DECLARATION

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ABSTRACT

Background:

Pneumocystis jirovecii is an opportunistic fungal pathogen that causes *Pneumocystis* pneumonia (PCP) in immunocompromized hosts**.** PCP is associated with substantial morbidity, and mortality rates range from 10% to 40%.

The diagnosis of PCP relies on the microscopic detection of *P. jirovecii* in stained clinical samples. Polymerase chain reaction (PCR) may provide better sensitivity than microscopy; therefore, evaluation and implementation of PCR assays are required for the detection of *Pneumocystis* infection.

P. jirovecii is not cultivatable, therefore molecular tools are used for characterizing *P. jirovecii* genotypes; common targets are the dihydropteroate synthase (DHPS) and mitochondrial large subunit rRNA (mtLSU rRNA) genes. DHPS is a therapeutic target; mutations may be associated with co-trimoxazole prophylaxis and treatment failure. Polymorphisms in mtLSUrRNA have been used for phylogenetic studies.

Aims: 1) to evaluate a real time PCR (rtPCR) assay for diagnosis of PCP by comparing the performance to immunofluorescence (IF) and 2) to describe the molecular epidemiology of *P. jirovecii* isolates from Tygerberg Hospital by analyzing DHPS and mtLSU rRNA genes.

Methods: Clinical samples from 305 children and adult patients at Tygerberg Hospital were collected, after testing using IF. DNA was extracted using the NucliSens easyMAG platform (Biomérieux). The rtPCR assay targeting the major surface glycoprotein (MSG) gene was evaluated to detect *P. jirovecii* DNA. The DHPS and mtLSU rRNA genes were amplified by nested PCR and analyzed by DNA sequencing.

Results: The SYBR Green rtPCR detected *P.jirovecii* in 57% of samples (175/305) compared to the 7% (21/305) detected by IF. Our rtPCR had a sensitivity of 100% and specificity of 46%, although this increased if the detection threshold increased. Of the 50 negative control samples used in this study, none tested positive for *P.jirovecii*.

There were 237 lower respiratory tract (LRT) and 58 upper respiratory tract (URT) samples. The yield of PCR in LRT samples was 55.3% (131/237) compared to 70.6% (41/58) in URT samples (p=0.03). In contrast, none of the URT samples were positive using IF, and 8.9% (21/237) of LRT samples were positive on IF.

DHPS was successfully amplified in 123 (70.3%) samples; and mtLSU in 126 (72%) samples. Genotype 1 (wild type) was the predominant DHPS genotype, and a mutation rate of 42.3% was recorded for this gene. The mtLSU genotype 3 was present in 50.8% of samples, genotype 1 (42%) was the next most common genotype. Mixed genotypes were detected in 2.4% of the samples analyzed for each gene. There was no clear association between DHPS polymorphisms and mtLSU genotype.

Conclusions: The SYBR Green rtPCR was more sensitive than IF for detection of *P. jirovecii*; especially in URT samples, which is comparable to previous studies. The DHPS mutation rate increased to 42% from 27% recorded in 2013 from our division. The increase in DHPS mutation rate may be a result of on-going co-trimoxazole use, for prophylaxis or treatment of PCP or other infections.

Our findings need to be linked to clinical data to better understand transmission dynamics and potential impact of strain variation on clinical outcome, and further studies are required to better describe the local strain diversity.

OPSOMMING

Agtergrond:

Pneumocystis jirovecii is 'n opportunistiese swampatogeen wat Pneumosistis longontsteking (PCP) veroorsaak in immuunonderdrukte pasiënte. PCP gaan gepaard met hoë morbiditeit; en sterftesyfers wissel van 10% tot 40%.

PCP word tans gediagnoseer deur middle van mikroskopiese ontleding van kliniese monsters. Die polimerasekettingreaksie (PCR) bied egter hoër sensitiwiteit vir die ontleding opsporing van Pneumosistis as mikroskopie. Dit is dus nodig om PCR toetse verder te evalueer voor suksessvolle implementasie daarvan in routine kliniese laboratoriums te bevorder.

P. jirovecii kan nie gekweek word nie, dus word daar ook van molekulêre metodes gebruik gemaak vir die genotipering van *P. jirovecii.* Die twee mees algemene teikengene is die dihidropteroaatsintase (DHPS) en mitochondriale groot subeenheid rRNA (mtLSU rRNA) gene in. DHPS is 'n terapeutiese teiken; mutasies in die geen gaan gepaard met kotrimoksasool profilakse en mislukte behandeling. Polimorfismes in mtLSU rRNA word gebruik vir filogenetiese tipering studies.

Doelwitte: 1) om 'n "real-time" PCR (rtPCR) toets vir die diagnose van PCP te evalueer deur dit met immunofluoressensie (IF) te vergelyk; en 2) om die molekulêre epidemiologie van *P. jirovecii* afkomstig van Tygerberg-hospitaal te beskryf, deur die DHPS en mtLSU rRNA gene te ontleed.

Metodes: Kliniese monsters (met IF resultate) van 305 kinders en volwasse pasiënte by Tygerberg-hospitaal is ingesamel. DNS is deur middel van die NucliSens easyMAG platform (BIOMERIEUX) geïsoleer. Die SYBR Green rtPCR toets wat die "major surface" glikoproteïen (MSG) geen amplifiseer, is evalueer. Laastens is die DHPS en mtLSU rRNA gene amplifiseer vir DNA volgorde bepaling.

Resultate: 57% van die monsters (175/305) het *P. jirovecii* DNS bevat, in vergelyking met die 7% (21/305) waargeneem deur IF. Die rtPCR het 'n sensitiwiteit van 100% en spesifisiteit van 46% gehad, waarvan die laasgenoemde verhoog kom word deur die deteksiedrumpel te verhoog. Nie een van die 50 negatiewe kontrole monsters het positief getoets vir *P. jirovecii* nie.

Van die 237 laer lugweg (LRT) monsters, het 55.3% daarvan positief getoets vir *P. jirovecii* in vergelyking met die 70.6% van boonste lugweg (URT) monsters (n=58). In teenstelling hiermee het geen van die URT monsters positief getoets met die IF toets nie, waar 8.9% van die LRT wel positief getoets het.

DHPS is suksesvol amplifiseer in 123 (70.3%) monsters; en mtLSU in 126 (72%) monsters. Genotipe 1 (wildetipe) was die oorheersende DHPS genotipe, en 'n mutasie tempo van 42.3% is vir hierdie geen aangeteken. Die mtLSU genotipe 3 was teenwoordig in 50.8% van die monsters, en genotipe 1 in 42%. Gemengde genotipes is in 2.4% van die monsters gevind vir elk van die twee gene. Geen verband is tussen DHPS polimorfismes en mtLSU genotipes gevind nie.

Gevolgtrekkings: Die SYBR Green rtPCR is meer sensitief as IF vir die opsporing van *P. jirovecii*; veral in URT monsters, wat vergelykbaar is met vorige studies. Die DHPS mutasietempo het toegeneem vanaf 27% tot 42%in 2013. Die toename in DHPS mutasie frekwensie kan moontlik toegeskryf word aan voortgesette kotrimoksasool gebruik, vir profilakse of behandeling van PCP of ander infeksies.

Ons bevindinge moet gekoppel word aan kliniese data om die oordragsdinamika en effek van stamvariasie op kliniese uitkoms te beter verstaan. Verdere studies word benodig om 'n beter beskrywing van die plaaslike stam diversiteit te beskryf.

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DEDICATION

To my mother Elizabeth Banda and all my family members for their sacrifice and support in my education

My lovely wife Mercy Mandandi Banda

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LIST OF ABBREVIATIONS

Abbreviation	Full name
Ala	Alanine
AIDS	Acquired immunodeficiency syndrome
ART	Anti-retroviral therapy
BAL	Bronchoalveolar lavage
CD	Cluster of differentiation
DNA	Deoxyribonucleic acid
DHFR	Dihydrofolate reductase
DHPS	Dihydropteroate Synthase
dNTPs	Dideoxynucleoside triphosphate
DTT	Dithiothreitol
f. sp.	formae speciales
fas	Folic acid synthesis
FRET	Fluorescence resonance energy transfer
GE	Gel electrophoresis
GERMS-SA	Group for Enteric, Respiratory and Meningeal disease Surveillance in South Africa
HIV	Human immunodeficiency virus
IF	Immunofluorescence
IS	Induced sputum
ITS	Internal transcribed spacerregions of the ribosomal RNA

LRT	Lower respiratory tract
MSG	Major surface glycoprotein
mtLSUrRNA	Mitochondrial large subunit ribosomal RNA
NADPH	Nicotinamide adenine dinucleotide phosphate
NHLS	National Health Laboratory Service
NPA	Nasopharyngeal aspirate
NPV	Negative predictive value
PCP	*Pneumocystis jirovecii* pneumonia
PCR	Polymerase Chain Reaction
PPV	Positive predictive value
Pro	Proline
rRNA	Ribosomal ribonucleic acid
Ser	Serine
SOP	Standard operating procedure
Taq	*Thermus aquaticus*
TB	Pulmonary tuberculosis
Thr	Threonine
T_m	Melting temperature
TMP-SMX	Trimethoprim-sulfamethoxazole
UNAIDS	United Nations Programme on HIV/AIDS
URT	Upper respiratory tract
WHO	World Health Organisation

CHAPTER 1
Literature review

1.1. Background

1.1.1. Introduction

Pneumocystis jirovecii (formerly *Pneumocystis carinii*) is a common, atypical opportunistic fungal pathogen, causing a severe, life-threatening disease called *Pneumocystis* pneumonia (PCP) in patient's immunosuppressed by HIV infection, malignancy, transplantation, or therapeutic immunosuppression (Saric et al., 1994; Aderaye et al., 2003)

The *Pneumocystis* organism was first discovered in 1909 in Brazil by Carlos Chagas in the lungs of guinea pigs, and later in the lungs of a patient who died of trypanosomiasis. Chagas misidentified the organism as a new schizogonic state of *Trypasonoma cruzi* and proposed a name of *Schizotrypanum* (Kovacs et al., 2009). In 1910, at the Pasteur Institute of Sao Paolo, Antonio Carinii found *Pneumocystis* in rat lungs and considered it a new type of trypanosome (Jang-Jih Lu et al., 2008.). In 1912, Delanoë & Delanoë recognized that the organism identified by both Chagas and Carinii was a new organism (Delanoë & Delanoë, 1912) and named the organism *Pneumocystis carinii* to highlight its lung tropism, cyst-like morphology and to give credit to Antonio Carinii, who provided the tissue samples (Delanoë & Delanoë, 1912).

1.1.2. Classification of *Pneumocystis*

For many years after it was discovered, it was believed that *Pneumocystis* was a protozoan because it displayed morphological features typical of protozoa and in addition, *Pneumocystis* is susceptible to anti-protozoan drugs, such as pentamidine, trimethoprim, and sulfamethoxazole. However, in 1988, after phylogenetic analyses of the 16S, 5.8S and 26S ribosomal RNA subunit, the *Pneumocystis* genus was placed in the fungal phylum Ascomycota, subphylum Taphrinomycotina, Order Pneumocystidales, Class Pneumocystidomycetes, Family Pneumocystidaceae, Genus *Pneumocystis* (Edman et al., 1999; Stringer et al., 2002; Stringer et al., 1989; Aliouat-Denis et al.,2008).

Genetic heterogeneity studies have shown differences in sequence composition of *Pneumocystis* DNA obtained from different mammalian host species suggesting that *Pneumocystis* may be a diverse complex of organisms comprising more than one species and

that *Pneumocystis* infection is host-species specific (Demanche et al., 2001; Keely & Stringer, 2004).

In 1999 the pathogen infecting humans was proposed to be renamed with a new binomial nomenclature as *Pneumocystis jiroveci* (formerly *P. carinii* f. sp. *hominis*) in honor of the Czech parasitologist Otto Jirovec, who is credit with describing the pathogen in humans (Stringer et al., 2002). In the 1940s and 1950s he reported the pathogen in undernourished and premature infants presenting with interstitial plasmacellular pneumonia in Europe (Stringer et al., 2002).

The current species described and named are *Pneumocystis jirovecii*, *P. carinii* f. sp. *mustalae*, *P. carinii*, *P. wakefieldae* (formerly *P. carinii* f. sp. *ratti*, also known as "variant"), *P. murina* (formerly *P. carinii* f. sp. *muris*) and *P. carinii* f. sp. *oryctolagi.*

1.1.3. Morphology and life cycle

Study of the morphology and life cycle of *Pneumocystis* has been hindered by the inability to isolate it in pure culture (Thomas and Limper, 2004). Nonetheless, a hypothesized *Pneumocystis* life cycle within the mammalian host lung is based on the various life cycle stages that have been identified by light and electron microscopy (Matsumoto & Yoshida, 1984; Yoshida, 1989).

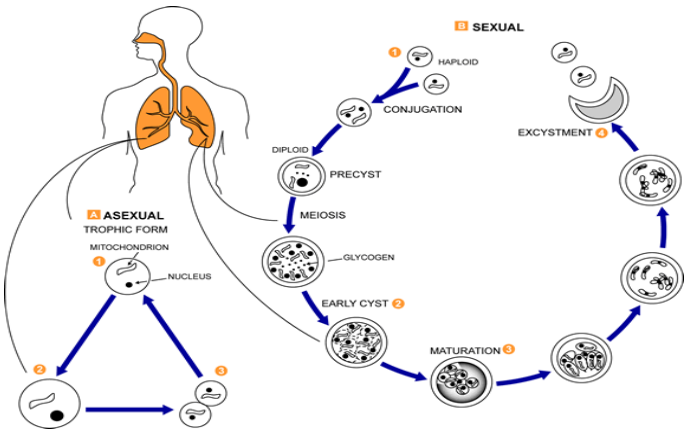


Figure 1.1: The proposed life-cycle of *Pneumocystis*. Based on the drawings by Ruffalo, 1998; and Larsen, 2004.

Three distinct morphologic stages or forms are predominant, namely the trophic form usually categorized into large and small (2-10 µm), the intermediate precyst form (5-8 µm) and the mature cystic form (5-10 µm) (Matsumoto & Yoshida, 1984; Cushion, 2004).

Pneumocystis is proposed to reproduce by two life cycles. One life cycle is characterized by the trophozoites reproducing asexually by binary fission and the other one consists of the sexual cycle when two trophozoites conjugate to form the cysts as shown in figure 1.1.

Trophic forms are thinwalled, mononuclear vegetative stages of *Pneumocystis.* They are haploid and appear to proliferate asexually by binary fission (Ruffolo, 1994, Cushion, 2004). The trophic forms mate conjugate, giving rise to diploid pre-cyst forms, which then enter the cyst-forming (sexual) phase of the life cycle (Yoshida, 1989; Ruffolo, 1994).
In the first meiotic division, the precyst yields 4 haploid daughter cells, which enter into the second meiotic division-giving rise to 4 intracystic bodies. These then undergo a post meiotic division, which leads to the formation of 8 haploid intracystic spores in a mature cyst. The mature cysts rupture to release the intracystic spores that develop into trophic forms completing this cycle (Yoshida, 1989).

The *pneumocystis* cyst and trophic forms are shown in figures 1.2a and 1.2b detected in bronchoalveolar specimens.

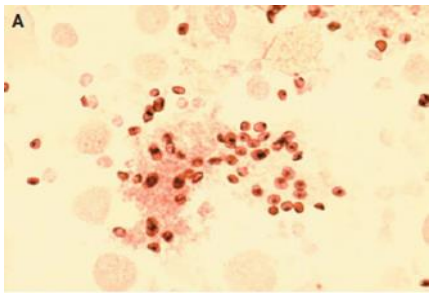


Figure 1.2a: The Cyst forms of *Pneumocystis* from bronchoalveolar specimen stained with Gomori methanamine silver (X 100) (Thomas and Limper, 2004)

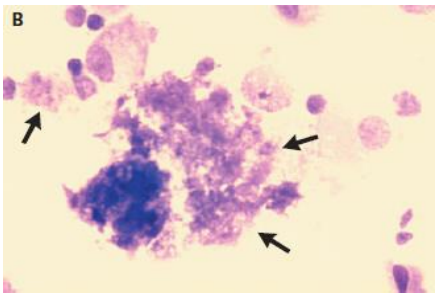


Figure 1.2b: The trophic form of *Pneumocystis* organism in bronchoalveolar specimen stained with Wright-Giemsa (X 100).

1.1.4. Transmission of the organism

Sero-epidemiological studies on *Pneumocystis* at Rosita Renard outpatient clinic in Santiago, Chile, showed that exposure to the fungus occurs early in life, with over 50% of 8-month-old and 85-100% of 2-year old children having specific anti-*Pneumocystis* antibodies (Vargas et al., 2001).

It is assumed that the acquisition of *P. jirovecii* by humans takes place via the respiratory tract (Miller et al., 2001; Chab´e et al., 2004). This theory is supported by accumulating evidence from animal models, the host specificity of *P. jirovecii* and the presence of *P. jirovecii* in the respiratory tract of both healthy and immuno-compromised individuals (Hauser et al., 2000; Chabe et al., 2004). Several experiments in animals have convincingly demonstrated that host-to-host transmission could occur via the airborne route and the most probable course of entry for the pathogen is through the respiratory tract during inhalation (Soulez et al., 1991).

In humans, development of *Pneumocystis* infection (PCP) could be due either to endogenous reactivation of latent infection present in the host because of earlier acquisition, or acquisition from an exogenous source (Morris et al., 2012). Endogenous reactivation would be triggered by immunosuppression of the host. Growing evidence indicates that person-to-person transmission from infected or colonized individuals does occur (Helweg-Larsen et al., 1998; Morris et al., 2012). A report from a genotypic study on HIV-infected patients who experienced two episodes of PCP showed that genetically distinct isolates were associated with each episode, suggesting that the recurrent episodes of PCP were caused by reinfection rather than by reactivation of a latent infection (Keely et al., 1997; Medrano et al., 2005). Another genotypic study of *Pneumocystis* organisms identified during an outbreak of PCP in renal transplant recipients also strongly indicates that inter-human transmission is the major route of infection (Yakazi et al., 2009). The results of these genetic epidemiological studies have challenged the hypothesis of reactivation of latent infection and supports person-to-person transmission of *Pneumocystis* (Morris et al., 2012; Beck et al., 2009).

1.1.5. Pathogenesis of *Pneumocystis jirovecii*

Pneumocystis has a high lung tropism; therefore, after transmission, the infective form binds tightly to type I alveolar epithelial cells and extracellular matrix proteins (Michelle et al., 2010; Nevez et al., 2008). The binding of *P. jirovecii* organisms promotes proliferation and destruction of epithelial cells (Limper et al., 1997).

Host responses are dependent on the physiological state of the particular host and range across a spectrum from intact defense to profound immunosuppression. Normally, in immunocompetent hosts, alveolar macrophages play a key role in the recognition, phagocytosis, and degradation of *Pneumocystis* (Limper et al., 1997). In addition, various

proinflammatory cytokines and chemokines released from activated macrophages and epithelial cells are essential for the optimal elimination of the organisms (Limper et al., 1997; Vassallo et al., 2000; Thomas et al., 2004). However, in immunocompromized hosts, the organisms are not cleared or contained, and are able to reproduce leading to a heavy pathogen load and subsequent pneumonitis (Kaneshiro and Baughman, 2001a).

The infection is progressive; the alveolar-epithelial membrane barrier becomes disrupted and results in the death of the epithelial cells which eventually leads to interstitial pneumonia, carnification of the interstitial tissue, damage to the alveolar lining, and accumulation of exudate within the alveoli. The accumulated exudate in the lungs blocks the gaseous exchange process (Cushion, 2004; Wright et al., 1999).

The inflammatory response to *P. jirovecii* not only promotes the essential clearance of this microorganism from the lungs but also causes the collateral damage to the lung tissue. This results in decreased efficacy of gaseous exchange and associated symptoms of respiratory distress (Wright et al., 1999; Thomas et al., 2004).

1.1.6. Clinical manifestations of *Pneumocystis* pneumonia

Pneumocystis pneumonia is a common AIDS-defining illness, but is also diagnosed in other immunosuppressed individuals (Thomas and Limper, 2004). The symptoms of PCP are non-specific and are shared amongst other infections including tuberculosis, cytomegalovirus, and other fungi; and by non-infectious pulmonary complications in the immunosuppressed, including drug-induced disease, pulmonary edema or pulmonary Kaposi's sarcoma.

Common symptoms of PCP include the subtle onset of progressive dyspnea, nonproductive cough, weight loss, low-grade fever with sweats. Acute dyspnea with pleuritic chest pain indicates the development of a pneumothorax. Physical examination typically reveals tachypnea, tachycardia, and normal findings on lung auscultation (Thomas and Limper, 2004; Speich et al., 2006). A chest radiograph shows diffuse bilateral infiltrates. However, the chest X-ray occasionally shows multiple nodules (Padley et al., 1999). PCP infection in immunosuppressed non-HIV-infected patients has a shorter duration of onset and fewer systemic symptoms than in HIV-infected individuals (Nuesch et al., 1999). HIV/AIDS patients with PCP have a high *Pneumocystis* organism load in their lungs, while broncho-

alveolar lavage clinical specimen from immunocompromized HIV negative patients shows lower concentrations of organisms but higher inflammatory response to the organism (Pagano et al., 2002; Matsumura et al., 2011). HIV-associated PCP usually develops in people whose CD4 cell count is less than 200 cells/µl (Phair et al., 1990). The outcome of PCP is more favorable in HIV-infected patients than in those without HIV infection (Roux et al., 2014). The differences in clinical features of PCP are thought to be due to the differences in the pathophysiology of the disease, and immune response of the host (Louis et al., 2015; Tasaka et al., 2015).

Overall, PCP mortality in the non-HIV-infected immunosuppressed patients is generally higher than that in HIV infection (Roux et al. 2014). The mortality rates of PCP range from 10% to 20% among HIV- infected patients, while it is 30%–60% among the non-HIV immunocompromized patients (Louis et al., 2015; Tasaka et al., 2015). Predictors of mortality in non-HIV infected patients include development of a pneumothorax, delay in intubation, longer duration of mechanical ventilation, and higher initial illness severity based on APACHE scores (Morris et al., 2012).

1.1.7. Epidemiology of *Pneumocystis* Pneumonia

With the beginning of the HIV epidemic in 1981, the incidence of PCP increased dramatically. Before systematic PCP prophylaxis was introduced, (Chaisson et al., 1992), PCP was reported as the AIDS defining event in 60% of HIV-1 infected patients and it was estimated that up to 80% of patients with CD4 counts less than 200 cells/µl would eventually develop PCP (Selik et al., 1987; Hay et al., 1988; Phair et al., 1990).

Although the incidence of PCP has decreased in HIV infected patients since the introduction of highly active antiretroviral therapy (HAART), PCP still remains a significant cause of morbidity and mortality globally especially in HIV positive patients unaware of their HIV status, or for those who do not have access to adequate medical care, particularly in developing countries (Montes-Cano MA et al., 2004; Morris et al., 2004).

The 2013 World Health Organization (WHO) UNAIDS Global report estimated that 35.3 (32.2-38.8) million people were living with HIV in 2012, of which 71% of them were from developing countries such as Asia and Sub-Saharan Africa (UNAIDS report on the global AIDS epidemic 2013).

There are a limited number of published articles regarding PCP in Sub-Saharan Africa, especially in adult HIV/AIDS patients.
A study of 83 HIV-infected patients who were admitted to the hospital in Uganda reported reported 38.6% of PCP (Worodia et al., 2003). Another study reported in 1995 by Malin et al. (1995) showed that 21 of 64 (33%) of hospitalized HIV-infected patients had PCP.

In South Africa, Wood et al. (1996), found 22% of HIV-positive clinic attendees in the Western Cape had PCP, the second highest HIV-related disease after TB (Wood et al., 1996). A post-mortem study performed on HIV/AIDS patients in the Eastern Cape found 9% PCP prevalence (Garcia-Jardon et al., 2010).

Children who are HIV positive in Africa have even higher rates of PCP than infected adults do. Autopsy investigations describe rates of PCP from 14% to 51.3%, depending on the age group studied. Ten percent of acute pneumonia cases in HIV-positive children were due to PCP in 1998 (Zar et al., 2000), and a similar study carried out in 2006 to 2008 found a PCP prevalence of 21% (Morrow et al., 2010). Ikeogu et al. found that in Zimbabwe, 19 (15.5%) of 122 HIV-infected children who died at <5 years of age had evidence of PCP at autopsy (Ikeogu et al., 1997). All cases except one were in infants < 6 months old. Similar results were found in autopsy studies from the early 1990s, with infants being more likely than older children to show signs of PCP (Lucas et al., 1996). The largest autopsy investigation examined 180 HIV-infected children in Zambia (Chintu et al., 2002). Twenty-nine percent of the children died of PCP, making PCP the third leading cause of death overall. Among children <6 months of age, PCP was the most common cause of pneumonia, detected in 51.3% of the children. Six of 84 HIV-negative children also showed evidence of PCP at autopsy. The most recent autopsy series reported that 10 (28.6%) of 35 HIV-infected children had PCP (Ansari et al., 2003)

There is now an increased recognition that the global burden of PCP infection is very large, and that PCP may be one of the most important cause of pneumonia in HIV-1 infected African adults and children (Ruffini et al., 2002).

PCP infection continues to be a life-threatening complication in immunocompromized patients. Therefore, early and accurate treatment based on rapid and certain detection is needed to prevent fatal PCP disease.

1.1.8. Radiographic Diagnosis of *Pneumocystis* Pneumonia

Radiological findings of *Pneumocystis* pneumonia are generally not specific and may range from a normal or near normal chest X-ray to different degrees of bilateral and usually symmetrical pulmonary infiltrates. At times clinical symptomatology, specifically in the absence of significant chest X-ray findings, initiates more sensitive radiological approaches such as high-resolution chest computed tomography (CT) which may show extensive ground-glass attenuation or cystic lesions (Gruden et al., 1997). Less-common features can include solitary or multiple nodules, pneumatoceles, pneumothorax, upper-lobe infiltrates and cystic lesions.

In daily practice, FDG-PET/CT and Ga-67 scintigraphy has been used already for many years in various fungal infections, such as *Pneumocystis jiroveci* aspergillosis, candidiasis, histoplasmosis, coccidioidomycosis and cryptococcosis (Glaudemans et al., 2013). However, these techniques have been most commonly used for the evaluation of other concomitant problems, with incidental or unexpected uptake in the lung prompting further diagnostic approaches, such as BAL to document *Pneumocystis pneumonia*. In the past, Ga-67 scintigraphy has been proposed as an adjunct for early detection of *Pneumocystis* (Carmona et al., 2011).

1.2. Laboratory Diagnosis of *Pneumocystis* Pneumonia

P. jirovecii cannot be cultured from clinical samples, and the standard laboratory diagnosis of PCP has relied on the use of various microscopic and molecular techniques for detection of cysts and trophozoites from both upper respiratory tract specimens (nasopharyngeal aspirate, oral washes, or nasal swab) and lower respiratory tract specimens (sputum, induced sputum, bronchoalveolar lavage (BAL), tracheal aspirate or lung biopsy) (Sing et al., 2000; Alvarez-Martinez et al., 2006; Durand-Joly et al., 2005).

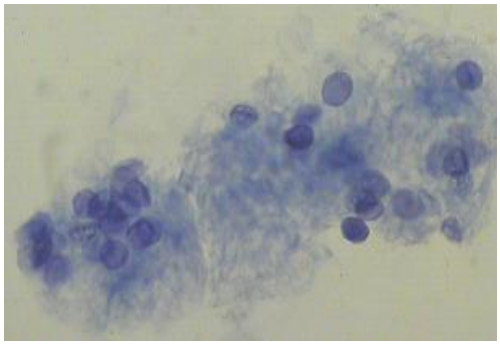
1.2.1. Microscopic Detection of *Pnemocystis*

The current method for diagnosis of PCP relies on the microscopic visualization of *P. jirovecii* in clinical specimens.

1.2.2. Light microscopy (Conventional staining)

The cysts or trophozoite forms of *Pneumocystis* can be visualized by staining samples with toluidine blue O (TBE), Gomori- methenamine silver nitrate (GMS), or methanol Giemsa stains (Flori et al., 2004; Morris et al., 2004). Methenamine silver and toluidine blue preparations stain only the cyst wall and do not allow detection of trophozoites. However, Giemsa stains detect all life stages of *P. jirovecii* (Gosey et al., 1985).

Identification of *Pneumocystis* organisms in clinical specimens therefore requires the systematic association of either TBE or GMS stained smears the same sample. Methanol-Giemsa allows differentiation of *Pneumocystis* organisms from other microorganisms and to identify the different *Pneumocystis* life-cycle stages.



Figrue 1.3a: *P. jrovecii* stained with Toluidine blue O (1000× magnification)

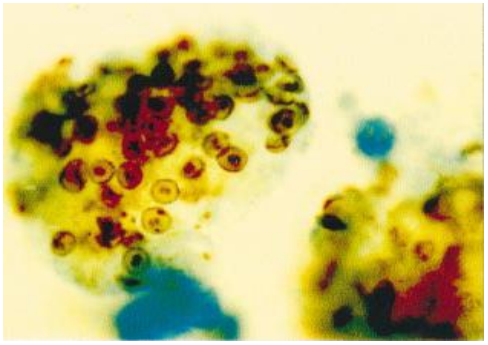


Figure 1.3b: Cysts of *P. jirovecii* with capsular dots (Grocott methylamine silver nitrate, original magnification ×1000). (Wazir et al., 2004)

The sensitivity and specificity of the different conventional staining techniques (Giemsa, TBE, and Grocott's methanamine silver nitrate) varies depending on the specimen type and experience of the microscopist (Table 1.2) (Procop et al., 2004).

1.2.3. Immunofluorescence

Higher sensitivity can be achieved by using commercialized fluorescein- or enzyme-labeled monoclonal or polyclonal *Pneumocystis* antibodies on respiratory samples (Durand-Joly et al., 2005). The detection of *Pneumocystis* using fluorescein-conjugated monoclonal antibodies is regarded as the gold standard test for diagnosis of PCP. Direct and indirect immunofluorescent assays (DFA, IFA) are specific for different life stages of *Pneumocystis*, depending on the antibody used (Wazir et al, 1994; Dei-Cas E et al 1998). These antibodies

have a higher sensitivity and specificity to detect *Pneumocystis* in respiratory samples than tinctorial stains (Flori et al., 2004). Comparative studies have shown DFA and IFA to be the most sensitive stains for *P. jirovecii* in sputum and bronchoalveolar lavage, with sensitivities of 97% and 90% respectively (Cregan et al., 1990).

After the clinical specimens are processed with monoclonal antibodies and stained, a UV microscope equipped with the correct wavelength filter is required, as well as an experienced microscopist for the laboratory diagnosis of *P. jirovecii*. An advantage of monoclonal antibodies is their ability to stain both trophic forms and cysts, which is important because the trophic forms are generally more abundant during PCP (Wazir et al., 1994; Kovac et al., 1998).

1.2.4. Serological tests for *P. jirovecii*

In recent years, inexpensive, non-invasive serological-based assays have also been studied for the diagnosis of PCP. It has been suggested that the level of serum β-D-glucan and/or S-adenosylmethionine is diagnostic for PCP within the appropriate clinical context (de Boer et al., 2011) and the level of lactate dehydrogenases is elevated at an early stage, offering diagnostic value despite its low specificity (Vogel et al., 2011).

Serum measurement of β-D-glucan (BDG) is based on the level of this polysaccharide that is present within the cell wall of *Pneumocystis* and other fungi (Morris et al., 2011). This polysaccharide triggers an innate immune response and can be measured in the serum and BAL specimens (Desmet et al., 2009; Morris et al., 2011). In the largest study to date, Sax et al. (2011), measured plasma BDG levels in 252 HIV-infected patients. They found that PCP patients had higher levels of β-glucan (median 408 pg/ml) compared to those without PCP (median 37 pg/ml). BDG had a sensitivity of 92% and a specificity of 65% for the diagnosis of PCP, using a cutoff value of 80 pg/ml (Sax et al., 2011).

An important point is that the β-D-glucan assay is not specific for *Pneumocystis* and may be elevated due to other fungal infections. In previous studies of neutropenic episodes, 70-80% of patients with positive β-D-glucan results had an invasive fungal infection (aspergillosis or candidiasis), but not PCP (Senn et al., 2008; Ellis et al., 2008).

An elevated serum lactate dehydrogenase level (LDH) has been related to PCP and probably reflects the underlying lung inflammation and injury rather than a specific marker for the disease (Quist et al., 1995; Zar et al., 2000).

S-adenosylmethionine (SAM) is a key component of the methylation reaction and polyamine synthesis (Merali et al., 2000). *Pneumocystis* lacks SAM synthetase (SAMS) activity and must rely on exogenous SAM from its human host. Studies have led to the hypothesis that *Pneumocystis* scavenges SAM from the lung and organism proliferation is enhanced and reduces the blood level of SAM in the host (Merali et al., 2000; Merali & Clarkson, 2004).

Reductions of plasma level of SAM in PCP patients followed by gradual increase in the plasma level of SAM after successful treatment of PCP have been reported (Skelly et al., 2003; Tasaka et al., 2007; Skelly et al., 2008). However, de Boer et al. (2011) studied 31 patients, 21 of whom were PCP-positive, and found that SAM levels did not discriminate between patients with and without PCP. Additionally, the use of SAM as a diagnostic marker for *Pneumocystis* pneumonia is still controversial due to some interfering factors including fasting status of patients, and the presence of intrinsic genes encoding SAM synthases (Kutty et al., 2008). The diagnostic utility of blood-based tests for PCP needs to be confirmed in a larger cohort of patients.

1.2.5. Molecular Methods

Several molecular techniques have been developed for the detection of *Pneumocystis*, including the Polymerase Chain Reaction (Leibovitz et al., 1995; Lu et al., 1995).

PCR can successfully amplify *Pneumocystis* DNA from various clinical specimens including sputum, BAL, NPA or tissue biopsy (Varela et al., 2011).

Several target genes have been used for PCR (Table 1.1), including the major surface glycoprotein gene (MSG), the mtLSU, the internal transcribed spacer gene (ITS), the DHPS, the dihydrofolate reductase gene (DHFR) (Durand-Joly et al., 2005, Alvarez-Martinez et al.,2006, Bandt et al., 2007), heat shock protein gene (HSP70) (Huggett et al., 2008), and the cell division cycle 2 gene (CDC2) (Arcenas et al., 2006).

Table 1.1: *Pneumocystis jirovecii* PCR target genes

Target gene	Copy number	Sensitivity (%)	Specificity (%)	Reference
MSG	multiple	98	93	Fischer et al.,2001,Lu et al., 2011
mtLSU	multiple	97	91	Wakefield et al., 1990
ITS	single	100	86	Lu et al.,2011
DHPS	single	94	81	Alvarez-Martinez et al., 2006
DHFR	single	23	100	Bandt et al., 2007
HSP70	single	98	96	Huggett et al., 2008
CDC2	single	100	86	McTaggartet al., 2012

The specificity and sensitivity of the PCR assays vary according to the primers used and the specific region of the *P. jirovecii* gene targeted. Multicopy genes such as mtLSU and MSG theoretically offer the greatest sensitivity for *P. jirovecii* detection (Robberts et al., 2007).

While conventional PCR techniques can detect low levels of *P. jirovecii* DNA, they are not quantitative. The introduction of real-time PCR (qPCR) in the diagnosis of pneumocystosis almost completely eliminates the risk for post-PCR carry-over (Larsen et al., 2002), and potentially more importantly offers to possibility of quantitating the organism load. During real-time qPCR, products are detected and quantified during amplification without opening of the reaction tube. Moreover, qPCR technology allows correlation of the copy number of genomes in the sample with microscopic counts of the organism (Brancart et al., 2005).

In addition to diagnosis, PCR has also proved valuable in the study of *Pneumocystis* colonization, defined as the detection of *Pneumocystis* organisms or their DNA in pulmonary specimens from individuals without signs or symptoms of pneumonia (Morris et al., 2008).

Several studies have shown that the copy number of *P. jirovecii*, measured by qPCR, interpreted in parallel with clinical manifestations and radiological and laboratory findings, may be useful in discriminating between colonization and infection (Alanio et al., 2011; Botterel et al., 2012). Quantitative real-time PCR assays, with defined upper- and lower-

quantitation thresholds of *P. jirovecii* copy number have been employed in an effort to distinguish true PCP infection from colonization (Larsen et al., 2004; Maillet et al., 2013). However, differentiation between carriage and active infection is contentious.

A simple strategy was developed to stratify patients when qPCR is positive and IFA negative, in which quantification of the fungal load in BAL specimens was combined with interpretation of the quantitative results using two thresholds and BDG detection. Quantification above the upper threshold (2×10^4copy/µl) was considered to represent infection, while those below the lower threshold (1.6×10^3copy/µl) indicated carriage. Between the two thresholds, a gray zone was defined, and classification of the case was then done by BDG detection in a concomitant serum sample. Patients with high BDG (>100 pg/ml) were reclassified as infection and low BDG (<100 pg/ml) as carriage (Damiani et al., 2013). However, if a threshold of 80 pg/ml for BDG were to have been applied, this strategy would not have been as discriminatory. Therefore, as in the case of qPCR, a two-threshold strategy may need to be applied (negative if less than 80 pg/ml, positive if more than 100 pg/ml, and inconclusive between 80 and 100 pg/ml). A more practical approach may be to select the lower thresholds for qPCR (1.6×10^3copy/µl) and BDG (80 pg/mL) and consider as high risk for PCP infection all cases harboring values higher than either of these two thresholds in order to avoid missing a diagnosis of PCP. Alternatively, some authors have used crude qPCR results (as quantification cycle, Cq, Ct, or Cp) (Fillaux J et al., 2008; Rohner et al., 2009), while others translate them into number of microorganisms based on count (for example, trophic form equivalent) (Alanio et al., 2011).

Another qPCR assay that targeted the *Pneumocystis* MSG multigene family was applied to oral wash (OW) samples, and revealed significant differences between infected and colonized subjects based on the number of MSG copies. The authors suggested a cutoff value of 50 MSG gene fragment copies/tube for distinguishing between the two conditions (Larsen et al., 2002). However, qPCR results appear difficult to apply in the field. The main problem has been the inability to control the sample volume. Another difficulty is related to differences in patient populations. It may be inappropriate to apply the same cutoffs to patients with HIV, patients with other underlying diseases or individuals receiving chemoprophylaxis against *Pneumocystis*.

Due to the heterogeneity of methods and diversity of chosen qPCR targets in published literature, generalizability of reported cut-off values is limited. Each institution must establish

and validate its own qPCR cut-off values appropriate to the preferred molecular method, as well as the population.

There is no international standardized qPCR assay or cutoff threshold. Large international studies are greatly needed for validation of this tool so that the use of qPCR for clinical interpretation of results can be reliably accepted and validated (Alanio et al., 2014).

In epidemiological investigations, PCR and direct sequencing or other typing methods can help to identify *Pneumocystis* and to explore the correlation between specific genotypes and virulence, transmissibility or drug susceptibility (Varela et al., 2011).

Table 1.2: Laboratory diagnostic methods for *Pneumocystis* pneumonia detection

Diagnostic performance of contemporary tests for diagnosing *pneumocystis* pneumonia					
Method	**Suitable kind of sample**	**Sensitivity (%)**	**Specificity (%)**	**Comments**	**Disadvantages**
Microscopic (Conventional tinctorial stains) (Procop et al., 2004)	BAL dried cytospin smear or biopsy)	49–79	99	Stains used: TBO CW, GMS, Diff-Quik	False positives (inexperienced staff)
Microscopic IFA (Cregan etal.,1990)	BAL, IS or sputum airdried cytospin smear	97	90	IFA stain used Merifluor pneumocystis	Cost; time consuming
PCR (Lu et al 2011; Fan et al 2013)	BAL, IS, OPW, NPA, biopsy	94–100	96–100	*Pneumocystis* targets used: the cdc2,DHFR, DHPS,HSP70,MSG,ITS, mtLSUgenes	Cost; positive in colonized patients
β-D-glucan (Watanabe et al., 2009)	Serum	96	87	Depends on study population	Positive in other deep fungal infections

Abbreviations: BAL, Bronchoalveolar lavage ; b-glucan, (1/3) b-D-Glucan; cdc2, Cell division cycle 2; CW, Calcofluor white;DHFR, Dihydrofolate reductase; DHPS, Dihydropteroate synthase; GMS, Grocott-Gomori methenamine-silver; mtLSU, Mitochondrial large subunit; ITS, Internal transcribed spacer HSP70, Heat-shock protein 70; IFA, Immunofluorescent antibody;NPA,Nasopharyngeal aspirate IS, Induced sputum; OPW, Oropharyngeal wash; PCP, *Pneumocystis* pneumonia; PCR, Polymerase chain reaction; PCR, Polymerase chain reaction; TBO: toluidine blue stain.

The use of molecular methods for the detection *P. jirovecii* can offer diagnostic objectivity and the potential for increased sensitivity and specificity compared with microscopic identification of organisms (Table 1.2).

1.3. Treatment of *Pneumocystis* Pneumonia

1.3.1.Trimethoprim-sulfamethoxazole (TMP-SMX)

Cotrimoxazole, a synergistic combination of trimethoprim and sulfamethoxazole (TMP-SMX) has been regarded as the first line agent for prophylaxis and treatment for PCP for many years (Meshnick et al., 1999; Fishman et al., 1998; Iliades et al., 2005). TMP-SMX in numerous comparative trials has been demonstrated to have efficacy superior to that of other agents (Hughes et al., 1993; Toma et al., 1993)

Sulfamethoxazole inhibits one of the integral enzymes in folic acid metabolism, DHPS, which catalyzes the condensation of para-aminobenzoic acid (PABA) and pteridine to form dihydropteroic acid (DHP)(Figure 1.5.). Dihydrofolic acid (DHF) is then produced by addition of glutamate to DHP by catalysis of dihydrofolate synthase (DHFS). Then, through reduction of DHFR, tetrahydrofolic acid can be produced from DHF (Figure 1.4) (Rao et al., 2013; Porollo et al., 2012).

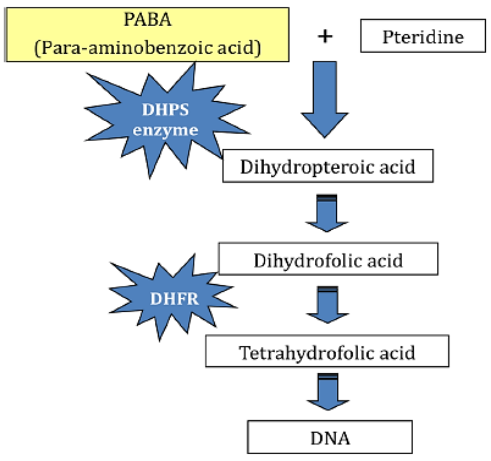


Figure 1.4: Folate synthesis pathway and the roles of the DHPS and DHFR enzymes (Huang et al., 2004)

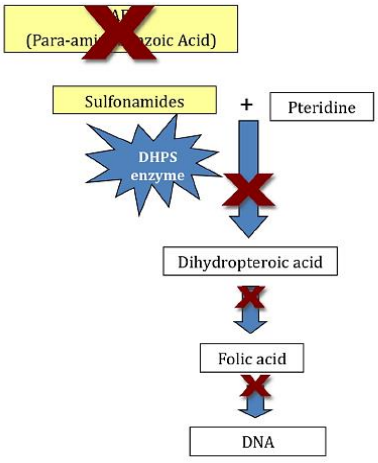


Figure 1.5: The inhibition of the folate synthesis pathway by sulfonamide (Huang et al., 2004)

Trimethoprim inhibits dihydrofolate reductase (DHFR), which catalyzes the reduction of 7,8-dihydrofolate to 5, 6,7,8-tetrahydrofolate in the presence of NADPH and prevents the formation of tetrahydrofolic acid. TMP-SMX effectively disrupts the metabolism in *P. jirovecii* and cause cell death, through the inhibition of folic acid, which plays an essential role for DNA synthesis in a cell's metabolism (Rao et al., 2013; Porollo et al., 2012).

Sulfamethoxazole is a widely used antibiotic, and bacteria resistant to this drug due to DHPS mutations are common (Huang et al., 2000; Crozier et al., 2011).

The DHPS inhibitors have been used for prophylaxis or treatment for PCP in hundreds of thousands of patients over the last 2 decades, and the development of sulfa-resistant strains of *P. jirovecii* was considered a serious concern (Meshnick et al., 1999). A number of studies have correlated prophylaxis failure with mutation in the *P. jirovecii* DHPS gene, but due to the inability to cultivate *P. jirovecii*, the link between DHPS mutation(s) and SMX susceptibility has not yet been definitively proven (Moukhlis et al., 2010).

Despite reports connecting DHPS mutations with prophylaxis failure, treatment failure, increased mortality, and selection by sulfa drug pressure, treatment failures have not yet been consistently reported in all studies (Navin et al., 2001; Alvarez-Martinez et al., 2008).

In 2001, Navin found no significant difference in the success of trimethoprim–sulfamethoxazole treatment, when comparing patients with the wild-type organism to those with mutants. Prior exposure to sulfonamide treatment and the geographical location of the patient have been identified as predictors of mutant genotypes (Ma et al., 1999; Huang et al., 2000). Support for the hypothesis of sulfa drug induced DHPS mutations can be derived from observations of patients with recurrent episodes of PCP. In 2003, Zingale et al. (2003) in a study of 70 bronchoalveolar lavage (BAL) specimens with positive PCR for *P. jirovecii* reported that DHPS mutations were significantly more common in patients exposed to sulfa drugs than in those un-exposed to sulfa drugs.

Further studies are required to determine the significance of the DHPS mutations in various populations and in different geographical locations. Nonetheless, the presence of molecular resistance is being increasingly observed, and clinicians should closely monitor clinical responses to standard *Pneumocystis* treatment regimens (Huang et al., 2004).

1.3.2. Second-line agents

1.3.2.1. Clindamycin/primaquine

In the case of patients who fail or cannot tolerate TMP-SMZ therapy for PCP, the combination of clindamycin and primaquine is an alternative choice for PCP therapy. Clindamycin interferes with protein synthesis of the organism by preferentially binding to its ribosome (Kouvela et al., 2006). The target of primaquine remains unknown. Clindamycin/primaquine combination shows comparable efficacy to TMP-SMZ for mild to moderate PCP (Toma et al., 1993).

1.3.2.2. Atovaquone

Atovaquone is an analog of ubiquinone (coenzyme Q). Atovaquone is clinically used to treat mild to moderate PCP, and its mechanism of action involves inhibition of mitochondrial electron transport and pyrimidine synthesis in *Pneumocystis* (Hughes et al., 1993). Atovaquone enters the mitochondria and acts on cellular respiration by specifically binding to the ubiquinol oxidation site of the cytochrome bc_1 complex (Kaneshiro et al., 2000; Larsen et al., 2005). Atovaquone resistant *Pneumocystis* strains have also emerged, and mutations in the *Pneumocystis* cytochrome *b* gene have been shown to confer this resistance (Kessl et al., 2004).

1.3.2.3. Dapsone

Dapsone is sometimes used as an alternative for preventive treatment to replace SMX. It inhibits the enzyme DHPS in similar way as TMP-SMX, although higher doses of dapsone may be required (Stein et al., 2004; Varela JM, 2011).

Dapsone, alone or associated with pyrimethamine, seems less effective than the TMP-SMX combination, but this is still questionable. It must be kept in mind that TMP-SMX intolerance is often predictive of dapsone intolerance, and should therefore not be considered as a replacement drug in these cases (Roux et al., 2014).

1.3.2.4. Pentamidine

Pentamidine is an aromatic compound that is used as an anti-protozoan drug. It is also used as an alternative against *Pneumocystis*, when TMP-SMX has failed Pentamidine is usually

parenterally administered, but it can also be applied in aerosol form to reduce the side effects (Fishman et al. 1998; Kaneshiro and Baughman, 2001b).

Pentamidine targets the metabolism of *para*-aminobenzoic acid, anaerobic glycolysis, inhibition of oxidative phosphorylation and impairs nucleic acid and protein synthesis (Miller et al. 1996). Although it is commonly used to treat PCP, it is highly toxic and has significant adverse effects such as hypoglycemia and nephrotoxicity (O'Brien et al., 1997).

1.4. Molecular epidemiology of *pneumocystis*

Study of the basic biology and epidemiology of *P. jirovecii* has been hampered by the lack of a reliable method for culture (Miller et al., 2005). Presently, no functional or morphological means for determination of phenotypic diversity of *P. jirovecii* exist. Molecular techniques have therefore been used to describe differences between isolates by comparison of DNA-sequence variation at a number of different genetic loci and thus to study the molecular epidemiology of the organism (Beard et al., 2004; Lee et al., 1998).

The genetic diversity of *P. jirovecii,* based on the identification of genotypes at single or multiple loci, has been described by PCR amplification and DNA sequencing analysis, restriction fragment length polymorphism (RFLP), multi-locus genotyping (MLG), single strand conformation polymorphism (SSCP) or type-specific oligonucleotide hybridization (Beard et al., 2000, 2004; Esteves et al., 2008, 2009). The detection of gene mutations such as deletions, insertions and even single-base substitutions can be achieved with the development of discriminatory methods for analyzing and comparing PCR products.

Several polymorphic loci have been studied with the purpose of understanding the epidemiological profile of *P. jirovecii* isolates including geographical distribution of genotypes, drug-susceptibility, virulence; and modes of transmission in distinct populations (Esteves et al., 2010). Different molecular targets, such as the MSG, ITS1 and ITS2, mtLSU-rRNA, b-tubulin, 26S RNA and DHPS have been used previously in analysis and characterization of *P. jirovecii* isolates (Lane et al., 1997; Maitte et al., 2013).

The DHPS and the mtLSU rRNA are among the most frequently targeted regions of the *P. jirovecii* genome (Matos et al., 2010).

The mtLSU rRNA and ITS loci are frequently used because they are assumed not to be under genetic selection, and are therefore useful for studies on molecular evolution in circulating strains (Lee et al., 1998; Esteves et al., 2008; van Hal et al., 2009).

1.4.1. *P. jiroveci* DHPS mutations and genotypes

In the *Pneumocystis* organism, DHPS is encoded by the folic acid synthesis (*fas*) gene, a multifunctional gene that encodes dihydroneopterin aldolase and hydroxymethyl-dihydropterin pyrophosphokinase (Volpe et al., 1992). The DHPS gene of *P. jirovecii* has been sequenced and the proteins it encodes are the enzymatic target of sulfa-based prophylactic and therapeutic regimens (Huang et al., 2004; Beck et al., 2009). However, there is still no consensus on the clinical significance of *Pneumocystis* DHPS mutations and their relationship to sulphonamide resistance as discussed in section 1.3.1.

The most common non-synonymous single nucleotide polymorphisms (nsSNPs) in the *P. jirovecii* DHPS gene are located at nucleotide positions 165 (A–G) and 171 (C–T), causing amino acid substitutions at codon 55 (Thr to Ala) and 57 (Pro to Ser) respectively, in a highly conserved region of one of the putative active sites of the enzyme (Lane et al., 1997; Kazanjian et al., 1998; Zar et al., 2004). According to Alvarez-Martínez et al. (2010), the frequent mutation seen at positions 165 and 171 of the DHPS region, leads to four possible different genetic alleles, as shown in Table 1.3.
Various mixed genotypes have also been described where at least one position has a mutation (Lane et al., 1997; Alvarez-Martínez et al., 2010).

Similar point mutations in conserved regions of the DHPS gene confer sulfonamide resistance by decreasing the affinity for sulphonamides and sulfones in pathogens such as Plasmodium falciparum, Neisseria meningitidis, Toxoplasma gondii, Streptococcus pneumoniae and Escherichia coli, raising concerns that similar resistance could potentially develop within *P. jirovecii* (Tyagi et al., 2008; Iliades et al., 2004; Moukhlis et al., 2010).

Table 1.3: The DHPS genotype classification

Locus	Genotype	Nucleotide (codon)
DHPS	1 (**Wt**)	165A(55Thr);171C(57Pro)
	2	165G(55Ala);171C(57Pro)
	3	165A(55Thr);171T(57Ser)
	4	165G (55Ala); 171 (57Ser)

The distribution of DHPS genotypes varies geographically. 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. As the use of sulfa drugs increases, so does the prevalence of DHPS mutations. DHPS gene mutations are also possibly caused by intrinsic epidemiological factors that influence the circulation and transmission of different genotypes, or perhaps because of differing use of sulphonamides for PCP prophylaxis (Lane et al., 1997; Esteves et al., 2008).

The prevalence of *P. jirovecii* DHPS mutations reported from different geographical areas ranges widely, from 0 to 81%. For example, DHPS mutation prevalence is lower in Europe and East Sweden (0%), Italy (8%), Paris (18%), Denmark (20%), Portugal (27%) and Japan (25%), but much higher in the USA (Atlanta (54%) and San Francisco (81%) (Larsen et al., 1999; Takahashi et al., 2000; Costa et al., 2003; Nahimana et al., 2003; Crothers et al.,2005; Le Gal et al., 2012; Magne et al., 2011; Beser et al., 2012; Yoon et al., 2013).

The prevalence of DHPS mutations has generally been reported to be lower in developing countries than in industrialized countries (Miller et al., 2003; Dini et al., 2006).

However, fewer studies have reported the prevalence from sub-Saharan Africa. A study conducted at University of Cape Town on *P. jirovecii* isolates obtained from HIV- infected children admitted for PCP, reported a 13.3% DHPS mutation prevalence (Zar et al., 2004). And 56% of isolates from 38 public hospitals and clinics in six provinces of South Africa in 2006 harbored mutant DHPS haplotypes (Dini et al., 2010). A similar study conducted in Gauteng Province observed 87% prevalence of *P. jirovecii* mutant genotypes. It used samples of patients aged between 2 months and 79 years obtained from seven provinces of South

Africa through the Group for Enteric, Respiratory and Meningeal disease Surveillance in South Africa (GERMS-SA) network (Mogoye et al., 2015).

The reasons for these geographic differences could be related to differences in use of sulfa-based prophylaxis, the adherence level to prophylaxis, or epidemiology of PCP transmission. Further research is needed to determine the importance of DHPS mutations and whether the geographic variation of *Pneumocystis* infection and of other mutations contributes to treatment failure.

1.4.2. *P. jirovecii* mtLSU mutations and genotypes

The mtLSU-rRNA gene, which is involved in basic metabolic functions, is highly conserved and is considered an informative marker for discerning associations between geographical locations and specific genotypes (Beard et al., 2000).

The clinical significance of mtLSUrRNA polymorphisms is unknown; however, these polymorphisms are commonly targeted for phylogenetic studies. The two most commonly found mtLSU-rRNA polymorphisms occur at nucleotide positions 85 and 248, and give rise to a total of four separate genotypes. A third variable position has also been reported, but it is rarely seen (Beard et al., 2004). Most of the conducted studies have detected the 4 polymorphisms shown in Table 1.5 (Gupta et al., 2011).

Table 1.4: The mtLSU genotype classification

Locus	Genotype	Nucleotide position/Identity
mtLSU rRNA	1	85C/248C
	2	85A/248C
	3	85T/248C
	4	85C/248T

The allelic frequency distribution of mtLSU-rRNA genotypes varies geographically. In one comparison study conducted in Portugal and Spain, Genotype 1 was most common in both countries (49% in Portugal and 64% in Spain) (Esteves et al., 2008). Genotype 2 was the second most frequent genotype among the Portuguese samples (22%) and the third most

frequent among the Spanish samples (11%). In contrast, Genotype 3 was the third most frequent genotype among the Portuguese samples (18%) and the second most frequent among the Spanish samples (25%). Genotype 4 was represented by only a single Portuguese sample (Esteves et al., 2008).

Mixed infections with more than one *P. jirovecii* mtLSU-rRNA genotype were detected in four (8%) Portuguese samples. Two (4%) yielded genotypes 1 and 2, and two others yielded genotypes 1 and 3. No mixed infections were detected among the Spanish samples (Esteves et al., 2008).

In contrast, in a study conducted in Cuba by de Armas et al. (2012), Genotype 3 was the most frequently detected (93.8%), while Genotypes 2 and 4 were not detected in paraffin-embedded postmortem lung specimens obtained at autopsy from HIV-positive patients (Esteves et al., 2008). Similar results were observed in a pediatric study in Cape Town, where Genotype 3 was the most common (30.3%), followed by Genotype 2 (18.1%) and Genotype 1 (6.1%), mixed genotypes accounted for 45.5% (Kumar et al., 2011).

These differences imply that genetic variations in *P. jirovecii* have a geographical component. It has been suggested that the epidemiological factors inherent in geographical locations may influence the transmission and circulation of different *P. jirovecii* genotypes (Beard et al., 2000; Esteves et al., 2008). To the best of our knowledge, there is no data describing the potential clinical significance of different mtLSU-rRNA genotypes.

1.5. Rationale

Pneumocystis pneumonia (PCP) has historically been one of the leading causes of morbidity and mortality in immunosuppressed individuals. This opportunistic infection has been associated with fatal outcomes in HIV/AIDS patients, and the burden of this pandemic is primarily in sub-Saharan Africa. Despite significant reductions in cases of PCP due to the use of *P jirovecii* prophylaxis and HAART in HIV/AIDS individuals, PCP remains a significant opportunistic infection in developing countries especially among HIV-infected patients who have less access to health care. PCP is still associated with morbidity and high mortality rates ranging from 10% to nearly 40% in those with severe pneumonia and respiratory failure.

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 such as tuberculosis which may hamper the diagnosis of PCP.

PCP is considered an HIV/AIDS indicator disease and should be suspected in all HIV-positive patients with respiratory symptoms. Thus, there is a need for local approaches to the global problem of managing HIV- related opportunistic diseases such as PCP by improving, developing and evaluating clinical algorithms for diagnosis, and primary prophylactic regimens directed against the most prevalent opportunistic infections.

1.6. Aim and objectives:

Aim: The study aims to improve the laboratory diagnosis of PCP and to describe the prevalence of DHPS and mtLSUrRNA mutations in *P. jirovecii* isolates obtained from children and adult patients of a study population in Cape Town, South Africa.

Objectives:

1. To compare 2 diagnostic real-time PCR assays (one using a hydrolysis probe, one using SYBR-green intercalating dye) for the detection of PCP.

2. To describe the molecular epidemiology of *P. jirovecii* in patients in Cape Town, South Africa by:

   a) Characterizing polymorphisms at DHPS and mtLSU-rRNA regions of the *P. jirovecii* genome

   b) Analyzing the different combinations of the 2 alleles (DHPS and mtLSU-rRNA) and evaluating the genetic diversity of *P. jirovecii* in study isolates

CHAPTER 2

Comparison of a probe vs. intercalating dye method for the detection of *Pneumocystis jirovicii* DNA in patients with suspected *Pneumocystis* Pneumonia

2.1. 1. Introduction

As mentioned in chapter 1 (section 1.2), the laboratory diagnosis of PCP relies heavily on microscopic detection of the organism's cyst and trophic forms in clinical specimens. However, the sensitivity of microscopic diagnosis is not ideal ranging from 49 to 97% depending on specimen type as shown in table 1.2 (page 14). Therefore, there is a need for more robust and sensitive rtPCR diagnostic assays to assist clinicians in the diagnosis of PCP.

Currently, two common formats of rtPCR are available in which the amplification of the target gene is monitored by an increased fluorescence signal. The first method uses intercalating fluorescent dyes such as SYBR Green, and the second utilizes fluorescent reporter probes (Kesmen et al., 2009).

The SYBR Green binds to all double–stranded DNA present, including any non-specific PCR products as well as primer dimers that may have formed (Figure 2.1).

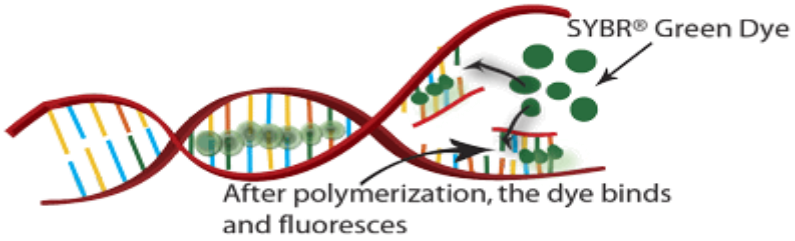


Figure 2.1: Simplified schematic illustrating the principle mechanism involved in a SYBR Green-based assay. (NFSTC Science Serving Justice® (2007). (http://projects.nfstc.org/pdi/Subject03/pdi_s03_m05_07_a.htm)

This real-time SYBR Green rtPCR assay works on the principle of measuring fluorescence signals generated by the binding of SYBR Green to double stranded products during the extension phase of each amplification cycle. The specificity of the fluorescent signal can be determined during subsequent melting curve analysis which results in a melting peak profile specific for the amplified target DNA (Ririe et al., 1997; Yang et al., 2004).

The melting temperature, defined as the temperature at which 50% of the DNA amplicon is in a double-stranded configuration, is dependent upon the length and the G+C content of the amplicon obtained (Ririe et al., 1997). The amplicons with a higher G+C content consequently have higher melting temperatures (Reese et al., 2010). In addition, a shorter amplicon will have a lower T_m than a longer amplicon as the covalent bonds between the two DNA molecules are weaker and will dissociate at a lower temperature (Ririe et al., 1997; Mackay et al., 2002).

The second real time PCR technique uses a fluorescent reporter probe-based approach, in which the probe binds specifically to the target DNA sequence during the annealing step of PCR. One such example is hydrolysis probes (Figure 2.2).

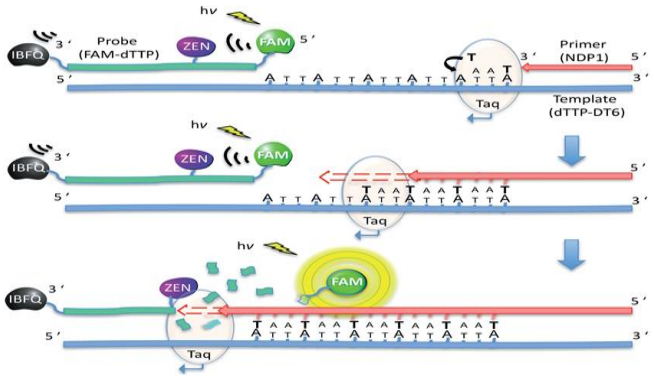


Figure 2.2: Simplified schematic illustrating the principle involved in the probe-based assay. Probes are labeled with a 5-carboxyfluorescein fluoro-phore (FAM) reporter dye on the 5′ end and two quenchers: Iowa Black (IBFQ) on the 3′ end and an internal ZEN (Wilson et al., 2011).

The probe is a short, sequence-specific oligonucleotide that contains a fluorescent reporter dye at 5'end and a quencher dye at the 3' end. During the PCR extension stage, the Taq polymerase cleaves the fluorescent reporter from the hydrolysis probe binding to the template DNA and this generates detectable fluorescent signal (Espy et al., 2006). The cleaved fluorescent dye accumulates exponentially after each temperature cycle and that allows results to be visualized in real-time, which in a clinical setting can be important (Kordo et al., 2013).

The aim of this component of the study was to improve the molecular diagnosis of PCP from isolates obtained from children and adult patients of a study population in Cape Town, South Africa and compare 2 diagnostic real-time PCR assays (one using a hydrolysis probe, one using SYBR-green intercalating dye) for the detection of *P. jirovecii*.

2.2. Materials and methods

2.2.1 Population and study samples.

Three hundred and five residual respiratory samples from adult and pediatric patients with clinical suspicion of PCP were included in the study. These samples had been sent to the NHLS Immunology laboratory at Tygerberg Hospital for routine diagnostic PCP investigations during the time period of March 2014 to January 2015. The Tygerberg hospital serves a drainage area of approximately half of Cape Town. The hospital acts as a referral centre for 4 regional hospitals (Karl Bremer, Paarl, Worcester and Helderberg Hospitals), 17 district hospitals and over 120 primary health care clinics. The population served is approximately 2.6 million, representing just under half the population of the Western Cape.

Residual clinical samples were stored in a -20°C freezer following routine IF examination and included tracheal aspirates (TA), induced sputum (IS), bronchoalveolar lavage fluid (BAL), bronchial washes, and nasopharyngeal aspirates. Limited demographic data including age and gender were stored on a secure Microsoft Excel patient database.
The patient demographics for both PCP suspects and the negative controls were included, to compare the age distribution of our populations.

2.2.2. pCR2.1 Plasmid *msg* control

The pCR-2.1 vector containing the *P. jirovecii* Major Surface Glycoprotein (MSG) gene (donated by J. Kovacs, NIH, USA) was used as a positive control for all molecular investigations. This plasmid was propagated in *E. coli* competent cells using a standard transformation protocol and was purified using the QiagenPlasmid Midi Kit (Valencia, USA) as described in Appendix H.

The quantity and purity of DNA in the neat sample of cloned DNA was measured using the BioDrop µLite (BioDrop, Cambridge, UK) system. The serial dilution was done by adding 10µl of a neat sample to 90µl nuclease-free water in the first tube. The tube was vortexed. Then, 10µl from the content of the first tube was transferred to the second tube containing 90µl nuclease-free water. The second tube was again vortexed to mix the tube contents. The serial dilution was then done from the neat sample down to 10^{-12}, which, in theory represented zero copies of DNA per microliter.

To avoid contamination of the lower standard, pipette tips were changed between each dilution during the tenfold serial dilution process.

To avoid and minimize the possibilities of contamination of the clinical samples with serial plasmid dilutions, the DNA extraction from our clinical samples and the serial plasmid dilutions were done in separate rooms. The non-template controls were also added in each runs to monitor contamination.

The tenfold dilution (10^{0} to 10^{-12}) of the pCR 2.1 vector (3929 bp) was made to determine the performance of the diagnostic PCR assays (section 2.3.2). The dilutions of the original concentration were repeated 2 times.

The theoretical number of molecules (N) per mole present in the pCR-2.1 vector sample was calculated according to the following equation:

$$N = nNa$$

$$= \left(\frac{C}{Mw}\right) Na$$

Na = the Avogadro constant = 6.023 x10^{23}

C = initial DNA concentration

n = number of mole = C/Mw

Mw = number of nucleotides in pCR 2.1 vector (3929 bp) x average molecular weight of a nucleotide (700)

The PCR assay detects the MSG gene which is present in multiple copies (approximately to 50-100 copies) in the *P. jirovecii* genome; therefore, the dilution range represented 50 to 250 x 10^{11} copies of the MSG gene per µl (Table 2.1).

Table 2.1: Number of molecules in each pure pCR 2.1 plasmid DNA dilution

Sample Dilution	Sample Concentration (ng/µl)	Number of molecules/µl
(10^{0})	4×10^{3}	4.9×10^{11}
(10^{-1})	4×10^{2}	4.9×10^{10}
(10^{-2})	4×10^{1}	4.9×10^{9}
(10^{-3})	4	4.9×10^{8}
(10^{-4})	4×10^{-1}	4.9×10^{7}
(10^{-5})	4×10^{-2}	4.9×10^{6}
(10^{-6})	4×10^{-3}	4.9×10^{5}
(10^{-7})	4×10^{-4}	4.9×10^{4}
(10^{-8})	4×10^{-5}	4.9×10^{3}
(10^{-9})	4×10^{-6}	4.9×10^{2}
(10^{-10})	4×10^{-7}	4.9×10^{1}
(10^{-11})	4×10^{-8}	4.9
(10^{-12})	4×10^{-9}	0.5

2.2.3. Specificity studies

Fifty respiratory samples positive for a respiratory virus, acquired from the Division of Virology at Tygerberg Hospital, were used as negative controls to assist with specificity studies. We assumed that patients with confirmed viral infection (by a multiplex PCR assay) would have a similar clinical presentation as PCP suspects. The fact that none of the patients from whom these samples were collected had PCP investigations requested suggests that there was little to no clinical suspicion of PCP disease in these patients. No clinical information was collected from these patients due to ethical constraints.

2.2.4. Direct immunofluorescence

Direct immunofluorescence following the manufacturer's kit instructions in Appendix E (Detect IF, Axis-Shield Diagnostics, UK), was performed as part of routine clinical practice on the clinical specimens according to the standard operating procedure (SOP) of the NHLS Immunology laboratory at Tygerberg Hospital and results recorded in the patient database.

2.2.5. Sample Preparation and DNA Extraction

Residual clinical samples (DTT treated and concentrated specimen following IF investigations) were thawed, followed by vortexing and centrifuging at 3 000 rpm for 5 minutes. The supernatant was discarded and the concentrated pellet was resuspended in 10 ml of 10x PBS (Diagnostic Media Products). Samples were then transferred to a 15 ml sterile tube and once again stored at -20°C until further use.

DNA was extracted in batches of 12 specimens using the automated NucliSENS easyMAG extraction kit- and platform (bioMérieux, Boxtel, Netherlands). This procedure uses patented BOOM technology which is based on the ability of positively charged magnetic silica beads to bind negatively charged nucleic acids in the presence of salts. The use of magnets allows the capture of these beads and heating them elutes the bound nucleic acids. 50 µL of each respiratory sample (IS, TA, BAL or NPA) was added to NucliSENS lysis buffer and incubated at room temperature for 10 minutes prior to the extraction process (Figure 2.3). Resultant DNA was stored at -20°C until further use.

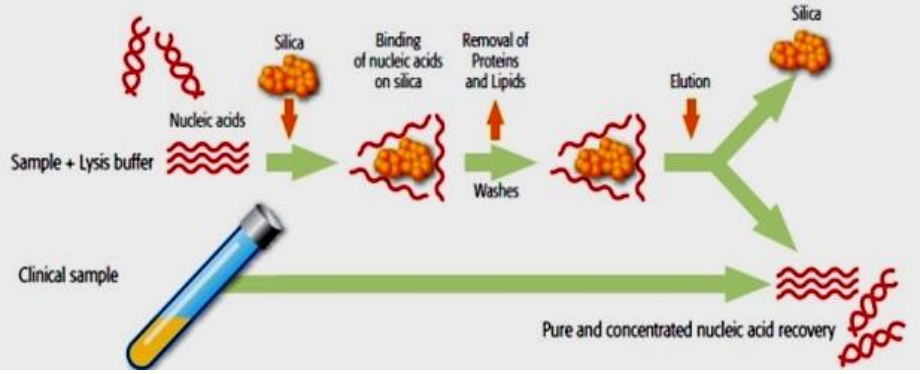


Figure 2.3: Nuclisens easyMag extraction principle

2.2.6. *P. jirovecii* detection by real-time PCR analysis

2.2.6.1. Probe vs intercalating dye based detection of *P. jirovicii* DNA

Two real-time PCR technologies, SYBR green intercalating dye and a hydrolysis probe, were compared on a dilution range of *P. jirovecii* DNA to determine the best performance for detection of *P. jirovecii* DNA.

Both methods employed a species specific primer set targeting a 250 bp region of the highly conserved MSG gene (Table 2.2).

Table 2.2: Primers and hydrolysis probe used for real-time PCR assays

Primers	Sequences 5′ - 3′	Size(bp)	Reference
MSG Heminested	JKK14/15 (5'-GAATGCAAATCYTTACAGAGACAACAG-3') JKK17(5'-AAATCATGAACGAAATAACCATTGC-3')	246-250	Huang, et al., 1999
Hydrolysis Probe	(5'-6-HEX/TGATGAAGG/ZEN/TCAGCCAGCCTATGC/ IBFQ -3')		Okholm et al., 2014

a) SYBR Green I intercalating dye method

The qualitative SYBR rtPCR was adopted from previous study conducted in our division. Two microliters of each dilution range of the positive control was added to a reaction mixture containing 1x Rotor-Gene SYBR Green PCR Master Mix (Qiagen, Whitehead Scientific, Cape Town, South Africa). Briefly, 12.5 µl Rotor-Gene PCR Mix, 0.5 µl (50µM) of each forward and reverse primer (final concentration of each primer in our reaction mix was 1µM) and 9 µl of nuclease-free water. The PCR reaction tube final volume of 25 µl was amplified in a Qiagen Rotogene Q analyzer with cycling parameters of 50 sec at 95°C, followed by 40 cycles at 95°C for 5 sec and 60°C for 60 sec.

Resultant amplicons were subjected to high-resolution thermal melt analysis (HRM) (Figure 2.3). The thermal denaturation profile was measured over the temperature range from 80°C to 90°C, and fluorometric readings were taken every 1°C. Rotorgene software was used to calculate the derivative of the intensity of fluorescence at different temperatures (dF/dT), thereby generating a plot where the derivative peak represented the T_m value of the MSG DNA fragment. The presence/absence of MSG DNA (thereby presence or absence of *P. jirovicii*) was automatically determined by the software according to the presence of the derivative peak located within a defined temperature bin (width ± 2°C). Optimal temperature bins were determined during optimization of the PCR and specificity of the bin for MSG DNA was confirmed by sequencing the resultant amplicon. A software defined threshold of fluorescence was set (see section 2.2.6.4) to define a positive result. Melting curves with peaks within the pre-defined temperature bins and, above the set threshold were deemed positive.

b) Hydrolysis probe method

The dilution series was amplified using the same primer set described in Table 2.2. The reaction mixture consisted of 12.5 µl Rotor-Gene PCR Mix, 0.5 µl (50µM) of each forward and reverse primer, 0.5 µl (5µM) of the probe, 2 µl of each dilution sample and 9 µl of nuclease-free water (final volume of 25µl). Cycling parameters included two initial holds, each for 2 min at 50°C and 95°C respectively, followed by 40 cycles at 95°C for 15 s and 60°C for 60 s. The florescence was detected in the HEX channel at the end of the annealing step of each cycle. The assay and data analysis was performed in a Qiagen Rotor-Gene Q thermo-cycler.

Separate rooms were used for each of the molecular procedures, including pre-PCR (DNA extraction), reagent preparation, addition of template DNA, thermo-cycling, and post-PCR steps (real-time PCR and gel electrophoresis). Non-template controls were included in PCR reactions to control and monitor reagent contamination.

2.2.6.2. Gel Electophoresis

During optimization, resultant amplicons were confirmed by gel electrophoresis on a 2% TAE agarose gel run for 40 minutes at 120V. 10µl of PCR product was mixed with 2µl of a fluorescent loading dye (Novel juice, GeneDirex, Taiwan). A 100 bp plus DNA ladder (Thermo Scientific-GeneRuler) was included to aid in identification of the 250bp target size. Specificity of product was further confirmed by sequencing analysis (Inqaba Biotech, South Africa).

2.2.6.3. Detection of *P. jirovecii* DNA from clinical specimens

Following the experiment in section 2.2.6.1a, all 305 clinical specimens and 50 negative control specimens were amplified using the SYBR Green I real-time method (rationale discussed in section 2.3.3). Positive and non-template controls were included in each run.

2.2.6.4. Rotogene Q Software Parameters

Fluorescence data from both the probe and intercalating dye based methods were analyzed using the Rotor- GeneQ software.

When using the SYBR Green I intercalating method, with subsequent melting curve analysis, certain software parameters may be defined, including the fluorescence threshold which is the amount of fluorescence considered to represent a positive result. Three different software thresholds (0.5, 1.5 and 3) were used to analyze all samples to determine which threshold provided optimum sensitivity and specificity. The 3 thresholds were assigned to fall within the lower 1/3rd to approximately half of the majority of the peak heights; and to exclude Baseline noise. As the assay was qualitative in nature and positivity for *P. jirovecii* DNA would be assigned based in part on the threshold level set, we aimed to determine the effect of threshold on sensitivity of the assay as compared to the gold standard.

Using the positive controls, an average melting temperature of 83.5 °C was defined and a software "bin" (with a range of 2 degrees in either direction) was created to simplify analysis. Any samples with melting temperatures within the defined temperature bin and with peaks above a threshold of 1, 1.5 or 3 (depending on analysis) were considered positive.

2.2.6.5. Data Analysis

The overall performance of the SYBR Green rtPCR assay on clinical specimen was assessed by calculating the sensitivity, specificity, positive predictive value, and negative predictive value using the standard formulae (Parikh et al., 2008).

All the statistical analyses were performed using STATA for Windows (version 14). The Fisher exact test was used to assess significant associations among categorical variables. A p-value of less than 0.05 was considered significant and tests were two-tailed.

2.2.7. Ethical approval

The patient samples obtained during the course of the study form part of the routine diagnostic work-up. Samples were anonymous and minimal clinical information was collected for analysis of the data. The PCR results were not reported to the clinicians or on the NHLS Laboratory Information System, but only used to validate and assess the assay.

This study was approved by the Health Research Ethics Committee of the University of Stellenbosch (#: S15/07/141)

2.3. Results

2.3.1. Study samples:

During the study period, (April 2014- November 2015), 305 clinical specimens including 9 bronchoalveolar lavage (BAL) or bronchial aspirate, 106 sputa, 122 tracheal (TA) and 58 nasopharyngeal aspirates (NPA) were collected from the NHLS Immunology laboratory at Tygerberg Hospital, South Africa (Figure 2.4).

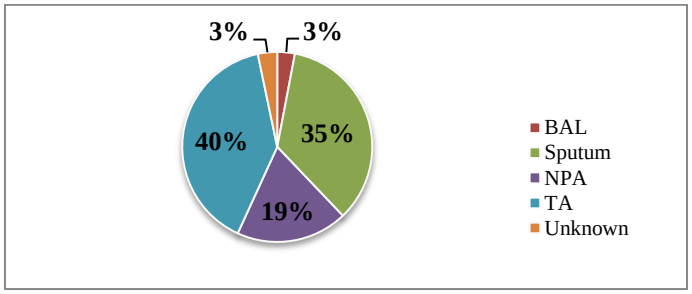


Figure 2.4: Pie chart illustrating proportion of different sample types

2.3.2. Patient demographics

The patients included children and adults. Clinical and demographic data were obtained from the clinical information system. There were 143 male and 155 female patients (sex ratio (M/F) of 0.93); in 7 patients the information on request forms did not specify sex. Age was not provided for 10 patients. Figure 2.5 shows the age distribution of the 295 study participants where age was available. The mean age of the patients was 18 years (range: 0.03–74 years), median age was 2.58 years; inter quartile range (3 months-3 years). For the negative control samples, the participant age ranged from 9 days to 77 years, the median was 0.58, the mean was 10.4, and the interquartile range was 5 months – 7 years.

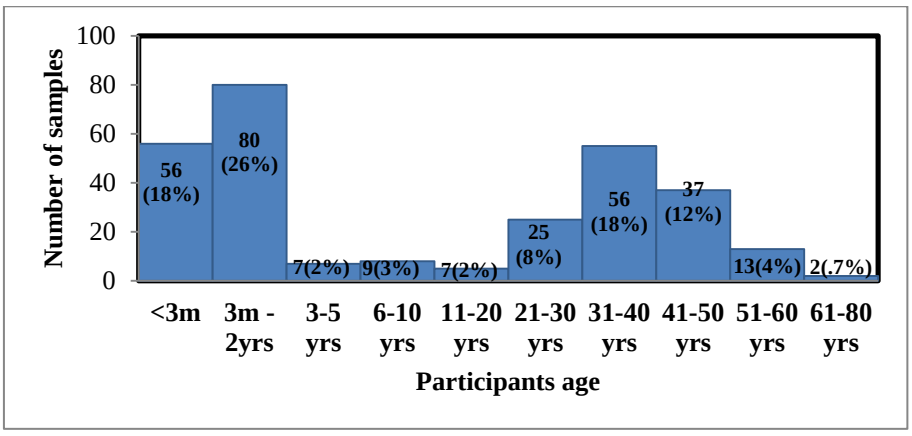


Figure 2.5: Participants age distribution and number of samples

Our study population of PCP suspects consisted of 155 paediatric (age<13 years) and 140 adult patients. Real time PCR successfully amplified a portion of MSG from 95 of the paediatric and 75 of the adult samples (61.3% and 53.6% respectively).

2.3.3. SYBR Green I PCR vs Hydrolysis probe based PCR

Two rtPCR detection chemistries were investigated for the detection of *P. jirovecii* DNA. Both methods were applied to a serial 10-fold dilution range (10^{-1} to 10^{-5}) of a plasmid carrying the MSG target gene. The characteristic sigmoidal shaped curves obtained are shown in figure 2.6a and figure 2.6b, and the post-PCR melting curve analysis for the SYBR green assay is shown in figure 2.6c.

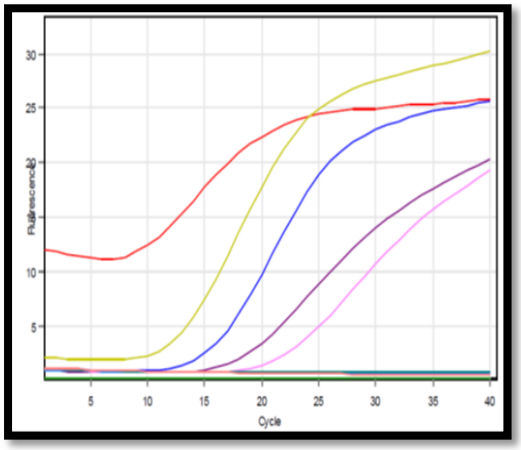


Figure 2.6a: Amplification curves of 10-fold serial dilutions of plasmid DNA using SYBR Green I PCR

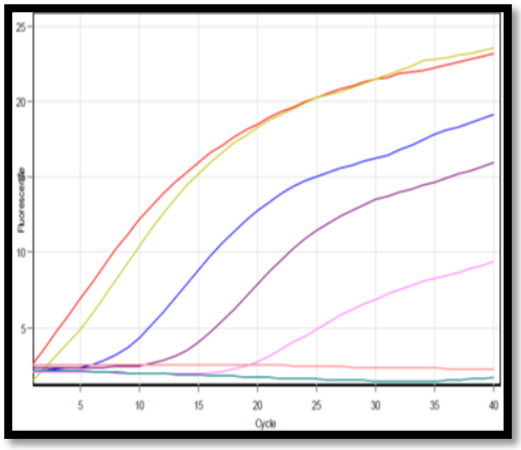
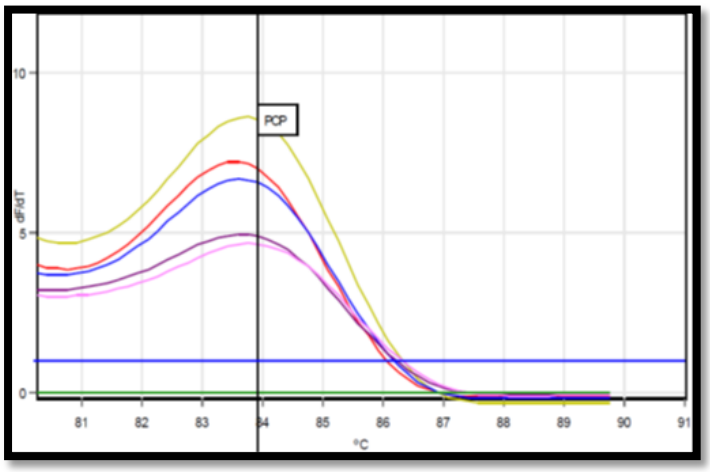


Figure 2.6b: Amplification curves of 10-fold serial dilutions of plasmid DNA using Hydrolysis probes



1		10^{-1}	4.9 x 10^{10}	83.65 (PCP)
2		10^{-2}	4.9 x 10^{9}	83.65 (PCP)
3		10^{-3}	4.9 x 10^{8}	83.65 (PCP)
4		10^{-4}	4.9 x 10^{7}	83.40 (PCP)
5		10^{-5}	4.9 x 10^{6}	83.60 (PCP)
6		Negative		
7		Negative		
8		Negative		

Figure 2.6c: Real-time MSG PCR melting curve analysis for plasmid dilutions (10^{-1} to 10^{-5})

The assays were compared using a relatively high concentration of plasmid DNA (10^{-5} corresponding to 4.9 x10^{6} molecules /µ). The main objective behind this initial comparison was to assess the workflows associated with each method, the ease of use and analysis, and the costs. Both assays showed similar performance for detection of the limited dilution range (10^{-1} to 10^{-5}).

However, the SYBR Green I based assay offered a number of advantages: (1) The SYBR Green dye protocol is simpler and more cost effective than the probe based protocol, (2) SYBR Green dye protocol can more easily be added to current laboratory work-flow, (3) primer dimers and other non-specific interactions can be readily visualized by melt curve analysis (Figure 2.6c). We therefore decided to continue our study using the SYBR Green I rtPCR technology.

2.3.5. SYBR Green I rtPCR sensitivity and limit of detection

To determine the performance of the SYBR Green I rtPCR assay, standard curves were obtained by amplification of 10-fold serial dilutions of the standard plasmids (10^{-1} to 10^{-12}) as shown in figure 2.7 a. The limit of detection was calculated as the lowest number of amplicon copies that could be detected by the PCR method. In our SYBR Green I rtPCR, the detection limit was 1 copy of plasmid per microliter (10^{-12} dilution). The repeatability of the SYBR Green rtPCR was determined by testing the serial dilutions of standard plasmids (10^{-1} to 10^{-12}) in duplicates as shown in figure 2.7b. Our SYBR Green I rtPCR using the manufacturer's recommended settings was successful on all positive control dilutions, confirming it appropriate to continue with clinical specimens.

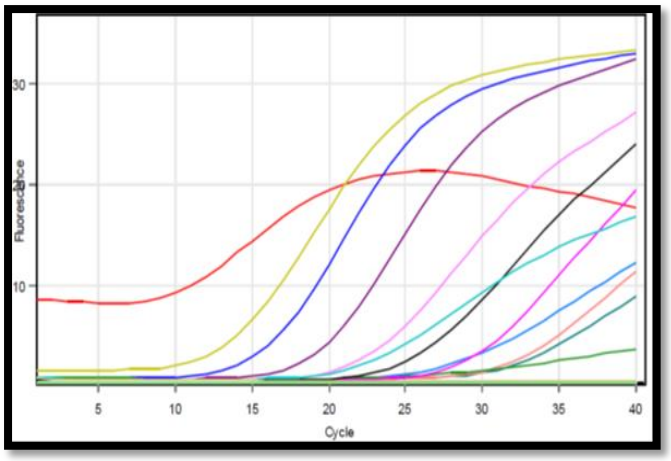


Figure 2.7a: Amplification curves of 10-fold serial dilutions of plasmid DNA (10^{-1} to 10^{-12}) using SYBR green rtPCR

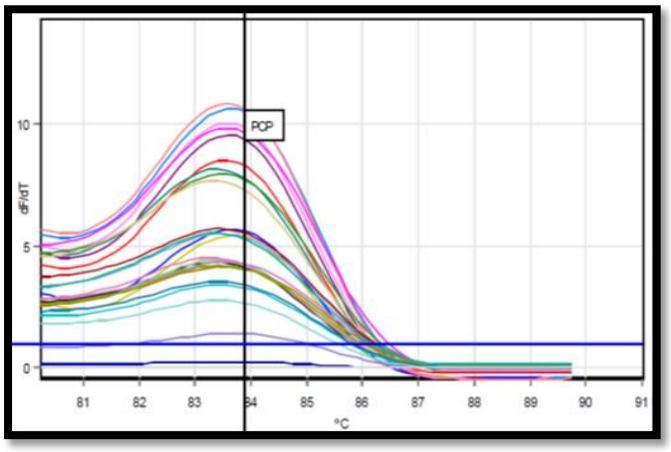


Figure 2.7b: Melting curve analysis of SYBR Green I rtPCR assay using 10-fold serial dilutions of plasmid DNA (10^{-1} to 10^{-12}) duplicates.

1		10^{-1}	83.25 (PCP)
2		10^{-2}	83.15 (PCP)
3		10^{-3}	83.35 (PCP)
4		10^{-4}	83.10 (PCP)
5		10^{-5}	83.40 (PCP)
6		10^{-6}	83.50 (PCP)
7		10^{-7}	83.50 (PCP)
8		10^{-8}	83.40 (PCP)
9		10^{-9}	83.75 (PCP)
10		10^{-10}	83.65 (PCP)
11		10^{-11}	83.35 (PCP)
12		10^{-12}	83.35 (PCP)
13		Negative	
14		Negative	

2.3.5. Detection of *P. jirovecii* DNA in clinical specimens

For detection of *Pneumocystis,* SYBR Green real-time PCR targeting the major surface glycoprotein of *P. jirovecii* was conducted on DNA extracted from all 305 specimens. Using 3 different thresholds, the SYBR Green PCR results are shown in table 2.3.

2.3.5.1 Patients with suspected PCP

Of the 305 specimens tested for *P. jirovecii* by immunofluorescence (IF) microscopy and real time PCR methods, IF was positive in 7% (21/305), while rtPCR recorded positives of 57% (175/305), 49 % (150/305) and 21 % (65/305) at thresholds of 1, 1.5 and 3 respectively.

IF-microscopy was deemed equivocal (ie less than 5 cysts observed) for specimens from 6 patients, and all of these yielded positive results with PCR at all 3 thresholds. Of the 21 samples positive by IF microscopy, only 3 were male, while 18 were female patients. By IF technique, 8.4% (12/143) sample positivity rate was recorded from the age group of between 3 month and 4 years, and 7.7% (9/117) sample positivity rate was recorded from age group of between 20 and 50 years. Similar for the PCR method, at the threshold of 1, 72 male and 93 female patients were positive. A 63% (90/143) positivity rate was recorded from the age group of 3 months to 4 years, while 54% (63/117) positivity rate was recorded in the age group 20 to 50 years.

Correlating the PCR result with microscopy, *P. jirovecii* DNA was detected in all of the IF-positive specimens using rtPCR analysed using all 3 thresholds. No false negatives were observer ("PCR−ve/IF+ve"). Table 2.3 provides the positivity correlation of the IF method and rtPCR using the 3 different thresholds.

There were 237 LRT samples and 58 URT samples. The yield of PCR in LRT samples was 55.3% (131/237) compared to 70.6% (41/58) in URT samples (p=0.03) In contrast, none of the URT samples were positive using IF, and 8.9% (21/237) of LRT samples were positive by IF. There was a significantly higher yield using PCR compared to IF for both URT and LRT samples (p<0.000001 in each case).

Table 2.3: Correlation of IF and PCR methods

		Positive	Negative	Total
Immunofluorescence (IF)		21 (7%)	284 (93%)	305
MSG SYBR-Green rtPCR	**Threshold 1**	175 (57%)	130 (43%)	305
	Threshold 1.5	150 (49%)	155 (51%)	305
	Threshold 3	65 (21%)	240 (79%)	305

Comparing SYBR green I rtPCR results to the IF test (gold standard), the sensitivity, specificity, positive and negative predictive values of the SYBR Green I rtPCR assay were calculated for different thresholds as shown in Table 2.4.

Table 2.4: SYBR Green PCR sensitivity, specificity and predictive values and 95% Confidence Intervals at 3 different thresholds

	PCR(1)	PCR(1.5)	PCR(3)
Sensitivity (95% Confidence Interval [CI])	100% (80.8-100%)	100% (80.8-100%)	100% (80.8-100%)
Specificity (95% CI)	46% (40-51.8%)	54.5% (48.5- 60.4%)	84.5% (79.6-88.4%)
Positive Predicitive Value (PPV) (95% CI)	12% (7.8-18%)	14% (9.1-20.8%)	32% (21.5-45.2%)
Negative Predictive Value (NPV) (95% CI)	100% (96.4-100%)	100% (97-100%)	100% (98-100%)

As the PCR threshold increased, the specificity and PPV increased as well, as one would expect. However, the sensitivity and NPV remained at 100% despite increasing the threshold.

P. jirovecii DNA was detected in many of the IF negative samples, which is then reflected by the relatively low specificity and positive predictive values of the test – however this presumes that the samples positive on PCR yet negative on IF are all false positives (ie patients did not have infection with *P. jirovecii*). If the sensitivity of the IF method is less than 100% (which is likely), then calculation of the specificity of PCR is more problematic. For this reason, an additional group of negative control samples were investigated.

2.3.6.2. Negative control samples

Fifty negative control (i.e. non- PCP suspects) samples including IS (n=5, 10%), BAL (n=2, 4%), TA/TS (n=11, 22%) and NPAs (n=32, 64%), were collected from 26 male and 24 female patients. The participant age ranged from 9 days to 77 years, the median was 0.58, the mean was 10.4, and the interquartile range was 5 months – 7 years. Of the 50 samples, 14 were positive for respiratory syncytial virus A, 15 positive for influenza virus A, 3 positive for human rhinovirus A/B/C, 2 positive for parainfluenza virus, 2 positive for adenovirus and 2 positive for metapneumovirus.

The 50 negative control samples were run separately from the samples of PCP suspects, but the serial dilution of 10^{-5} was included as a positive control in each run.

None of these nagtive control samples tested positive for *P. jirovecii* using the SYBR green PCR assay.

2.4. Discussion

In the present study, we compared two rtPCR assays (SYBR green intercalating dye and a hydrolysis probe based method) on a dilution range of *P. jirovecii* DNA to determine the best performance for detection of *P. jirovecii* DNA. Results obtained showed that the limit of detection for both assays was similar using dilution range (10^{-1} to 10^{-5}) of a plasmid carrying the MSG target gene as shown in section 2.3.3.
We elected to test the stored clinical samples in this study using the The SYBR Green assay since it was 1) easier to optimize, and 2) the relatively cheaper than the hydrolysis probe.

In this study SYBR Green real-time PCR detected more *P.jirovecii* positive in samples than microscopy using IF. Out of 305 samples the gold standard IF detected only 7% (21/305) of specimen as positives, while real-time PCR detected up to 57% (depending on the software threshold set). For both the IF and PCR techniques, the highest sample positivity rates were recorded from patients between 3 months and 4 years of age. This is consistent with the previous reports that indicate that *P.jirovecii* usually infects children as early as 3 months to 4 years of age (Pifer et al., 1978; Huang et al., 2011). We also detected a high number of cases in the 20 to 50 year age group, which could be linked to the higher rates of HIV/AIDS amongst this demographic in South Africa.

Specimens from the URT usually have a low organism load, making it more difficult to make a reliable diagnosis by microscopy. Therefore, IF is primarily done on LRT samples (BAL, IS and TA) to improve the sensitivity of the assay (Caliendo et al., 1998). However, PCR based assays have the ability to detect *P. jirovecii* DNA in both URT and LRT specimens, with a reported sensitivity of 86-100% (Cartwright et al., 1994; Murdoch et al., 2003; Huang et al., 2006).

Our PCR assay was as a qualitative assay, but not quantitative assay. Therefore this was difficult to ascertain whether upper respiratory tract colonization may be associated with low levels of *P.jirovecii* DNA, since we did not quantify our samples to know the levels of levels of *P.jirovecii* DNA loads.

Several studies have compared paired URT and LRT samples for the detection of *P. jirovecii* using PCR, and the results are mixed (Oz et al., 2000; Nyamande et al., 2005; Samuel et al., 2011; To et al., 2013).

A study by Samuel et al. (2011) on paired LRT and URT samples obtained from children with suspected PCP, found no significant difference between the detection sensitivity of PCR on LRT samples compared to URT samples.

In our study, the results indicate that the MSG-rtPCR assay has improved sensitivity over IF for the detection of *P. jirovecii* DNA, particularly in URT samples. There were 237 LRT samples and 58 URT samples. The yield of PCR in LRT samples was 55.3% (131/237) compared to 70.6% (41/58) in URT samples ($p=0.03$). In contrast, none of the URT samples were positive using IF, and 8.9% (21/237) of LRT samples were positive on IF. There was a significantly higher yield using rtPCR compared to IF for both URT and LRT samples ($p<0.000001$ in each case).

Our improved PCR yield results on URT specimens are consistent with the 48% PCR yield from the URT samples obtained from children with suspected PCP, reported by Samuel et al. (2011). A study by Wakefield reported a 78% PCR yield from oropharyngeal specimens obtained from 31 HIV-infected patients with PCP (Wakefield et al., 1993). Another study by Tamburrini et al. (1997) examined oropharyngeal specimens from 27 HIV-infected patients (18 with PCP; nine without PCP), reported a PCR yield of 55% in detecting *P. jirovecii* DNA.

In contrast to our results, a study by Gupta et al. (2009) reported no detection of *P.jirovecii* DNA in 65 nasoharygeal aspirate samples, when yield of different samples was compared. The respiratory samples obtained from patients with different categories and immunosuppression. Another study conducted in the USA, used 258 URT samples from 86 individuals without discernible respiratory infection, no *Pneumocystis* organisms were found by the IF method and the PCR technique could not detect any *P. jirovecii* DNA (Oz et al., 2000).

The differences in the PCR sensitivity values reported by different authors may be attributed to variations in the methodology (single or nested PCR), the cohort of the participants (HIV-infected or uninfected), specimens and collection, target gene (multicopy or single-copy), PCR primers and probe binding sites (Botterel et al., 2012). However, the use of rtPCR on easily obtainable non-invasive URT specimens, without the need for more difficult and hazardous bronchoscopy and biopsy can be a cost-effective way to provide a diagnosis of PCP as reported by Harris et al. (2011).

Correlating the rtPCR result with IF microscopy, despite using 3 different detection thresholds, all 21 IF microscopy positive samples were also rtPCR positive. The rtPCR detected a large number of additional positive results (amongst the IF-negative samples) (Table 2.3). These data suggest a higher sensitivity of PCR compared to IF as has been found in previous studies (Alli et al., 2012).

The results of our SYBR Green I rtPCR had an analytical sensitivity of 100% on all 3 different detection thresholds as compared to IF (gold standard). The large number of "false" positive results was reflected in the decreased specificity and PPV of our PCR assay. The low specificity values of 46% and 54.6% at thresholds of 1 and 1.5 respectively was expected, but it is much poorer than similar rtPCR experiments such as that of Larsen et al. (2004) which had a clinical sensitivity of 88% and a specificity value of 85% (albeit it on oral wash specimens), and Huggett et al. (2008) who found a sensitivity of 98% and a specificity of 96%. The specificity in our assay increased to 84.5% as we increased the detection threshold to 3 (Table 2.4), although this is still not ideal, with a positive predictive value of only 32%.

Of the 50 negative control samples used in this study, none of the samples tested positive for *P. jirovecii* DNA. This does suggest that the false positive results in our PCR assay may not be purely a result of colonization with *P. jirovecii*, and that a proportion of the positive PCR results, regardless of threshold, may reflect true *P. jirovecii* infection. However, this needs to be further investigated. The negative control group used in this study was not ideal and was chosen primarily for its convenience. In future, a negative control group that is prospectively enrolled and matched to suspected cases, with appropriate clinical outcomes described would be required.

Given its high sensitivity, low risk of contamination, ease of performance, speed and reproducibility, the SYBR green rtPCR has potential to replace IF as a diagnostic test for PCP, but the questions around specificity need to be better resolved using a more robust negative control cohort. The main drawback to PCR detection of *P. jirovecii* is the problem with the high analytical sensitivity, and the difficulties in distinguishing colonization from clinical disease.

The IFA method has been reported to be an imperfect gold standard reference. Using an imperfect gold standard reference often leads to misclassification of the disease status in a substantial portion of subjects, which can lead to biased estimates of index test performance

and disease prevalence. Therefore to evaluate the performance of the PCR assay, a composite reference standard should be considered. This composite reference standard would include the IFA results, but would also take into account the clinical and radiological presentation, response to cotrimoxazole, and presence or absence of alternative diagnoses. This ideally would require a prospective study which standardizes investigation and follow up of all participants, and if performed properly may improve the accuracy estimation of performance of the PCR test.

Although the foundation has been set for the use of rtPCR in routine diagnostic laboratories, some issues have to be addressed. In order for rtPCR to be diagnostically useful, further considerable optimizations are required. Additional prospective cohort studies should focus on standardization of quantitative PCR, and determine the cutoff of qPCR results. PCP PCR results should be interpreted in parallel with clinical manifestations, radiological and laboratory findings to exclude the issue of *Pneumocystis* colonization.

Further attention is required for implementation of a more cost-effective molecular testing in routine diagnostics. A more-rapid diagnosis can reduce number of hospitalization days, decrease risk of nosocomial spread, and decrease use of antibiotics.

Specific and sensitive results with reduced time turnaround are important in a clinical setting, and therefore, a PCR assay could improve patient management by appropriate therapy after rapid diagnosis of a pneumocystis infection.

CHAPTER 3

Molecular Epidemiology of *Pneumocystis jiroveci*

3.1. Introduction:

The study of *P. jirovecii* organism biodiversity has been hampered by the lack of a reliable culture-based system. Therefore, molecular methods are employed for typing, determining strain genotypes and to study the molecular epidemiology of the *Pneumocystis* organism (Beard et al., 2004; Miller et al., 2005).

The mtLSU rRNA, ITS and DHPS loci are frequently targeted. The mtLSU rRNA and ITS loci are considered to be highly informative, and have been used to analyze the clustering of *P. jirovecii*, the route of transmission, and association with severity of disease (Lu et al., 1994; Miller et al., 1999). The DHPS locus has been extensively studied in recent years because of the occurrence of point mutations at codons 55 and 57, which may be due to prior exposure to sulphonamides (Kazanjian et al., 1998). DHPS mutations have been associated with emergence of sulphonamide resistance and subsequent failure of treatment and prophylaxis (Navin et al., 2001; Huang et al., 2000), although these associations have not been consistently demonstrated (section 1.4.1).

The aim of this component of the study was to describe the molecular epidemiology of *P. jiroveci* in samples obtained from children and adult patients of a study population in Cape Town, South Africa. Multilocus genotyping was reported as an efficient system for *P. jirovecii* characterization (Esteves et al., 2008), and genotyping of the cohort was carried out by analysing the dihydropteroate synthase (DHPS) and the mitochondrial large subunit rRNA (mt LSU rRNA) loci.

3.2. Materials and methods

3.2.1. Study sample

For this study, we used 175 clinical samples confirmed as PCP positive by MSG real time PCR, as described in chapter 2. DNA was extracted using the easyMAG system (BioMérieux) as explained in section 2.2.5.

The samples consisted of 5 (2.9 %) broncheoalveolar lavage (BAL), 56 (32%) induced sputum (IS), 41(23.4%) nasopharyngeal aspirate (NPA), 70 (40%) tracheal aspirate and 3 (1.7%) unknown sample types.

3.2.2. Nested PCR for DHPS and mtLSU gene amplifications

3.2.2.1. DHPS gene amplification

Nested PCR of the DHPS gene was performed as previously described (Lane et al., 1997), using the primers shown in table 3.1. These nested primers amplify a 370bp fragment, and the primer binding positions are shown in figure 3.1

Table 3.1: Primers used for DHPS and mtLSU nested PCR assays

DHPS			
First	**F1 (5'- CCTGGTATTAAACCAGTTTTGCC-3')** **B45(5'-CAATTTAATAAATTTCTTTCCAAATAGCATC-3')**	**870**	**Lane et al., 1997**
Nested	**A (Hum)(5' GCGCCTACACATATTATGGCCATTTTAAATC-3')** **Bn(5'-GGAACTTTCAACTTGGCAACCAC -3')**	**370**	
mtLSU rRNA			
First	**pAZ 102 E (5'-GATGGCTGTTTCCAAGCCCA-3')** **pAZ 102 H (5'-GTGTACGTTGCAAAGTACTC-3')**	**260**	**Wakefield et al., 1990**
Nested	**pAZ 102X (5'-GTGAAATACAAATCGGACTAGG-3')** **pAZ 102Y (5'-TCACTTAATATTAA TTGGGGAGC-3')**	**267**	

The reaction mixture (applicable for both rounds of the PCR) contained KAPA Taq ReadyMix (1.25 U/µL KAPA HotStart Taq Polymerase, 0.2 mM dNTP's, 1.5 mM $MgCl_2$, 1x KAPA HotStart Buffer, 0.4 µM of each primer and 3 µL of template DNA, made up to 25 µL with nuclease-free water.

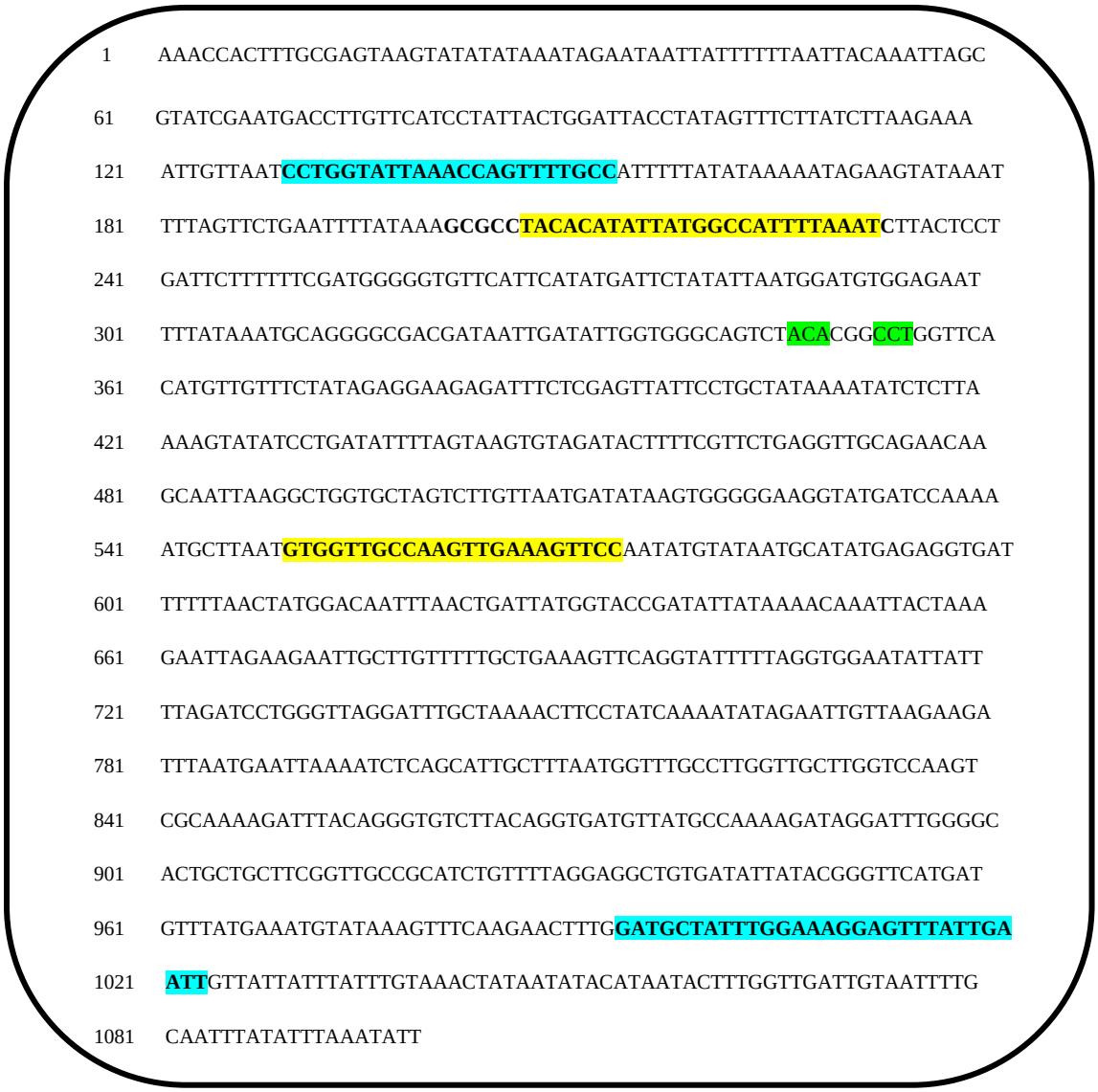


Figure 3.1: A segment of the *fas* gene showing primer positions flanking the outer region (shaded in blue), and nested primers flanking the inner region (shaded in yellow). The codons of amino acids 55 and 57 are shaded in green.

As part of the optimization process, different annealing temperatures for the 1st reaction of the nested PCR were used, ranging from 52°C to 58°C. Similarly, annealing temperatures ranging from 58°C to 65°C were used to optimize the 2nd reaction.

The cycling conditions for the first round were set to 94°C for 5 minutes, followed by 35 cycles at 92°C for 30 seconds, 58°C for 30 seconds and 72°C for 60 seconds with a final extension step at 72°C for 5 minutes. For the nested round, initial denaturation was also at 94°C for 5 minutes, followed by 35 cycles at 92°C for 30 seconds, 65°C for 30 seconds and

72°C for 60 seconds with a final extension step at 72°C for 5 minutes. Non-template controls were included in each batch of PCRs to control for contamination.

After successful completion of the initial runs, one of the amplicons was sent for DNA sequencing, confirmed as the *fas* gene target using the Basic Local Alignment and Sequencing Tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi) and used as a positive control for all subsequent PCR reactions.

3.2.2.2. mtLSUrRNA gene amplification

All MSG PCR positive samples were amplified targeting a region of the mtLSU rRNA using a nested PCR protocol. The reverse and forward primers used to amplify 267bp band are shown in table 3.1.

Amplification was performed in a total volume of 25 µl which contained KAPA Taq ReadyMix (1.25 U/µL KAPA HotStart Taq Polymerase, 0.2 mM dNTP's, 1.5 mM $MgCl_2$, 1x KAPA HotStart Buffer, 0.4 µM of each primer and 3 µL of template DNA (PCR positive PCP samples), with nuclease-free water. For the nested round PCR, 3µL of 10x diluted first-round product was added into the reaction mixture. PCR conditions for the first round PCR and nested PCR were maintained.

The thermo cycler settings from Gupta et al, 2009, were modified as follows: for the first round initial denaturation of 94°C for 5 minutes was followed by 35 cycles at 94°C for 3 minute, 62°C for 60 seconds and 72°C for 60 seconds. The final extension step was at 72°C for 5 minutes (Gupta et al., 2009). The cycling conditions for the second round of PCR were initial denaturation at 94°C for 5 minutes, followed by 35 cycles of 95°C for 60 seconds, 65°C for 60 seconds and 72°C for 60 seconds. The final extension step was at 72°C for 5 minutes.

After successful completion of the initial runs, one of the amplification products was sent for DNA sequencing, confirmed as mtLSUrRNA with the BLAST tool from Pubmed (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and used as a positive control for all subsequent PCR reactions. Non-template controls were included in each batch of PCRs to control for contamination.

3.2.3. Gel electrophoresis for DHPS and mtLSUrRNA

The DHPS and mtLSUrRNA nested PCR products of about 370 bp and 267 bp respectively, were detected by agarose gel electrophoresis with 10000 bp KAPA universal ladder or 100 bp plus DNA ladder (Thermo Scientific GeneRuler) for 35 min at 120V in 1.5% agarose gel, using 10µL PCR product loaded with 2 µL 6X fluorescent loading dye.

3.2.4. Sequence analysis of DHPS and mtLSUrRNA regions

All amplification products, from both assays, were submitted to the core DNA sequencing facility at the University of Stellenbosch for automated DNA sequence analysis. The recommended concentration (20ng/ µL) of each sample was sent with forward primers A (Hum) (3ng/ µL) to sequence DHPS, and pAZ 102-X (3ng/µL) to sequence the mtLSUrRNA. The DNA sequences obtained were edited and analyzed using BioEdit Sequence Alignment Editor (version 7.2.5). The DNA sequences were aligned with the corresponding reference genes in the NCBI GenBank database for the detection of mutations by using BLASTn (NCBI GenBank accession: for DHPS; AJ586567.1 and for mtLSUrRNA; JX855937.1). Insertions and deletions were not considered.

3.3. Results

Of the 175 samples positive by MSG real-time PCR using threshold of 1, a portion of the DHPS region in the *fas* gene was successfully amplified from 123 (70.3%) samples (figure 3.2). Despite at least 3 attempts to amplify the DHPS gene, amplification was unsuccessful in 52 samples and these were excluded from the study. Of the 52 excluded samples, 63.5% (33/52) were MSG PCR positive at detection thresholds of 1.5 compared to 36.5% (19/52) positive at a threshold of 3. This is similar to the proportion of DHPS positive samples that were MSG PCR positive at a threshold of 3 (p=NS).

The mtLSU gene was successfully amplified in 126 (72%) samples, while amplification failed in 49 samples despite at least 3 attempts at amplification. Of these 49 samples, 75.5% (37/49) were positive at MSG PCR detection thresholds of 1 and 1.5 while only 24.5% (12/49) samples were positive by all 3 (1,1.5 and 3) MSG PCR detection thresholds. Again, this was not significantly different from the proportion of mtLSU positive samples that were MSG PCR positive at a threshold of 3.

Samples from the lower respiratory tract would be expected to contain greater quantities of target organism, and thus one would expect a greater yield on lower respiratory tract samples. However, the success of PCR for the both DHPS and mtLSU rRNA did not differ substantially based on specimen type, as shown in table 3.2.

Table 3.2: Unsuccessful amplification of DHPS and mtLSU genes depending on the sample type

	DHPS PCR positive (n/%)	**mtLSU PCR positive (n/%)**
LRT (n=131)	95 (72, 5%)	93 (70, 9%)
URT (n=41)	26 (63, 4%)	30 (73, 1%)

The success rate for PCR amplification of the DHPS and mtLSU loci also depends on the primer hybridization sites on the target DNA, therefore mutations on the primer hybridization sites may have contributed to the unsuccessful amplification in some clinical samples. The nested PCR conditions used may also have influenced the amplification success.

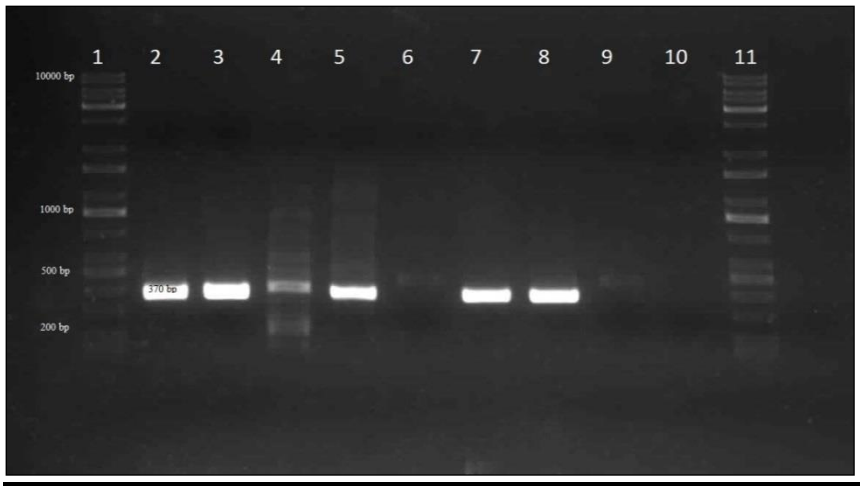


Figure 3.2: Agarose gel showing PCR-amplified 370 bp product of the *P. jirovecii* DHPS gene. Lanes 1and 11: 1000 bp KAPA universal ladder, lanes 2-5, 7-8 positive samples, lanes 6 and 9 negative samples and lane 10 PCR negative control

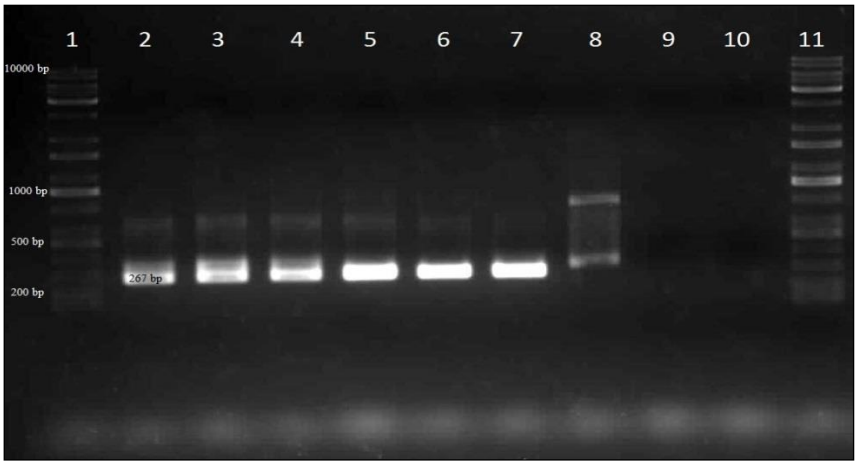


Figure 3.3: Agarose gel showing PCR -amplified 267 bp product of *P. jirovecii* mtLSU gene. Lanes 1 and 11: 1000 bp KAPA universal DNA ladder, Lane 2 positive control, Lanes 3-8 positive samples, Lane 9: Negative sample, Lane 10: Negative control

3.3.2. Sequence alignment and analysis of the DHPS gene

The sequencing analysis was performed on the DHPS genes from the successfully amplified samples. The DHPS nucleotide sequence inserts obtained after direct sequencing were aligned with a wild type DHPS reference sequence (NCBI GenBank accession: AJ586567.1). The determination of genotypes for DHPS is dependent on the amino acid substitutions at codon positions 55 and 57 as described previously (Huang et al., 2004).

Figure 3.3 illustrates an example of electropherograms of a typical mixed infection (mutations at both codon 55 and 57).

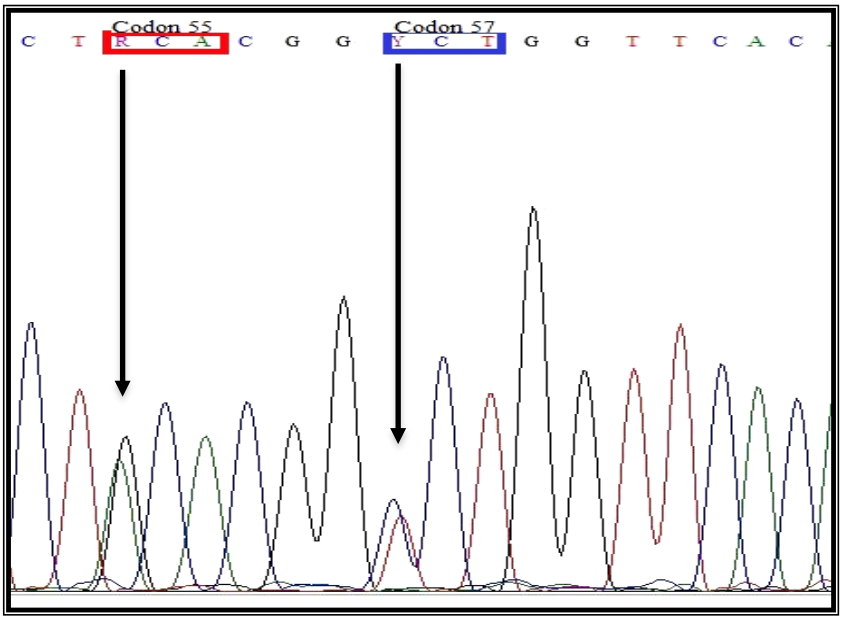


Figure 3.4: Electropherogram showing DHPS sequence data for a mixed genotype sample with double peaks, arrows pointing at indistinguishable nucleotide bases at both position 55 and 57 codons

In this study, the sequence variations were observed at codon positions 55 and 57. The DHPS gene mutation rate was 42% (52/123), and distribution of different genotypes are shown in table 3.3.

Table 3.3: *P. jirovecii* genotypes detected at the DHPS locus in 123 samples

DHPS Genotype	Nucleotide(amino-acid) position	No. of isolates	Percentage of Population (%)
1(Wt)	165 (55)/A (Thr); 171 (57)/C (Pro)	71	57.7
2	165 (55)/G (Ala); 171 (57)/C (Pro)	2	1.6
3	165 (55)/A (Thr); 171 (57)/T (Ser)	40	32.5
4	165 (55)/G (Ala); 171 (57)/T (Ser)	7	5.7
Mixed	-	3	2.4

3.3.3. Analysis of mtLSU rRNA locus using multiple sequence alignment

The mtLSU rRNA primers produced an amplification product of 267bp from 126 (72%) of the 175 clinical specimens.

By using BioEdit sequence alignment software, the mtLSU rRNA sequence inserts obtained after nested PCR and sequencing, were aligned with already known *P. jirovecii* mtLSU rRNA wild-type genotype sequence (NCBI GenBank accession JX855937.1).

In our analysis of 126 samples, the multiple sequence alignment showed variations in the mtLSU rRNA of *P. jirovecii* only at nucleotide position 85, while all strains had a cysteine nucleotide at position 248.

In the mtLSU rRNA gene, all the genotypes were determined on the basis of polymorphisms at nucleotide positions 85 and 248. The recorded mutation prevalence was 58% (73/126), and distribution of genotypes is shown in table 3.4.

Table 3.4: *P. jirovecii* mtLSU rRNA locus genotypes detected in 126 specimens

mtLSU rRNA Genotype	Nucleotide position/identity	No. of isolates	Percentage of population (%)
1	85C; 248C	53	42
2	85A; 248C	6	4.8
3	85T; 248C	64	50.8
4	85C; 248T	0	0
5	85A; 248T	0	0
Mixed	-	3	2.4

3.3.4. Correlation of DHPS genotypes with mtLSU genotypes

Both the DHPS and mtLSU genes were successfully amplified in 90 of 175 samples, while DHPS only was amplified from 33 samples, and mtLSU only from 36. The correlation of the DHPS genotypes with mtLSU genotypes was assessed in the 90 samples where both targets were amplified and noted in Table 3.5.

Table 3.5: Correlation of DHPS mutations with mtLSU genotypes

	DHPS genotype	
mtLSU genotype	Genotype 1(Wt)	Mutant genotypes
Genotype 1	21(23.3%)	14(15.6%)
Genotype 2	4(4.4%)	1(1.1%)
Genotype 3	27(30%)	20(22.2%)
Genotype 4	0	0
Genotype 5	0	0
Mixed	2(2.2%)	1(1.1%)

Using Fisher's exact test, there was no clear association between DHPS polymorphisms and mtLSU genotypes ($p = 0.921$).

3.3.5. Comparison of genotypes in samples from padiatric and adult patients

Our study population consisted of 155 pediatric (age<13 years) and 140 adult patients. The DHPS and mtLSU genes were succesfully amplified from 69 and 66 pediatric samples respectively. In adult patients, DHPS and mtLSU were successfully amplified from 50 and 60 samples respectively. The distribution of genotypes in adults and children is shown in Table 3.6(a) and (b).

Table 3.6: Distribution of DHPS (a) and mtLSU (b) genotypes between paediatrics and adult patients

	Age category	
DHPS genotype	**Adult**	**Pediatrics**
Genotype 1(**Wt**)	26(52%)	43(62.3%)
Genotype 2	2(4%)	0
Genotype 3	17(34%)	22(31.8%)
Genotype 4	3(6%)	3(4.3%)
Mixed	2(4%)	1(1.4%)

a) Comparison of DHPS genotypes distribution between pediatric and adult patients

	Age category	
mtLSU genotype	**Adult**	**Pediatric**
Genotype 1	24(40%)	30(45.5%)
Genotype 2	5(8.3%)	1(1.5%)
Genotype 3	29(48.3%)	34(51.5%)
Genotype 4	0	0
Genotype 5	0	0
Mixed	2(3.3%)	1(1.5%)

b) Comparison of mtLSU genotypes distribution between pediatric and adult patients

Using Fisher's exact test, there was no statistically significant difference in the distributions of either DHPS or mtLSU rRNA genotypes between adult and pediatric patients (p=0.4 and p=0.29 for DHPS and mtLSU rRNA respectively).

3.4. Discussion

The uncultivable nature of *P. jirovecii* complicates many research studies of the organism. This characteristic has made it difficult to study the genetic diversity, complexity, and evolution of this organism in humans. In addition, the genome sequence of *P. jirovecii* was only recently published (Alanio et al., 2016).

In recent years, mtLSU and DHPS genes have been of great interest for studying the genetic biodiversity of *P. jirovecii*. The mtLSU ribosomal RNA sequence data has been frequently used in detecting intraspecific differences between populations of diverse organisms, therefore useful for studies on molecular evolution in circulating strains (Beard et al., 2000).

The DHPS gene has been implicated in the emergence of antimicrobial resistance to anti-*Pneumocystis* drugs, because of the strong link between mutations at the gene level and sulfa exposure (Ma et al., 1999; Huang et al., 2000).

Through molecular epidemiological investigations, the prevalence of mutations in both mtLSU and DHPS genes have been reported from different parts of the world and the distribution of DHPS and mtLSU rRNA genotypes varies geographically. The aim of the present study was to describe the prevalence of DHPS and mtLSU genes mutations in Cape Town, South Africa. In this study the DHPS gene mutation prevalence was 42.3%, while the mutation prevalence for mtLSU was 58%.

3.4.1. DHPS mutation and genotype prevalence

The frequency of DHPS mutations among PCP patients may be reflection of exposure to sulfa medication, used either prophylactically against PCP or for treatment of other infections. Many studies have suggested that mutation at DHPS gene leads to drug resistance in *P.jirovecii*. However, the confirming evidence of DHPS mutations association with treatment failure is still lacking, since a number of studies have also shown contradictory results as mentioned in section 1.3.1.

Given the above interest in the relationship between DHPS and resistance, the prevalence of DHPS mutations has been reported from both developed and developing countries worldwide.

In developed countries, the DHPS mutation rates range widely, from 3.7% to 81% (Matos et al., 2010). A United States (San Francisco) study found a mutation prevalence of 81% in HIV-infected patients with PCP (Crothers et al., 2005). The DHPS mutation prevalences are reported in some of the European studies as 40% in Italy (Zingale et al., 2003), 27 % in Portugal (Costa et al., 2003), 20% in Sweden (Besser et al., 2011), 17% in Spain (Alvarez-Martinez et al., 2010) and 33 % in France (Rabodonirina et al., 2006).

Few studies have been performed in developing countries, and most of these report low prevalences of mutations in the DHPS gene. A study in Brazil showed no DHPS mutations (Wissmann et al., 2006). A similar result of no DHPS mutation was observed in a study conducted in China between 2007 and 2008 (Li et al., 2009). Other studies from China and India reported DHPS mutation prevalence of 7% and 4.1% respectively (Kazanjian et al., 2004; Tyagi et al., 2011). In a Colombian study, 7.7% of the samples studied had DHPS mutations (Muñoz et al., 2012). A recent study from Iran reported the frequency of mutation to be 14.7% (Sheikholeslami et al., 2015).

A low rate of DHPS mutations has also been observed from some centres in Africa. A study from 2003, based on a small sample size of 14 patients, reported a prevalence of *P. jirovecii* DHPS mutations in 7% of Zimbabwean HIV-infected adults with PCP (Miller et al., 2003). A study conducted by Zar et al. (2004) on 30 samples from HIV-Infected South African children with *P. jiroveci* pneumonia, found a DHPS mutation prevalence of 13.3%. Another study by Robert et al. (2005) conducted in South Africa, reported a DHPS mutation rate of 8%. In 2008, Govender et al. (2008) screened 45 sputum samples obtained in Port Elizabeth for the presence of *P. jirovecii* DHPS mutations, and detected no resistance associated mutations.

It was assumed that the low prevalence of DHPS mutations in *P. jirovecii* from developing countries was a result of limited availability of sulfa drug prophylaxis against PCP. More recently however, higher prevalence rate of DHPS mutations were reported in South Africa. In 2010, a South African study by Dini et al. (2010) reported a mutation prevalence of about 56%. In addition, a recent study by Mogoye et al. (2015) in the Gauteng province reported an overall prevalence of 87% for DHPS mutations from 671 characterized samples.

These higher rates of DHPS mutation are more in keeping with our results, where we found a mutation rate of 42.3% in the DHPS gene, which includes any non-synonymous mutations. A similar study conducted in our division in 2013 found a *P. jirovecii* DHPS mutation prevalence of 26.7% from 30 clinical samples collected from August to October 2013. The 2 studies had similar patient characteristics. However, given the small sample size of the 2013 study, we cannot state unequivocally that there has been an increase in DHPS mutation.

In the present study, the DHPS locus revealed that the frequency of the wild-type genotype was higher than that of the mutant. This is consistent with the results reported from most other studies carried out across the world (van Hal et al., 2009; Larsen et al., 1999; Zar et al., 2004; Robert et al., 2004).

A comparable study conducted by Esteeves et al. (2008) on Spanish and Portuguese samples, showed that the wild- type genotype was the predominant genotype, in 93% and 78% of Portuguese and Spanish samples analyzed, respectively. A recent study by Kim et al. (2015) in Korea reported 100% of wild-type *P. jirovecii* DHPS genotype distribution in 95 samples analyzed.

In our study, among the mutant genotypes, genotype 3(32.5%) was the most common genotype, followed by genotype 4 (5.7%) and genotype 2(1.6%). This was consistent with a previous South African study (Dini et al. 2010), conducted in 2006 and 2007, where genotype 3 was the most common mutant genotype, in 18% of the analysed samples.
A mixed genotype was only detected in 2.4% of our samples. This is comparable to the overall results reported on *P. jirovecii* DHPS genotypes in different countries.
A *P. jrovecii* DHPS genotypes analysis by Montes-Cano et al. (2004) in Spain revealed that 64.5% of their isolates were wild-type. Genotypes 2, 3 and 4 occurred in 12.9%, 9.6% and 3.2% of isolates, respectively; while 9.6% of the 31 cases analyzed showed a mixed genotype. A similar study in Cuban children observed 82% wild-type genotype, 12% for both genotypes 2 and 3, while only 6% contained mixed genotype from the 16 samples ananlyzed for DHPS mutations (Monroy-Vaca et al., 2014).

Detection of mixed infections or multiple genotypes in a single sample suggests the presence of genetically different strains in the same host. This is an important issue for interpretation

of *P. jirovecii* transmission dynamics data (Hauser et al., 2001; Esteves et al., 2008, 2010b). The analysis of complex single stranded conformation polymorphism patterns shows the presence of several alleles in a single specimen of the mixed genotypes (Nahimana et al., 2000). This reveals that patients may become reinfected with new genotypes or coinfected with different genotypes of the organism during a single episode of pneumonia (Helweg-Larsen et al., 2001).

Differences in mutant prevalence in one city compared to another may be related to the difference of sulfonamide selection pressure and levels of *P. jirovecii* circulation within human communities (Le Gal et al., 2012; Totet et al., 2004).

3.4.2. mtLSU rRNA genotype prevalence

The present study was conducted to determine the genotype distribution of *P. jirovecii* in our population by sequence analysis of the mt LSU gene. Based on polymorphisms at 2 nucleotide positions of 85 and 248, our study identified a high frequency of genotype 3 (85A/85C) with a rate of 50.8%. Our findings are similar to the results obtained from a Zimbabwean study where genotype 3 accounted for 57% of their population (Miller et al., 2003). Genotype 3 was also the most frequent type in immunosuppressed patients in Cuba as well as in Northern Ireland (de Armas et al., 2012; Curran et al., 2013), whereas in contrast, it has been less frequently observed in other regions of the world. It was the second most common genotype as reported by Gupta et al. (2011) in an Indian study.

Most of the studies from Europe report genotype 1 as the most prevalent type (Miller et al., 2005; Montes-Cano et al., 2004; Esteves et al., 2008). In one comparison study conducted in Portugal and Spain, the distribution of genotypes showed that Genotype 1 was the most predominant in both groups of samples (49% in Portugal and 64% in Spain). Similarly, a recent study from Australia showed that genotype 1 accounted for 87% of all types (van Hal et al., 2009). However, in our study, genotype 1 was the second most common and accounted for 42.1% of all samples.

In our study genotype 2 accounted for only 4.8%; however, a study conducted in India showed that Genotype 2 was most common accounting for 42% of all samples (Gupta et al, 2011) and it was reported as a most common genotype among the immunocompromised patients in France and Italy (de Armas et al., 2012; Dimonte et al., 2013; Maitte et al., 2013).

The absence or low frequency of genotype 4 and 5 was reported in most of the studies, our study observed neither genotype 4 nor genotype 5, and Gupta et al. (2011) also reported genotype 4 to be the least frequent genotype in an Indian study with a frequency of 8%. Genotype 5 was reported as an infrequent type in Portugal and Japan (Esteves et al., 2010; Matsumura et al., 2011).

Mixed infections accounted for 2.4% of all our samples, similar to the DHPS findings. These mixed infections (with more than one genotype in a sample) in *Pneumocystis* have been reported by several authors (Hauser et al., 2001; Beard et al., 2000; Miller et al., 2003). Hauser et al. (2001) reported mixed infection with 2 types in 50% of their isolates. In the USA, Spain and India the mixed genotypes were reported as 10.2%, 3.7% and 6% respectively (Beard et al., 2000; Montes-Cano et al., 2004; Gupta at al., 2011). The samples with mtLSU mixed genotypes, probably contains two different *P. jirovecii* strains or a single *P. jirovecii* strain containing more than one mitochondrion per organism (with genotypic variations between the copies) (Dimonte et al., 2013).

The different prevalence rates of *P. jirovecii* genotypes observed in different parts of the world indicate that the epidemiological factors inherent to a particular area (geographical and climate characteristics) may influence the distribution, circulation, and transmission of different *P. jirovecii* types.

CHAPTER 4

General conclusion

4.1. Conclusions

The results of this study revealed that real time PCR is more sensitive in detecting *P. jirovecii* than the current gold standard IF method. The SYBR green PCR improved the detection of *P. jirovecii*; especially in URT samples, compared to IF and this is consistent with previous studies. Our results suggest that rtPCR may be able to replace IF for diagnostic purposes. The ability of rtPCR to detect *P. jirovecii* in URT samples with high sensitivity, offers a good diagnostic technique as this will reduce the risk of complications associated with the use of bronchoscopy and biopsy, which requires specialiazed personel and equipment for sample collection, especially in children and critically ill adults.

The high analytical sensitivity of the PCR has been a concern, as PCR may detect *P. jirovecii* DNA in asymptomatic individuals without PCP. Therefore, the rtPCR methods must be further evaluated in order to set an accurate quantitative cut-off value which will be able to discriminate PCP from asymptomatic colonization. However, in our study we tested 50 samples from patients with suspected respiratory illness other than PCP, as our negative control group, of which no samples tested positive for *P. jirovecii* DNA.

After further validation and evaluation rtPCR has a potential to replace microscopic methods, and to become the gold standard method for diagnosis of PCP, especially in high burden HIV and resource limited settings.

For our molecular epidemiology study, the targeted genes were successfully amplified in 123 (70.3%) samples for DHPS; and 126 (72%) mtLSU rRNA samples. The DHPS mutation prevalence increased to 42% from the 27% recorded from a previous study in this setting in 2013. The increased DHPS mutation rate in this study may be a result of on-going sulfa-drug use, for prophylaxis or treatment of PCP or other infections. The need to continue efforts to estimate the true burden of PCP in South Africa must be emphasised. In 126 samples analyzed for the mtLSU gene, a mutation prevalence of 58% was observed. The high frequency of mtLSUrRNA polymorphisms among PCP patients in our study population may be important for phylogenetic or population genetic models to elucidate *P. jirovecii* interspecies strain relatedness.

4.2. Limitations of the study

There are several limitations to this study. First, it is the lack of the universal standardized limit of detection for *P. jirovecii.* The variability of the target genes and unit expressions used for detection described in research papers makes it problematic or impossible to compare the sensitivities and specifities of the different methods.

Secondly, the on-going debate of standardized cut-off values determination for quantitative real time PCR is problematic. We did not quantify the *P. jirovecii* burden of our clinical samples; therefore, we cannot comment on whether organism load was related to the difficulties in amplifying DHPS or mtLSU in some clinical samples. However, by using the 3 different detection thresholds, it was observed that more samples which were only positive by 1 and 1.5 MSG PCR detection thresholds, failed to amplify for both DHPS and mtLSU genes, compared to samples which were positive on all 3 (1,1.5 and 3) MSG PCR detection thresholds. The samples positive by all 3 detection thresholds were more likely to be successfully amplified, as the higher fluorescent signal above the baseline may correlate to the higher concentration of the initial template in the samples.

Thirdly, our SYBR Green I rtPCR assay specificity was affected due to lack of a universally accepted definition for the diagnosis of PCP, and we were unable to distinguish between colonization and clinical significant disease. We attempted to use a negative control cohort to better understand the specificity of our assay; however, this negative control group was a convenience sample, and a more robust prospective control cohort is required.

4.3. Future studies

Based on results of this study, further studies are required to; 1) evaluate, validate and implement qPCR for the detection of *P. jirovecii*; and 2) improve understanding of the current molecular epidemiology of *P. jirovecii* in the Western Cape and countrywide, including in low- or middle-income communities.

At present, PCP can occur with pathogen densities below the detection limit of microscopic visualization methods. Therefore, an easy to use and highly sensitive technique such as real time PCR is required. The future studies for implementation of molecular detection methods;

need to include short turnaround times without losing sensitivity and/or specificity for the optimal integration of molecular technologies in clinical care.

Future prospective studies are needed to investigate whether quantitative PCR results can be employed to differentiate between clinical significant PCP and colonization of *P. jirovecii*.
In the future studies to estimate the accuracy of the PCR test, the use of a composite reference standard (IF results, clinical information and patient's response to cotrimoxazole) should be considered, since the IFA method is an imperfect gold standard.
The outcome of a molecular test should, as with every diagnostic method, be translated into clinical signs and symptoms of the patient in order to determine whether a positive test clarifies the aetiology of the illness of the patient.

The standardization of the PCR methodology for the detection of PCP is required, to enable comparison of results between different laboratories and the potential for establishing a computer database of results. This can be achieved by monitoring the performance of the different in-house PCR assays currently used for the detection of PCP in microbiology laboratories; and the availability of quality control panels is of utmost importance. The similar assays described by different researchers could serve as reference assays in the development and maintenance of such panels.

In our setting, further studies are also required to better describe the local strain diversity, using additional targets such as the internal transcribed spacer regions (ITS). If possible, ITS based phylogenetic analysis can be constructed for the strains of *P. jirovecii* found in Cape Town in order to epidemiologically study the divergences and correlations between strains worldwide (Hsueh et al., 2001; Marcilla et al., 2002).

The mutation prevalence data based on the different targeted genes need to be further linked to clinical data to better understand transmission dynamics and the potential impact of strain variation on clinical outcome. Since DHPS gene mutations at codon positions 55 and 57 have been correlated with prior sulfonamide usage (Totet et al., 2004), patients' medical histories will be needed to further investigate whether the presence of mutations in this gene are associated with disease severity, prior TMP-SMX exposure or treatment failure.

There are many potential difficulties in achieving widespread implementation of PCR for PCP research, including cost and the need for specialized molecular laboratories, equipment,

and personnel. Therefore, more data is needed to overcome current questions regarding costs and clinical value of PCR for PCP research in clinical microbiology.

APPENDICES

APPENDIX A: Hydrolysis probe details

Hydrolysis probe

The hydrolyis probe was designed to anneal to the conserved region of *P.jirovecii* genome. . For MSG detection, the following probe was commercially synthesized (Integrated DNA Technologies) and labelled with fluorophores: 23-bp oligonucleotide hybridization modified probe with HEX on its 5', a ZEN internal quencher and Iowa Black FQ (IBFQ) on its 3'.

(5'-6-HEX/TGATGAAGG/ZEN/TCAGCCAGCCTATGC/ IBFQ -3')

The two non-emissive (dark) quenching molecules incorporated into the detection probes include the Iowa black fluorescein quencher (IBFQ; absorption max. =531nm) and ZEN (non-abbreviation; absorption max 532 nm). The fluorescent label utilized was HEX (HEX: hexachloro-6-carboxyfluoroscein; excitation max. =535 nm, emission max. =556 nm).

APPENDIX B: Agarose gel electrophoresis details

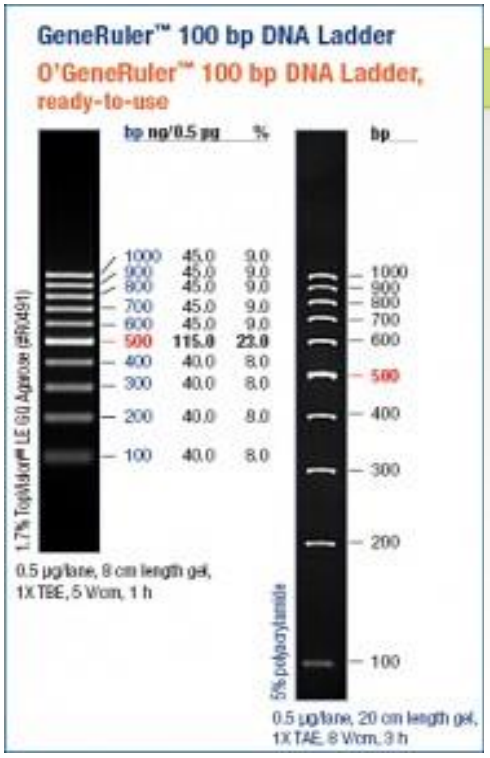
Agarose gel

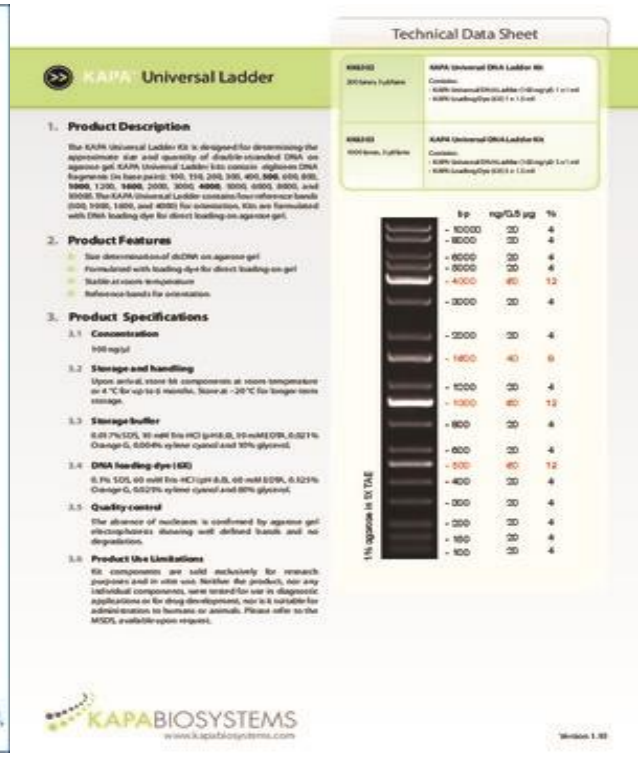
The 1.5 % gel is prepared by adding approximately 100 mL of 1x TBE buffer to 1.5 g of SeaKem LE Agarose powder (Lonza). The mixture is heated in a microwave until the powder is dissolved and the fluid level is at 100 mL. The mixture is carefully poured into a gel template, and combs are inserted to create wells. Any bubbles in the gel can be removed with a pipette tip. After approximately thirty minutes, the gel solidifies sufficiently to remove the combs. The gel, still on its template, is then placed into the BioRad gel chamber and covered with 1x TBE buffer, before samples are loaded into the wells. DNA is negatively charged, and will therefore move through pores in the wells toward the positive electrode. Smaller fragments move further and ladders with known fragment sizes can therefore be used to estimate size and confirm a product.

APPENDIX C: Molecular weight ladder and loading dye details

Molecular weight ladder

Thermo Scientific Fermentas O'GeneRuler 100bp Plus and KAPA 1000bp universal DNA Ladders were used for size reference purposes during all gel electrophoresis runs.





Loading dye

Novel Juice is manufactures by GeneDirex and is a non-mutagenic alternative to Ethidium-Bromide staining. It is used in the same way as a loading dye and is therefore timesaving and safer. It is supplied in 1 mL vials and contains three dyes for visible tracking of DNA migration. Signal is detected by UV light.

APPENDIX D: Patient details

Patients' characteristics

(n =305)

Female 155

Male 143

Mean age for participants was 18 years

Median age was 2.58 years

Inter quartile range (IQR) (0.25-35) years

Participants age	No. of samples (%)
<3m	56 (18.4%)
3m -2years	80 (26.2%)
3-5 years	7 (2.3%)
6-10 years	8 (2.6%)
11-20 years	5(1.6%)
21-30 years	25 (8.2%)
31-40 years	55 (18%)
41_50 years	37(12%)
51-60 years	13(4.3%)
61-80 years	2(0.66%)

APPENDIX E: Detect IF Pneumocystis carinni KIT

AXIS-SHIELD

DETECT IF

Pneumocystis carinii

IVD

Σ 60

REF FIPC200

CE

FOR PROFESSIONAL USE ONLY

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ENGLISH INTENDED USE

The Detect IF *Pneumocystis carinii* (*P. carinii*) test is an indirect qualitative immunofluorescence kit for the detection of *P. carinii* oocysts in human bronchoalveolar lavage fluid and induced sputum. It is intended to aid in the diagnosis of suspected *P. carinii* infection. Results should be interpreted in light of all clinical and diagnostic information.

INTRODUCTION

Pneumocystis carinii is a eukaryotic microorganism of uncertain taxonomy. Recent ribosomal RNA homology studies have shown identical nucleic acid sequences with some fungi, but classification of the organism is still the subject of discussion.[1]

P. carinii is ubiquitous, infecting man and other mammals; the route of infection is presumed to be airborne.[1] It is a major pathogen in the immunocompromised, especially patients with AIDS[2,3] where it is an established cause of pulmonary infection. Starvation, haematological malignancies, collagen vascular diseases, primary cellular immune deficiency and immunosuppressive therapy, for example in transplant patients and leukaemic patients on cytotoxic drugs are factors that increase the likelihood of infection with *P. carinii* pneumonia.

Onset of *P. carinii* pneumonia may be apparently rapid or occur insidiously. When clinically evident, features are increased respiration rate and spiking fever. Chest films show a diffuse infiltrate; pulmonary function tests show alveolar-capillary block resulting from impaired gas exchange in alveoli, causing hypoxaemia and hypercapnia.

Currently, *P. carinii* pneumonia may be diagnosed by the observation of *P. carinii* in either open lung or transbronchial lung biopsy material, bronchoalveolar lavage[4,5] or induced sputum. It can be visualised with a variety of non-specific stains including Gomori methenamine silver, toluidine blue-O, Gram-Weigert, Giemsa and Wright-Giemsa. Because all these stains react with yeasts and other structures, *P. carinii* must be distinguished on the basis of morphology. Staining techniques are time consuming and often require a high level of technical expertise in the interpretation of results. Monoclonal antibodies specific for *P. carinii* oocysts have become available, allowing the development of immunofluorescent techniques to rapidly and unambiguously identify *P. carinii* oocysts in bronchoalveolar material[6,7] and induced sputum.[8,9] The Detect IF *P. carinii* test uses a murine monoclonal antibody reactive with both human and rodent *P. carinii* in a simple and rapid test for the detection and identification of *P. carinii* in human bronchoalveolar lavage fluid (BAL) and induced sputum (IS).

PRINCIPLE OF THE ASSAY

Bronchoalveolar lavage fluid or pre-treated induced sputum specimens are centrifuged and washed. The pellets are resuspended, placed on slides and fixed. The specimens are Enzyme-digested. Murine anti-*P. carinii* antibody and fluorescently labelled anti-mouse antibody are added in turn after incubation, rinsing, wicking, and air-drying steps. On viewing with a fluorescence microscope, oocysts show as medium bright to bright apple green and may be evenly or unevenly labelled. The presence of *P. carinii* oocysts in bronchoalveolar lavage fluid or induced sputum indicates *P. carinii* infection.

KIT COMPONENTS

REAG A	Anti-*P. carinii* Monoclonal Antibody	1 × 1 mL	Murine anti-*P. carinii* monoclonal antibody, bovine serum albumin, 0.1% (w/v) sodium azide. **Ready-to-use.**
REAG B	FITC-Conjugated Anti-Mouse Antibody	1 × 1 mL	Fluorescein-isothiocyanate (FITC) conjugated anti-mouse antibody, Evans Blue counterstain. **Ready-to-use.**
REAG C	Enzyme (Lyophilised)	1 vial	Pre-treatment enzyme for clinical specimens. **Reconstitute with 200 µL 0.001M HCl (supplied) and dilute before use.** N.B. DANGER
REAG D	Dilute Hydrochloric Acid (0.001M HCl)	1 × 0.5 mL	For Enzyme reconstitution. **Ready-to-use.**
REAG E	Enzyme Diluent	1 × 3 mL	Tris buffer with enzyme activator. **Ready-to-use.**
SPCM SLD	Patient Specimen Slides	25 slides	PTFE-coated (yellow) slides with four square specimen wells.
REAG F	Mounting Medium	2 × 3 mL	Phosphate-buffered glycerol, Citifluor photobleaching retardant. **Ready-to-use.**

STORAGE OF REAGENTS

Handling and Procedural Notes

1. Store kit components at 2-8°C and use until the expiry date on the labels. Do not use expired reagents.
2. Do not mix different kits.
3. Do not freeze kits.
4. The lyophilised Enzyme must be reconstituted before use, see Preparation for the Assay section. All other reagents are ready-to-use.
5. After reconstitution with 200 µL 0.001M HCl, the lyophilised Enzyme is stable for up to 3 months from the date of reconstitution if stored at 2-8°C .
6. Do not expose Mounting Medium to direct light during storage. Store at 2-8°C or at 18-25°C.
7. Patient Specimen Slides can be stored at 18-25°C.
8. A precipitate may form in the Enzyme Diluent. Should this occur do not try to redissolve it, there is no detrimental effect on the efficacy of the test.
9. Avoid contamination of reagents. Use a new disposable pipette tip for each reagent or sample manipulation.

Specimen Collection, Storage and Pre-treatment

The assay is for use with human bronchoalveolar lavage and induced sputum specimens. Ideally, up to 30 mL bronchoalveolar lavage and 2-4 mL induced sputum should be collected into sterile vessels by appropriate procedures, and tested as soon as possible after collection. To inactivate any human immunodeficiency virus that may be present, it is strongly advised that the suspension of clinical material is diluted with an equal volume of absolute ethanol and incubated for ten minutes at room temperature (18-25°C) before processing. Dispose of waste materials in accordance with local regulations.

Sputum specimens should be pre-treated (homogenisation or incubation) by the addition of "Sputasol", "Sputolysin" or similar mucolytic agent for ten minutes at room temperature (18-25°C) before assay.

WARNINGS AND PRECAUTIONS

For *in vitro* diagnostic use only.

Safety Precautions

1. Adhere strictly to the instructions in this booklet, particularly for handling and storage conditions for kit reagents and clinical samples.
2. All patient samples should be considered potentially infectious and handled with the same precautions as any other potentially biohazardous material. The CDC/NIH Health Manual "Biosafety in Microbiological and Biomedical Laboratories", 5th Edition, 2007, describes how these materials should be handled in accordance with Good Laboratory Practice.[14]
3. Do not pipette by mouth.
4. Do not smoke, eat, drink or apply cosmetics in areas where kits and samples are handled.
5. Any skin complaints, cuts, abrasions and other skin lesions should be suitably protected.
6. The Anti-*P. carinii* Antibody and FITC-Conjugate contain sodium azide which can react with lead and copper plumbing to form highly explosive metal azides. On disposal, drain with large quantities of water to prevent azide build-up.
7. Material safety data sheets for all hazardous components contained in this kit are available on request from Axis-Shield Diagnostics.

Reagent A	EUH032 -	Contact with acids liberates very toxic gases.
DANGER **Reagent C**	**WARNING** H334 -	May cause allergy or asthma symptoms or breathing difficulties if inhaled.
	PREVENTION P261 -	Avoid breathing dust/fumes/gas /mist/vapours/spray.
	P285 -	In case of inadequate ventilation wear respiratory protection.
	RESPONSE P304+341 -	**IF INHALED:** If breathing is difficult, remove victim to fresh air and keep at rest in a position comfortable for breathing
	P342+311 -	If experiencing respiratory symptoms: Call a POISON CENTER or doctor/ Physician.

PREPARATION

Materials/Equipment Required but not Provided

1. Precision pipettes to dispense 5 µL, 15 µL, 20 µL and 200 µL.
2. Centrifuge for volumes up to approximately 30 mL at 3,000 x g.
3. Distilled/ultrapure water.
4. Wash bottle containing distilled/ultrapure water.
5. Analar or equivalent grade acetone.
6. Incubator at 37°C.
7. Humidified slide incubation chamber at 37°C.
8. Microscope coverslips, 18 x 18 mm and 50 x 20 mm.
9. Ultraviolet microscope equipped for viewing fluorescein and Evans Blue fluorescence.
10. Cytospin (e.g. Cytospin 2), optional.
11. Timer for 5 to 30 minutes.
12. Wicking/tissue material.
13. Appropriate mucolytic agent for induced sputum specimens, e.g. Sputolysin (Behring Diagnostic) or Sputasol (Oxoid). Use as recommended by the manufacturer; alternatively, use 0.1% Dithiothreitol (w/v) solution in a 1:1 ratio with specimen volume and incubate at 37°C for as long as required.
    N.B. Dithiothreitol can irritate eyes and skin. If contact with skin or eyes occurs irrigate with water for at least 10 minutes. If discomfort exists, seek medical attention.

Preparation for the Assay

Allow all reagents to equilibrate to room temperature.

Reconstitute lyophilised Enzyme with 200 µL 0.001M HCl; this results in a 10X concentrate. Record the reconstitution date on the label and allow to stand at room temperature (18-25°C) for ten minutes. Mix gently by inversion, ensuring all particulate material is in solution. Reconstituted enzyme is stable for 3 months at 2-8°C.

ASSAY PROTOCOL

Pre-treatment of Specimens

Patient specimens should be tested as soon as possible after collection. When performing an assay be aware of the potential HIV status of specimens and take all the recommended precautions for dealing with such specimens.

Induced sputum specimens should be pre-treated with a mucolytic agent, e.g. Sputasol. Non-mucoid specimens such as BAL will normally not require the mucolytic procedure.

Protocol

1. Centrifuge specimens for 15 minutes at 3,000 x g, and wash the particulate/pelletable material in distilled/ultrapure water. Repeat once or twice, ensuring the pellet is fully resuspended between washes.
2. Resuspend the final pellet in a small amount of distilled/ultrapure water, such that the density of the material is not excessive, and vortex.
3. Spread 10-20 µL over the entire area of one or more Patient Specimen Slide wells. Evaporate to dryness at 37°C. If a Cytospin (e.g. Cytospin 2) is available, spin 0.4 to 0.5 mL BAL or IS at 900 rpm, using one white and one tan filter.
4. Fix specimens by overlaying 1-2 drops Analar (or equivalent quality) acetone. Allow to evaporate at room temperature.
5. **Rinse Cytospin preparations with a stream of distilled/ultrapure water to remove salts from the specimen, as they reduce the efficacy of enzyme digestion.**
6. Air-dry slides.
7. **Dilute** the reconstituted Enzyme **1 in 10** (1+9) **with Enzyme Diluent.** Dilute only enough reconstituted Enzyme for immediate requirements.
8. Overlay dried and fixed specimens with **20 µL** diluted Enzyme. Ensure the entire well area is covered by reagent.
9. Incubate slides for EXACTLY 30 minutes in a humidified chamber set at 37°C. Over-digestion of oocysts will result if incubation is continued for more than 30 minutes. Oocysts may become less characteristic and less readily identifiable.
10. Rinse slides with distilled/ultrapure water by running a stream of water over the surface of the wells. Do not direct the jet directly at the specimen.
11. Wick and air-dry the slides.

12. Add **15 µL** anti-*P. carinii* Antibody to specimens. Ensure that the entire well area is covered by reagent. Incubate in a humidified chamber for 15 minutes at 37°C.
13. Rinse wells as described in step 10, wick and air-dry.
14. Add **15 µL** FITC-Conjugated Anti-Mouse Antibody to specimens. Ensure that the entire well area is covered by reagent. Incubate in a humidified chamber for 15 minutes at 37°C.
15. Rinse wells, wick and air-dry.
16. Place a drop of Mounting Medium onto every well in use and apply a coverslip of appropriate size. Invert the slide on an absorbent tissue and gently press to exclude excess Mounting Medium and air bubbles.
17. Examine specimens for bright to medium bright apple-green oocysts, which may be evenly or unevenly labelled. Cellular debris and other material may be counterstained with Evans Blue, which will fluoresce red. Examine the entire specimen area.

INTERPRETATION OF RESULTS

POSITIVE RESULT - Five or more fluorescent oocysts over the whole slide.

EQUIVOCAL RESULT - One to five fluorescent oocysts.

NEGATIVE RESULT - No fluorescent oocysts. If *P. carinii* infection is still suspected, repeat the assay with a heavier inoculum.

PERFORMANCE DATA

CENTRE 1[10]

223 BAL and IS specimens from HIV patients with respiratory tract symptoms were evaluated and compared with modified Grocott stain. Overall agreement = 90.6%.

Of 21 (9.4%) discrepant results, six subsequent specimens were obtained, and five out of six results were positive by both tests.

CENTRE 2[8]

135 IS specimens were evaluated and compared with Grocott stain. Overall agreement = 88.9%.

Fifteen results (11.1%) were Detect IF *P. carinii* positive/equivocal and Grocott negative. The authors concluded that this indicates an increased sensitivity for *P. carinii* in cytological preparations of IS with the immunofluorescence technique compared to conventional stains.

CENTRE 3[9]

254 BAL and IS specimens from 75 patients with AIDS, other immunocompromised patients, including transplant patients, and patients diagnosed as 'atypical pneumonia' were evaluated and compared with Grocott stain. Overall agreement = 94.1%.

Fifteen results (5.9%) were Detect IF *P. carinii* positive/equivocal and Grocott negative. The authors concluded that the Detect IF test was more reliable and sensitive than the Grocott technique.

CENTRE 4[6]

50 BAL and 50 IS specimens were tested for *P. carinii* infection using indirect immunofluorescence, direct immunofluorescence, modified Wright-Giemsa stain and modified silver stain. A positive specimen was defined as any smear which was positive by two or more methods.

Using this definition, the sensitivity and specificity of DETECT IF *P. carinii* were as follows.

BAL Sensitivity = 86% Specificity = 100%

IS Sensitivity = 97% Specificity = 100%

CENTRE 5

152 BAL specimens from patients with clinical evidence of *P. carinii* pneumonia were evaluated and compared with Grocott stain. Results for each method were compared to the clinical evidence of *P. carinii* pneumonia (PCP). In five cases, the clinical evidence result was equivocal; four were Detect IF positive and Grocott negative, one was Grocott positive and Detect IF negative.

Overall agreement of DETECT IF with clinical evidence of PCP = $\frac{146}{147}$ = 99.3% (one result was equivocal with IF)

Overall agreement of Grocott stain with clinical evidence of PCP = $\frac{140}{147}$ = 95.2%

Overall agreement of DETECT IF with Grocott = 94.5%

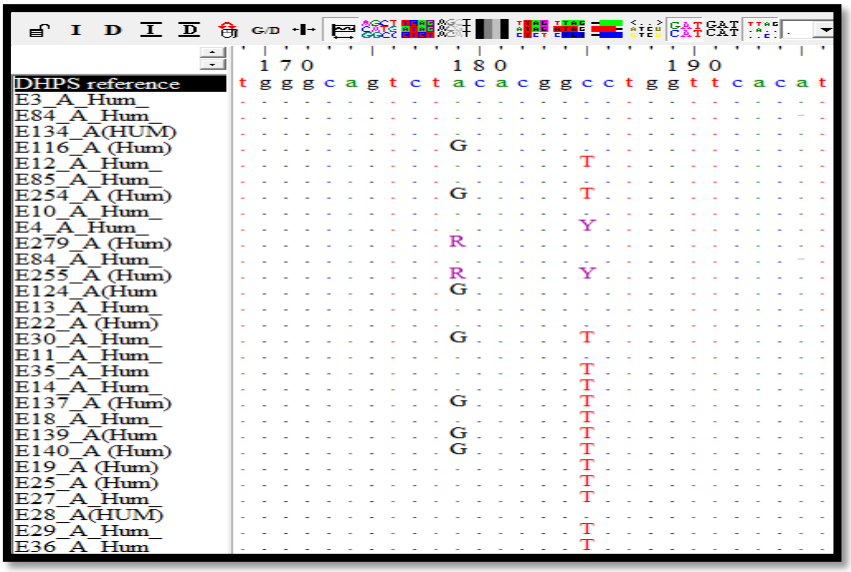
LIMITATIONS OF USE

1. A negative result does not exclude the possibility of *P. carinii* infection. Results should be interpreted in light of all clinical and diagnostic information. If necessary, obtain a further specimen.
2. Excess mucous in specimens may prevent adequate staining.
3. The FITC-conjugated anti-mouse antibody has the potential to cross-react with *Candida albicans* when present in patient samples which can be misinterpreted as false positive results.[15]

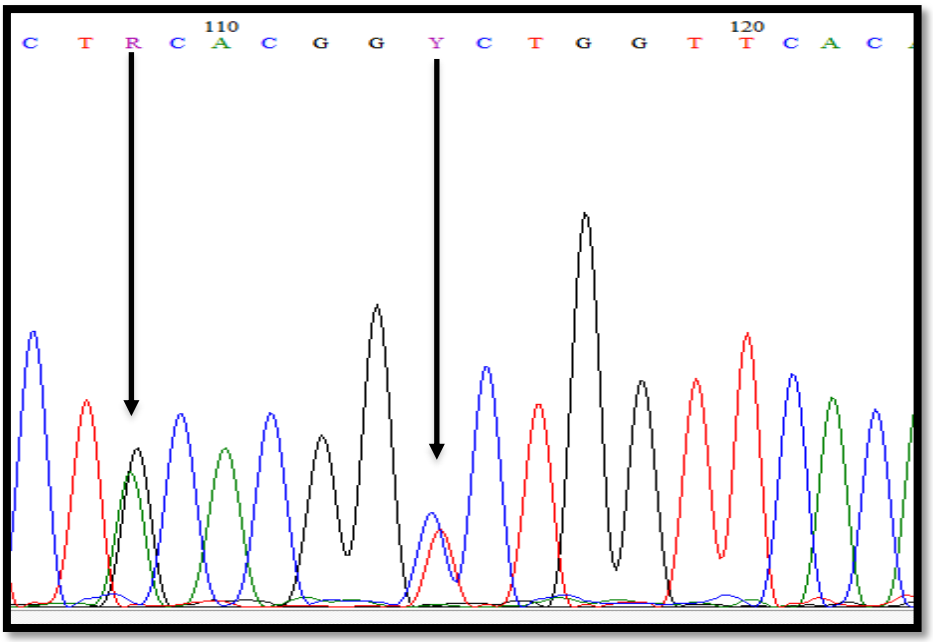
APPENDIX F: Sequence alignments

Sequence alignments

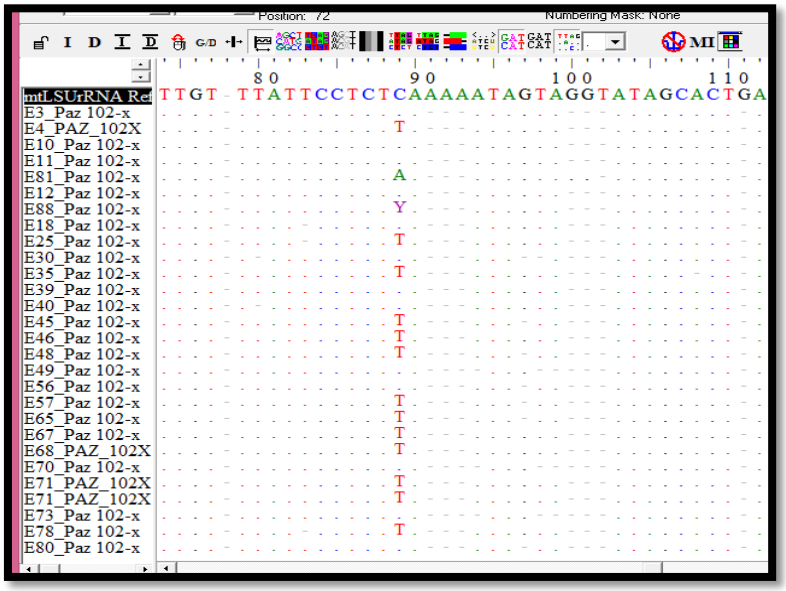
The figures below show sequencing results, converted to alignments to verify fragments against the reference gene (wild type) and control (first two rows). The dots underneath the reference sequence indicate identical nucleotides. If the the nucleotide at a certain position differs from the reference sequence, its symbol (A, T, G or C) is given. When results are inconclusive (more than two bases are amplified at that position) and R or Y is indicated. To verify which nucleotides were amplified at these positions (possible mixed genotypes) the sequence files with chromatograms of individual samples were observed.



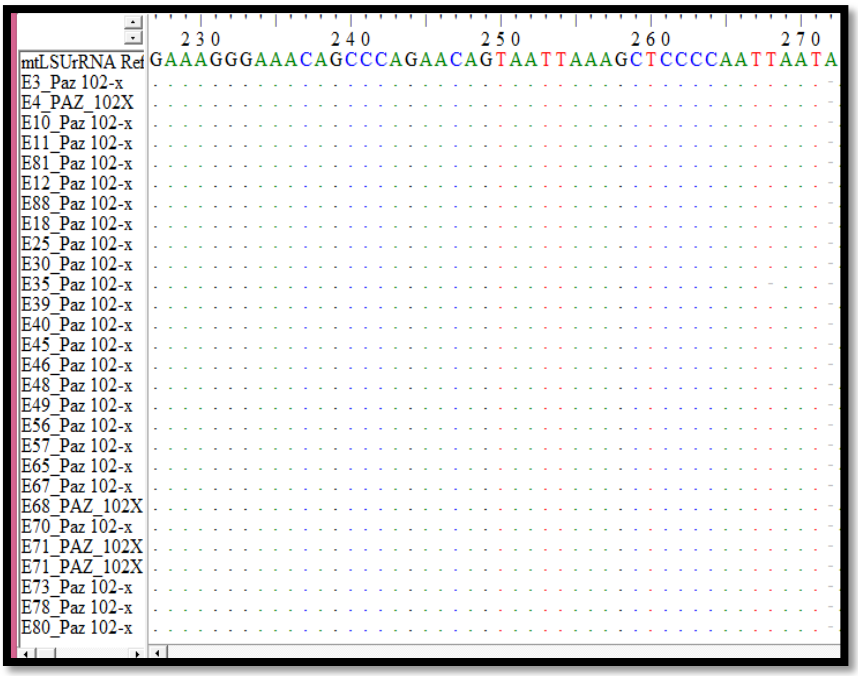
Partial Alignment of first set of clinical sample sequences to reference sequence, showing codons of interest and mutations.



Electropherogram showing sequence data for sample E255 corresponding to E255 with mixed genotype with double peaks showing indistinguishable nucleotide bases at both position 55 and 57 codons.



Multiple sequence alignment on 30 mtLSU amplified gene inserts with a wild-type mtLSU rRNA reference sequence using Bioedit sequence alignment editor. The figure reveals mutations on nucleotide 85. The dots underneath the reference sequence indicate identical nucleotides.



Multiple sequence alignment done on 30 mtLSU amplified gene inserts with a mtLSU rRNA reference sequence using Bioedit sequence alignment editor. The samples corresponding to this figure reveals no mutations on nucleotide 248

APPENDIX G– Ethics clearance certificate

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Ethics Letter

26-Nov-2015

Ethics Reference #: S15/07/141
Clinical Trial Reference #:
Title: The molecular epidemiology of Pneumocystis jirovecii in Cape Town, South Africa.

Dear Mr Derrick Banda,

The HREC approved your application for a protocol amendment dated 17 September 2015.

If you have any queries or need further assistance, please contact the HREC Office 0219389657.

Sincerely,

REC Coordinator
Franklin Weber
Health Research Ethics Committee 1

APPENDIX H: Plasmid transformation and purification using the QIAGEN Plasmid Midi Kit (QIAGEN)

Protocol: Transformation of Competent *E. coli* Cells

Important points before starting

- When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

Equipment and reagents to be supplied by user

- Competent *E. coli* BL21 strain cells, e.g., BL21 (DE3) Competent Cells (Sigma, cat. no. B 8808), BL21 (DE3) pLysS Competent Cells (Sigma, cat. no. B 8933), BL21 (DE3) pLysE Competent Cells (Sigma, cat. no. B 9058). Alternatively, competent cells can be produced using the protocol in Appendix B, page 55.
- QIAgenes Expression Construct DNA in solution (refer to Product Sheet for resolubilization protocol).
- LB agar plates containing 50 µg/ml kanamycin (and 25 µg/ml chloramphenicol if using *E. coli* BL21 cells containing the pLysS or pLysE plasmid).
- Psi broth.
- Heating block or water bath set to 42°C.

For composition of media and solutions, see Appendix A, page 50.

Procedure

1. **Transfer an aliquot of the QIAgenes Expression Construct DNA (1 µl or less) into a cold sterile 1.5 ml microcentrifuge tube, and keep it on ice.**
1. **Thaw an aliquot of frozen competent *E. coli* cells on ice.**
2. **Gently resuspend the cells and transfer 100 µl of the cell suspension into the microcentrifuge tube with the QIAgenes Expression Construct DNA, mix carefully, and keep it on ice for 20 min.**
3. **Transfer the tube to a 42°C water bath or heating block for 90 sec.**
4. **Add 500 µl Psi broth to the cells and incubate for 60–90 min at 37°C.**
   Shaking increases transformation efficiency.
5. **Plate out 50, 100, and 200 µl aliquots on LB-agar plates containing 50 µg/ml kanamycin (and 25 µg/ml chloramphenicol if using *E. coli* BL21 cells containing the pLysS or pLysE plasmid). Incubate the plates at 37°C overnight.**

Positive control to check transformation efficiency:

6. **Transform competent cells with 1 ng of the QIAgenes control plasmid (undigested) in 20 µl of TE. This plasmid expresses the 20 kDa protein 6xHis-TNFα.**
   Plate 1/100 and 1/10 dilutions of the transformation mix (diluted in prewarmed Psi broth) as well as undiluted transformation mix on LB-agar plates containing 50 µg/ml kanamycin (and 25 µg/ml chloramphenicol if using E. coli BL21 cells containing the pLysS or pLysE plasmid). The cells should yield 106 transformants per microgram of plasmid.

Negative control to check antibiotic activity:

7. **Transform cells with 20 µl of TE.**
   Plate at least 200 µl of the transformation mix on a single LB agar plate containing the appropriate antibiotics.

Protocol: Plasmid or Cosmid DNA Purification using QIAGEN Plasmid Midi and Maxi Kits

This protocol is designed for preparation of up to 100 µg of high- or low-copy plasmid or cosmid DNA using the QIAGEN Plasmid Midi Kit, or up to 500 µg using the QIAGEN Plasmid Maxi Kit. For additional protocols, such as for purification of very low-copy plasmids or cosmids of less than 10 copies per cell, see page 29 or visit www.qiagen.com/goto/plasmidinfo.

Low-copy plasmids that have been amplified in the presence of chloramphenicol should be treated as high-copy plasmids when choosing the appropriate culture volume.

Table 3. Maximum recommended culture volumes*

	QIAGEN-tip 100	QIAGEN-tip 500
High-copy plasmids	25 ml	100 ml
Low-copy plasmids	100 ml	500 ml

* For the QIAGEN-tip 100, the expected yields are 75–100 µg for high-copy plasmids and 20–100 µg for low-copy plasmids. For the QIAGEN-tip 500, the expected yields are 300–500 µg for high-copy plasmids and 100–500 µg for low-copy plasmids.

Important points before starting

- New users are advised to familiarize themselves with the detailed protocol provided in this handbook. In addition, extensive background information is provided on our plasmid resource page www.qiagen.com/goto/plasmidinfo.
- If working with low-copy vectors, it may be beneficial to increase the lysis buffer volumes to increase the efficiency of alkaline lysis, and thereby the DNA yield. In case additional Buffers P1, P2, and P3 are needed, their compositions are provided in Appendix B: Composition of Buffers, on page 44. Alternatively, the buffers may be purchased separately (see page 49).
- **Optional**: Remove samples at the indicated steps to monitor the procedure on an analytical gel (see page 41).
- Blue (marked with a ▲) denotes values for QIAGEN-tip 100 using the QIAGEN Plasmid Midi Kit; red (marked with a ●) denotes values for QIAGEN-tip 500 using the QIAGEN Plasmid Maxi Kit.

Plasmid Midi and Maxi Kits

Things to do before starting

- Add the provided RNase A solution to Buffer P1 before use. Use 1 vial RNase A (centrifuge briefly before use) per bottle Buffer P1 for final concentration of 100 µg/ml.
- Check Buffer P2 for SDS precipitation due to low storage temperatures. If necessary, dissolve the SDS by warming to 37°C.
- Pre-chill Buffer P3 at 4°C.
- **Optional**: Add the provided LyseBlue reagent to Buffer P1 and mix before use. Use 1 vial LyseBlue reagent per bottle Buffer P1 for a final dilution of 1:1000 (e.g., 10 µl LyseBlue into 10 ml Buffer P1). LyseBlue provides visual identification of optimum buffer mixing thereby preventing the common handling errors that lead to inefficient cell lysis and incomplete precipitation of SDS, genomic DNA, and cell debris. For more details see "Using LyseBlue reagent" on page 14.

Procedure

1. **Pick a single colony from a freshly streaked selective plate and inoculate a starter culture of 2–5 ml LB medium containing the appropriate selective antibiotic. Incubate for approx. 8 h at 37°C with vigorous shaking (approx. 300 rpm).**

   Use a tube or flask with a volume of at least 4 times the volume of the culture.

2. **Dilute the starter culture 1/500 to 1/1000 into selective LB medium. For high-copy plasmids, inoculate ▲ 25 ml or ● 100 ml medium with ▲ 25–50 µl or ● 100–200 µl of starter culture. For low-copy plasmids, inoculate ▲ 100 ml or ● 500 ml medium with ▲ 100–200 µl or ● 250–500 µl of starter culture. Grow at 37°C for 12–16 h with vigorous shaking (approx. 300 rpm).**

   Use a flask or vessel with a volume of at least 4 times the volume of the culture. The culture should reach a cell density of approximately 3–4 x 10^9 cells per milliliter, which typically corresponds to a pellet wet weight of approximately 3 g/liter medium.

3. **Harvest the bacterial cells by centrifugation at 6000 x *g* for 15 min at 4°C.**

   ⊗ If you wish to stop the protocol and continue later, freeze the cell pellets at –20°C.

4. **Resuspend the bacterial pellet in ▲ 4 ml or ● 10 ml Buffer P1.**

   For efficient lysis, it is important to use a vessel that is large enough to allow complete mixing of the lysis buffers. Ensure that RNase A has been added to Buffer P1.

   If LyseBlue reagent has been added to Buffer P1, vigorously shake the buffer bottle before use to ensure LyseBlue particles are completely resuspended. The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain.

5. **Add ▲ 4 ml or ● 10 ml Buffer P2, mix thoroughly by vigorously inverting the sealed**

tube 4–6 times, and incubate at room temperature (15–25°C) for 5 min.

Do not vortex, as this will result in shearing of genomic DNA. The lysate should appear viscous. Do not allow the lysis reaction to proceed for more than 5 min. After use, the bottle containing Buffer P2 should be closed immediately to avoid acidification from CO_2 in the air.

If LyseBlue has been added to Buffer P1, the cell suspension will turn blue after addition of Buffer P2. Mixing should result in a homogeneously colored suspension. If the suspension contains localized colorless regions or if brownish cell clumps are still visible, continue mixing the solution until a homogeneously colored suspension is achieved.

6. **Add ▲ 4 ml or ● 10 ml of chilled Buffer P3, mix immediately and thoroughly by vigorously inverting 4–6 times, and incubate on ice for ▲ 15 min or ● 20 min.**

Precipitation is enhanced by using chilled Buffer P3 and incubating on ice. After addition of Buffer P3, a fluffy white material forms and the lysate becomes less viscous. The precipitated material contains genomic DNA, proteins, cell debris, and KDS. The lysate should be mixed thoroughly to ensure even potassium dodecyl sulfate precipitation. If the mixture still appears viscous, more mixing is required to completely neutralize the solution.

If LyseBlue reagent has been used, the suspension should be mixed until all trace of blue has gone and the suspension is colorless. A homogeneous colorless suspension indicates that the SDS has been effectively precipitated.

7. **Centrifuge at ≥20,000 x *g* for 30 min at 4°C. Remove supernatant containing plasmid DNA promptly.**

Before loading the centrifuge, the sample should be mixed again. Centrifugation should be performed in non-glass tubes (e.g., polypropylene). After centrifugation the supernatant should be clear.

Note: Instead of centrifugation steps 7 and 8, the lysate can be efficiently cleared by filtration using a QIAfilter Kits or Cartridges (see www.qiagen.com/products/plasmid/LargeScaleKits).

8. **Centrifuge the supernatant again at ≥20,000 x *g* for 15 min at 4°C. Remove supernatant containing plasmid DNA promptly.**

This second centrifugation step should be carried out to avoid applying suspended or particulate material to the QIAGEN-tip. Suspended material (causing the sample to appear turbid) can clog the QIAGEN-tip and reduce or eliminate gravity flow.

Optional: Remove a ▲ 240 µl or ● 120 µl sample from the cleared lysate supernatant and save for an analytical gel (sample 1) to determine whether growth and lysis conditions were optimal.

9. **Equilibrate a ▲ QIAGEN-tip 100 or ● QIAGEN-tip 500 by applying ▲ 4 ml or ● 10 ml Buffer QBT, and allow the column to empty by gravity flow.**

Flow of buffer will begin automatically by reduction in surface tension due to the presence of detergent in the equilibration buffer. Allow the QIAGEN-tip to drain completely. QIAGEN-tips can be left unattended, since the flow of buffer will stop when the meniscus reaches the upper frit in the column.

10. **Apply the supernatant from step 8 to the QIAGEN-tip and allow it to enter the resin by gravity flow.**

The supernatant should be loaded onto the QIAGEN-tip promptly. If it is left too long and becomes cloudy due to further precipitation of protein, it must be centrifuged again or filtered before loading to prevent clogging of the QIAGEN-tip.

Optional: Remove a ▲ 240 µl or ● 120 µl sample from the flow-through and save for an analytical gel (sample 2) to determine the efficiency of DNA binding to the QIAGEN resin.

11. **Wash the QIAGEN-tip with ▲ 2 x 10 ml or ● 2 x 30 ml Buffer QC.**

Allow Buffer QC to move through the QIAGEN-tip by gravity flow. The first wash is sufficient to remove contaminants in the majority of plasmid DNA preparations. The second wash is especially necessary when large culture volumes or bacterial strains producing large amounts of carbohydrates are used.

Optional: Remove a ▲ 400 µl or ● 240 µl sample from the combined wash fractions and save for an analytical gel (sample 3).

12. **Elute DNA with ▲ 5 ml or ● 15 ml Buffer QF.**

Collect the eluate in a 15 ml or 50 ml tube (not supplied). Use of polycarbonate centrifuge tubes is not recommended as polycarbonate is not resistant to the alcohol used in subsequent steps.

Note: For constructs larger than 45–50 kb, prewarming the elution buffer to 65°C may help to increase yield.

Optional: Remove a ▲ 100 µl or ● 60 µl sample of the eluate and save for an analytical gel (sample 4).

⊗ If you wish to stop the protocol and continue later, store the eluate at 4°C. Storage periods longer than overnight are not recommended.

13. **Precipitate DNA by adding ▲ 3.5 ml or ● 10.5 ml (0.7 volumes) room-temperature isopropanol to the eluted DNA. Mix and centrifuge immediately at ≥15,000 x *g* for 30 min at 4°C. Carefully decant the supernatant.**

    All solutions should be at room temperature to minimize salt precipitation, although centrifugation is carried out at 4°C to prevent overheating of the sample. Alternatively, disposable conical bottom centrifuge tubes can be used for centrifugation at 5000 x *g* for 60 min at 4°C. Isopropanol pellets have a glassy appearance and may be more difficult to see than the fluffy, salt-containing pellets that result from ethanol precipitation. Marking the outside of the tube before centrifugation allows the pellet to be more easily located. Isopropanol pellets are also more loosely attached to the side of the tube, and care should be taken when removing the supernatant.

14. **Wash DNA pellet with ▲ 2 ml or ● 5 ml of room-temperature 70% ethanol, and centrifuge at ≥15,000 x *g* for 10 min. Carefully decant the supernatant without disturbing the pellet.**

    Alternatively, disposable conical-bottom centrifuge tubes can be used for centrifugation at 5000 x *g* for 60 min at 4°C. The 70% ethanol removes precipitated salt and replaces isopropanol with the more volatile ethanol, making the DNA easier to redissolve.

15. **Air-dry the pellet for 5–10 min, and redissolve the DNA in a suitable volume of buffer (e.g., TE buffer, pH 8.0, or 10 mM Tris·Cl, pH 8.5).**

    Redissolve the DNA pellet by rinsing the walls to recover the DNA, especially if glass tubes have been used. Pipetting the DNA up and down to promote resuspension may cause shearing and should be avoided. Overdrying the pellet will make the DNA difficult to redissolve. DNA dissolves best under slightly alkaline conditions; it does not easily dissolve in acidic buffers.

Determination of yield

To determine the yield, DNA concentration should be determined by both UV spectrophotometry at 260 nm and quantitative analysis on an agarose gel. For reliable spectrophotometric DNA quantification, A_{260} readings should lie between 0.1 and 1.0.

Agarose gel analysis

We recommend removing and saving aliquots during the purification procedure (samples 1–4). If the plasmid DNA is of low yield or quality, the samples can be analyzed by agarose gel electrophoresis to determine the stage of the purification procedure where the problem occurred (see page 41).

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