The use of a quantitative assay to differentiate between *Pneumocystis jirovecii* colonisation and disease among hospitalised patients, South Africa 2015-2016

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Declaration

I, Jessica Yun, declare that this research report is my own work, unless stated otherwise. The report is submitted in partial fulfilment of the requirements for the degree of Master of Science (MSc) Epidemiology with the University of the Witwatersrand, Johannesburg, South Africa. It has not been submitted previously for any other degree or examination at any other institution.

Ethical clearance was granted by the University of the Witwatersrand Human Research Ethics Committee: 1911144

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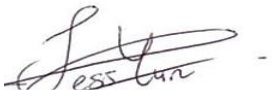

(Signature of Jessica Ann Lai Yun)

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Date: 25 June 2021

Dedication

I would like to dedicate this to my mother, friends and other family relatives for their endless encouragement, support and sacrifices they made throughout this journey.

Abstract

Background: *Pneumocystis jirovecii* pneumonia (PJP) is responsible for infant and adult mortality predominantly among those who are immunocompromised. Due to the use of quantitative real-time polymerase chain reaction (qPCR) yielding a high sensitivity, distinguishing between *P. jirovecii* colonisation and active clinical disease is challenging because *P. jirovecii* DNA is detected regardless of its association with illness. By establishing *P. jirovecii* qPCR DNA load cut-off value using beta-D-glucan (BDG) levels a biomarker of fungal infection, *P. jirovecii* colonisation and disease could be differentiated. This could prompt healthcare providers to initiate treatment in a timely manner as well as to assess the "true" prevalence of PJP.

Aim: To establish *P. jirovecii* qPCR DNA load cut-off value for the differentiation between *P. jirovecii* colonisation and disease among hospitalised patients admitted for severe respiratory illness in South Africa, using BDG level as a biomarker of fungal infection.

Methodology: The study was a cross-sectional study design utilizing secondary data. Study population included hospitalised patients enrolled in the pneumonia surveillance programme from two of the five participating sentinel sites (KwaZulu-Natal and North West provinces) from 2015-2016. A univariable and multivariable random effect (on surveillance hospital) logistic regression was used to evaluate the demographic and clinical factors associated with patients with and without available BDG level results. To determine the optimal *P. jirovecii* DNA load cut-off value of the qPCR, using the BDG as the gold standard for fungal infection, a receiver operating characteristic (ROC) analysis was conducted. Descriptive analysis to describe the demographic, clinical and epidemiological characteristics of those who were *P. jirovecii* diseased, colonised and negative was conducted.

Results: The total study population was 1542 patients enrolled in the pneumonia surveillance programme who had sputum samples tested for *P. jirovecii* and had available qPCR results. HIV was the most prevalent underlying condition with 70.43% (131/186) of patients living with HIV had BDG results as compared to those without BDG results (51.48%, 680/1321). A qPCR load cut-off value of 550 copies/5 µL was established which correctly classified 75% of patients with PJP. This yielded a high specificity of 91.67% and a moderate sensitivity of 57.14%, respectively. The positive and negative predictive values were 100.0% and 81.6%, respectively. Overall, the PJP prevalence was 5.12% and the prevalence of *P. jirovecii* colonisation was 11.28% from 2015

to 2016. On multivariable analysis adjusting for surveillance hospital clustering, patients who were infected with *P. jirovecii* compared to those who were colonised with *P. jirovecii* were more likely to be female (Adjusted relative risk ratio (aRRR) 1.91; 95% CI:1.04-3.49; p=0.04), to be living with HIV (aRRR 4.30; 95% CI:1.82-10.16; p<0.01) and receive cotrimoxazole at time of hospital admission (aRRR 5.19; 95% CI:2.24-12.06; p<0.001). Interestingly, patients who were negative for *P. jirovecii* compared to those who were colonised with *P. jirovecii* were more likely to present with fever (aRRR, 1.44; 95% CI:1.01-2.05; p=0.04). Furthermore, patients infected with *P. jirovecii* compared to those who were colonised with *P. jirovecii* were at an increased risk of in-hospital death (aRRR 2.63; 95% CI:0.98-7.05; p=0.05); therefore, indicating the severity of PJP.

Conclusions & recommendation: The cut-off value cannot be universally applied but rather it may only be used in pilot studies with a similar population that consists of adults who are living with HIV. Irrespective of not establishing a true gold standard for diagnostic tests to diagnose PJP, the established cut-off value could be used in conjunction with clinical presentation as well as to ruling in patients who have PJP (ruling diagnoses). Although the PCR assays are considered to yield a higher sensitivity, more intensive specimen collection is needed to estimate the true burden of PJP in South Africa.

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Nomenclature/List of Abbreviations and Symbols

β	beta
copies/5 μL	copies per 5 microlitres
AIDS	acquired immune deficiency syndrome
ARDS	acute respiratory distress syndrome
ART	antiretroviral therapy
AUC	area under the curve
BALF	bronchoalveolar lavage fluid
BDG	beta-D-glucan
CD4	cluster of differentiation 4
CI	confidence interval
COPD	chronic obstructive pulmonary disease
CRDM	Centre for Respiratory Diseases and Meningitis
DNA	deoxyribonucleic acid
GERMS-SA	Group for Enteric Respiratory and Meningeal Disease Surveillance in South Africa
HAART	highly active anti-retroviral therapy
HIV	human immunodeficiency virus
ICU	intensive care unit
LDH	lactate dehydrogenase
mL	mililitres
Msg	major surface glycoprotein
NICD	National Institute for Communicable Diseases
PCP	*Pneumocystis carinii* pneumonia
PCR	polymerase chain reaction
PJP	*Pneumocystis jirovecii* pneumonia
qPCR	quantitative real-time polymerase chain reaction
RSV	respiratory syncytial virus
SAM	s-adenosylmethionine
SD	standard deviation
TMP-SMX	trimethoprim-sulfamethoxazole
TB	tuberculosis

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Chapter 1: Introduction

1.1 Introduction

In this chapter, the background, description of the primary study and problem statement for the study were presented. The background section provides an overview of *Pneumocystis jirovecii* pneumonia (PJP), its transmission dynamics and prevalence within sub-Saharan Africa, surveillance programmes used in the monitoring of PJP in South Africa and the current diagnostic techniques used for the detection of *Pneumocystis jirovecii* (*P.jirovecii*). To conclude the chapter, the study justification, research question, hypothesis and objectives of the study were described.

1.2 Background

The opportunistic atypical fungus, *Pneumocystis jirovecii* (*P. jirovecii*, formerly known as *Pneumocystis carinii*), is presumably transmitted by the airborne route (1,2). PJP is responsible for infant and adult mortality predominantly among those who are immunocompromised due to infection with the human immunodeficiency virus (HIV) or acquired immune deficiency syndrome (AIDS) (3–5). Since the roll-out of highly active antiretroviral therapy (HAART) to patients living with HIV, the incidence of PJP has shifted to those who are immunocompromised due to organ transplant, malignancy, treatment-related immunosuppression or connective tissue diseases (3–7). According to a systematic review and meta-analysis among adults in sub-Saharan Africa, the pooled prevalence of PJP was 18.8% among hospitalised patients presenting with respiratory illness (8). PJP mainly occurs among those living with HIV and those who do not have readily available access to healthcare services (9). Healthy individuals are able to harbour the fungus and be asymptomatic (a process known as colonisation); therefore, playing a major role in the transmission of *P.jirovecii* (9).

Surveillance of PJP in South Africa was initially conducted between 2006 and 2010 through GERMS-SA, a laboratory-based sentinel surveillance programme (10). Subsequently, PJP was monitored through the pneumonia surveillance programme, a hospital-based sentinel surveillance programme, from June 2012 to December 2016. Both surveillance programmes were conducted by the National Institute for Communicable Diseases (NICD) (11,12).

One diagnostic technique used in the detection of invasive fungal pathogens such as *P.jirovecii* is the use of assays that detect the fungal antigen, (1→3)-β-D-glucan (beta-D-glucan or BDG), which is

derived from a component of the fungal cell walls (13). *P. jirovecii* DNA in respiratory specimens such as bronchoalveolar lavage fluid (BALF), induced sputum and nasopharyngeal and oropharyngeal samples, can also be detected with the use of qPCR (3,9). In addition, the qPCR assay provides an estimate of the *P. jirovecii* DNA load (number of DNA copies) in the specimen tested. Due to the unthresholded qPCR having a low specificity for *P. jirovecii* disease, BDG assays may be helpful when used in accompaniment with qPCR.

1.3 Description of primary study

1.3.1 Description of the pneumonia surveillance programme

The pneumonia surveillance programme was initiated by the NICD in 2009 and performs prospective, hospital-based sentinel surveillance for severe respiratory illness (SRI) (14). The main aim of the surveillance programme is to describe the epidemiology of SRI cases at sentinel surveillance sites and determine the relative contribution of influenza and other respiratory pathogens to the disease presentation in a high HIV prevalence setting (15). The primary study objectives are:

1. To estimate the incidence of and proportion of patients with Pneumocystis jirovecii, Mycobacterium tuberculosis (TB), Streptococcus pneumoniae, Bordetella pertussis, Haemophilus influenza type B (Hib) and atypical bacterial causes of pneumonia (Legionella species, Chlamydophila pneumoniae and Mycoplasma pneumoniae) in HIV-positive and negative adult and paediatric patients hospitalised with SRI and describe factors associated with positivity for these infections, at selected sites.
2. To determine the extent of specific respiratory viral co-infections with P. jirovecii, TB, S. pneumoniae, B. pertussis, Hib and atypical bacterial causes of pneumonia and describe how these co-infections relate to patient morbidity and outcome, at selected sites.
3. To determine the incidence of and proportion of hospitalised patients with influenza, other respiratory viruses, S. pneumoniae, B. pertussis, Hib and atypical bacterial causes of pneumonia among patients admitted with fever who do not meet the SRI case definition and describe factors associated with positivity for these infections at one site.
4. To determine the incidence of bacteraemia particularly typhoid fever amongst patients admitted with fever at one site

As part of the surveillance programme, nasopharyngeal and oropharyngeal samples were collected and tested for respiratory pathogens. In addition to nasopharyngeal samples tested for respiratory pathogens as part of the pneumonia surveillance programme, sputum samples (induced or expectorated) for expanded testing for *P. jirovecii* were collected by trained surveillance officers at the Edendale (KwaZulu-Natal Province) and Klerksdorp-Tshepong sites (North West Province) from February 2012 to December 2016. Sputum samples were collected from patients who presented with severe respiratory illness (SRI) and were enrolled as part of the pneumonia surveillance programme. Clinical specimens (nasopharyngeal, oropharyngeal and sputum samples) were transported to the NICD for *P. jirovecii* testing (11). *P. jirovecii* mitochondrial large subunit (MtLSU) rRNA was targeted on qPCR assay in order to determine fungal load. The PCR targeting MtLSU was used because it was a well-established, validated method in the laboratory at the time. The Fungitell assay was used to test BDG levels in serum collected from SRI patients from 2015 to 2016. If BDG levels are above 80 pg/mL, it indicates invasive fungal disease and is used as a serological biomarker for non-specific fungal infection (16). For this study, patients with existing PCR and BDG results from sputum and sera samples were included. Figure 1 shows the types of laboratory tests that were conducted during the surveillance programme study period.

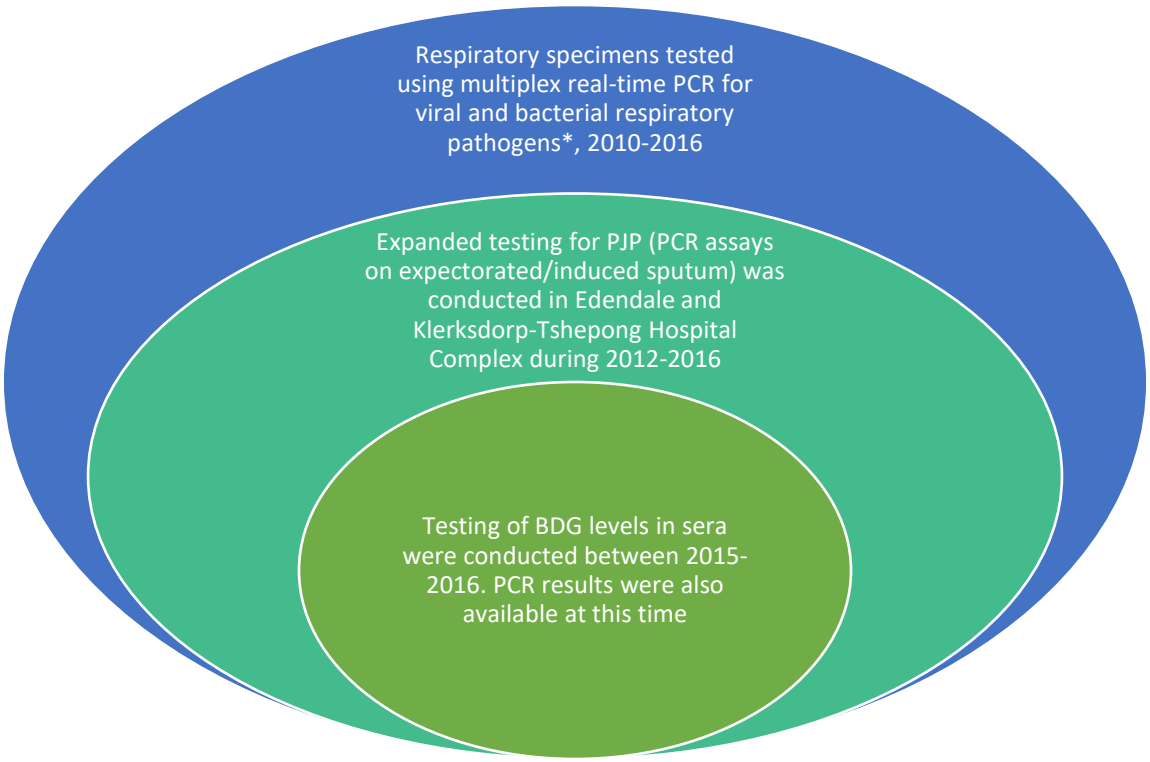

Figure 1: Laboratory tests for *P. jirovecii* during 2015 and 2016

*Respiratory pathogens tested include influenza A & B virus, human metapneumovirus, enterovirus, rhinovirus, *Bordetella pertussis* (*B. pertussis*), parainfluenza 1, 2 and 3, adenovirus, *Mycoplasma pneumonia*, respiratory syncytial virus (RSV), *Legionella* spp, *Chlamydophila pneumonia*, *Streptococcus pneumoniae*, tuberculosis (TB). Testing for *P. jirovecii* was later conducted between 2012 and 2016.

1.3.2 Case definitions for pneumonia surveillance programme

A case of severe respiratory illness (SRI) was defined as a hospitalised person who met age-specific clinical inclusion criteria irrespective of the duration of symptoms. Children aged 2 days to <3 months were eligible to be included in the surveillance programme if they were admitted, irrespective of signs and symptoms, with suspected sepsis or medically diagnosed lower respiratory tract infection (LRTI), including pneumonia, pleural effusion, bronchitis and bronchiolitis. Any patient hospitalized with doctor-diagnosed LRTI was included as a case in children aged 3 months to <5 years. Any admitted patient presenting with a manifestation of acute lower respiratory tract infection with a fever (temperature ≥38°C) or history of fever and cough was included in a case between aged ≥5 years. Hospitalised cases at Edendale Hospital or Klerksdorp-Tshepong Hospital complex who were medically diagnosed of suspected pulmonary TB, irrespective of symptoms, were also eligible for enrolment (15).

1.4 Problem statement

Despite the development of sensitive and specific PCR assays for the detection of *P. jirovecii*, differentiation between colonisation and disease using these assays remains challenging. As a result of qPCR being highly sensitive, both *P. jirovecii* colonisation and disease are detected, which may lead to less valuable diagnostic results and low specificity for disease identification (17). Previous studies from the northern hemisphere have suggested the use of DNA copy number cut-off values of qPCR assays to differentiate between *P. jirovecii* disease and colonisation; however, these cut-off values need to be standardised for routine use (9,18,19). Furthermore, there is limited literature establishing clear clinical prediction rules or DNA copy cut-off values for the broader South African population (patients living with and without HIV), where the burden of communicable diseases is substantial and often driven by social and biological determinants (20,21). For this analysis, BDG levels will be used as the gold standard for diagnosing those with a fungal infection. Subsequently, BDG levels will be used to determine a cut-off value for the qPCR DNA load in order to differentiate between *P. jirovecii* colonisation and disease among qPCR-positive patients.

1.5 Justification

This study will identify cut-off values for qPCR DNA load to differentiate between *P. jirovecii* colonisation and disease among hospitalised patients with severe respiratory illness within a South African setting. Differentiating between *P. jirovecii* colonisation and disease could prompt healthcare providers to initiate treatment in a more timely manner (22). Finally, this study will also contribute to a better understanding of PJP epidemiology by allowing assessment of the "true" prevalence of PJP.

1.6 Research question

What is the *P. jirovecii* qPCR DNA load cut-off value for the differentiation between *P. jirovecii* colonisation and disease among patients hospitalised for severe respiratory illness in South Africa, using BDG level as a biomarker of fungal infection?

1.7 Hypothesis

Null hypothesis:

There is no difference in the qPCR *P. jirovecii* DNA load among patients with *P. jirovecii* disease as compared to those with *P. jirovecii* colonisation.

Alternate hypothesis:

Patients with *P. jirovecii* disease have a higher qPCR *P. jirovecii* DNA load compared to those with *P. jirovecii* colonisation.

1.8 Study objectives

1. Compare the demographic, clinical (fever, dyspnoea, cough, hypoxia, underlying medical conditions) and epidemiological characteristics (person, time and place) of enrolled patients with and without an available BDG result.
2. Establish the possible cut-off values for qPCR loads considering sensitivity, specificity, and positive and negative predictive values in the differentiation between *P. jirovecii* colonisation and disease among hospitalised patients with pneumonia, using BDG as a standard.
3. Estimate the prevalence of *P. jirovecii* colonisation and infection among patients with available qPCR and BDG results enrolled in the pneumonia surveillance programme.
4. To describe the demographic and clinical characteristics of hospitalised patients enrolled in the pneumonia surveillance programme, stratified by patients who were negative for *P. jirovecii*, patients colonised with *P. jirovecii* and patients infected with *P. jirovecii*.

Chapter 2: Literature Review

In the literature review chapter, a detailed discussion of the clinical and laboratory diagnostic techniques used for the detection of *P.jirovecii*, prevalence of PJP in sub-Saharan Africa and South Africa, proposed cut-off values of biomarker beta-D-glucan (BDG) assay and proposed cut-off values of quantitative polymerase chain reaction (qPCR) deoxyribonucleic acid (DNA) used to differentiate between *P. jirovecii* colonisation and disease among hospitalised patients, was given.

2.1 History of PJP

At the beginning of the 20th century, *P. jirovecii* was initially regarded as a pulmonary parasite with unknown biological significance. This pathogen became a significant public health problem since the start of the HIV/AIDS pandemic and was a prominent opportunistic pathogen in immunocompromised patients (23,24). In 1909, C. Chagas was the first to discover *Pneumocystis* while he was studying a new disease (thought to be trypanosomiasis) that affected the railroad workers in Minas Gerais in Brazil (23). Lungs of guinea pigs were inoculated with the blood of patients with trypanosomiasis and C. Chagas came to the conclusion that it was caused by a new life stage of the protozoan flagellate, identified as *Trypanosoma cruzi*. In 1910, Antonio Carini also reported a similar description of cysts found in rat lungs and believed them to be infected by a new type of trypanosome, *Trypanosoma lewisi* (23).

At the Institute Pasteur in 1912, P. Delanoë and M. Delanoë concluded that their observations of similar pulmonary cysts in sewer rats were a new, unknown microorganism that was not related to trypanosomes. As a result, they proposed to name the new microorganism *Pneumocystis carinii* (*P. carinii*) due to the tropism to the lungs and their characteristic shape, as well as honouring Antonio Carini who, upon initial discovery, sent the tissue samples to Institute Pasteur (23). *P. carinii* was considered non-pathogenic until 1951, when J. Vanek and O. Jirovec found the organism in the lungs of infants and neonates with interstitial plasma cell pneumonitis (25,26). *Pneumocystis* was believed to be a protozoan, but in 1988, ribosomal RNA gene sequencing confirmed it to be a fungus (27). The nomenclature of *Pneumocystis* was also updated when taxonomic details became available. The human-derived *Pneumocystis* was named *Pneumocystis jirovecii* in honour of parasitologist Otto Jirovec in 1976 and *Pneumocystis carinii* referred to the form which infected rats (28,29). Originally, PCP (*Pneumocystis carinii* pneumonia) was used; however, to avoid confusion with the name change to

Pneumocystis jirovecii pneumonia, the abbreviation of PCP was kept in current literature and subsequently stands for *Pneumocystis* pneumonia (30). For the purpose of this paper, the abbreviation PJP will be used instead.

2.2 Infection and transmission

Studies have shown that the species of *Pneumocystis* are highly host specific; although the exact route of *P. jirovecii* transmission remains largely unknown (31). Literature has suggested two key theories. The first theory proposes that *P. jirovecii* infection is caused by the reactivation of a latent infection acquired during infancy (22). The organism is assumed to reside in the host and is subsequently reactivated to cause PJP once the immune function of the host has been compromised (22). Earlier studies have reported the detection of immunoglobulin response to *P. jirovecii* in healthy children aged 6 months to 8 years (32,33). Later studies have challenged this theory, indicating that the same strain of *P. jirovecii* has not been observed in many immunocompetent individuals on follow-up; therefore, suggesting a possibility of re-infection (34–36). Additionally, animal studies have reported that mice who recovered from PJP failed to reactivate the infection after the depletion of CD4+ cells for up to 84 days or depleted of CD4+ cells and treated with corticosteroid for 35 days. As a result, the immune response to *P. jirovecii* can completely clear the organism from the host and supports the hypothesis that the development of PJP could be due to new exposure to an exogenous source of *P. jirovecii* (37).

Secondly, it has been assumed that *P. jirovecii* infection is an outcome of a new exposure or recent transmission via person-to-person spread (i.e. patients with active PJP are able to transmit *P. jirovecii* to susceptible persons) (22). PJP outbreaks among transplant patients support this theory of nosocomial inter-human transmission alternatively to the latent infection reactivation theory (1,31,38–40). In a hospital setting in France, results have suggested nosocomial inter-human transmission from HIV patients with *P. jirovecii*, who were not isolated, to renal transplant patients due to patients sharing the same hospital building, and 70% of renal transplant patients harboured the same molecular form of *P. jirovecii* as those found in PJP patients (31). Studies have also acknowledged the probability of a common environmental source; however, air sampling was unsuccessful and the presence of *P. jirovecii* colonization in healthcare staff or nephrology patients

who were asymptomatic was not evaluated (1,38). Therefore, the possibility of indirect transmission of the infection cannot be ruled out.

Healthy individuals who are non-immunocompromised have been shown to harbour *P. jirovecii* without disease and are a potential reservoir for transmitting the fungus to immunocompromised patients susceptible to PJP (41). Colonisation has been described as the detection of *P. jirovecii* DNA in respiratory secretions among individuals who do not display specific symptoms (42). In a study conducted in Iran, *P. jirovecii* colonisation was found to occur among non-hospitalised patients with mild immunosuppression and underlying pulmonary diseases (who were referred for bronchoscopy at Shariati Hospital). Furthermore, colonisation rates were higher among patients with predisposing factors such as malignancy, and among the recipients of organ transplants (21.7% and 20.3% of cases, respectively). This indicates that higher rates of colonisation could be as a result of the severity of their predisposing factors and impaired immunity (42). Studies have shown that an increased risk of *P. jirovecii* colonisation is correlated with HIV infection, with prevalence ranging between 40% and 44.8% among patients living with HIV (including patients hospitalised and outpatients) (43–45). Similarly, hospitalised patients with autoimmune inflammatory diseases from Germany, especially those above 60 years of age, displayed a 28.5% prevalence of colonisation with *P. jirovecii* (46). In comparison, a study conducted in China found that *P. jirovecii* colonisation was found in 69.7% (281/403) of hospitalised patients living without HIV (47).

Health care workers (HCW) were also identified as possible asymptomatic carriers in *P. jirovecii* transmission. Studies that examined anti-*Pneumocystis* antibody responses showed that HCW (with presumed exposure to PJP) had higher levels of antibodies to the surface glycoprotein (Msg) fragments, such as MsgC, as compared to nonclinical workers without PJP exposure. Overall, antibodies against MsgC can also be a potential marker of *P. jirovecii* exposure and colonisation in immunocompetent individuals (41,48). This further supports the theory that HCWs may serve as reservoirs and colonisation may occur in them.

While it is difficult to rule out a particular environmental cause of infection, the route of human infection is believed to be airborne (22,49–51). In an animal study, *P. jirovecii* DNA was found in air samples one week after infection and increased until week 4-5 after infection (50). Air samples from

Parisian hospitals have shown that *P. jirovecii* was identified one metre away from infected patients in 79% of air samples and has decreased with distance from the patient (51). Diffusion of *P. jirovecii* was concentrated mainly in the patient's room; however, *P. jirovecii* was also detected in hospital corridors, which could pose as a risk to immunocompromised patients as well as HCW (51). Comparatively, another study showed that *P. jirovecii* DNA was not detected at one metre in air samples but it was detected at five metres. It is hypothesised that the air movement from an open door My have rasied the air burden of *P. jirovecii* by five metres, thus, making it easier to detect the fungus at a further distance (49). Overall, the source of infection is disputable. The ubiquitous nature of this fungus may be due to a combination of all of the above serving as possible reservoirs of infection.

2.3 Different diagnostic techniques used to diagnose and detect *P. jirovecii*

2.3.1 Clinical and radiological diagnosis

The respiratory disease, PJP, can be clinically suspected when two of the following are present: (1) progressive dyspnea, (2) unproductive cough, (3) fever, (4) chest pain, (5) arterial partial pressure of oxygen (PaO_2) lower than 65 mm Hg (resulting in shortness of breath), and (6) bilateral, perihilar interstitial shadowing presented in chest radiographs (6,24,52). Patients who present with the above symptoms should be further assessed and tested if they could have PJP. Oral thrush and substantial weight loss were seen as common symptoms among AIDS patients with *P. jirovecii*, whilst patients living without HIV displayed milder symptoms (7). Progression of PJP symptoms seems to be quicker and immunocompromised patients living without HIV have reported higher mortality rates relative to patients living with HIV (6,7).

Initial chest radiographs of patients with early presentation of PJP may show no changes related to the onset of symptoms (7,24,52). In individual patients, clinical images are complex and many infectious and non-infectious diseases may be viewed identically (i.e., nonspecifically) (24). Radiological patterns have been shown to be very similar between immunocompromised individuals living with and without HIV (6,7). Therefore, high resolution computed tomography scans are preferable as they are more sensitive than chest x-rays and a more detailed description of the radiological pattern that differs from other pulmonary infection may be given (7).

2.3.2 Laboratory diagnosis

Due to PJP patients presenting with non-specific symptoms, the coexistence of other infections as well as the inability to culture *P. jirovecii*, there is a reliance on molecular methods or microscopic visualisation of the organism in respiratory samples for *P. jirovecii* diagnosis (53). As patients with respiratory failure or patients living with HIV are frequently unable to undergo invasive procedures such as bronchoscopy for pulmonary specimen collection, microscopic visualisation of *P. jirovecii* from induced sputum, BALF, or lung biopsy is not desirable. This is due to higher risks of associated complications (24). Relative to direct microscopic visualization, molecular methods have increased the sensitivity and specificity (53).

Various staining techniques have historically been used to distinguish *Pneumocystis* organisms. Gram-Weigert, Giemsa, known as modified Papanicolaou stains, were used to distinguish the trophic form of *P. jirovecii*. In comparison, cresyl echt violet, calcofluor white, Gomori methenamine silver or toluidine blue O were used to visualise the cyst forms (54). Among improved diagnostic techniques, the most common technique used in laboratories is fluorescein-conjugated monoclonal antibodies, commonly known as immunofluorescence (IF) microscopy/stain technique (54). With the use of the ultraviolet light-equipped microscope, laboratory technicians are able to detect fluorescing antibody-antigen complexes; therefore, categorising the presence of *P. jirovecii* as weak positive, positive or strongly positive (20). The sensitivity of these assays depends on the quality of samples received by the laboratories and the number of *P. jirovecii* organisms detected in the samples. Due to low fungal burden, especially in patients living without HIV , caution has been advised as all direct organism visualisation methods can result in false-negative results. Furthermore, the diagnosis of PJP through direct organism visualisation methods depends on the experience and skills of the observer (55). The IF technique also tends to be time-consuming and costly, and invasive procedures are required to obtain either BALF or sputum to yield reliable results (54,56,57).

In recent years, BDG levels have been used as an indirect diagnostic biomarker for invasive fungal infections such as PJP. In cell walls of most fungi, BDG is present and detected (in plasma or serum specimens and BALF specimens) with the use of commercial kits such as the Fungitell test (Associates of Cape Cod, Inc., East Falmouth, MA), Wako turbidimetric assay (WakoPure Chemical Industries, Ltd., Tokyo, Japan) or Fungitec-G test (Seikagaku, Kogyo, Tokyo, Japan) (58).

The BDG assay has been shown to be a useful biomarker for PJP, especially when combined with qPCR and immunofluorescent staining (18,52,59,60). More specifically, the Fungitell assay (Associates of Cape Cod, Inc., East Falmouth, MA) is a chromogenic kinetic test and is based on the modification of the Limulus Amebocyte Lysate (LAL) pathway (61). The Fungitell reagent is modified to eliminate bacterial endotoxin reactivity (through Factor G, a serine protease zymogen). Therefore, in the presence of BDG, Factor G, is activated. This activates the clotting enzyme which in turn cleaves para-nitroanilide (pNA) from the chromogenic peptide substrate, BocLeu-Gly-Arg-pNA, creating a chromophore, para-nitroaniline (62). The assay is for the presumptive diagnosis of invasive fungal disease through detection of elevated levels of (1→3)-β-D-glucan in serum. BDG levels above 80 pg/mL indicates invasive fungal disease and is used as a serological biomarker for non-specific fungal infection (16). It should be used in conjunction with other diagnostic procedures. Testing for this biomarker remains an option for *P. jirovecii* diagnosis as it shortens the time between suspected infection and confirmed diagnosis (63). One of the main limitations of the BDG assay is that the presence of PJP cannot be proven by BDG levels alone (due to its non-specific nature). It has been advised that it should be interpreted in conjunction with clinical results and other complementary diagnostic techniques such as qPCR (63).

Studies have assessed the usefulness of the metabolite S-adenosylmethionine (SAM) and lactate dehydrogenase (LDH) as potential biomarkers for the diagnosis of PJP (7,64,65). *P. jirovecii* was believed to have lacked the enzyme SAM synthase and was acquired from its human host (65). Despite the observations of patients with PJP having low SAM levels, sensitivity analysis has demonstrated that the biomarker was unable to differentiate between patients with and without PJP (low diagnostic robustness) (64,66). Additionally, the differences in SAM levels between studies may be attributable to the use of different laboratory methodologies and sampling (24,64,66). In the bronchoalveolar space, LDL has been known to be released after damage to the cytoplasmic cell membrane. High LDH levels could reflect the presence of other underlying lung injury or inflammation due to other respiratory pathogens (64). Resultantly, raised serum levels of LDH have been associated with PJP, but there is a possibility of false-positive findings. Conclusively, the use of LDH as a biomarker was not recommended and BDG was considered to be the most reliable biomarker in PJP diagnosis (7,64).

The use of molecular techniques such as real-time PCR in combination with other diagnostic techniques such as IF microscopy and BDG assays is becoming more widely accepted (20,53,67). With the use of qPCR assays, medical scientists are able to quantify fungal load (DNA copies of specific gene/s) in BALF, induced sputum, nasopharyngeal and oropharyngeal samples, and these assays are proven to be highly sensitive (3,9,11,68). Consequently, diagnosis with the use of qPCR complements clinical diagnosis as it can detect low numbers of *P. jirovecii* organisms among asymptomatic patients (11). The most common gene targets used for the detection of *P. jirovecii* on qPCR include Msg, mtLSU rRNA gene, heat shock protein (HSP)70 gene, the beta-tubulin gene and dihydropteroate synthetase (DHPS) gene in previous literature (16,42,66,69). One of the main advantages of using qPCR for the detection of *P. jirovecii* is that it can operate on a large number of samples at a time in comparison with immunofluorescent antibody testing (11,57). Despite qPCR being cost-efficient, it is not routinely available, especially within resource-constrained settings, therefore leading to a possible underestimation of PJP cases (5). The use of sputum for PCR testing has become a more viable alternative to invasive testing (53). Due to this molecular technique yielding a higher sensitivity, differentiating between colonisation and active clinical disease is challenging because *P. jirovecii* DNA is detected regardless of its association with illness (17,68). As a result, this diagnostic technique could lead to overestimation of PJP in comparison with IF microscopy (68).

2.4 Management of PJP

Routine PJP prophylaxis is recommended for all patients who are at moderate to high risk of developing PJP, including those who are immunocompromised due to malignancy, HIV, use of corticosteroids, organ transplant or treatment-related immunosuppression (69). Trimethoprim-sulfamethoxazole (TMP-SMX, also known as cotrimoxazole) remains the recommended first-line treatment or prophylaxis of mild, moderate and severe PJP in adults and children (24,69–71). The use of TMP-SMX can also provide additional protection against other infections, such as nocardiosis, toxoplasmosis, and many other bacterial infections (69). TMP-SMX inhibits the two steps in the biosynthesis of essential nucleic acids by blocking the production of tetrahydrofolic acid from dihydrofolic acid by binding to and reversibly inhibiting the required enzyme, dihydrofolate reductase and proteins essential to *P. jirovecii* replication (72). The recommended dose for adults is two double-strength tablets (160 mg/800 mg) every 6 hours over the course of 14-21 days to treat

PJP. For administering PJP prophylaxis in adults, a single-strength tablet (80/mg/400mg) per day was recommended. TMP-SMX dosage is determined by weight in children and the recommended liquid formulation is 40 mg/200 mg TMP-SMX per 5 mililitres (mL) (73,74).

It has been suggested that PJP prophylaxis should be administered or reintroduced to all immunocompromised patients with CD4 cell counts below 200 cells/mm^3 (73). In adult and adolescent patients who have reacted to ART with an increase in CD4 counts of above 200 cells/mm3 for >3 months, primary PJP prophylaxis should be discontinued (caution needs to be exercised when taken by young children). This is to reduce the risk of drug toxicity, associated costs, drug interactions and to prevent the possibility of acquiring drug-resistant pathogens (73,74). For patients who are unable to tolerate or fail to respond to TMP-SMX, alternative regimens include dapsone, aerosolised pentamidine (through a nebuliser) dapsone plus trimethoprim and atovaquone (54,73,74). A common side effect associated with TMP-SMX is a rash, and this can be treated with a prescribed antihistamine. Alternative drugs should also be considered if a patient experiences other serious side effects including neutropenia, pancreatitis, nephritis, aseptic meningitis and hepatitis (71).

Treatment with TMP-SMX should be initiated immediately when PJP is suspected (71). Intravenous pentamidine still remains the second-line therapy after TMP-SMX in patients with serious infection. Caution has been advised, especially among islet or pancreas transplant recipients, numerous toxicities and possible islet cell necrosis can complicate its use (74). Adjunctive corticosteroids such as prednisone should be given with antimicrobial therapy within 72 hours after initiation of PJP therapy in patients suffering from hypoxemia (pAO2 <70 mmHg in room air) and have moderate-to-severe disease, for optimum gain (73,74). Dapsone plus trimethoprim, clindamycin plus primaquine, and atovaquone suspension are the alternative treatment regimens for patients with mild-to-moderate PJP. Clindamycin-primaquine or pentamidine given intravenously are alternative treatment regimens for patients with mild to serious illness (73).

Overall, the recommended length of therapy for PJP should be 21 days for serious infections and 14 days for mild to moderate infections, regardless of the regimen (73,74). Based on data that was collected between 2006 and 2010 from a South African laboratory-based surveillance study of PJP,

35% of PJP hospitalised patients received TMP-SMX prophylaxis at least one week prior to admission, and 82% of cases received TMP-SMX on discharge. Prophylaxis failure rates in South Africa were relatively high in 32% of children below 5 years old, and in 38% of adults and children above 5 years old (10). As a result, PJP remains a common cause of hypoxic pneumonia in South Africa, despite widespread availability of TMP-SMX prophylaxis and HAART.

2.5 Prevalence of PJP in Sub-Saharan Africa & South Africa

Arising from the HIV/AIDS epidemic, there was a drastic increase in *P. jirovecii* disease. The overall global prevalence has decreased recently due to effective public health interventions, namely the availability of ART and PJP prophylaxis (8,56). The pooled PJP prevalence among hospitalised patients in sub-Saharan Africa according to a systematic review and meta-analysis study declined from 28% in 1990 to 9% in 2005 (8). The prevalence of PJP remains high in South Africa, due to the high burden of HIV/AIDS as well as limited access to healthcare services (3,5). One of the major gaps within South African literature is that the majority of South African PJP prevalence studies mainly focused on infants and children (3,5,75,76). The prevalence of PJP among hospitalised South African children with pneumonia ranged from 7-54% where most PJP cases occurred in children living with HIV (3,5,75). The case fatality rate among South African children was 32.1% (3). It is noteworthy that the variability in PJP prevalence could be attributable to the types of specimens collected as well as the varying sensitivity of diagnostic methods used.

A South African study that used sentinel-based surveillance laboratory data reported that the majority of PJP cases in South Africa appeared in infants aged ≤1 year and in adults aged 26–45 years. Moreover, there were significantly more females aged 26-45 years who were infected than males (10). Among South African miners, the PJP prevalence was 3.9%; however, 32.6% of those who presented with histological features of PJP had another concomitant respiratory infection, namely cryptococcal pneumonia (46.7%), bacterial pneumonia (34.6%) or tuberculosis (TB) (13.1%) (77). Interestingly, in 89% of the deaths associated with PJP, the infection was unsuspected prior to death, therefore, suggesting accuracy of the clinicians' diagnoses in the miners with PJP at autopsy was poor (77). PJP is a common and life-threatening cause of severe pneumonia and is associated with elevated mortality among African children living with HIV, especially in South

Africa. As suitable diagnostic instruments and up-to-date prevalence data are missing, the number of PJP cases may be grossly underestimated (8).

2.6 Proposed cut-off values of biomarker BDG assay

According to manufacturer's recommended cut-off values, BDG levels greater than 80 pg/mL are considered as an indicator for fungal infection, 60-79 pg/mL is undetermined and less than 60 pg/mL is *P. jirovecii* negative (16). Studies conducted in Germany and Belgium have also proposed similar cut-off values to manufacturers' recommended cut-off values that ranged between ≥70-100 pg/mL to get a sensitivity range of 91%-100% and specificity range of 79%-96% (18,58). In comparison, higher cut-off values have been proposed to discriminate positive *P. jirovecii* that ranged between ≥200-400 pg/mL and yielded a sensitivity range of 91%-100% and a specificity range of 83%-100% (19,52,63,67,78). All studies indicated the usefulness of using BDG levels for diagnosis of *P. jirovecii*; however, optimal cut-off values differ. The potential sources of heterogeneity in optimal cut-off values among the included studies are study design, type of samples used (serum and BALF), different BDG detection assays used or BDG level variation among different study populations including immunocompromised patients (patients living with HIV, haematological malignancies, acute respiratory distress syndrome (ARDS)) (18,19,52,63,67,78). A majority of studies concluded that the combination of serum BDG levels and qPCR assays was useful to discriminate between those who were *P. jirovecii* diseased, colonised and negative (16,59,60,67). Overall, BDG levels and DNA copies were significantly greater in patients with *P. jirovecii* disease as compared to those colonised with *P. jirovecii* (59,60).

2.7 Proposed cut-off values of *P. jirovecii* qPCR DNA copy number

Since the development of qPCR assays, several studies were conducted to evaluate the use and establishment of cut-off values of qPCR DNA copy load and BDG levels to differentiate *P. jirovecii* colonisation and disease. However, the cut-off values differ across all studies due to methodological differences, study populations, and the incorporation of a "grey zone" for indeterminate results (9,79). In previous literature, cut-off values were expressed in various manners such as copies/mL, trophic form equivalents/mL, copies per 5µL and copies/mL; therefore, making it difficult to standardise a cut-off value. Studies conducted in Japan and France proposed qPCR DNA copy load cut-off values for the differentiation between *P. jirovecii* disease and colonisation ranging between

1300-31600 copies/mL, which yielded sensitivities of 80-100% and specificities of 70-100% (59,80). A South African study among hospitalised patients proposed a qPCR DNA copy load cut-off value of 78 copies/5 µL with a sensitivity and specificity of 94.6% and 89.1% respectively. Despite qPCR DNA copy load cut-off values having been suggested for South Africa previously, this study was only conducted among adults living with HIV and the comparison test was IF, which yields poor sensitivity (20). In comparison, a study conducted in France assessed *P. jirovecii* disease and colonisation by expressing positive qPCR results as trophic form equivalents using cut-off values of 1,900 trophic form equivalents/mL and 120 trophic form equivalents/mL respectively, yielding 100% sensitivity and 96.9% specificity (9). Despite the usefulness of these diagnostic tests, there was variability in PCR assay measurements (DNA copies and trophic form equivalents (TFEq)/mL) as well as the use of different specimens (bronchoalveolar lavage fluid, serum and induced sputum), which do not allow for a good comparison of cut-off values between studies. Furthermore, to our current knowledge, there is limited literature in South Africa that has established qPCR cut-off values for diagnosis of *P. jirovecii* (20).

2.8 Clinical relevance of establishing *P. jirovecii* DNA cut-off values

Treatment options are often based on the ability to distinguish disease from colonisation. Establishing the qPCR DNA load cut-off values for *P. jirovecii* disease and colonisation will allow physicians to avoid unnecessary treatment, therefore limiting the associated adverse effects, the development of resistant colonising strains and reducing the cost of treatment (22,81,82). The true clinical relevance of *P. jirovecii* colonisation remains unknown; thus there is a lack of understanding of the epidemiology of PJP (22).

Chapter 3: Methodology

3.1 Study design

The study is a cross-sectional study design utilising secondary data derived from the pneumonia surveillance programme in South Africa conducted by the National Institute for Communicable Diseases (NICD) during 2015 and 2016.

3.2. Data source

Data were obtained from the pneumonia surveillance programme, a prospective, active, hospital-based sentinel surveillance programme implemented on February 2009 to date by the Centre for Respiratory Diseases and Meningitis (CRDM), NICD.

3.3 Study setting

Data were collected at sentinel hospitals located in two provinces in South Africa (KwaZulu-Natal and North West provinces). Further details of the pneumonia surveillance programme are described in chapter 1.

3.4 Study population

The study population included hospitalised patients enrolled in the pneumonia surveillance programme from two of the five participating sentinel sites, who had a sputum sample taken for *P. jirovecii* laboratory confirmation from 2015-2016. Hospitalised patients who refused consent to participate in the study or were not hospitalised in Edendale or Klerksdorp-Tshepong hospitals were excluded.

3.5 Study sample

For this study, no sub-sampling was done; however, the sample power was calculated. The prevalence of *P. jirovecii* infection was over 20% amongst inpatients presenting with respiratory symptoms in sub-Saharan Africa (8) and the number of participants enrolled in the pneumonia surveillance programme was 1542. To estimate 20% detection of *P. jirovecii* among hospitalised patients in the pneumonia surveillance, with a 95% confidence interval (CI), a 5% desired absolute precision and 1.5 hospital clustering effect, will require 369 patients be enrolled. The sample size was calculated using the following formula:

$$n = \frac{[Deff * Np(1-p)]}{\left[\frac{d^2}{Z^2_{1-\alpha/2}} * (N-1) + p * (1-p)\right]}$$

Where:

- n: sample size
- N: population size for finite population correction
- p: expected prevalence in the population
- d: desired absolute precision
- Deff: design effect

3.6 Data collection

3.6.1 Measuring instruments

No primary data collection was conducted for this study. In the pneumonia surveillance programme, all patients who met the surveillance case definitions and consented to be enrolled in the study had a specimen collected for diagnosis of respiratory pathogens and a case investigation form (CIF) was completed by a structured interview with the patient and a medical record review. This was conducted by trained surveillance officers located at the participating sentinel sites. The CIF was used to collect data on patient demographics, clinical presentation, underlying conditions and management received during hospital admission. Accompanied by the CIF, a form was used to collect test results conducted at the admitting hospitals and an outcome form was used to record the outcome of hospitalisation.

3.6.2 Measurement of variables

Description of the variables used for analysis is detailed below.

3.6.2.1 Demographic variables

- **Age of participant:** This was calculated from the date of birth to the interview date. The variable was recorded as a continuous variable but was later categorised into the following: “0-4 years”, “5-14 years”, “15-24 years”, 25-44 years”, “45-64 years”, and “≥65 years”.
- **Sex:** This variable was a nominal categorical variable defined as either “female” or “male”.
- **Race:** This was a nominal categorical variable defined as: “Black” and “Non-black”

- **Province:** This was a nominal categorical variable defined as “KwaZulu-Natal” and “North West”.
- **Year of enrolment:** This was a nominal categorical variable defined as “2015” and “2016”. This indicates which year the patient was enrolled at the pneumonia surveillance programme.

3.6.2.2 Clinical variables

- **Presenting symptoms:** In the original study, patients were asked a series of questions indicating whether they presented with respiratory-related symptoms such as “Fever”, “Cough”, “Difficulty breathing”, “Chest pain” and “Chills” upon admission at one of the participating hospitals. These five variables were recorded as binary variables (yes or no response).
- **Symptom duration before hospital admission:** Symptom duration was recorded as a continuous variable (in days) and was later recoded into the following: “≤7” and “>7” days
- **Known underlying conditions:** This consists of a series of variables related to known underlying conditions that are prevalent in South Africa. This is further described below.
    - **HIV status:** This was a nominal categorical variable defined as “Positive” and “Negative”.
    - **Diabetes:** This was a nominal categorical variable defined as “Yes” and “No”.
    - **Tuberculosis:** This was a nominal categorical variable defined as “Positive” and “Negative”. Tuberculosis status was based on the results of tuberculosis testing at current hospital admission of enrolled patients. A laboratory-confirmed case of tuberculosis was identified by the GeneXpert MTB/ RIF test if an individual had a positive result for Mycobacterium tuberculosis on microscopy, culture or polymerase chain reaction (PCR) (Cepheid, Sunnyvale, California).
    - **Other underlying conditions:** Patients were asked whether they had any other underlying conditions not stated above including “asthma”, “other chronic lung disease”, “stroke”, “chronic heart disease” (, coronary heart disease, heart failure or valvular heart disease excluding hypertension), “anaemia”, “seizures”, “liver disease” (liver failure or cirrhosis), “renal disease” (chronic renal failure or nephrotic syndrome), “immunocompromising conditions excluding HIV infection”

(immunoglobulin deficiency, autoimmune disease, immunosuppressive therapy), "pregnancy", "asplenia", "burns", "obesity", "malignancy", "organ transplant", "chronic obstructive pulmonary disease (COPD)/emphysema" & "neurological disease" (neuromuscular conditions, spinal cord injury). These were originally recorded as binary variables (yes or no) indicating the presence or absence of each of these conditions separately. These series of variables were later recoded into a single binary variable (yes or no) indicating whether a patient had any of the underlying conditions as previously listed.

- **Other respiratory infections:** Three pathogen-based respiratory specimens from the pneumonia surveillance sites were screened for: influenza, RSV and *B. pertussis*. These were all recorded as binary variables and defined as "Positive" and "Negative".
- **Management received during admission:** Surveillance officers were tasked to look through patient medical files or ask the patient during the enrolment stage and indicate whether the patient received any treatment during admission. This includes whether the patient received antibiotics, steroids, were undergoing ventilation, received oxygen therapy and/or cotrimoxazole. These were all recorded as binary variables (yes or no) indicating whether the patient received any of the treatments listed above.
- **Duration of hospitalisation:** This was recorded as a continuous variable and was later recoded into the following: "<3 days", "3-7 days" and "≥7 days".
- **Intensive Care Unit (ICU) admission:** This was recorded as a binary variable defined as "yes" and "no".
- **In-hospital mortality:** Patient outcome was recorded as a binary variable defined as "yes" and "no".

3.6.2.3 Main outcomes and exposures for each study objective

Objective 1: To compare the demographic, clinical (fever, dyspnoea, cough, hypoxia, underlying medical conditions) and epidemiological characteristics (person, time and place) of enrolled patients with and without available BDG results.

- **Outcome variable:** Available BDG results as a binary variable (yes or no).
- **Exposure variables:** Age, sex, race, province, year of enrolment, presenting symptoms (fever, cough, difficulty breathing, chest pain and chills), duration of symptoms before

hospital admission, known underlying conditions (HIV status, diabetes, tuberculosis, other underlying conditions leading to immunosuppression), other respiratory illness (influenza, RSV, *B. pertussis*), management received during admission (ventilation, steroid, antibiotics, oxygen therapy and cotrimoxazole), duration of hospitalisation, ICU admission and in-hospital mortality.

Objective 2: **To establish the possible cut-off values for qPCR loads considering sensitivity, specificity, positive and negative predictive values in the differentiation between *P. jirovecii* colonisation and disease among hospitalised patients with pneumonia, using BDG as a standard.**

- **Outcome variables:** BDG results (binary variable defined as positive and negative) and qPCR loads (continuous variable)

Objective 3: To estimate the prevalence of *P. jirovecii* colonisation and infection among patients with available qPCR and BDG results enrolled in the pneumonia surveillance programme.

- **Outcome variables:** Number of patients who are positive and colonised for *P. jirovecii* using the qPCR DNA load cut-off established under Objective 2.

Objective 4: **To describe the demographic and clinical characteristics of hospitalised patients enrolled in the pneumonia surveillance programme stratified by patients who were qPCR negative for *P. jirovecii*, patients colonised with *P. jirovecii* and patients infected with *P. jirovecii*.**

- **Outcome variable:** patients negative for *P. jirovecii*, patients colonised with *P. jirovecii* and patients infected with *P. jirovecii* as a nominal categorical variable after applying the established cut-off values for qPCR loads (as described in objective 2).
- **Exposure variables:** Age, sex, race, province, year of enrolment, presenting symptoms (fever, cough, difficulty breathing, chest pain and chills), duration of symptoms before hospital admission, known underlying conditions (HIV status, diabetes, tuberculosis, other underlying conditions leading to immunosuppression), other respiratory illness (influenza, RSV, *B. pertussis*), management received during admission (ventilation, steroid,

antibiotics and oxygen therapy), duration of hospitalisation, ICU admission and in-hospital mortality.

3.7 Data management and analysis

Data were managed and analysed using STATA version 15 (Stata Corp, LP Texas USA).

3.7.1 Data management

In the primary study (i.e. pneumonia surveillance conducted by NICD), double data entry was conducted into a Microsoft Access database by independent data clerks and any discrepancies were corrected. In preparation for secondary analysis, the data were re-examined and checked for any missing and/or miscoded data. The initial sample size of 2630 patients enrolled at the selected sites during 2015-2016 was reduced to 1542 patients with available induced sputum and qPCR results.

3.7.2 Data analysis

Objective 1: Compare the demographic, clinical and epidemiological characteristics of enrolled patients with and without available BDG results

This analysis was conducted to assess differences in the study population for patients included and excluded in the primary analysis (i.e. qPCR load value to differentiate between PJ infection vs. colonization). Frequency tables were conducted to summarise the distribution of demographic, clinical and epidemiological characteristics of patients with and without available BDG results. Furthermore, a univariate and multivariable logistic regression was conducted to evaluate the demographic and clinical factors associated with patients with and without available BDG level results. A random effect on surveillance hospital was accounted for potential differences in the service population and the quality of care of each hospital. For the multivariable model, all variables that were significant at p-value<0.2 were assessed on univariate analysis and dropped non-significant factors with manual forward selection. A p-value <0.05 was considered statistically significant. A descriptive analysis of patients with and without available BDG results was conducted to determine whether any bias in relation to the study population was introduced before the ROC analysis was conducted.

A separate analysis was conducted for patients who had a positive BDG result but had a negative qPCR result. Individual patient records submitted on the NICD Trakcare system were examined

to establish what other tests were requested by the attending physician. This is to have a better understanding whether patients were infected with any other fungal pathogen.

Objective 2: Establish the possible cut-off values for qPCR loads considering sensitivity, specificity, positive and negative predictive values in the differentiation between *P. jirovecii* colonisation and disease among hospitalised patients with pneumonia, using BDG as a standard

A receiver operating characteristic (ROC) analysis was used to determine the optimal *P. jirovecii* DNA load cut-off value of the qPCR using the BDG as the gold standard for fungal infection. To conduct the ROC analysis, patients who had available BDG and qPCR-positive results were analysed. A graphical presentation of the ROC curve was presented and used to estimate the area under the curve (AUC). The optimum cut-off points for the best trade-off between specificity and sensitivity using the BDG results as the reference variable was established. The AUC was used to measure the ability of the qPCR load to differentiate between individuals who were *P. jirovecii* colonised and diseased.

Objective 3: Estimate the prevalence of *P. jirovecii* colonisation and infection among patients with available qPCR and BDG results enrolled in the pneumonia surveillance programme.

Using the established qPCR load cut-off values as established in objective 2, the PJP prevalence was calculated over the study period: total number of participants who were colonised and diseased with PJP /total number of participants who enrolled at the same sentinel surveillance hospitals with available *P. jirovecii* results.

Objective 4: To describe the demographic and clinical characteristics of hospitalised patients enrolled in the pneumonia surveillance programme stratified by patients who were negative for *P. jirovecii*, patients colonised with *P. jirovecii* and patients infected with *P. jirovecii*.

To describe the demographic, clinical and epidemiological characteristics of those who were *P. jirovecii* diseased, colonised, and negative, the established qPCR load cut-off values were re-applied back into the original subset population who were enrolled into the pneumonia sentinel sites between 2015 and 2016. Frequency tables were used. To evaluate the demographic and clinical factors associated with patients who were infected, colonised and those who were negative

for *P. jirovecii*, a univariable and multivariable multinomial logistic regression was conducted using the patients colonized with *P. jirovecii* group as the reference group for inter-group comparison. Random effects for the univariate and multivariable multinomial logistic regression model could not converge; thus, a priori criteria was used to account for clustering (on surveillance hospital). For the multivariable model, variables with a significant p-value below 0.2 were assessed and non-significant factors were dropped using manual forward selection. A p-value <0.05 were considered statistically significant.

3.8 Ethical considerations

Ethical approval for the ongoing activities of the pneumonia surveillance programme is covered by the Human Research Ethics Committee (Medical), University of the Witwatersrand (clearance number M140824). Data were de-identified before analysis was conducted. Before commencing this secondary analysis, ethical approval was received from the University of the Witwatersrand Human Research Ethics Committee (M1911144).

Chapter 4: Results

For the period 2015 through 2016, 2630 patients were enrolled in the syndromic pneumonia surveillance programme conducted at the Edendale Hospital and Klerksdorp-Tshepong Hospital Complex. Of those, 1542 (58.63%) had sputum samples tested for *P. jirovecii* and had available qPCR results (Figure 1). Of the samples that were tested, 190 (12.32%) had both BDG and qPCR load results available in comparison with 1352 (87.68%) that did not have available BDG results. Of the 1542 patients with available qPCR results, 254 (16.47%) patients were positive for *P. jirovecii* (qPCR DNA load >0 copies/5 µL) and 1288 (83.53%) patients were negative for *P. jirovecii* (qPCR DNA load ≤0 copies/5 µL). Of the 190 patients with available BDG results, 64 (4.15%) were positive for a fungal infection, 120 (7.78%) were negative and 6 (0.39%) were classified as undetermined.

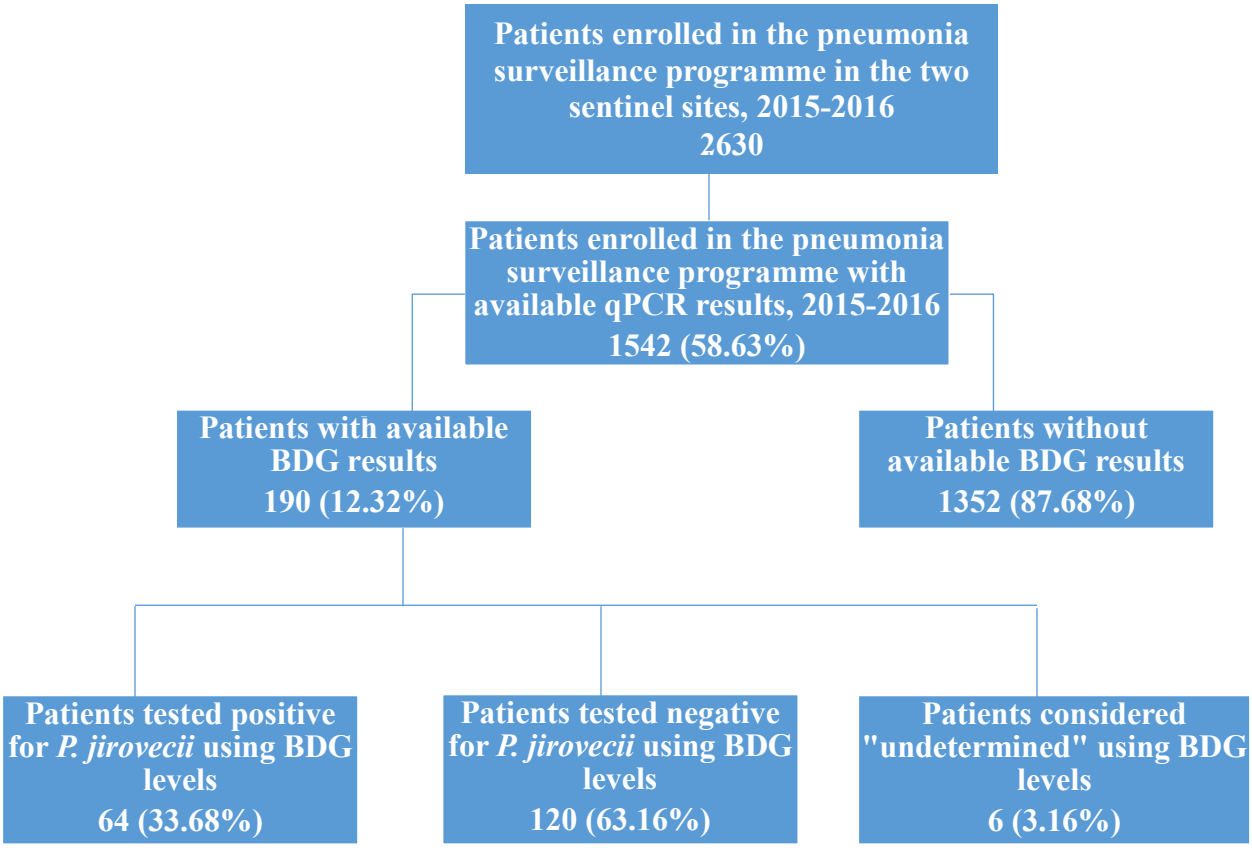

Figure 2: Individuals who had an induced sputum and sera sample collected in the pneumonia surveillance programmes, South Africa, 2015-2016

4.1 Demographic, clinical and epidemiological characteristics of individuals enrolled in pneumonia surveillance programme at the two sentinel sites with an available qPCR result, 2015-2016

Of the 1542 patients with available qPCR results, a higher proportion of individuals were in the 25-44 year age group (36.25%, 559/1542), male (52.53%, 810/1542) and black (97.47%, 1503/1542). The median age of all patients was 33 years (range: 0 days to 80 years). Nine hundred induced sputum specimens were collected from Edendale Hospital (58.37%) and six hundred and forty-two were collected from Klerksdorp-Tshepong Hospital Complex (41.63%). Fifty-four percent (840/1542) were enrolled in the pneumonia surveillance programme in 2016. A higher proportion of patients presented with a cough (94.81%, 1462/1542), difficulty breathing (61.54%, 949/1542) and chest pain (59.92%, 924/1542) at hospital admission. HIV results were available for 1507 (97.73%) of all patients who had an induced sputum specimen collected; 53.82% (811/1507) of patients were living with HIV. In-hospital mortality was at 4.93% (76/1542) and 0.65% (10/1542) were admitted into ICU.

4.2 Demographic, clinical and epidemiological characteristics of individuals with and without available BDG results

Of the 1542 patients enrolled in the pneumonia surveillance programme, a majority of patients with and without available BDG results were in the 25-44 year age group (44.74%, 85/190; 35.06%, 474/1352) (Table 1). Of those who had available BDG results, a majority of patients were male and black (55.26%, 105/190; 96.84%, 184/190, respectively). The number of specimens collected varied by year, with the greatest number received in 2016 among patients with and without BDG results available (64.74%, 123/190 and 53.03%, 717/1352).

HIV was the most prevalent underlying condition (in comparison to diabetes, tuberculosis and other underlying conditions) with a prevalence of 70.43% (131/186) among patients with BDG results and a prevalence of 51.48% (680/1321) among patients without BDG results. More than half of patients without available BDG results were hospitalised between 3-7 days (53.34%, 687/1288) as compared to those with BDG results (46.59%, 82/176). The in-hospital mortality among patients with available BDG results was 4.95% (9/182) in comparison to those without BDG results (5.12%, 67/1309); however, this was not statistically significant (p-value>0.05)

(Table 1 & Table 2). Other factors such as race, presenting clinical symptoms at hospital admission, duration of symptoms, other respiratory infections, management received during admission, ICU admission and underlying conditions including TB, diabetes and others listed in Table 2 were not statistically significant in the univariable and multivariable model (p-value>0.05).

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Table 1: Demographic and clinical characteristics of hospitalised patients enrolled in the pneumonia surveillance at two sites stratified by availability of BDG results, South Africa 2015-2016 (N=1542)

	Descriptive analysis	
Demographic and clinical characteristics	Patients with available BDG level results n/N (%) N=190	Patients without available BDG level results n/N (%) N=1352
Age group, years		
0-4	23/190 (12.11)	377/1352 (27.88)
5-14	2/190 (1.05)	52/1352 (3.37)
15-24	9/190 (4.74)	69/1352 (5.10)
25-44	85/190 (44.74)	474/1352 (35.06)
45-64	57/190 (30.00)	294/1352 (21.75)
≥65	14/190 (7.37)	86/1352 (6.36)
Female sex	85/190 (44.74)	647/1352 (47.86)
Race		
Black	184/190 (96.84)	1319/1347 (97.92)
Non-black	6/190 (3.16)	28/1347 (2.08)
Year of enrolment		
2015	67/190 (35.26)	635/1352 (46.67)
2016	123/190 (64.74)	717/1352 (53.03)
Presenting symptoms		
Fever	104/189 (55.03)	592/1348 (43.92)
Cough	179/189 (94.71)	1283/1341 (95.67)
Difficulty breathing	107/189 (56.61)	842/1341 (62.79)
Chest pain	132/166 (79.52)	792/967 (81.90)
Chills	0/122 (0.00)	0/656 (0.00)
Duration of symptoms before hospital admission (days)		
≤7	102/185 (55.14)	690/1312 (52.59)
>7	83/185 (44.86)	622/1312 (47.41)
Known underlying conditions		
HIV	131/186 (70.43)	680/1321 (51.48)
Diabetes	4/190 (2.11)	36/1352 (2.66)
Tuberculosis	21/175 (12.00)	149/1226 (12.15)
Other underlying conditions *	13/190 (6.84)	126/1352 (9.32)
Other respiratory infections		
Influenza**	7/190 (3.68)	80/1351 (5.92)
RSV	6/190 (3.16)	106/1351 (7.85)
Bordetella pertussis	1/106 (0.94)	12/960 (1.25)
Management received during admission		
Ventilation	0/188 (0.00)	9/1338 (0.67)
Steroid	1/190 (0.53)	5/1326 (0.38)
Antibiotics	178/189 (94.18)	1278/1343 (95.16)
Oxygen therapy	93/188 (49.47)	655/1340 (48.88)
Cotrimoxazole	12/189 (6.35)	62/1343 (4.62)
Duration of hospitalisation (days)		
<3	36/176 (20.45)	241/1288 (18.71)
3-7	82/176 (46.59)	687/1288 (53.34)
>7	58/176 (32.95)	360/1288 (27.95)
ICU admission	0/189 (0.00)	10/1344 (0.74)
In-hospital mortality	9/182 (4.95)	67/1309 (5.12)

Abbreviations: BDG=β-D-glucan, RSV=respiratory syncytial virus
*Underlying conditions included any of the following: anaemia, asthma, other chronic lung disease, stroke, chronic heart disease (valvular heart disease, coronary heart disease, or heart failure excluding hypertension), seizures, liver disease (liver failure or cirrhosis), renal disease (nephrotic syndrome, chronic renal failure), immunocompromising conditions excluding HIV infection (autoimmune disease, immunosuppressive therapy, immunoglobulin deficiency), pregnancy, asplenia, burns, obesity, malignancy, organ transplant, chronic obstructive pulmonary disease (COPD)/emphysema & neurological disease (spinal cord injury, neuromuscular conditions).
**All influenza subtypes were grouped into one category including influenza A (H1N1)pdm09, influenza A (H3N2), influenza A untyped and influenza B.
Note: Influenza, RSV and *Bordetella* pertussis were tested from nasopharyngeal swabs.

Table 2: Demographic and clinical characteristics associated with the availability of BDG results among hospitalised patients enrolled in the pneumonia surveillance, South Africa 2015-2016 (N=1542)

	Univariate analysis		Multivariate analysis	
Demographic and clinical characteristics	OR (95% CI)	P-value	Adjusted OR (95% CI)	P-value
Age group, years				
0-4	1	1	-	-
5-14	0.28 (0.06-1.27)	0.10	-	-
15-24	1.04 (0.44-2.47)	0.93	-	-
25-44	1.35 (0.77-2.35)	0.30	-	-
45-64	1.50 (0.84-2.66)	0.17	-	-
≥65	1.47 (0.69-3.11)	0.31	-	-
Female sex	0.82 (0.60-1.11)	0.21	-	-
Race				
Black	1	1	-	-
Non-black	0.81 (0.33-2.01)	0.65	-	-
Year of enrolment				
2015	1	1	1	1
2016	1.97 (1.42-2.73)	<0.001	1.98 (1.42-2.76)	<0.001
Presenting symptoms				
Fever	1.12 (0.81-1.55)	0.49	-	-
Cough	0.88 (0.43-0.48)	0.72	-	-
Difficulty breathing	1.06 (0.77-1.46)	0.73	-	-
Chest pain	1.13 (0.74-1.72)	0.57	-	-
Chills	-	-	-	-
Duration of symptoms before hospital admission (days)				
≤7	1	1	-	-
>7	0.91 (0.66-1.25)	0.56	-	-
Known underlying conditions				
HIV	1.47 (1.03-2.11)	0.03	-	-
Diabetes	0.74 (0.26-2.15)	0.58	-	-
Tuberculosis	0.81 (0.49-1.34)	0.42	-	-
Other underlying conditions *	0.78 (0.42-1.42)	0.41	-	-
Other respiratory infections				
Influenza**	0.60 (0.27-1.33)	0.21	-	-

RSV	0.65 (0.28-1.54)	0.33	-	-
Bordetella pertussis	0.55 (0.07-4.31)	0.57	-	-
Management received during admission				
Ventilation	0.37 (0.02-6.40)	0.50	-	-
Steroid	0.76 (0.09-6.55)	0.80	-	-
Antibiotics	0.90 (0.46-1.77)	0.77	-	-
Oxygen therapy	1.16 (0.85-1.59)	0.36	-	-
Cotrimoxazole	1.13 (0.59-2.18)	0.71	-	-
Duration of hospitalisation (days)				
<3	1	1	-	-
3-7	0.85 (0.55-1.30)	0.45	-	-
>7	1.18 (0.75-1.87)	0.48	-	-
ICU admission	-	-	-	-
In-hospital mortality	0.92 (0.45-1.92)	0.83	-	-

Abbreviations: BDG=β-D-glucan, RSV=respiratory syncytial virus, CI=confidence intervals

*Underlying conditions included any of the following: anaemia, asthma, other chronic lung disease, stroke, chronic heart disease (valvular heart disease, coronary heart disease, or heart failure excluding hypertension), seizures, liver disease (liver failure or cirrhosis), renal disease (nephrotic syndrome, chronic renal failure), immunocompromising conditions excluding HIV infection (autoimmune disease, immunosuppressive therapy, immunoglobulin deficiency), pregnancy, asplenia, burns, obesity, malignancy, organ transplant, chronic obstructive pulmonary disease (COPD)/emphysema & neurological disease (spinal cord injury, neuromuscular conditions).

**All influenza subtypes were grouped into one category including influenza A (H1N1)pdm09, influenza A (H3N2), influenza A untyped and influenza B.

Note: Influenza, RSV and *Bordetella* pertussis were tested from nasopharyngeal swabs.

4.3 Description of the 28 patients who were positive on BDG results, but had negative qPCR results

Overall, 28 patients had a positive BDG result but had a negative qPCR result. Further examination of patient records was conducted. A majority of patients were between the ages of 25-44 years (11/28, 39.39%) and male (16/28, 57.14). Most patients did not get tested for fungal pathogens (25/28) and one patient tested positive for *Candida albicans*. Two patients had a high BDG level according to Fungitell assay results; however, no further tests were conducted. Interestingly, 64.29% (18/28) of patients were living with HIV and 14.29% (4/28) patients had a co-infection of TB. Seven patients had confirmed bacterial infections: Gram-positive cocci were detected in four patients, Gram-negative bacilli were detected in one patient and mild toxic granulation was found in two patients. Two patients tested positive for hepatitis B virus.

4.4 Accuracy and predictive values of qPCR DNA load value cut-offs for *P. jirovecii* among hospitalised patients, South Africa 2015-2016

Of the 190 sera, 120 were negative for BDG, 64 were positive and 6 were considered undetermined based on the manufacturer's recommended cut-off values as described in the literature review (BDG levels greater than 80 pg/mL are considered as an indicator for fungal infection, 60-79 pg/mL is undetermined and less than 60 pg/mL is *P. jirovecii* negative). A ROC analysis was conducted using results from patients with available qPCR-positive and BDG results (n=59). Once the ROC curve was applied, the AUC was 0.70 and the model was considered to be "fair" at differentiating those who are colonised & infected with *P. jirovecii* (Figure 3). A qPCR load cut-off value of 550 copies/5 µL was established which correctly classified 71% of qPCR results. This yielded a high specificity of 91.67% and a moderate sensitivity of 57.14%, respectively. Using the prevalence of PJP in Sub-Saharan Africa (18.8%), the positive and negative predictive values were 100.0% and 81.6%, respectively.

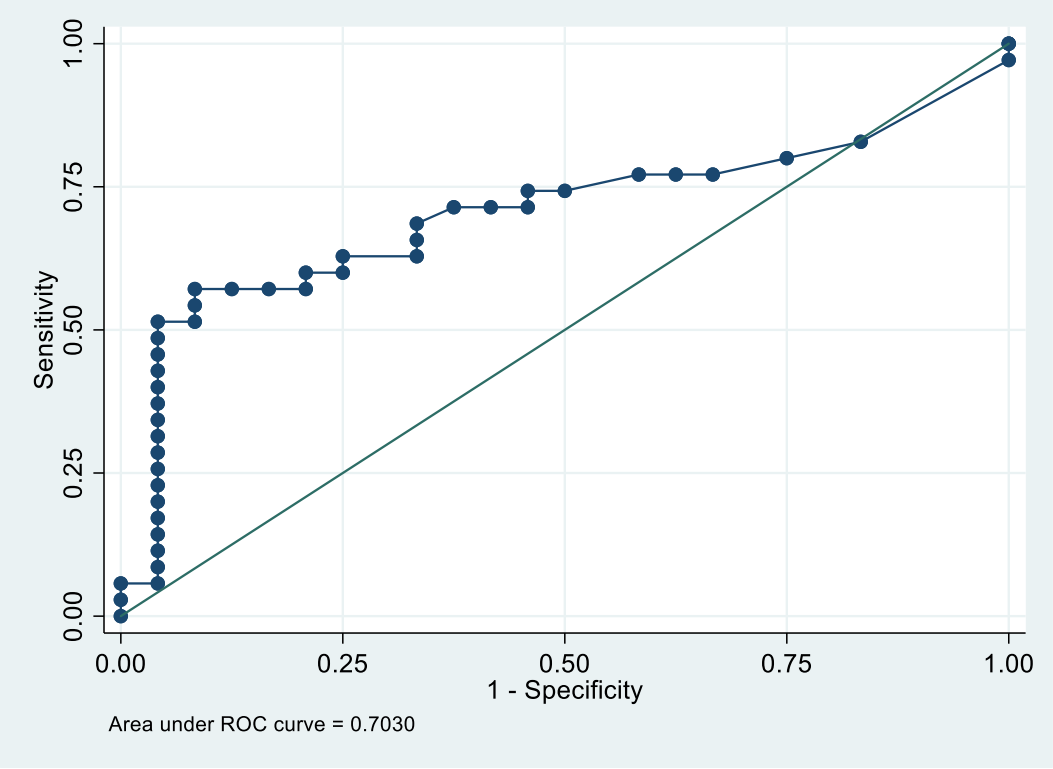

Figure 3: Sensitivity-specificity plot for the validity of established qPCR DNA load value cut-offs for *P. jirovecii* among hospitalised patients

4.5 Prevalence of *P. jirovecii* colonisation and infection among patients with available qPCR and BDG results enrolled in the pneumonia surveillance programme

Based on the established qPCR DNA load cut-off value, patients with a qPCR load of 0 copies/5 µL were classified as *P. jirovecii* negative, those with a qPCR load between 1-549 copies/5 µL were classified as patients colonised with *P. jirovecii* and patients with a qPCR load above 550 copies/5 µL were classified as *P. jirovecii* infected. Overall, the annual PJP prevalence from 2015 to 2016 was 5.19% (80/1542*100). Furthermore, the prevalence of colonisation with *P. jirovecii* was 11.28% (174/1542*100).

4.6 Demographic and clinical characteristics of hospitalised patients enrolled in the pneumonia surveillance stratified by patients who were negative for *P. jirovecii*, patients colonised with *P. jirovecii* and patients infected with *P. jirovecii*, South Africa 2015-2016

Once the established cut-off value was re-applied back into the study population with available qPCR results between 2015 and 2016 (n=1542), 80 patients were classified as infected with *P. jirovecii* (5.19%, 80/1542), 174 patients as colonised with *P. jirovecii* (11.28%, 174/1542) and 1288 patients as negative for *P. jirovecii* (83.53%, 1288/1542) (Table 3). Across all three categories, a large proportion of patients was in the 25-44 year age group. Across the three groups (patients infected with *P. jirovecii*, patients colonised with *P. jirovecii* and patients who were *P. jirovecii* negative), a majority of patients were black (96.25%, 77/80; 98.84%, 171/173; 97.67%, 1255/1285, respectively).

On multivariable analysis adjusting for surveillance hospital clustering, patients who were infected with *P. jirovecii* compared to those who were colonised with *P. jirovecii* were more likely to be female (Adjusted relative risk ratio (aRRR) 1.91; 95% CI:1.04-3.49; p=0.04), to be living with HIV (aRRR 4.30; 95% CI:1.82-10.16; p<0.01) and receive cotrimoxazole at time of hospital admission (aRRR 5.19; 95% CI:2.24-12.06; p<0.001) (Table 3). However, patients who were infected with *P. jirovecii* compared to those who were colonised with *P. jirovecii* were less likely to be enrolled into the pneumonia surveillance programme in 2016 as compared to 2015 (aRRR 0.48; 95% CI: 0.27-0.88; p=0.02) and present with a cough (aRRR 0.20; 95% CI:0.05-0.85; p=0.03).

On multivariable analysis adjusting for surveillance hospital clustering, patients who were negative for *P. jirovecii* compared to those who were colonised with *P. jirovecii* were less likely to be enrolled into the pneumonia surveillance programme in 2016 as compared to 2015 (aRRR 0.70; 95% CI:0.50-0.99; p=0.04) and receive cotrimoxazole at time of hospital admission (aRRR 0.47; 95% CI:0.22-1.01; p=0.05) (Table 3). Interestingly, patients who were negative for *P. jirovecii* compared to those who were colonised with *P. jirovecii* were more likely to present with fever (aRRR, 1.44; 95% CI:1.01-2.05; p=0.04)

More than two-thirds of patients infected with *P. jirovecii* had a symptom duration of >7 days before hospital admission (63.64%, 49/77) (Table 3). Among those who were colonised with *P. jirovecii*, 52.69% (88/167) had a symptom duration >7 days. Interestingly, 54.67% (685/1253) of patients who were negative for *P. jirovecii* had a symptom duration of ≤7 days. Overall, those who were either colonised or infected with *P. jirovecii* presented with a longer symptom duration. However, this observation was not statistically significant in the multivariable analysis (p>0.05).

Among *P. jirovecii* infected patients, 2.5% (2/80) were admitted to ICU as compared to patients who were colonised (0.58%, 1/173) and those who were negative for *P. jirovecii* (0.55%, 7/1274) (Table 3). Patients infected with *P. jirovecii* compared to those who were colonised with *P. jirovecii* were at an increased risk of in-hospital death (aRRR 2.63; 95% CI:0.98-7.05; p=0.05).

Table 3: Demographic and clinical characteristics of hospitalised patients enrolled in the pneumonia surveillance stratified by patients who were negative for *P. jirovecii*, patients colonised with *P. jirovecii* and patients infected with *P. jirovecii*, South Africa 2015-2016 (n=1542)

Demographic and clinical characteristics	Patients colonised with *P. jirovecii* n/N (%) N=174 Reference[a]	Patients negative for *P. jirovecii*				Patients infected with *P. jirovecii*			
		n/N (%) N=1288	RRR[b] (95% CI)	ARRR[c] (95% CI)	P-value	n/N (%) N=80	RRR[b]	ARRR[c] (95% CI)	P-value
Age group, years									
0-4	36/174 (20.69)	359/1288 (27.87)	1	-	-	5/80 (6.25)	1	-	-
5-14	2/174 (1.15)	50/1288 (3.88)	3.17 (0.73-13.81)	-	-	2/80 (2.50)	11.11 (1.24-99.78)	-	-
15-24	8/174 (4.60)	65/1288 (5.05)	1.00 (0.43-2.32)	-	-	5/80 (6.25)	6.60 (1.49-29.09)	-	-
25-44	76/174 (43.68)	427/1288 (33.15)	0.71 (0.44-1.15)	-	-	56/80 (70.00)	8.19 (2.89-23.23)	-	-
45-64	39/174 (22.41)	301/1288 (23.37)	0.97 (0.57-1.64)	-	-	11/80 (13.75)	3.07 (0.94-10.04)	-	-
≥65	13/174 (7.47)	86/1288 (6.68)	0.78 (0.39-1.58)	-	-	1/80 (1.25)	0.75 (0.08-7.11)	-	-
Female sex	78/174 (44.83)	604/1288 (46.89)	1.11 (0.81-1.53)	1.22 (0.87-1.71)	0.24	50/80 (62.50)	2.09 (1.21-3.60)	1.91 (1.04-3.49)	**0.04**
Race									
Black	171/173 (98.84)	1255/1288 (97.44)	1	-	-	77/80 (96.25)	1	-	-
Non-black	2/173 (1.16)	29/1288 (2.25)	2.62 (0.61-11.18)	-	-	3/80 (3.75)	4.13 (0.66-25.83)	-	-
Year of enrolment									
2015	69/174 (39.66)	589/1288 (45.73)	1	1	-	44/80 (55.00)	1	-	-
2016	105/174 (60.34)	699/1288 (54.27)	0.74 (0.53-1.02)	0.70 (0.50-0.99)	**0.04**	36/80 (45.00)	0.51 (0.30-0.88)	0.48 (0.27-0.88)	**0.02**
Presenting symptoms									
Fever	69/174 (39.66)	600/1284 (46.73)	1.57 (1.12-2.20)	1.44 (1.01-2.05)	**0.04**	27/79 (34.18)	0.86 (0.48-1.53)	0.97 (0.50-1.88)	0.92
Cough	170/174 (97.70)	1220/1277 (95.54)	0.49 (0.17-1.36)	0.37 (0.11-1.19)	0.10	72/79 (91.14)	0.24 (0.07-0.83)	0.20 (0.05-0.85)	**0.03**
Difficulty breathing	104/174 (59.77)	787/1277 (61.63)	0.96 (0.69-1.34)	-	-	58/79 (73.42)	1.78 (0.98-3.24)	-	-
Chest pain	118/138 (85.51)	745/921 (80.89)	0.66 (0.39-1.09)	-	-	61/74 (82.43)	0.67 (0.31-1.46)	-	-
Chills	0/100 (0.00)	0/643 (0.00)	-	-	-	3/35 (0.00)	-	-	-
Duration of symptoms before hospital admission (days)									
≤7	79/167 (47.31)	685/1253 (54.67)	1	-	-	28/77 (36.36)	1	-	-
>7	88/167 (52.69)	568/1253 (45.33)	0.74 (0.53-1.02)	-	-	49/77 (63.64)	1.56 (0.90-2.72)	-	-

Known underlying conditions									
HIV	105/170 (61.76)	636/1258 (50.56)	0.73 (0.51-1.03)	0.81 (0.56-1.18)	0.27	70/79 (88.61)	6.44 (2.94-14.10)	4.30 (1.82-10.16)	<**0.001**
Diabetes	8/174 (4.60)	30/1288 (2.33)	0.50 (0.22-1.11)	-	-	2/80 (2.50)	0.54 (0.11-2.59)	-	-
Tuberculosis	19/160 (11.88)	141/1169 (12.06)	1.09 (0.65-1.83)	-	-	10/72 (13.89)	1.25 (0.55-2.86)	-	-
Other underlying conditions*	15/174 (8.62)	119/1288 (9.24)	1.04 (0.59-1.83)	-	-	5/80 (6.25)	0.69 (0.24-1.97)	-	-
Other respiratory infections									
Influenza**	9/174 (5.17)	77/1287 (5.98)	1.17 (0.57-2.38)	-	-	1/80 (1.25)	0.23 (0.03-1.86)	-	-
RSV	13/174 (7.47)	95/1287 (7.38)	0.82 (0.44-1.52)	-	-	4/80 (5.00)	0.56 (0.17-1.82)	-	-
Bordetella pertussis	1/114 (0.88)	9/896 (1.00)	1.20 (0.15-9.64)	-	-	3/56 (5.36)	6.59 (0.67-65.01)	-	-
Management received during admission									
Ventilation	0/173 (0.00)	8/1273 (0.63)	-	-	-	1/80 (1.25)	-	-	-
Steroid	1/171 (0.58)	4/1266 (0.32)	-	-	-	1/79 (1.27)	-	-	-
Antibiotics	165/174 (94.83)	1215/1278 (95.07)	1.02 (0.49-2.09)	-	-	76/80 (95.00)	1.01 (0.30-3.39)	-	-
Oxygen therapy	80/174 (45.98)	615/1274 (48.27)	1.05 (0.76-1.45)	-	-	53/80 (66.25)	2.26 (1.30-3.92)	-	-
Cotrimoxazole	10/174 (5.75)	35/1278 (2.75)	0.50 (0.24-1.04)	0.47 (0.22-1.01)	**0.05**	29/80 (36.25)	10.28 (4.64-22.79)	5.19 (2.24-12.06)	<**0.001**
Duration of hospitalisation (days)									
<3	27/166 (16.27)	243/1222 (19.89)	1	-	-	7/76 (9.21)	1	-	-
3-7	80/166 (48.19)	654/1222 (53.52)	0.88 (0.56-1.40)	-	-	35/76 (46.05)	1.66 (0.66-4.17)	-	-
>7	59/166 (35.54)	325/1222 (26.60)	0.59 (0.36-0.96)	-	-	34/76 (44.74)	2.17 (0.85-5.52)	-	-
ICU admission	1/173 (0.58)	7/1274 (0.55)	0.89 (0.11-7.35)	-	-	2/80 (2.5)	4.23 (0.38-47.51)	-	-
In-hospital mortality	8/165 (4.85)	55/1248 (4.41)	0.92 (0.43-1.97)	1.08 (0.50-2.36)	0.84	13/78 (16.67)	3.96 (1.57-10.02)	2.63 (0.98-7.05)	**0.05**

Abbreviations: BDG=β-D-glucan, RSV=respiratory syncytial virus, CI=confidence intervals

[a] Patients colonised with *P. jirovecii* were used as a reference for multinomial model analysis (ie. comparing patients colonised with *P. jirovecii* to patients infected with *P. jirovecii* and patients negative for *P. jirovecii*

[b] Unadjusted relative risk ratio (RRR) at multivariable analysis

[c] Adjusted relative risk ratio (ARRR) at multivariable analysis

*Other conditions leading to immunosuppression include tuberculosis, chronic obstructive pulmonary disorder and diabetes.
**All influenza subtypes were grouped into one category including influenza A (H1N1)pdm09, influenza A (H3N2), influenza A untyped and influenza B.
Note: Influenza, RSV and *Bordetella pertussis* were tested from nasopharyngeal swabs.

Chapter 5: Discussion

In the discussion chapter, the main study findings were summarised and the established qPCR DNA load cut-off value was further compared to previous studies. The study strengths and limitations were outlined. To conclude the study, the conclusion and recommendations were made based from the study findings.

5.1 Performance of the established qPCR cut-off value

Due to PJP patients presenting with non-specific symptoms and a lack of a true gold standard in terms of diagnostic tests for PJP diagnosis, there is a reliance on molecular methods in respiratory samples for *P. jirovecii* diagnosis (53). This study aimed to establish *P. jirovecii* qPCR DNA load cut-off values to differentiate between *P. jirovecii* colonisation and disease among hospitalised patients for severe respiratory illness in South Africa, using BDG level as a biomarker of fungal infection. The use of BDG levels has been used as an indirect diagnostic biomarker for invasive fungal infections, whereas qPCR quantifies fungal load (DNA copies of specific gene/s) (3,9,11,68).

The optimal qPCR load cut-off value of 550 copies/5 µL was established according to the ROC analysis which correctly classified 71% of qPCR results. This yielded a high specificity of 91.67% and a moderate sensitivity of 57.14%, respectively. The ROC analysis model was considered to be "fair" at differentiating between those who were colonised & those infected with *P. jirovecii*. As a result of the model yielding a high specificity (ability to detect an individual negative with *P. jirovecii*), this could also indicate that the use of the established cut-off value may result in few false-positive results. Therefore, fewer cases of PJP are missed and may prevent unnecessary and invasive follow-up tests/specimen collection such as BALF. However, due to the tests slightly lower sensitivity, the trade-off would be that the test has a decreased capacity to detect "real" outbreaks of PJP which could also lead to a delay or omission for many outbreaks. Ideally, the established cut-off value could be useful in screening and ruling in patients who have PJP (ruling diagnoses).

Previous literature has suggested cut-off values to differentiate between *P. jirovecii* infection and colonisation, with others including an indeterminate zone (diagnosis between *P. jirovecii* infection

and colonisation could not be established). In addition, cut-off values were expressed in various manners such as copies/mL, trophic form equivalents/mL, copies per 5μL and copies/mL. Studies conducted in Japan (targeting the DHPS gene) and France (targeting the Msg gene) proposed qPCR DNA copy load cut-off values for the differentiation between *P. jirovecii* disease and colonisation ranged between 1300-31600 copies/mL, which yielded sensitivities of 80-100% and specificities of 70-100% (59,80). A South African study among hospitalised patients proposed a qPCR DNA copy load cut-off value of 78 copies/5 μL with a sensitivity and specificity of 94.6% and 89.1% respectively whilst a study conducted in France proposed two cut-off values of 120 and 1900 trophic form equivalents/mL to discriminate active pneumonia from colonization, with an inclusion of a "grey zone" (sensitivity of 100% and specificity of 96.9%). Both studies used qPCR targeting the MtLSU gene (9,20). However, caution is needed when applying previous cut-off values to other clinical situations due to multiple factors that may influence the sensitivity and specificity of the test including (1) specimen type and quality collected, (2) study population and (3) use of different assays and various gene targets.

With regards to specimen collection type and quality, induced sputum and BALF samples are the preferred specimen type for *P. jirovecii* testing as it also yields a higher sensitivity (diagnostic yield) (83). However, other studies have demonstrated that oropharyngeal washings using nested PCR could also yield a sensitivity of 95.6% (45). Studies have also shown that patients living with HIV had a higher fungal burden as compared to immunocompromised patients living without HIV (9,43). This reflects the distinct pathogenesis between the two population groups. Considering PJP is prevalent in infants, paediatric patients may have a higher fungal loads compared to adults (3,5,10). Studies have used other assays in the identification of *P. jirovecii* such as direct immunofluorescence, nested PCR and qPCR targeting the Msg gene and the DHPS gene (9,20,44). The preferred assay for the detection of *P. jirovecii* is qPCR and the Msg target has proven to have the highest specificity as compared with other PCR assays (9). Overall, fungal loads may vary depending on the study population, the variability of PCR assay measurements, the use of different specimens and quality. Therefore, previous studies results may not allow for a good comparison of cut-off values (20).

Based on the demographic and clinical characteristics of the study population (higher proportion of individuals living with HIV and adults between 25 to 44 years), the established cut-off value of 550 copies/5 µL could be applied to differentiate between *P. jirovecii* infection and colonisation among adults living with HIV and not the entire South African population. While the use of qPCR in conjunction with Fungitell assays is useful, the cut-off values cannot be universally applied but should be used in similar study populations.

5.2 Comparison of *P. jirovecii* colonisation and PJP prevalence

Using the established qPCR DNA load cut-off value, the PJP prevalence was 5.12% and the prevalence of *P. jirovecii* colonisation was 11.28%. Overall, the PJP prevalence was lower as compared to a systematic review and meta-analysis among hospitalised adults presenting with a respiratory illness in sub-Saharan Africa that reported a pooled prevalence of PJP of 18.8% (8). Similarly, the prevalence of colonisation with *P. jirovecii* obtained from our study results was underestimated as compared to studies that reported *P. jirovecii* colonisation prevalence that ranged between 28.5% and 44.8% among patients who were immunocompromised (patients living with HIV and those with autoimmune inflammatory diseases) (43–46). A limitation to note with the above mentioned studies conducted by Pereira et al.; Gutierrez et al. and Riebold et al., are that nested PCR and staining methods on oropharyngeal washings were used to assess colonization among patients living with HIV; therefore, detection of a fungal load may differ as compared to our study results (43–45). Another contributing factor to the variability in *P. jirovecii* colonisation prevalence is the differences in the geographical study population as highlighted by Gutierrez et al. (44). A study conducted in two French hospitals were able to successfully differentiate patients with PJP (13/17, 76.47%) and *P. jirovecii* colonisation (19/29, 65.52%). The lower and upper bound established cut-off value ranged between 1.6 x 10^3 copies/µL and 2 x 10^4 copies/µL. These established cut-off values achieved a 100% sensitivity and 100% specificity having the ability to distinguish PJP from *P. jirovecii* colonisation in 96% of cases. The main limitation with establishing an upper and lower cut-off value is that values in between the range represent an indeterminate zone resulting in 14 patients' diagnosis between PJP and *P. jirovecii* colonisation could not be established (60).

Matsumura et al. used a cut-off value of 1300 copies/mL to differentiate *P. jirovecii* colonisation and infection with an AUC of 0.96, the sensitivity of 100% and specificity of 80%. Therefore, 11 patients found to have definite PJP (8.59%), 42 with probable PJP (32.81%), 15 colonised with *P. jirovecii* (11.72%) and 60 who were negative for *P. jirovecii* (46.88%) (59). This is similar to our study methodology as the authors combined BALF and induced sputum samples to evaluate the qPCR. Conclusively, by applying the established cut-off value, 550 copies/5 µL, to a database from a respiratory syndromic surveillance programme between a smaller time period (2015-2016), the PJP prevalence may be underestimated as this includes patients with SRI who were hospitalised. As a result, patients who were not ill enough to be admitted to hospital but were colonised with *P. jirovecii* may have been missed. Another contributing factor to the low prevalence of PJP detected in our study is that our model yielded slightly lower sensitivity; therefore, the test has a decreased capacity to detect those with definite PJP. Despite qPCR displaying high accuracy for discriminating between *P. jirovecii* colonisation and infection, cut-off values used in these assays remain to be standardised.

5.3 Discrepancies of the 28 patients who were positive on BDG results, but had negative qPCR results

Within the study population, 28 patients had a positive BDG result and negative qPCR result. Due to BDG being a non-specific biomarker for a fungal pathogen, there could be a possibility that patients had other types of fungal infections (other than PJP). Upon further examination, two patients had a high BDG level according to Fungitell assay results; however, no further qPCR tests were conducted. Due to both patients living with HIV and presenting with either a fever or a cough (clinical symptom of PCP), TMP/SMX was administered as a prophylactic treatment.

5.4 Differences in demographic, clinical and epidemiological characteristics between those infected and colonised with *P. jirovecii*

Based on the results that described the demographic, clinical and epidemiological characteristics of those who were *P. jirovecii* diseased, colonised and negative, we found that patients who were infected with *P. jirovecii* compared to those who were colonised with *P. jirovecii* were more likely to be female (adjusted relative risk ratio (aRRR) 1.91; 95% CI:1.04-3.49; p=0.04) and to be living with HIV (aRRR 4.30; 95% CI:1.82-10.16; p<0.01). Similarly, a PJP laboratory-based

surveillance study reported that the highest prevalence of PJP occurred in adults aged 25–44 years with significantly more females affected than males (84). The current study findings are also supported by other literature that has reported that patients living with HIV are at highest risk to to *P. jirovecii* due to their low CD4 count (5,8,10,45). Patients who were infected with *P. jirovecii* compared to those who were colonised with *P. jirovecii* were less likely to be enrolled into the pneumonia surveillance programme in 2016 as compared to 2015 (aRRR 0.48; 95% CI:0.27-0.88; p=0.02). This could possibly indicate that there was a higher transmission of PJP in 2015 as compared to 2016.

By definition, *P. jirovecii* colonisation occurs in individuals who do not present with any signs or symptoms of acute pneumonia (85). Therefore, it is interesting to find that study results highlighted that patients who were negative for *P. jirovecii* compared to those who were colonised with *P. jirovecii* were more likely to present with fever (aRRR, 1.44; 95% CI:1.01-2.05; p=0.04). However, patients who were infected with *P. jirovecii* compared to those who were colonised with *P. jirovecii* were less likely to present with a cough (aRRR 0.20; 95% CI:0.05-0.85; p=0.03). More than two-thirds of patients infected with *P. jirovecii* had a symptom duration of >7 days before hospital admission (63.64%, 49/77). Among those who were colonised with *P. jirovecii*, had a symptom duration >7 days. Interestingly, 54.67% (685/1253) of patients who were negative for *P. jirovecii* had a symptom duration of ≤7 days. Patients who were either colonised (52.69%, 88/167) or infected with *P. jirovecii* (63.64%, 49/77) presented with a longer symptom duration. However, this observation was not statistically significant in the multivariable analysis (p>0.05). Due to the study population that includes inpatients presenting with an acute lower respiratory tract infection characterized with a temperature ≥38°C or history of fever and cough, clinical signs and symptoms could be attributed to another respiratory-related pathogen.

Results have also shown that among *P. jirovecii* infected patients, more (2.5%, 2/80) were admitted to ICU as compared to patients who were colonised (0.58%, 1/173) and those who were negative for *P. jirovecii* (0.55%, 7/1274). This indicates that patients with PJP are usually severely ill and therefore, contribute to the burden on the South African healthcare system, but this is dependent on the duration of treatment and severity. Patients infected with *P. jirovecii* compared to those who were colonised with *P. jirovecii* were at an increased risk of in-hospital death (aRRR 2.63; 95%

CI:0.98-7.05; p=0.05). This is reflective to previous South African studies that reported a PJP-related mortality rate that ranged between 6.5% and 25.4% (4,6,8).

On multivariable analysis adjusting for surveillance hospital clustering, patients who were infected with *P. jirovecii* compared to those who were colonised with *P. jirovecii* were more likely to have received cotrimoxazole at time of hospital admission (aRRR 5.19; 95% CI:2.24-12.06; p<0.001). Considering that a majority of *P. jirovecii* infected patients had a co-infection of HIV (88.61%, 70/79), cotrimoxazole may have been used as prophylaxis as a precautionary measure for physicians. It should be noted that cotrimoxazole has the potential to reduce the burden of viable *P. jirovecii* but does not eliminate non-replicating forms of the pathogen. Therefore, *P. jirovecii* concentration levels will remain high on qPCR after successful treatment or prophylaxis due to qPCR detecting dead and viable *P. jirovecii*. To reduce transmission of *P. jirovecii*, initiating prophylaxis to those infected and colonised should be recommended for patients with immunosuppressive therapy; however, the potential risk of long terms administration of cotrimoxazole should be considered (9).

5.5 Strengths and limitations

Several limitations warrant discussion. Results may not be truly representative of South Africa's population as a limited number of specimens were collected and tested on BDG and qPCR for *P. jirovecii* from sentinel surveillance sites located in North West and KwaZulu-Natal provinces. Furthermore, the prevalence of PJP may be underestimated due to the use of sentinel data. Due to the small sample size, qPCR DNA load cut-off values could not be established when stratified by age groups and HIV status.

The study results further support the idea of improving qPCR diagnosis using a qPCR cycle threshold cut-off value thereby distinguishing PJP from *P. jirovecii* colonisation. Despite the fact that established cut-off values cannot be universally applied, the cut-off value could be used in a larger South African study population over a longer period of time in order to get a true representation of PJP prevalence in South Africa.

5.6 Conclusions and recommendations

Overall, PJP is a common opportunistic infections among immunocompromised patients and the use of qPCR is a good diagnostic tool for *P. jirovecii*, especially when an accurate cut-off value is used to differentiate between colonisation and infection. However, the cut-off value cannot be universally applied but rather it may only be used in pilot studies with a similar population that consists of adults living with HIV. Irrespective of not establishing a true gold standard for diagnostic tests to diagnose PJP, the established cut-off value could be used in conjunction with clinical presentation. Although the PCR assay is considered to yield a higher sensitivity, more intensive specimen collection is needed to estimate the true burden of PJP in South Africa as well as to improve the knowledge on associated demographic, clinical and epidemiological factors between patients with PJP, those who are colonised and patients who tested negative for *P. jirovecii*. This could assist healthcare providers in initiating and managing treatment in a more timely manner.

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Appendices

Annex 1: Senate plagiarism policy

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

FACULTY OF HEALTH SCIENCES

PLAGIARISM DECLARATION TO BE SIGNED BY ALL HIGHER DEGREE STUDENTS

SENATE PLAGIARISM POLICY: APPENDIX ONE

I Jessica Ann Lai Yun (Student number: 1779814) am a student registered for the degree of MSc (Epidemiology) in the academic year 2021.

I hereby declare the following:

- I am aware that plagiarism (the use of someone else's work without their permission and/or without acknowledging the original source) is wrong.
- I confirm that the work submitted for assessment for the above degree is my own unaided work except where I have explicitly indicated otherwise.
- I have followed the required conventions in referencing the thoughts and ideas of others.
- I understand that the University of the Witwatersrand may take disciplinary action against me if there is a belief that this is not my own unaided work or that I have failed to acknowledge the source of the ideas or words in my writing.
- I have included as an appendix a report from "Turnitin" (or other approved plagiarism detection) software indicating the level of plagiarism in my research document.

Signature: _____ Date: 25 June 2021

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Annex 2: HREC certificate

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

R14/49 Miss Jessica Ann Lai Yun

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M1911144

NAME: (Principal Investigator)	Miss Jessica Ann Lai Yun
DEPARTMENT:	Epidemiology and Biostatistics National Institute for Communicable Diseases
PROJECT TITLE:	The use of a quantitative assay to differentiate between Pneumocystis jirovecii colonisation and disease among hospitalised patients, South Africa, 2015-2016
DATE CONSIDERED:	29/11/2019
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Prof John Frean
APPROVED BY:	CB Penny Dr CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	29/11/2019

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary on the Third Floor, Faculty of Health Sciences, Phillip Tobias Building, 29 Princess of Wales Terrace, Parktown, 2193, University of the Witwatersrand. I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**. The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. In this case, the study was initially reviewed in **November** and will therefore be due in the month of **November** each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature

Date 09/12/2019

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Annex 3: Approval letter to use database from the Centre for Respiratory Diseases and Meningitis

NATIONAL INSTITUTE FOR COMMUNICABLE DISEASES
Division of the National Health Laboratory Service

Centre for Respiratory Diseases and Meningitis National Institute
1 Modderfontein Road, Sandringham, 2131
Tel: +27 (0)11 386 6593

30th October 2019

To whom it may concern:

RE: Permission for Jessica Yun to use the CRDM pneumonia and influenza-like illness surveillance programmes' databases for her MSc Epidemiology

I, Prof Cheryl Cohen, head of the Centre for Respiratory Diseases and Meningitis (CRDM) at the National Institute for Communicable Diseases (NICD), hereby grant Jessica Yun (Student number: 1779814) permission to use the pneumonia and influenza-like illness surveillance programmes databases for her two research projects entitled "The use of a quantitative assay to differentiate between *Pneumocystis jirovecci* colonisation and disease among hospitalised patients, South Africa, 2015-2016" and "Evaluation of the pneumonia and influenza-like illness surveillance programmes for the detection of pertussis in South Africa, 2016-2019", required for the fulfilment of the research component for the SA Field Epidemiology and Laboratory Training Programme and the University of the Witwatersrand Master of Science in Field Epidemiology degree (MSc, SAFETP track). She will be conducting secondary data analysis.

Kind regards

Prof Cheryl Cohen
Head of the Centre for Respiratory Diseases and Meningitis
National Institute for Communicable Disease
011 386 6593
Cherylc@nicd.ac.za