken, were excluded from analysis

**Statistical test influenced by small numbers

A summary of all results of this project is illustrated in Figure 3.10.

Figure 3.10 Summary of results from *P. jirovecii* DHPS mutation study, 2005-2009, Gauteng, South Africa



*The IFA test could not be completed for one specimen due to a laboratory error that occurred during processing

CHAPTER 4 - DISCUSSION

Pneumocystis pneumonia is often diagnosed clinically and radiologically in SA, which explains why there are limited data sources from which prevalence can be determined. However, we do know that 10.5% of the South African population is HIV positive [135], and since PCP is known to be one of the most common opportunistic infections in HIV/AIDS patients, we can presume that a substantial number of PCP cases will emerge from these 5.24 million people. This study, carried out at CHB Hospital in Gauteng Province, SA, found a 67% prevalence of PCP (including possible cases of colonisation) in a group of patients clinically suspected of having PCP.

The average age in the study population was 35 years which falls within the main age group affected by HIV/AIDS, namely the 15-49 year age group [135]. The majority (73%) of patients were female; however this is in accordance with the national higher prevalence of HIV in females, and the fact that there are generally more females registered at CHB Hospital [personal communication with Dr Michelle Wong, CHB Hospital].

There was no seasonal trend observed; however, patient recruitment throughout the collection period was inconsistent due to variable availability of the clinician and study nurse. In 2008 and 2009 there was a marked increase in patient enrolment, most likely attributable to the appointment of a new, dedicated study nurse.

4.1 Validation of the RMPC

When comparing the RMPC to the Qiagen method (Experiment 3), one IFA-negative sample was found to have 14 copies of DNA following extraction by the Qiagen method.

As the IFA method is not as sensitive as PCR, this result was more likely to be a true positive rather than a contaminated specimen. A noteworthy finding made through this validation was that shaking the cartridges, in order to settle the magnetic beads to the bottom of the well, increased the DNA yield substantially. The increased starting and loading volumes and the choice of protocol also affected the DNA yield, highlighting the importance of consistency in methodology, as minor technical changes do affect results. Following the validation of the RMPC, the technical findings were incorporated into the development of a standardised method.

The RMPC was comparable to the Qiagen method in terms of repeatability and showed no possibility of cross-contamination between specimens, as was found by another study [136]. Although PCR is an indirect measure of DNA concentration, the qPCR results showed that the RMPC does not extract as much DNA from a clinical specimen as the Qiagen method, especially at high DNA concentrations. This is most likely due to the RMPC method using magnetic bead technology to extract the DNA; the beads are limited and can therefore be overloaded at high DNA concentrations [74]. In addition, this method uses cartridges containing wells in which the DNA is passed from one to the next, and loss of DNA may occur during this transfer. However, when the bacterial protocol was used, there was no significant difference in DNA yield between the RMPC and the Qiagen method. For this reason, along with the other strengths of the method, namely ease of use and speed, the RMPC method was selected for use. Two similar studies which compared automated extraction methods against manual methods both found the Roche MagNA Pure (a higher throughput version of the RMPC which uses the same magnetic bead technology) comparable to the manual extraction method [136;137].

4.2 Diagnostic methods

Despite the advances in molecular biology, at present resource limitations in many laboratories rule out PCR as an option. As a result staining methods, particularly immunofluorescent antigen staining, are still commonly used for the diagnosis of *P. jirovecii* [60]. In this study the IFA method identified 51% of specimens as positive. Of the three IFA-positive specimens that were found to be negative by qPCR, two showed PCR inhibition. On careful examination of the slide from the remaining specimen, it was evident that while there was a clear presence of fluorescence, the morphology of the fluorescent material was not consistent with that of *P. jirovecii* and therefore this was most likely a false positive IFA result. Non-specific staining is a limitation of IFA kits for *P. jirovecii* [69], especially if coupled with inexperienced microscopists. The IFA stain was shown to have the highest sensitivity but the lowest specificity when compared to three other staining methods [60]. However, Khan *et al.* [71] concluded that immunofluorescence should not be used solely for the diagnosis of PCP, and that PCR should be used when the IFA result is negative and the patient is suspected of having PCP.

In this study, the qPCR assay identified 67% of specimens as positive for *P. jirovecii*. It had a good sensitivity (98%) and negative predictive value (96%) when compared to the IFA test as the gold standard. The lower specificity (70%) and positive predictive value (80%) were influenced by the large number of apparent false positive results. However, these are most likely to be true cases, as PCR is known to be more sensitive than IFA. As these specimens had an overall lower copy number of DNA, compared to the true positive results, they may be from patients who are colonised rather than infected with *P. jirovecii* [71]. If this is true, then these patients probably have another respiratory disease as they were recruited because of their clinical presentation. A study that compared a PCR assay to

an IFA method for *P. jirovecii* diagnosis, found the PCR assay had a low sensitivity (55%) and specificity (41.2%) [69]. However, when the discrepant results were analysed using clinical data and further laboratory testing the sensitivity and specificity of the PCR increased substantially. Following this study, the qPCR assay for *P. jirovecii* is being added to the list of routine tests offered by the PRU, NICD.

A major problem when evaluating a test for PCP diagnosis is the lack of a true gold standard. This is due to the non-existence of a culture system for *P. jirovecii*. The IFA test was chosen as the gold standard in this project because it is the current routine diagnostic test for PCP, used by the laboratory. In the absence of a good gold standard, it has been suggested that other analytic approaches, such as composite reference standards, be used [138]. Such approaches draw on the strengths of a number of different methods to compensate for the imperfectness of the individual methods themselves. In addition, the use of clinical and radiological criteria may serve as a good gold standard for *P. jirovecii* diagnosis, as was used by Fujisawa *et al*. [139]. Consequently, it must be noted that the use of an imperfect gold standard in this project, negatively affected the statistical measures, especially the specificity, of the qPCR assay.

A very important question remains unanswered with respect to using qPCR for PCP diagnosis: what is the appropriate cut-off value for colonisation versus infection? In order to determine accurate cut-offs, many criteria need to be analysed along with the Ct value/DNA copy number, such as patients' clinical presentation and chest x-rays. For this project, only the results of two diagnostic tests (i.e. IFA and qPCR) were available for analysis. The results showed a good correlation between the median DNA copy number and IFA result i.e. low copy number for IFA-negative specimens and increasingly higher

copy numbers for IFA-possibly positive and IFA-positive specimens (+, ++ and +++). The median copy number was used instead of the mean to reduce the effect of outliers. However, there was a large overlap in the range of copy numbers in each category and this makes it difficult to determine clear cut-offs. Based on these results, proposed arbitrary cut-offs are less than or equal to 100 copies of DNA for colonisation, between 100 - 1 000 copies indeterminate and greater than or equal to 1 000 copies for infection. As previously discussed, the IFA test is not a reliable gold standard because it does not always distinguish true infection from colonisation; therefore these cut-offs are highly subjective.

While the majority of studies comment on the need for cut-off values, only a few provide possible values. Arbitrary cut-offs of 10, 30 and 50 copies/tube have been applied in studies [63;139-141]. Most of these reports also state the important fact that higher cut-offs increase specificity but decrease sensitivity of the real-time assay. It is strongly advised that the test result be combined with the clinical presentation of the patient before a diagnosis of PCP is made [69]. Linssen *et al*. suggested that patients be divided into three groups on the basis of a positive or negative laboratory test result and the presence or absence of compatible clinical symptoms [142]. Patients with positive test results and symptoms will have PCP, patients with negative test results and no symptoms will not have PCP and patients with positive test results and no symptoms will be carriers. If there are patients with PCP symptoms and negative test results, the test should be repeated and the patient monitored.

Although the nPCR was not used as a diagnostic test in this study but rather to facilitate sequencing of the DHPS gene, statistical comparisons with the IFA and qPCR tests were performed. The nPCR had a high sensitivity (94%), but a low specificity (55%) compared

to the IFA, but as discussed previously, the IFA is not a good gold standard. The qPCR positive specimens that were nPCR negative had mostly low copy numbers, which has been found in a previous study [143]. The authors of this study found that when qPCR and nPCR were compared to microscopy, the qPCR and nPCR had the same sensitivity but the qPCR had a statistically better specificity compared to the nPCR. This was due to the high number of false positives with the nPCR. Such comparisons cannot be done in this study because not all specimens were processed by nPCR, only the qPCR positives. Excluding the costs of the thermocycler qPCR is cheaper than nPCR, four times faster, quantifies positive results and is less susceptible to contamination.

4.3 DHPS genotypes

The majority of specimens (64%) harboured a mutant DHPS genotype. This is unusual compared to most other studies carried out across the world, in which the wild type DHPS genotype was most predominant in nearly all samples tested [101;108;116;124;128]. Four studies that found higher rates of mutations (65% to 81%) were all carried out in the United States [37;112;114;119]. It is strongly suggested that DHPS mutations arise as a result of sulfa drug pressure, and as these drugs are not widely used in most developing countries the prevalence of DHPS mutations is low [127]. As the use of sulfa drugs increases, so does the prevalence of DHPS mutations in many organisms, including *P. jirovecii* [110]. In SA, sulfa drugs (particularly cotrimoxazole) have become widely used in recent times because of HIV-related indications, as well as general use for a wide variety of diseases, including cholera, some bacterial infections and urinary tract infections {Anonymous, 2010 148 /id}. This widespread use of sulfa drugs is the most likely reason for the increase of *P. jirovecii* DHPS mutant genotypes in our population.

Further to the high prevalence of mutant DHPS genotypes, we found a large proportion (24%, 41/173) of irresolvable mixed genotypes. This was also the case in the previous study done by our group in SA, in which 36% (31/85) of mutant genotypes were of the irresolvable category [129]. This highlights the need to resolve these genotypes in order to accurately identify the predominant DHPS genotypes circulating in our population. In contrast, the majority of other studies found mostly wild type and basic genotypes; a few mixed genotypes were found, and these were mostly resolvable mixed genotypes [109;116;117].

RFLP analysis and primary sequencing are commonly used to determine DHPS genotypes. The limitation of these methods is that any irresolvable mixed genotypes cannot be discerned from the results. To clone and resequence allows for unambiguous results to be obtained for all specimens. The number of colonies screened and sequenced is very important and, if too few, may bias the results. Other groups that used the cloning method to determine DHPS genotypes screened either five colonies [14;126]; three to eight colonies [116] or ten colonies [120] for each specimen. We chose to screen ten colonies for each specimen to increase sensitivity. However, even using ten colonies, some of our secondary sequencing results showed basic and resolvable mixed genotypes only. As these specimens clearly contained *P. jirovecii* with mixed genotypes from the primary sequencing results, this could mean that the number of colonies screened was too few to identify the genotypes present in low volumes. This could be seen as a limitation in this project.

The most common DHPS mutant genotypes found in this sample of patients were the wild type + M2 (21%), wild type + M1 (9%), M3 (9%) and M2 (7%) genotypes. This was

consistent with the previous study we did in SA, in 2006 to 2007, where the most common genotypes were M3 (18%), wild type + M1 (18%) and wild type + M2 (12%) genotypes [129]. Interestingly, in this project there were no M1 genotypes, which we found previously. Overall, when comparing the results of *P. jirovecii* DHPS studies carried out in different countries, there does seem to be more basic genotypes present than mixed genotypes. In the USA in particular there appears to be a predominantly large number of M3 genotypes. Distribution trends of the *P. jirovecii* DHPS genotypes have not been well documented.

Only mutations at nucleotide positions 165 and 171 in codons 55 and 57 respectively, were analysed in this project, as these mutations lead to specific amino acids changes that have been linked to sulfa drug resistance [37]. In the *E. coli* DHPS enzyme, threonine at codon 62 is homologous to threonine at codon 55 in *P. jirovecii*. When a mutation results in an amino acid change from threonine to alanine in this position, one of the two hydrogen bonds between the DHPS enzyme and the substrate pteridine is lost [106]. The adjacent amino acid in both *P. jirovecii* and *E. coli* is arginine at codons 56 and 63, respectively. This amino acid is also involved in the binding of pteridine; therefore amino acid changes on either side of it may affect its positioning and thus affect binding to the substrate. Another group in SA looked for any mutations in codons 55 and 57 of the *P. jirovecii* DHPS gene [124]. They found 13% (4/30) of specimens with mutations, one wild type + M1 genotype and three with mutations at position/s 166 and/or 172.

Repeat specimens were collected from 40 patients; mainly due to the clinician strongly suspecting PCP despite the initial negative laboratory result. Two patients were readmitted but the other 38 had two specimens collected on their initial admission. The interval

between sample collections from the latter group of patients ranged from the same day to 29 days. Repeat specimens collected from the same patients showed that some patients had one positive and one negative result in their respective specimens; this may be due to variable specimen quality. It was also found that some patients had different DHPS genotypes in the two specimens. As stated by Miller *et al*. [35], if PCP is due to a reactivation of a latent infection, the genotypes should remain the same. However, it is possible that the organism is mutating in the host under the influence of the sulfa drugs [14]. Alternatively, the patients may have been re-infected with a new strain, but this seems unlikely as most repeat specimens were taken within one to two days.

4.4 DHPS genotypes and in-hospital patient outcome

The results from this project show that there appears to be no significant association between DHPS genotype and adverse patient outcome. Published data provide conflicting evidence on this topic. The data for patient outcome were based on the in-hospital data; further follow-up could alter the data as patients may have died after discharge. On the other hand, the in-hospital deaths could have been due to other causes, and not necessarily the PCP infection. If it is concluded that the outcome and mutations are not linked, what would be the value of investigating the mutations? For the mutations to have significance they must be associated with clinical impact.

When individual DHPS genotypes were compared with the wild type genotype, the M2 genotype was associated with a worse patient outcome. However, as the numbers in many of the DHPS genotype groups were small, these results may not be indicative of a true association. If such a result were confirmed with a larger set of data in future, the need to determine the DHPS genotype would be justified.

The problem of PCP drug alternatives may arise, if it is found conclusively that DHPS mutations are associated with sulfa drug resistance. Dapsone is the second choice prophylactic drug for PCP but it offers little and no protection against toxoplasmosis and bacterial infections, respectively, unlike cotrimoxazole which is active against both {Anonymous, 2010 148 /id}. It has not been irrefutably determined that patients with *P. jirovecii* with DHPS mutations fail treatment or have a worse outcome. Some studies have shown that treatment with sulfa drugs was unsuccessful in these patients [110], while others have shown that patient outcome is not affected by the presence of *P. jirovecii* DHPS mutations [36].

4.5 Future studies

It is important to continue monitoring the *P. jirovecii* DHPS mutations in those infected with PCP in SA, especially as sulfa drug usage increases. In future, it would be beneficial to investigate the DHPS mutations in HIV-negative patients with PCP as well, especially because of the higher mortality and worse presentation of the disease in these patients [54].

Further to this project, the larger study aims to investigate the associations between the *P. jirovecii* DHPS mutations and PCP prophylaxis, treatment failure, and 3-month patient outcome. It will also explore risk factors for PCP, the effect of exposure to HAART and PCP prophylaxis, mixed infections and PCP prophylactic drug usage, dosage and adherence thereof. Correlation between clinical and radiological findings and laboratory diagnosis will be analysed. These data will be paramount to establishing information regarding PCP infection in HIV-positive patients in SA.

CHAPTER 5: CONCLUSIONS

With respect to DNA extraction methodology, from the experiments carried out for the RMPC validation, it can be concluded that DNA extraction with the RMPC instrument is useful in a high-throughput diagnostic laboratory as the process is automated and saves a great amount of time compared to the Qiagen manual extraction method. Quantitative PCR is a good diagnostic tool for *P. jirovecii* especially if an accurate cut-off value to differentiate between colonisation and infection is determined. An arbitrary cut-off of 100 – 1 000 copies of DNA has been proposed but this may be inaccurate.

Pneumocystis jirovecii pneumonia is one of the most common opportunistic infections in HIV/AIDS patients. In SA the burden of this disease remains largely unknown, mainly because the disease is frequently diagnosed and treated empirically. In this study population, we found a 67% positivity rate in specimens from patients suspected of PCP. The need to continue efforts to estimate the true burden of PCP in our country must be emphasised.

The majority of specimens collected in this study contained *P. jirovecii* mutant DHPS genotypes. This provides further evidence that these mutations do exist in developing countries and are most likely increasing because of the widespread use of sulfa drugs. There are conflicting data regarding the importance of these mutations, and the results of this project support the argument that DHPS mutations do not appear to have an effect on patient outcome. However, this does not diminish the need to continually examine the impact, if any, of these mutations. The possibility that they do cause clinical resistance to

sulfa drugs will have an immense impact on health care and therefore warrants further investigation.

Appendix A – Informed consent form

Patient information sheet for an adult patient or guardian of a minor:

Hello. My name is___*(name of interviewer)*___and I would like to invite you to participate in a study. Your participation in this study is completely voluntary. If you decide not to participate it will not affect your treatment in any way. If you decide to participate, it will not affect your treatment in any way and you may also change your mind at any time. As we discuss the information please feel free to ask me any questions.

We would like to take an induced sputum sample from you to test if you have *Pneumocystis* pneumonia. This is a serious disease and it is important for us to know if the medicines we are using to treat it are working well. You will need to breathe in vapour using a mask and this will make you cough, so that the sputum will come out of your lungs.

I would like to ask you a few questions about your medical history, what medicines you have taken and how you are feeling, if you agree. If we find something interesting we will need to look at your hospital records and laboratory tests. We will keep all of your information confidential by using a coded study number. Only the people involved in this study will be able to link your name to the information.

After 3-4 months we would like to contact you by telephone to ask you questions about your health. Please could you give us your contact telephone number and the name and contact number of your next of kin.

I would like to take a 2 ml (½ teaspoon) blood sample from your vein, if you agree. Your blood will be tested for an enzyme called SAM that is linked to pneumonia. I will need to take a 2 ml (½ teaspoon) blood sample from you every 2 days for 2 weeks, if you agree.

(Only to be asked if an HIV test has not previously been performed). I would like to offer you an HIV test. If you agree, I will take a 5 ml (1 teaspoon) blood sample from your vein for testing. You will be given pre and post test counselling.

Thank you for your time. Once you have asked me any questions you may have, there is a form you need to sign if you agree to take part in the study.

If you would like any more information on this study you can contact Dr Michelle Wong at this telephone number: (011) 933-8933, or Bhavani Poonsamy at this telephone number: (011) 555-0313/04.

PCP Study 2008 1

Informed consent form (copy for patient):
I understand the contents of the information sheet and understand that I have been invited to participate in this study. I understand that my agreeing to participate is fully voluntary and I can withdraw at any time.

Study no:_____

Consent given for induced sputum, interview questions, record review, 4 month follow up, SAM testing and HIV test:

Patient's/Guardian's name & signature:	Date:
Witness's name & signature:	Date:

Consent given for induced sputum, interview questions and record review, 4 month follow up, SAM testing, but not HIV testing:

Patient's/Guardian's name & signature:	Date:
Witness's name & signature:	Date:

Patient's contact telephone number:_____

Next of kin name and relationship:_____

Next of kin contact telephone number:_____

Interviewer's name & signature:_____

Appendix B – Clinician case report form

PCP Study 2009 **Clinician's** Clinical Case Report Form

STUDY NO: _____

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

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

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

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

Medication in last 3 mo:_____

_____

_____

Smoker: no ☐ yes ☐

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

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

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

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

1

PCP Study 2009 **Clinician's** Clinical Case Report Form

STUDY NO: _____

EXAMINATION:

Temp: _____

BP: _____

Pulse: _____

RR: _____

Oral thrush : ☐ yes ☐ no

Chest auscultation:

_____

_____

_____

Other significant clinical findings :

_____

_____

_____

_____

_____

_____

_____

_____

_____

2

PCP Study 2009 **Clinician's** Clinical Case Report Form

STUDY NO: _____

INVESTIGATIONS

Blood investigations:

	Date	Result
FBC		
U&E		
CD4		

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

CXR: Date: _____ No. _____

_____

_____

Sputum:

	Date	Result
MC&S		
TB auramine		
TB culture		

3

PCP Study 2009 **Clinician's** Clinical Case Report Form

STUDY NO: _____

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

Specimen	Date	Result

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

TREATMENT

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

Co-trimoxazole:

Route	Dose	Start date	End date

Steroids:

Route	Dose	Start date	End date

Other drugs:

Route	Dose	Start date	End date

4

PCP Study 2009 **Clinician's** Clinical Case Report Form

DAILY PROGRESS

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

COMPLICATIONS

Respiratory failure: no ☐ yes ☐

Pneumothorax: no ☐ yes ☐ Spontaneous ☐ Iatrogenic ☐
Right ☐ Left ☐ Date: _____
Rx: O_2 ☐ Heimlich valve ☐ IC drain ☐

Sulpha allergy: no ☐ yes ☐

Other: _____

_____

_____

_____

OUTCOME

Discharged ☐ Date: _____ Follow-up appointment: _____

Died ☐ Date: _____

Excluded ☐ Date: _____

5

Appendix C – Reagent recipes

To prepare DTT solutions

- 65 mM stock solution (50 ml) — Add 0.5 g of DTT to 50 ml of distilled water
- 6.5 mM DTT working solution — Add 1 part stock solution to 9 parts of phosphate buffered saline pH 7.2 (DMP)

To prepare qPCR standards

- Prepare fish sperm DNA working solution by adding 5 µl fish sperm DNA (Roche Diagnostics) to 4495 µl water into a 15 ml tube and vortex well.
- Aliquot 90 µl of the fish sperm DNA working solution into a 1.5 ml tube labelled '10^{-5}', and 360 µl into tubes labelled '10^{-6}', '10^{-7}', '10^{-8}', '10^{-9}' and '10^{-10}' tubes.
- Add 10 µl of standard 10^{-4} (donated by the Swedish Institute for Infectious Diseases) to the 90 µl of fish sperm DNA working solution in the standard 10^{-5} tube, pulse vortex 3 times and centrifuge (1 minute at maximum speed).
- Add 40 µl of standard 10^{-5} to 360 µl of fish sperm DNA working solution in the standard 10^{-6} tube, vortex and centrifuge (as above).
- Continue making 1 in 10 dilutions and then dispense into 17 µl aliquots.
- Standards should not be refrozen; therefore these aliquots are single use, allowing for three replications per plate.

To prepare TAE buffer solutions

- 10x TAE stock solution — Add 48.4 g Tris (Sigma Aldrich, Steinheim, Germany) and 7.44 g of EDTA (Merck Chemicals, Gauteng, South Africa) to 11.42 ml of acetic acid. Make up to 1 litre with distilled water.

- 1x TAE buffer — Add 1 part stock solution to 9 parts of distilled water.

To prepare X-gal

• 40 mg/ml X-gal solution	Add 1.25 ml of DMSO (Merck Chemicals) to 50 mg of X-gal, vortex and store at 20°C.

To prepare ampicillin

• 50 mg/ml ampicillin solution	Add 1 ml of PBS pH 7.2 (DMP) to 50 mg of ampicillin (Sigma Aldrich), vortex and store at 4°C.

To prepare LB, with 50 µg/ml ampicillin

• Luria Broth (1 L)	Combine 10 g Bactotryptone (Duchefa, Haarlem, The Netherlands), 10 g NaCl (Merck Chemicals) and 5 g of yeast extract (Oxoid Ltd, Hampshire, England). Make up to 1 litre with water. Autoclave, cool, add 1 ml of 50 mg/ml ampicillin and refrigerate.
• Luria broth plates (~25 plates)	Add 11.25 g of Bactoagar (Becton, Dickinson and Company, Sparks MD 21152, USA) to 750 ml of LB. Mix well, autoclave, cool slightly and add 750 µl of 50 mg/ml ampicillin. Pour aseptically into Petri dishes, allow to cool and refrigerate.

Appendix D – Extraction method with the Qiagen DNA Mini Kit

1. Aliquot 50 µl of washed sample into a 1,5 ml labelled tube.

2. Add 150 µl of ATL buffer.

3. Add 20 µl Proteinase K (Roche Diagnostics), mix by vortexing, and incubate at 56°C for 2 hours. Vortex occasionally during incubation to disperse the sample.

4. Briefly centrifuge the tubes to remove drops from the inside of the lid. Add 200 µl Buffer AL to the sample, mix by pulse-vortexing for 15 seconds, and incubate at 70°C for 10 minutes.

5. Briefly centrifuge the tube. Add 200 µl ethanol (96-100%) to the sample, mix by pulse-vortexing for 15 seconds and centrifuge briefly again.

6. Carefully apply the mixture (including the precipitate, ±650 µl) to QIAamp spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 minute. Place the QIAamp spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.

7. Carefully open the QIAamp spin column and add 500 µl Buffer AW1 without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 minute. Place the QIAamp spin column in a clean 2 ml collection tube (provided), and discard the collection tube containing the filtrate.

8. Carefully open the QIAamp spin column and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x g; 14,000 rpm) for 4 minutes.

9. Place the QIAamp spin column in a new 2 ml collection tube (not provided, use 1.5 ml tube with lid removed) and discard the collection tube containing the filtrate. Centrifuge at full speed for 1 minute.

10. Place the QIAamp spin column in a clean labelled 1.5 ml microcentrifuge tube (not provided), and discard the collection tube containing the filtrate. Carefully open the QIAamp spin column and add 100 µl Buffer AE. Incubate at room temperature for 5 minutes and then centrifuge at 6000 x g (8000 rpm) for 5 minutes.

11. Discard the column and store at -70°C. The final volume is 100 µl.

Appendix E – Ethics clearance certificate

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Dini

CLEARANCE CERTIFICATE **PROTOCOL NUMBER M040612**

PROJECT Survey and Management of Drug Resistant Pneumocystis jiroveci Pneumonia in Gauteng South Africa

INVESTIGATORS	Mrs L Dini
DEPARTMENT	Parasitology Reference Unit
DATE CONSIDERED	04.06.25
DECISION OF THE COMMITTEE*	Approved unconditionally

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

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

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Prof J Feam

DECLARATION OF INVESTIGATOR(S)

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

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendix F: Results of the RMPC validation experiments

Experiment 1: Cross-contamination in the RMPC

Specimen	qPCR copy number
Positive A	11,600,000
Positive B	5,000,000
Positive C	7,700,000
Positive D	1,100,000
Negative A, B, C and D	0

Experiment 2: Comparison of the RMPC versus the manual Qiagen method

Specimen number	IFA Result	RMPC qPCR copy number	Qiagen qPCR copy number
002	Negative	0	0
003	Negative	0	0
004	Negative	0	0
005	Negative	0	0
006	Negative	0	0
007	Negative	0	0
008	Negative	0	0
009	Negative	0	0
010	Negative	0	14
011	Negative	0	0
012	Negative	0	0
013	Negative	0	0
014	+	868,108	1,800,000
015	+	467	32,098
016	+++	19,347	9,330,000
017	+++	681,156	1,260,000
018	++	32,344	24,902
019	+	32	122
020	+++	984,027	5,160,000
021	+++	702,019	3,050,000
022	+++	119	803
023	+++	1,220	98,057
024	++	240	1,032
025	++	4,483	4,682

Using a Wilcoxon matched-pairs signed-rank test, p-value = 0.01 (significant).

Experiment 3: Technical optimisation of the use of the RMPC reagents

	Exp.2	Exp.3
	Cartridges not shaken	Cartridges shaken
Specimen number	qPCR, copy number	
014	272,926	603,090
015	214	2,087
016	15,460	319,251
017	325,562	876,149
018	14,087	143,476
019	0	1,497
020	419,126	1,070,000
021	253,133	857,057
022	20	170
023	642	9,776
024	46	1,877
025	2,515	2,525

Using a Wilcoxon matched-pairs signed-rank test, p-value < 0.05 (significant).

Experiment 4: Repeat of the comparison of the RMPC versus the manual Qiagen method experiment

	RMPC (Exp.3)	Qiagen (Exp.2)
	Cartridges shaken	-
Specimen number	qPCR, copy number	
014	1,160,000	1,320,000
015	1,606	2,288
016	395,457	6,650,000
017	1,320,000	1,030,000
018	194,287	12,654
019	884	0
020	738,078	3,650,000
021	973,967	2,580,000
022	128	819
023	6,054	53,619
024	1,085	693
025	1,618	3,846

Using a Wilcoxon matched-pairs signed-rank test, p-value = 0.20 (not significant).

Experiment 5: Robustness

Starting volume	**50µl**	**20µl**	**50µl**	**20µl**
Loading volume*	**200µl**		**100µl**	
Copy number	115	71	53	8
All negatives were undetermined				
*Setting on robot could not be changed and was set at 200µl				

Experiment 6: Repeatability

		RMPC		**Qiagen**	
Specimen number		**Copy number**	**Average copy number**	**Copy number**	**Average copy number**
035	Low 1	12	23	0	46
	Low 2	35		24	
	Low 3	0		68	
036	Med 1	269	494	341	301
	Med 2	704		192	
	Med 3	509		371	
037	High 1	99,930	88,216	231,798	202,318
	High 2	70,367		191,118	
	High 3	94,350		184,039	

Using a paired t test, p-value = 0.09 (not significant).

Experiment 7: Varying instrument protocols

		RMPC-Bacterial protocol		**RMPC-Total NA protocol**		**Qiagen**	
Specimen number		**Copy number**	**Average copy number**	**Copy number**	**Average copy number**	**Copy number**	**Average copy number**
038	Low 1	0	N/A	0	N/A	0	N/A
	Low 2	0		0		0	
	Low 3	0		0		0	
039	Med 1	2,359	2,378	1,242	1,601	4,082	3,115
	Med 2	2,195		2,272		2,942	
	Med 3	2,581		1,290		2,321	
040	High 1	443,354	420,891	265,262	311,026	930,288	711,790
	High 2	397,383		275,765		806,058	
	High 3	421,937		392,052		399,024	

Using a Dunn's multiple comparison test, there is only a significant difference between the RMPC - Total NA protocol and the Qiagen method

Appendix G: PCR protocol to detect inhibition in a clinical sample

This assay amplifies the human ribonuclease P (RNase P) gene.

1. Prepare a 25 µl reaction mixture consisting of
   - 12.5 µl of 2x TaqMan gene expression master mix (Applied Biosystems),
   - 1 µl of primer RNaseP-F (5'-CCA AGT GTG AGG GCT GAA AAG-3') mix at 10 µM,
   - 1 µl of primer RNaseP-R (5'-TGT TGT GGC TGA ACT ATA AAA GG-3') mix at 10 µM,
   - 0.5 µl of RNaseP-Probe [5'VIC-CC CCA GTC TCT GTC AGC ACT CCC TTC-3'NFQ (MGB Probe)] at 5 µM,
   - 7.5 µl of water and
   - 2.5 µl of DNA.

   Prepare for three extra samples, two for a positive and negative control and one for pipetting errors. Add all regents except the DNA, vortex and centrifuge briefly.

2. The master mix was pipetted into 96 well MicroAmp plates (Applied Biosystems). Add the DNA templates accordingly. Plates were covered with an adhesive optic film or caps (Applied Biosystems) and centrifuged at 3000 rpm for 10 seconds.

3. Load the plate into the real-time PCR instrument; select an absolute quantification (standard curve) assay with the Applied Biosystems SDS v 1.3.1 programme. Select detector VIC and apply to the appropriate wells.

4. The PCR assay parameters are as follows: 2 minutes at 50°C, 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C.

5. A positive result (Ct value >40) indicates that there is no inhibitory substances in the sample, while a negative result (Ct value <40) indicates the presence of inhibitory substances in the sample which is likely to inhibit other PCR assays.

Appendix H: Results of the 40 repeat specimens taken

Patient number	Date of specimen collection 1 and 2 respectively	IFA result	qPCR copy number	Nested PCR result	DHPS genotype
1	14 March 2005 31 March 2005	Negative Negative	0 0		
11	18 May 2005 20 May 2005	Negative Positive	33 825 156	Positive Positive	WT WT + M2
21	02 August 2005 04 August 2005	Negative Positive	12 270 192 794	Positive Positive	WT + M2 + M3 WT + M3
59	27 October 2006 03 November 2006	Negative Positive	22 93	Negative Negative	
60	01 November 2006 02 November 2006	Possible Positive	2 566 388	Positive Positive	M2 WT + M2
77	24 January 2007 25 January 2007	Negative Negative	0 0		
81	19 February 2007 20 February 2007	Negative Positive	0 0		
82	19 February 2007 21 February 2007	Negative Negative	0 0		
94	17 May 2007 18 May 2007	Negative Negative	0 0		
99	04 July 2007 05 July 2007	Negative Positive	0 467	 Positive	 WT
100	05 July 2007 11 July 2007	Negative Positive	29 453	Negative Positive	 WT
103	18 July 2007 20 July 2007	Negative Negative	0 0		
108	25 September 2007 25 September 2007	Negative Negative	0 0		
109	26 September 2007 27 September 2007	Possible Negative	0 3	 Negative	
118	21 November 2007 22 November 2007	Negative Negative	0 0		
120	29 November 2007 29 November 2007	Positive Negative	0 0		
123	04 January 2008 08 January 2008	Negative Negative	0 0		
128	08 February 2008 12 February 2008	Negative Negative	0 0		
136	21 May 2008 23 May 2008	Negative Negative	0 0		
138	27 May 2008 30 May 2008	No result* Positive	622 7	Positive Negative	WT + M1 + M2

142	09 July 2008	Negative	0		
	10 July 2008	Negative	0		
148	25 July 2008	Negative	2	Not done**	
	28 July 2008	Positive	62 727	Positive	WT
151	26 August 2008	Negative	0		
	27 August 2008	Negative	0		
157	30 August 2008	Negative	18	Negative	
	02 September 2008	Negative	0		
162	11 September 2008	Negative	352	Positive	WT + M1
	15 September 2008	Negative	0		
167	22 September 2008	Possible	585	Positive	WT
	23 September 2008	Negative	475	Positive	WT
176	07 October 2008	Negative	20	Negative	
	09 October 2008	Negative	638	Positive	M3
196	12 November 2008	Negative	0		
	14 November 2008	Negative	0		
200	19 November 2008	Possible	873	Positive	M2 + M3
	21 November 2008	Negative	45	Positive	M3
201	24 November 2008	Negative	5	Negative	
	26 November 2008	Negative	0		
228	20 February 2009	Possible	202	Negative	
	22 February 2009	Possible	1 085	Positive	WT + M2
244	03 April 2009	Negative	826	Positive	WT + M1
	06 April 2009	Possible	939	Positive	WT + M1
254	13 May 2009	Possible	401	Positive	WT
	15 May 2009	Negative	1 705	Positive	WT + M2
258	18 May 2009	Possible	396	Positive	WT
	20 May 2009	Positive	1 714	Positive	WT
259	21 May 2009	Negative	4	Negative	
	22 May 2009	Negative	0		
267	26 June 2009	Possible	60	Negative	
	29 June 2009	Positive	6 529	Positive	WT + M2

* No result due to error while processing
** Not done due to very low copy number

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*Organism names italicised as per original publication.

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