

**PREVALENCE OF *Pneumocystis jirovecii* IN SMEAR
NEGATIVE SPUTUM SAMPLES AT THE COAST
GENERAL TEACHING AND REFERRAL HOSPITAL IN
KENYA**

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**Prevalence of *Pneumocystis jirovecii* in Smear Negative Sputum
Samples at the Coast General Teaching and Referral Hospital in
Kenya**

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**A Thesis Submitted in Partial Fulfilment of the Requirements for
the Degree of Master of Science in Molecular Medicine of the Jomo
Kenyatta University of Agriculture and Technology**

DECLARATION

This thesis is my original work and has not been presented for a degree in any other University

Signature.....Date.....

Anne Sein Saitoti

This thesis has been submitted for examination with our approval as the University Supervisors

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DEDICATION

I would like to dedicate this work to my parents, Mr. and Mrs. Saitoti, for their constant financial support, as well as my family, my husband, Mwakio, and my children, Ethan and Shighadi, for their psychological and emotional support and encouragement during my Master's Degree journey.

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TABLE OF CONTENTS

DECLARATION.....	ii
DEDICATION.....	iii
ACKNOWLEDGEMENT.....	iv
TABLE OF CONTENTS	v
LIST OF TABLES	x
LIST OF FIGURES	xi
LIST OF APPENDICES	xii
ABBREVIATIONS AND ACRONYMS	xiii
ABSTRACT	xv
CHAPTER ONE	1
INTRODUCTION.....	1
1.1 Background Information	1
1.2 Statement of the Problem.....	2
1.3 Justification.....	3
1.4 Objectives of the Study	4
1.4.1 General Objective	4
1.4.2 Specific Objectives	4
1.5 Research Questions	4

1.6 Limitation of the Study	5
CHAPTER TWO	6
LITERATURE REVIEW	6
2.1 Taxonomy of <i>Pneumocystis jirovecii</i>	6
2.2 Phylogeny of <i>Pneumocystis jirovecii</i>	6
2.3 Ecology of <i>Pneumocystis jirovecii</i> (Habitat)	7
2.4 Life Cycle of <i>Pneumocystis jirovecii</i>	8
2.5 Epidemiology of <i>Pneumocystis jirovecii</i> Pneumonia	9
2.6 Cause of Disease and Effects.....	11
2.7 Coinfections with Other Diseases.....	13
2.8 Risk Factors Associated with Pneumocystis Pneumonia	14
2.9 Economic Impact of Pneumocystis Pneumonia (PCP).....	15
2.9.1 Diagnostic Approaches for Pneumocystis Pneumonia	15
2.9.2 Management of Pneumocystis Pneumonia (PCP).....	20
CHAPTER THREE	22
MATERIALS AND METHODS	22
3.1 Study Site.....	22
3.2 Study Design.....	22
3.3 Study Population and Clinical Parameters.....	23

3.3.1 Target Population.....	23
3.4 Selection Criteria	23
3.4.1 Inclusion Criteria.....	23
3.4.2 Exclusion Criteria	24
3.5 Sample Size Determination	24
3.6 Sampling Technique	24
3.7 Recruitment of Patients.....	25
3.7.1 Study Participants	25
3.8 Data Collection Tools.....	26
3.8.1 Evaluation of Socio-Demographic and Risk Factors.....	26
3.9 Laboratory Methods	26
3.9.1 Sample Collection.....	26
3.9.2 Toluidine Blue O Staining Procedure and Microscopic Evaluation...	27
3.9.3 DNA Extraction	27
3.9.4 PCR Amplification	28
3.9.5 Electrophoresis.....	30
3.9.6 Prevalence Assessment	30
3.8.7 Sensitivity and Specificity Assessment	30
3.10 Statistical Data Analysis.....	31

3.10.1 Social Demographic Data Analysis	31
3.10.2 Prevalence Analysis	31
3.10.3 Diagnostic Performance of Polymerase Chain Reaction and Microscopy Analysis	32
3.11 Ethical Consideration	32
CHAPTER FOUR.....	33
RESULTS	33
4.1 Socio-Demographic Characteristics.....	33
4.1.2 Clinical Presentation of <i>Pneumocystis jirovecii</i>	35
4.2 Prevalence, Detection and Diagnostic of <i>Pneumocystis jirovecii</i>	37
4.3 Diagnostic Performance of PCR vs. Microscopy	38
4.4 Microscopy Results	38
4.5 Molecular Results	40
CHAPTER FIVE	43
DISCUSSION, CONCLUSION AND RECOMMENDATION.....	43
5.1 Discussion.....	43
5.1.1 Socio-Demographic Characteristics of <i>P. jirovecii</i> -Positive Patients	43
5.1.2 Prevalence of <i>Pneumocystis jirovecii</i> Pneumonia among Tuberculosis Smear-Negative and Retreatment Patients	50

5.1.3 Diagnostic Performance of Nested PCR Compared with Toluidine Blue O Staining.....	53
5.2 Conclusion.....	55
5.3 Recommendations	56
REFERENCES.....	58
APPENDICES	74

LIST OF TABLES

Table 3.1: Primers Used for Amplification of the Mitochondrial LSU rRNA Gene	29
Table 3.2: Amplification Conditions for <i>Pneumocystis jirovecii</i> Gene	29
Table 4.1: Association between Social Demographic Factors and <i>Pneumocystis jirovecii</i> Status	33
Table 4.2: Environmental Factors Associated with <i>Pneumocystis jirovecii</i> Status ..	34
Table 4.3: Association between Clinical and Health Status Factors and <i>Pneumocystis jirovecii</i> Status	36
Table 4.4: Detection and Diagnostic Performance of Toluidine Blue O Staining Compared to Nested PCR for <i>Pneumocystis jirovecii</i>	38

LIST OF FIGURES

Figure 2.1: Proposed Life Cycle of <i>Pneumocystis jirovecii</i>	8
Figure 3.1: Map of the Study Area	22
Figure 4.1: Microscopic Appearance of <i>Pneumocystis jirovecii</i> Cysts in Sputum following Toluidine Blue O Staining (100× Magnification)	39
Figure 4.2: Characteristic Cystic Structures of <i>Pneumocystis jirovecii</i> at 100× Magnification	39
Figure 4.3: Gel Showing PCR Amplification of different Sputum Samples.....	40
Figure 4.4: Representative Image Showing Positive Results under Nested PCR.....	41

LIST OF APPENDICES

Appendix I: Informed Consent (English Version).....	74
Appendix II: Informed Consent (Kiswahili Version).....	77
Appendix III: Structured Questionnaire	79
Appendix IV: SOPs of Sputum Collection and Processing	81
Appendix V: SOPs for Microscopy Technique and Stain	82
Appendix VI: Approval Letters	84
Appendix VII: Publication	88

ABBREVIATIONS AND ACRONYMS

AFB	Acid Fast Bacillus
BAL	Broncho alveolar Lavage
CGTRH	Coast General Teaching and Referral Hospital
C.I	Confidence Intervals
COPD	Chronic Obstructive Pulmonary Disease
DFA	Direct Fluorescence Antibody
DPX	Distyrene Plasticizer Xylene
ERC	Ethical Reviewing Committee
GM-CSF	Granulocyte Macrophage Colony stimulating Factor
GMS	Grocott's Methenamine Silver nitrate
HAART	Highly Active Antiretroviral Therapy
HR CT	High Resolution CT
IF	Immunofluorescence
KEMRI	Kenya Medical Research Institute
LDH	Lactate Dehydrogenase
MSG	Major Surface Glycoprotein's
Mt LSU Rrna	Mitochondrial Large subunit rRNA
OR	Odds Ratio
PCP	Pneumocystis pneumonia
PJP	<i>Pneumocystis jirovecii</i> pneumonia
PCR	Polymerase Chain Reaction

PTB	Pulmonary Tuberculosis
TB	Tuberculosis
TBO	Toluidine Blue O
TMP-SMX	Trimethoprim-sulfamethoxazole

ABSTRACT

Pneumocystis jirovecii pneumonia (PJP) is a significant opportunistic fungal infection among tuberculosis (TB) smear-negative and retreatment patients, especially in resource-limited settings. The overlap in clinical symptoms between PJP and tuberculosis can lead to delayed or incorrect diagnosis, resulting in ineffective therapy and poor patient outcomes. The aim of this study was to compare the socio-demographic characteristics and potential risk factors associated with *Pneumocystis jirovecii* infection, determine the prevalence of PJP, and assess the diagnostic performance of Toluidine blue O staining versus nested polymerase chain reaction (PCR) among TB smear-negative and retreatment patients at Coast General Hospital. A cross-sectional study of 100 TB smear-negative and retreatment patients was performed. Toluidine Blue O staining was used to evaluate sputum samples, followed by nested PCR targeting *P. jirovecii*'s mitochondrial large subunit ribosomal RNA (mtLSU rRNA). The socio-demographic and clinical data were described using frequencies and percentages. Bivariate logistic regression was used to investigate associations between *P. jirovecii* infection and predictor factors. Odds ratios (ORs), 95% confidence intervals (CIs), and p-values were provided. The diagnostic performance characteristics, including sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV), were computed with nested PCR as the reference standard. Of the 100 participants, 41% tested positive for *P. jirovecii* by nested PCR, while 29% tested positive using Toluidine blue O staining. Nested PCR uncovered an additional 12 positive cases from samples that were negative according to TBO staining. Most participants were male (63%), employed (84%), and aged between 20 and 49 years (23%). Fever was reported in 59% of participants, night sweats in 61%, and reduced EuroQol status in 74%. Bivariate logistic regression analysis showed no statistically significant association between *P. jirovecii* infection and socio-demographic factors, including age, gender, employment status, environmental factors such as housing type, flooding exposure, and health risk factors such as TB treatment status, fungal growth, fuel type, and smoking habit ($p > 0.05$). Similarly, clinical risk factors such as fever (OR = 1.63, 95% CI: 0.72–3.77), night sweats (OR = 0.71, 95% CI: 0.31–1.60), and EuroQol status (OR = 0.93, 95% CI: 0.38–2.34) were not significantly associated with *P. jirovecii* infection. Toluidine blue O staining demonstrated a sensitivity of 70.7% and specificity of 98.3% compared with nested PCR. The study found a rather high prevalence of *P. jirovecii* (41% among TB smear-negative and retreatment patients). Although no significant socio-demographic or clinical risk factors were identified, toluidine blue O staining demonstrated high sensitivity and acceptable specificity, indicating its potential use as a screening tool in resource-constrained settings where molecular diagnostic methods are not readily available. Using sensitive molecular diagnostic tools and target screening for *Pneumocystis jirovecii* before starting TB treatment may improve early detection, guide appropriate treatment, and improve patient outcomes in high-risk populations.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Pneumocystis jirovecii pneumonia (PJP) is a life-threatening fungal infection caused by the opportunistic pathogen *Pneumocystis jirovecii* (formerly classified as *Pneumocystis carinii*) which primarily affects immunocompromised individuals (Truong and Ashurst, 2023). Earlier misclassified as a parasite, this organism is now recognized as a fungus that colonizes the lungs of healthy individuals without causing symptoms (Bateman, Oladele, and Kolls, 2020). However, individuals with compromised immune systems, like HIV/AIDS patients, cancer survivors, or transplant recipients undergoing immunosuppressive treatment, are particularly susceptible (Gálvez *et al.*, 2025). *Pneumocystis jirovecii* can trigger severe pneumonia characterized by inflammation, fluid accumulation, and difficulty breathing (Carmona and Limper, 2010). The *P. jirovecii* spreads through airborne transmission, though its environmental reservoir remains unknown. Without timely diagnosis and therapy, PJP carries a high mortality rate, rendering it a substantial threat in clinical settings, especially in regions with high HIV prevalence (Truong and Ashurst, 2023).

The clinical relevance of PJP became widely recognized during the HIV/AIDS epidemic of the 1980s when it emerged as one of the leading causes of death among untreated patients (Chakaya *et al.*, 2004). Before that, there were occasional reports of cases, particularly in malnourished infants during the post-World War II era (Jakab *et al.*, 2025). Today, PJP is still a serious infection that mainly affects people with CD4 counts under 200 cells/ μ L, highlighting how important a strong immune system is for fighting this infection (Harris *et al.*, 2011). Beyond HIV, other vulnerable groups include leukemia and lymphoma patients, organ transplant recipients, and individuals on long-term corticosteroids or biological therapies (Chawla *et al.*, 2011). Symptoms usually include fever, dry cough, progressive dyspnea, and hypoxia, which can rapidly progress to respiratory failure if not treated (Harris *et al.*, 2011).

1.2 Statement of the Problem

The Kenya National Strategic Plan for Tuberculosis, Leprosy, and Lung Health (2023–2028) highlights the significant challenge of diagnosing smear-negative tuberculosis (TB) among HIV-positive individuals. Despite advancements in TB detection, diagnosing smear-negative TB remains a significant challenge, particularly in immunocompromised patients (Mohamed *et al.*, 2022). Despite its widespread use, smear microscopy is not sensitive in identifying TB in patients with low bacillary loads, such as those who are HIV-positive. This leads to increased misdiagnosis or under-diagnosis of smear-negative TB patients (Guto, Bii, and Denning, 2016).

Tuberculosis and other infections that take advantage of weakened immune systems, like PJP, show similar symptoms, making it harder to diagnose them (Dereje *et al.*, 2019). The successful diagnosis of PCP requires an evaluation of the clinical signs in at-risk patients and radiographic evidence; however, the clinical and radiographic findings are nonspecific, and they may overlap with those of other lower respiratory tract infections. Current diagnostic algorithms and limited resources in peripheral health facilities hinder prompt differentiation between TB and PJP (Bateman, Oladele, and Kolls, 2020).

The Kenya TB Strategic Plan emphasizes the need to improve diagnostic accuracy using molecular diagnostics, radiological tools, and HIV care. Addressing the coexistence of TB and PJP is crucial to reducing TB deaths, especially in areas where HIV is common.

Despite the high burden of HIV and tuberculosis in Kenya, little information exists on the prevalence of *Pneumocystis jirovecii* among smear-negative and retreatment TB patients. Because PJP and TB share similar clinical manifestations, patients may be misdiagnosed and treated inappropriately. Limited access to sensitive diagnostic methods such as PCR further complicates accurate diagnosis. This study therefore sought to determine the prevalence of *P. jirovecii* and evaluate the diagnostic performance of Toluidine Blue O staining compared with PCR among smear-negative

and retreatment TB patients at Coast General Teaching and Referral Hospital.

1.3 Justification

The diagnosis of respiratory illnesses in smear-negative patients in Kenya is a significant challenge, with the Kenya National TB Strategic Plan focusing on tuberculosis (TB). However, diagnostic efforts often overlook other serious opportunistic infections like *Pneumocystis jirovecii* pneumonia (PJP), which presents similar clinical features and is poorly diagnosed in routine care settings. PJP is life-threatening and often misdiagnosed as smear-negative TB or remains undetected, leading to inappropriate treatment, disease progression, and high mortality (Mbithi *et al.*, 2014).

The Kenyan Ministry of Health recognizes pneumonia, including PJP, as a top mortality driver among HIV patients, particularly in resource-limited settings where healthcare access is constrained (Kairu *et al.*, 2021). Accurate data on PJP prevalence remains scarce in Kenya, reflecting broader diagnostic challenges (Khehra, Padda, and Swift, 2023). The signs of PJP, such as fever, cough, trouble breathing, and low oxygen levels, are similar to those of tuberculosis (TB) and bacterial pneumonia, presenting a diagnostic challenge (Mbithi *et al.*, 2014).

Current routine practices rely heavily on microscopy-based TB diagnosis, which fails to detect PJP, leaving many patients undiagnosed until advanced stages (K Chepkondol *et al.*, 2020). While molecular techniques like polymerase chain reaction (PCR) are more accurate, they are not widely available in local medical settings, so most healthcare facilities still use traditional staining methods that are less reliable (Harizanov *et al.*, 2023).

This study aims to bridge the gap in diagnosis by comparing traditional staining methods with PCR, giving important information to help in better clinical decisions in low resource settings. Detecting PJP in smear-negative sputum can lead to more timely and precise therapy, reducing morbidity and mortality rates in immunocompromised individuals. Strengthening laboratory capacity for PJP detection will save lives and

align with global efforts to achieve equitable healthcare access for vulnerable populations.

1.4 Objectives of the Study

1.4.1 General Objective

To determine the prevalence of *Pneumocystis jirovecii* pneumonia and compare the diagnostic accuracy of traditional staining methods versus polymerase chain reaction (PCR) in sputum samples from tuberculosis smear-negative and retreatment cases at Coast General Teaching and Referral Hospital (CGTRH).

1.4.2 Specific Objectives

- i. To determine the socio-demographic characteristics of *Pneumocystis jirovecii* Pneumonia (PJP)-positive patients among tuberculosis smear-negative and retreatment cases at Coast General Teaching and Referral Hospital.
- ii. To determine the prevalence of *Pneumocystis jirovecii* pneumonia in sputum samples from tuberculosis smear-negative and retreatment cases at Coast General Teaching and Referral Hospital.
- iii. To compare the sensitivity and specificity of polymerase chain reaction and Toluidine Blue O staining for detecting *Pneumocystis jirovecii* in sputum samples from tuberculosis smear-negative and retreatment cases at Coast General Teaching and Referral Hospital.

1.5 Research Questions

- i. What are the socio-demographic differences between *P. jirovecii* pneumonia-positive patients among tuberculosis smear-negative cases and retreatment cases at Coast General Teaching and Referral Hospital?
- ii. What is the prevalence of *Pneumocystis jirovecii* pneumonia in sputum samples from tuberculosis smear-negative and retreatment cases at the Coast General Teaching and Referral Hospital?

- iii. What is the sensitivity and specificity of polymerase chain reaction (PCR) testing compared to Toluidine Blue O staining in detecting *Pneumocystis jirovecii* in sputum samples from tuberculosis smear-negative and retreatment cases at Coast General Teaching and Referral Hospital?

1.6 Limitation of the Study

The study had several limitations that could potentially introduce bias in the interpretation of its findings.

The lack of HIV status data represents a major weakness, given the well-documented association between HIV and PJP.

The study was conducted in one referral hospital and this may limit the generalizability of the findings to other settings. The cross-sectional design enabled us to estimate the prevalence of *Pneumocystis jirovecii* infection but not to determine causal relationships between risk factors and infection. Selection bias may have been introduced by consecutive sampling and reliance on sputum samples may have led to underestimation of prevalence due to lower sensitivity compared with invasive respiratory specimens. Moreover, the follow-up of participants and their molecular characterization could not be continued due to resource limitations. The inability to reliably culture *P. jirovecii* has made diagnosis primarily dependent on microscopy, which can be less sensitive than ideally desired.

CHAPTER TWO

LITERATURE REVIEW

2.1 Taxonomy of *Pneumocystis jirovecii*

Pneumocystis jirovecii is a fungal organism categorized within the phylum Ascomycota, class Saccharomycetes, and order Pneumocystidales (Apostolopoulou & Fishman, 2022). Initially classified as a protozoan parasite, advancements in molecular biology have reclassified it as a member of the fungal kingdom. *Pneumocystis jirovecii* is responsible for causing Pneumocystis pneumonia, a severe pulmonary infection that predominantly affects individuals with compromised immune systems, especially those living with HIV/AIDS (Brooks, Kaplan, and Fitzpatrick, 2016).

The taxonomy of this organism has been refined through ribosomal RNA gene sequencing, which has helped to distinguish it from other fungal species (Cartagena *et al.*, 2025). It is important to note that *P. jirovecii* is recognized as a separate species, although it has historically been referred to as *Pneumocystis carinii* in humans, a term that is now considered obsolete (Lécuyer *et al.*, 2024).

2.2 Phylogeny of *Pneumocystis jirovecii*

Pneumocystis jirovecii is a highly specialized fungal pathogen closely aligned with the Taphrinomycotina class within the Ascomycota (Guto, Bii, and Denning, 2016). It has a more remote relationship with other pathogenic fungi, highlighting its unique ecological and pathogenic characteristics. *Pneumocystis* is a genus of ascomycetous fungus that is particularly pathogenic in immunocompromised humans and other mammals (Bateman, Oladele, and Kolls, 2020).

Pneumocystis is difficult to propagate in vitro, which limits our understanding of its pathobiology (Mills *et al.*, 2011). Because *Pneumocystis* shares many genetic similarities with other fungi, it has some key differences, like having cholesterol in its cell membrane instead of ergosterol, which is what drugs like amphotericin B

and ketoconazole target. These drugs, commonly used as therapeutics in infections caused by other fungi, are ineffective in treating symptoms triggered by *P. jirovecii* (Carmona and Limper, 2010).

Further DNA analyses have revealed that *Pneumocystis* species infecting the lungs of various mammalian species are quite different and their infection is host-specific (Chawla *et al.*, 2011). In 1994, an interim trinomial nomenclature was introduced: *Pneumocystis carinii* f. sp. *hominis* for the species infecting humans, and *P. carinii* f. *carinii* for one of the two species infecting rats. In 2002, the human pneumocystis organism was renamed *Pneumocystis jirovecii* in honor of Otto Jirovec, who is credited for describing the microbe in humans (Carmona and Limper, 2010).

Pneumocystis spp. is a unicellular, eukaryotic organism occurring in the lungs of many mammals (Fitzpatrick, Kunisaki, and Morris, 2017). Five species-specific *Pneumocystis* species have been identified: *Pneumocystis carinii* and *Pneumocystis wakefieldiae* in rats, *Pneumocystis murina* in mice, *Pneumocystis oryctolagi* in rabbits, and *Pneumocystis jirovecii* in humans (Eitner *et al.*, 2010).

2.3 Ecology of *Pneumocystis jirovecii* (Habitat)

Pneumocystis jirovecii predominantly resides in the lungs of mammals, including humans, where it is regarded as an obligate pathogen (Brooks, Kaplan, and Fitzpatrick, 2016). It is classified as an environmental microorganism and is transmitted via airborne spores (Elst *et al.*, 2020). This organism can inhabit a variety of hosts, such as humans, non-human primates, and other mammals, indicating a wide host range (WHO, 2022).

Pneumocystis jirovecii is commonly found in environments with immunocompromised individuals, such as hospitals. It is most frequently transmitted through direct person-to-person contact, likely via the inhalation of airborne cysts (Truong and Ashurst, 2023). The organism is sensitive to environmental conditions, including temperature and humidity, and its presence in the lungs is believed to be closely regulated by the host's immune response (Yuksel, Gelmez, and Yildiz-Pekoz,

2023).

2.4 Life Cycle of *Pneumocystis jirovecii*

Pneumocystis jirovecii has a complex life cycle that includes both asexual and sexual phases, although the sexual stage has not been distinctly observed in human (Harizanov *et al.*, 2023). The cycle begins when individuals inhale infectious cysts from environmental sources or infected persons as illustrated in figure 2.1 below.

Once inside the lungs, the cysts break open, releasing trophozoites which adhere to the alveolar of epithelial cells of the host (Gálvez *et al.*, 2025). The trophozoites undergo asexual reproduction via binary fission and may develop into new cysts, which are subsequently expelled from the lungs primarily by coughing, allowing the infection to spread, thus continuing the cycle of infection (Eitner *et al.*, 2010). While the function of sexual reproduction within this life cycle is poorly understood, it is believed to play a role in genetic diversity (Harris *et al.*, 2011). However, this has only been documented in animal studies and not conclusively in those involving human infection.

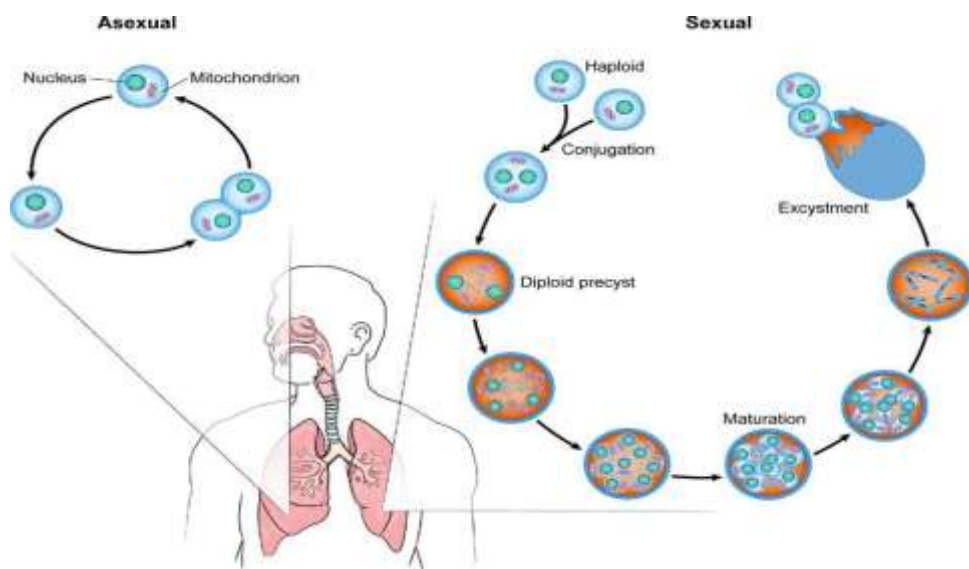


Figure 2.1: Proposed Life Cycle of *Pneumocystis jirovecii*

Source: (Skalski, Kottom, and Limper, 2015)

Pneumocystis spp. has a biphasic life cycle with two unique physical forms: haploid trophozoites, which represent the proliferative and asexual phases of the lifecycle, and cysts, which represent the reproductive stage (Yuksel, Gelmez, and Yildiz-Pekoz, 2023). During the sexual phase, trophozoites conjugate, resulting in the formation of cysts (Skalski, Kottom, and Limperc, 2015).

Trophozoites are small, thin-walled, pleomorphic yeast that binds to the surface of type 1 alveolar cells, primarily through the 95-140 kDa major surface glycoproteins (Lécuyer *et al.*, 2024). These glycoproteins play a crucial role in interacting with the host and eliciting immunological responses (Truong and Ashurst, 2023). Trophozoites are primarily haploid; however, diploid forms have also been documented (Rath and Steinmann, 2014).

Conversely, the cystic forms (also called asci or sporangium) are thick-walled, oval structures that can contain up to eight daughter cells and serve as the reproductive of the life cycle (Yuksel, Gelmez, and Yildiz-Pekoz, 2023).

During infection, trophozoites prevail in the lungs while cysts play the primary role of *Pneumocystis* proliferation and transmission (Neal *et al.*, 2022). The attachment of *P. jirovecii* to lung epithelial cells after inhalation of trophozoites is crucial for initiating illness in susceptible hosts (Gálvez *et al.*, 2025). This attachment may be mediated by critical host molecules like fibronectin, vitronectin, laminin, and mannose receptors (Brooks, Kaplan, and Fitzpatrick, 2016).

2.5 Epidemiology of *Pneumocystis jirovecii* Pneumonia

Pneumocystis jirovecii pneumonia remains a major cause of morbidity and mortality globally, particularly among those with weakened immune systems, such as HIV/AIDS patients, organ transplant recipients, and those undergoing immunosuppressive therapy (Jakab *et al.*, 2025). Before the widespread use of antiretroviral therapy (ART), PJP was the leading cause of death in HIV-positive individuals, particularly in areas with high HIV prevalence (Fitzpatrick, Kunisaki, and Morris, 2017). While ART has substantially reduced PJP incidence in high-income

countries, the disease continues to be a major threat in low- and middle- income countries (LMICs), where access to ART remains inconsistent (Mfinanga *et al.*, 2014).

Beyond HIV-associated cases, PJP also affects other immunocompromised populations, highlighting its broader public health significance (Ibrahim *et al.* 2022). Sub-Saharan Africa, South Asia, and Eastern Europe bear a disproportionate burden of PJP due to high HIV prevalence and limited healthcare resources. In Sub-Saharan Africa, where access to antiretroviral therapy (ART) is often limited or delayed, PJP is particularly prevalent among patients with advanced HIV (CD4 counts below 200 cells/ μ L) (Sasani *et al.*, 2023).

The co-occurrence of TB and HIV further exacerbates immunosuppression, increasing susceptibility to PJP (Yonge *et al.*, 2017). Studies report mortality rates of 30–50% among affected individuals, with PJP contributing significantly to respiratory disease-related deaths in HIV patients (Mfinanga *et al.*, 2014). Case in point, autopsy studies in Kenya and Côte d'Ivoire identified PJP as the primary cause of death in 0–2% of HIV-infected individuals, while prevalence among those with respiratory symptoms ranged from 1% in Tanzania to 22% in Zimbabwe (Mills *et al.*, 2011). In Zimbabwe, *P. jirovecii* was detected in 33% of smear-negative TB patients, underscoring diagnostic challenges in differentiating PJP from TB (Weldearegawi *et al.*, 2020). Similarly, in Ethiopia, polymerase chain reaction (PCR) testing revealed PJP in 42.7% of HIV-positive patients with atypical chest X-rays and smear-negative sputum (Arefaine *et al.*, 2020).

A systematic review that examined the burden of clinically suspected or laboratory-confirmed PCP in sub-Saharan Africa from 1995 to 2015 reported a pooled PCP prevalence of 19% among HIV-positive adults presenting with respiratory disease (Wasserman *et al.*, 2016). However, significant heterogeneity exists in reported PCP rates ranging from 1 to 77% reflecting differences in the populations studied and difficulties associated with both clinical and laboratory PCP diagnosis (Gelaw *et al.*, 2023).

In Kenya, where an estimated 1.6 million people are living with HIV, PJP prevalence is high, particularly among individuals with advanced disease (CD4 <200 cells/ μ L) (Kairu *et al.*, 2021). Despite ART rollout efforts, untreated or late-presenting patients, especially in rural areas remain vulnerable (WHO, 2021). Studies in Kenya have detected *P. jirovecii* in 27.4–37.2% of HIV/AIDS patients with bilateral pulmonary infiltrates and smear-negative sputum (Chakaya *et al.*, 2004). Although initiatives like PEPFAR and Kenya’s Ministry of Health have expanded prophylaxis and ART access, challenges in achieving comprehensive access to care persist throughout the country (Guto, Bii, and Denning, 2016).

Although previous studies from Kenya, Zimbabwe and Ethiopia have reported varying prevalence rates of PJP among HIV-positive and smear-negative TB patients, differences in study populations, diagnostic methods and geographical settings limit direct comparison. Furthermore, limited data exist from the Kenyan coastal region, creating a need for locally generated evidence.

2.6 Cause of Disease and Effects

Pneumocystis pneumonia generally presents with a gradual emergence of symptoms, which may include fever, a dry cough, dyspnea (difficulty in breathing), fatigue, and hypoxia (Li *et al.*, 2024). As the infection advances, patients may encounter heightened respiratory distress, cyanosis (a bluish discoloration of the skin due to insufficient oxygen), and potential respiratory failure (Harizanov *et al.*, 2023).

The underlying pathophysiological process involves the invasion of the alveoli in the lungs by *Pneumocystis jirovecii*, which proliferate and incite inflammation (Skalski, Kottom, and Limper, 2015). This inflammatory response leads to the accumulation of fluid and cellular debris within the alveolar spaces, thereby impairing gas exchange and resulting in the observed hypoxia (Apostolopoulou and Fishman, 2022). If not adequately addressed, alveolar damage may lead to chronic respiratory complications and pulmonary fibrosis (Carmona and Limper, 2010).

Members of the *Pneumocystis* genus are distinct from other medically significant fungi

because they appear to undergo sexual replication in their mammalian hosts, who are then able to transmit the infection, unlike other fungi (e.g., *Aspergillus*) where the host is a dead end (Bateman, Oladele, and Kolls, 2020). Transmission of *P. jirovecii* cysts takes place through the airborne route, and usually, its presence in lungs is asymptomatic (Arefaine *et al.*, 2020). However, individuals with impaired immunity, especially those with CD4⁺ T cell count below 200/μl often development of *Pneumocystis pneumonia* due to *P. jirovecii* invasion (Harizanov *et al.*, 2023).

Pneumocystis jirovecii trophozoites proliferate and attach to type 1 alveolar pneumocytes causing desquamation (Ibrahim *et al.*, 2022). This leads to the formation of foamy eosinophilic exudates visible on hematoxylin eosin (H&E) staining, giving lung tissue a characteristic honeycomb appearance. Both antibody and cell mediated immunity are believed to be involved in host protection (Neal *et al.*, 2022). Although specific virulence factors are poorly defined, *P. jirovecii* induces selective alterations in lung surfactant expression and biophysical activity, (Lécuyer *et al.*, 2024). It may also have a negative effect on non-epithelial lung cells, such as alveolar macrophages and lung monocytes, by downregulating important transcription factors (Arefaine *et al.*, 2020). Additionally, the pathogen escapes immune detection by coating itself with host-derived glycoproteins from the pulmonary microenvironment, such as surfactant protein-A and a soluble form of macrophage mannose receptor. This molecular delay immune recognition and destruction, enabling proliferation (Mohamed *et al.*, 2022).

Pneumocystis jirovecii is generally not associated with clinical disease in healthy individuals. An effective host response to *P. jirovecii* infection requires concerted effects of cellular and molecular components of innate and adaptive immunity, with CD4⁺ T lymphocytes playing a key role (Yuksel, Gelmez, and Yildiz-Pekoz, 2023). Other contributing immune cells include CD8⁺ lymphocytes, antibody producing B-lymphocytes, natural killer (NK) cells, and alveolar macrophages. Both T helper type 1 and 2 cytokines including tumor necrosis factor- α , Interleukin-1 (IL-1), Interleukin-6 (IL-6), and granulocyte-macrophage colony-stimulating factor (GM-CSF) are critical for effective pathogen clearance of *P. jirovecii*; (Yonge *et al.*, 2017). *Pneumocystis jirovecii* replicates extracellularly, impairing oxygen diffusion across

the alveolar-capillary membrane (Ullah *et al.*, 2025). The resulting inflammation causes host cell damage, resulting in lysis and rupture (Carmona and Limper, 2010). Damage to the lung basement membrane generates a characteristic foamy exudate and interstitial leukocytic infiltration, further impairing alveolar capillary permeability (Gálvez *et al.*, 2025).

Individuals who recover from severe cases of PJP may experience lasting respiratory complications, including chronic obstructive pulmonary disease (COPD), pulmonary fibrosis, or reduced lung function (Homayouni *et al.*, 2017). These complications can manifest as persistent shortness of breath or a lowered ability to engage in physical activities (Hosseini-kargar *et al.*, 2024). Without prompt treatment, patients may become susceptible to secondary bacterial infections or worsening of pre-existing HIV/AIDS conditions (K Chepkondol *et al.*, 2020). Post-infection management may require extended respiratory therapy, pulmonary rehabilitation, or treatment for comorbid conditions.

2.7 Coinfections with Other Diseases

Pneumocystis pneumonia (PJP) frequently occurs alongside other opportunistic infections, particularly in immunocompromised individuals such as those with advanced HIV/AIDS (Tufa and Denning, 2019). Common co-infections include cytomegalovirus (CMV), which is prevalent in patients with low CD4 counts, as well as bacterial pneumonias caused by pathogens like *Streptococcus pneumoniae* and *Staphylococcus aureus*. Pneumonia represents a significant factor in the mortality rates of young children, contributing to 15% of global deaths among those under five years of age in 2014 (Mbithi *et al.*, 2014). Mycobacterium tuberculosis is acknowledged as a leading cause or a contributing factor to pneumonia cases in children residing in areas where tuberculosis (TB) is endemic (Kazi *et al.*, 2022). These infections can exacerbate the severity of PJP, resulting in more complicated clinical courses. Moreover, fungal infections such as candidiasis often occur with PJP, further complicating treatment strategies and recovery (Homayouni *et al.*, 2017).

2.8 Risk Factors Associated with Pneumocystis Pneumonia

Pneumocystis pneumonia (PCP) is a serious opportunistic infection caused by the fungus *Pneumocystis jirovecii*, primarily affecting immunocompromised individuals particularly those with advanced HIV infection. Among HIV-positive patients the risk is aggravated when their CD4 count drops below 200 cells/ μ L, (Guto, Bii, and Denning, 2016), making PCP both a common AIDS-defining illness and a critical indicator of disease progression. These individuals benefit substantially from chemoprophylaxis with trimethoprim-sulfamethoxazole (TMP-SMX), which has become a cornerstone of HIV management in high-risk patients (WHO, 2021).

Beyond HIV/AIDS, numerous other conditions predispose individuals to PCP. Organ transplant recipients face heightened risk due to immunosuppressive regimens required to prevent graft rejection (Romain Lécuyer *et al.*, 2024). Similarly, cancer patients undergoing chemotherapy—especially those with neutropenia—may also need TMP-SMX to reduce their risk of opportunistic infections, particularly *Pneumocystis jirovecii* pneumonia (PJP), to which they are predisposed because of chemotherapy-induced immunosuppression that compromises host immune defenses and increases susceptibility to pneumocystis infection (Bateman, Oladele, and Kolls, 2020). Long-term or high-dose use of corticosteroids can impair immune function, putting patients with asthma or autoimmune diseases at greater risk (Gálvez *et al.*, 2025). Primary immune deficiencies, such as DiGeorge syndrome and Severe Combined Immunodeficiency, are at a heightened risk.

Early initiation of prophylactic treatments, most notably trimethoprim-sulfamethoxazole (TMP-SMX) is a common practice in the management of affected children. (Mfinanga *et al.*, 2014). Chronic lung diseases, such as COPD, asthma, and lung fibrosis, further impair lung function and increase the likelihood of respiratory infections. Prompt diagnosis and treatment are essential, with high-dose TMP-SMX as the first-line treatment and corticosteroids for severe cases (WHO, 2022). Understanding age and HIV- related factors is vital in assessing PCP risk in immunocompromised individuals (Zhang *et al.*, 2024).

2.9 Economic Impact of Pneumocystis Pneumonia (PCP)

The economic implications of PJP are considerable, particularly in low-income nations such as Kenya. These include direct costs associated with hospitalization, diagnostic procedures (e.g. chest X-rays, PCR tests, and broncho alveolar lavage), and medications, especially second-line treatments like pentamidine, as well as outpatient services (Kairu *et al.*, 2021).

In Kenya, the financial burden associated with antibiotics, diagnostic equipment, and therapies for advanced infections places significant strain on both healthcare systems and patients. For those lacking access to antiretroviral therapy (ART) or other preventive measures, the strain is even more exacerbated (Guto, Bii, and Denning, 2016).

Kenya recorded about 90,841 TB cases in 2022, a significant rise from 77,854 cases in 2021 (Kairu *et al.*, 2021). This increase suggests that the economy and healthcare system are under increasing strain. In the same year, 756 drug-resistant TB cases were reported, which makes treatment even more difficult and raises medical expenses. Direct medical expenses are only one aspect of the economic impact. TB-affected households frequently spend more than half of their income on TB-related costs, which is significantly higher than the 20% threshold that is deemed catastrophic (Kairu *et al.*, 2021). Increased poverty and decreased productivity can result from this financial strain, especially in rural areas with limited access to healthcare services.

Additionally, long-term costs associated with managing severe cases or complications, along with the lost productivity due to extended illness, further amplify the economic impact on families and communities (Guto, Bii, and Denning, 2016).

2.9.1 Diagnostic Approaches for Pneumocystis Pneumonia

The diagnosis of Pneumocystis pneumonia generally relies on a multifaceted approach integrating clinical evaluation, radiological imaging, and microbiological confirmation (Rath and Steinmann, 2014).

The identification of *P. jirovecii* presents a considerable obstacle due to the inability to cultivate the organism outside of a living host. As a result, laboratory identification relies on the direct observation of the organism in respiratory samples through microscopic analysis using staining methods like Gomori meth enamine silver (GMS), Toluidine Blue O, Giemsa, and Diff-Quik stains (Sadegh Khodavaissy *et al.*, 2015). To enhance detection, immunofluorescence assays may also be employed to emphasize the distinctive cyst forms of the pathogen. Nevertheless, the efficacy of these traditional techniques can be limited, especially in patients with low *pneumocystis jirovecii* loads, making it necessary to adopt more sensitive molecular methods such as polymerase chain reaction (PCR).

The primary diagnostic methods include microscopy, polymerase chain reaction (PCR), immunohistochemistry (IHC), serum biomarkers such as β -D-glucan, and Chest imaging, each with distinct advantages and limitations (Bateman, Oladele, and Kolls, 2020). Microscopy remains the gold standard; samples from broncho alveolar lavage (BAL) or sputum are stained and examined for *Pneumocystis jirovecii* cysts. The PCR technique, a molecular diagnostic tool, provides high sensitivity for identifying pneumocystis DNA in respiratory specimens. In contrast, IHC involves the application of antibodies to stain tissue samples specifically for the organism. Serum assays for β -D- glucan detect components of the pneumocystis cell wall and serve as a valuable non-invasive diagnostic option (Cartagena *et al.*, 2025). Although each method provides distinct advantages, combining them frequently improves the overall accuracy of the diagnosis.

The diagnosis of Pneumocystis pneumonia (PCP) generally requires an integrated approach that combines clinical evaluation, radiological imaging, and microbiological testing to verify the presence of *Pneumocystis jirovecii*, the pathogen responsible for the disease. The primary diagnostic techniques employed include:

2.9.1.1 Microscopy

In clinical microbiology laboratories, the most used stains for respiratory secretions

are Calcofluor white, Giemsa-like stains, and immune fluorescent assays. Other stains, such as hematoxylin and eosin, periodic Acid-Schiff, Papanicolaou's, and Grocott's Methenamine Silver, are more routinely performed in anatomical pathology laboratories, particularly those specializing in cytology-based assays. This technique entails the analysis of respiratory specimens, such as Broncho alveolar lavage (BAL) fluid or sputum, following staining with specific dyes like Gomori's methenamine silver (GMS) or Wright-Giemsa stain (Harris *et al.*, 2011). This process allows for the identification of the distinctive cysts or trophic forms of *Pneumocystis jirovecii*.

Hematoxylin and eosin (H&E) stain is used to visualize *Pneumocystis* forms in lung tissues but lacks sufficient sensitivity for detection due to its inability to bind to the organisms' cell walls. It highlights the frothy eosinophilic exudate characteristic of PCP, with trophic forms possibly seen upon close examination (Cartagena *et al.*, 2025).

Papanicolaou's stain, while less sensitive, effectively enhances the contrast of cystic forms in cytological samples, resulting in blue or green structures against a pink background (Dugan *et al.*, 1988). Periodic acid-Schiff (PAS) stain targets polysaccharides, producing magenta coloration that highlights *P. jirovecii* cyst walls, best used with other diagnostics (Matouri *et al.*, 2023).

Giemsa stain, applied to BAL fluid and lung tissues, effectively visualizes both ascus and trophic forms, staining nuclei reddish-purple and cytoplasm light blue, although its sensitivity may decrease in samples with low organism loads (Cartagena *et al.*, 2025).

Calcofluor white is a fluorescent stain that binds to the polysaccharide components of the fungal organism's cell wall. Under ultraviolet light, calcofluor fluoresces, allowing for rapid visualization of cysts as kidney bean-shaped (double parenthesis-shaped or comma-shaped) structures (Procop *et al.*, 2004). The quick turnaround of results makes it a valuable diagnostic tool for PCP, although its requirement for a fluorescent microscope may limit its availability in some laboratories.

Toluidine Blue O (TBO) stain is particularly advantageous due to its ability to highlight cystic forms of *P. jirovecii*. By binding to nucleic acids and certain polysaccharides, it results in a blue coloration of the cysts observable against a contrasting background. TBO's sensitivity is lower than that of other stains and requires multiple slides to ensure adequate sensitivity (Li *et al.*, 2024).

A modified version of TBO, the modified TBO (mTBO), provides cleaner background but the use of toxic reagents, the fact that trophic forms are not visualized, and the overall challenge with interpretation of the stain has decreased the use of TBO and mTBO in most clinical labs (Cartagena *et al.*, 2025).

Microscopy offers high specificity, but its sensitivity is moderate (40–60%), influenced by sample quality and skilled interpretation. False negatives may occur in cases of low fungal burden or improper staining. Despite these limitations, microscopy remains widely used due to its cost-effectiveness and reliability in resource-limited settings (Luísa Tomás and Matos, 2018).

2.9.1.2 Polymerase Chain Reaction (PCR)

PCR stands out as one of the most sensitive approaches for diagnosing PJP, molecular testing is typically conducted at reference laboratories or in laboratories with advanced infrastructure, particularly in instances where other diagnostic tests are inconclusive, with sensitivity rates reported between 80-90% (Chawla *et al.*, 2011).

This molecular diagnostic approach detects nucleic acids in respiratory specimens and offers high sensitivity and specificity. PCR methods have dramatically improved both the sensitivity and specificity of diagnoses compared with traditional staining techniques, enabling accurate detection in clinical specimens such as BAL fluid, induced sputum, and lung tissue biopsies, and even blood, making it particularly advantageous in instances where other diagnostic methods are ineffective or when the organism is present in low quantities (Li *et al.*, 2024).

PCR amplifies specific DNA sequences of *P. jirovecii* and can detect very small

quantities of the organism that may not be seen on staining techniques such as Gomori meth enamine silver, Giemsa or Toluidine Blue O stains. Different PCR formats such as conventional PCR, nested PCR and real time quantitative PCR (qPCR) are available with nested PCR and qPCR having higher sensitivity (Dini *et al.*, 2010).

However, although PCR demonstrates high sensitivity, its high cost and limited availability, especially in resource-constrained settings, pose significant barriers (Khehra, Padda, and Swift, 2023).

Besides PCR, other NAATs, including loop-mediated isothermal amplification (LAMP), have been developed for the diagnosis of PCP (Singh *et al.*, 2015).

2.9.1.3 Immunohistochemistry (IHC)

This method employs monoclonal antibodies that specifically target antigens on the surface of *Pneumocystis jirovecii*, allowing for detection of the organism in lung tissue samples, with near-perfect specificity (Harris *et al.*, 2011). IHC is particularly useful in lung biopsy or autopsy specimens but is limited by its invasive nature and requirement for specialized laboratory facilities (Cartagena *et al.*, 2025). While IHC is more sensitive than microscopy, it lacks the broad applicability of PCR and is typically reserved for cases where other tests are inconclusive (Iturrieta-González *et al.*, 2024).

2.9.1.4 Serum Biomarkers

The identification of *Pneumocystis* antigens or markers in blood, such as the β -D-glucan assay, represents a non-invasive diagnostic approach. Elevated β -D-glucan levels strongly suggest fungal infection, however false positives can occur with other fungal diseases or inflammatory conditions (Chawla *et al.*, 2011). Clinicians should interpret this test in the context of clinical findings and confirm it with diagnostic methods.

2.9.1.5 Chest Imaging

Radiological techniques particularly high-resolution computed tomography (HRCT)

could reveal characteristic findings such as diffuse ground-glass opacities and cystic lesions in advanced cases. While imaging cannot definitively diagnose PCP, it helps differentiate it from other pulmonary infections and guides further testing. In resource-limited settings, chest X-rays may show bilateral infiltrates, though their sensitivity is lower than CT (Azoulay *et al.*, 2020).

2.9.2 Management of Pneumocystis Pneumonia (PCP)

Selection of an initial anti-*Pneumocystis* regimen depends on the severity of the patient's illness, other comorbid conditions, and the patient's ability to tolerate a specific agent. Mild disease is defined as partial O₂ pressure (pO₂) of greater than 70 mm Hg while breathing room air and an alveolar-arterial gradient of less than 35 mm Hg (A. Morris and Norris, 2012).

First-line therapy for PCP consists of trimethoprim-sulfamethoxazole (TMP-SMX), which demonstrates significant efficacy when administered promptly during the initial stages of the illness. In cases where patients exhibit allergy to TMP-SMX or suspected resistance, alternative treatment regimens include clindamycin combined with primaquine, pentamidine, or atovaquone. For individuals with HIV whose CD4 counts fall below 200 cells/ μ L, prophylactic treatment with TMP-SMX is recommended to avert the onset of PJP (Fitzpatrick, Kunisaki, and Morris, 2017). While TMP-SMX is relatively inexpensive and widely accessible, particularly in various regions including Kenya, second-line options such as pentamidine and atovaquone are slightly costly and inaccessible, posing significant challenges to patients in low-resource environments (Chawla *et al.*, 2011). Timely access to diagnosis and antiretroviral therapy (ART) is essential for mitigating both the incidence and severity of PJP (Luísa Tomás and Matos, 2018).

There is no universally agreed approach on the initial management of patients with suspected PCP (Hosseini-kargar *et al.*, 2024). Disease severity classification guides therapeutic decisions and determines the need for adjuvant corticosteroids (Huang *et al.*, 2023). Current protocols categorize PCP as mild, moderate, or severe based on

clinical presentation and oxygenation status (Cartagena *et al.*, 2025). Patients with mild disease can be treated orally with co-trimoxazole as out-patients if they are willing to attend the out-patient clinic for regular review, able to cope at home, and there is clinical and radiographic evidence of recovery (Mfinanga *et al.*, 2014). If, despite clinical recovery, oral treatment is not tolerated, the regimen may be switched to alternative oral therapies, although close monitoring is still required (Fitzpatrick, Kunisaki, and Morris, 2017). Patients with moderate and severe PCP should always be managed initially in hospital with either co-trim oxazole IV or pentamidine IV together with adjuvant corticosteroid therapy (Yonge *et al.*, 2017). If patients fail to respond to either regimen within 7-10 days then, where feasible, treatment should be switched to the other drug, allowing at least 48hrs overlap for intrapulmonary accumulation of the new drug to occur (Carmona and Limper, 2010). However, such patients have a very poor outcome. Patients with moderate and severe PCP who still do not respond should be treated with clindamycin IV with oral primaquine, or intravenous trimetrexate with folinic acid. Eflornithine is a third choice (Chakaya *et al.*, 2004). There has been recent interest in the potential to use newer antifungal agents such as echinocandins for treatment or prophylaxis of *Pneumocystis*. These agents target BG synthesis. Case reports of PCP patients treated with echinocandins in addition to standard PCP therapy have reported mixed outcomes (Alison Morris *et al.*, 2004). Current data do not support use of these agents for PCP treatment or prophylaxis. The use of adjunctive corticosteroids has significantly improved outcomes in patients with moderate to severe PCP studies have shown that HIV-infected patients with severe respiratory impairment benefit from glucocorticoid therapy, which improves survival by reducing lung inflammation caused by dying organisms and preventing clinical deterioration during treatment. A commonly recommended regimen is oral prednisone given at decreasing doses over 21 days. Although evidence is limited for non-HIV patients, corticosteroids are often used in immunosuppressed individuals with PCP (Melkamu *et al.*, 2020).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Site

The research was carried out at Coast General Teaching and Referral Hospital TB referral Clinic where tuberculosis retreatment, relapse, or smear-negative are referred to. The hospital serves a significant population from Kilifi, Kwale and Mombasa County. The samples were analyzed at the Kenya Medical Research Institute—Centre for Microbiology Research, Mycology Laboratory in Nairobi.

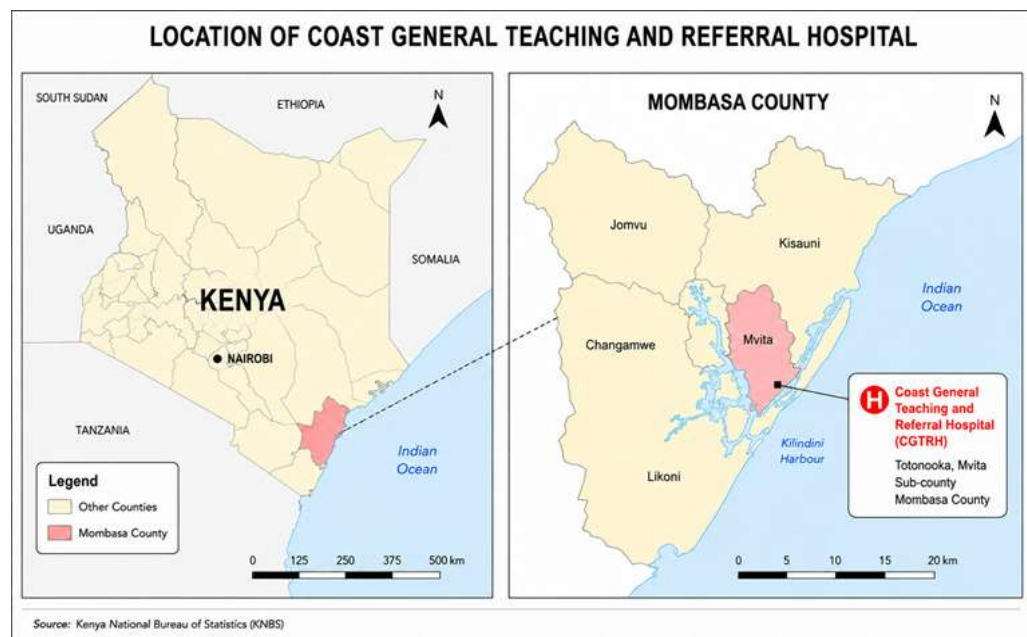


Figure 3.1: Map of the Study Area

Source: (KNBS, 2019)

3.2 Study Design

This was a cross sectional study conducted between November 2017 and December 2021. Patients were consecutively sampled at the TB clinic during their initial diagnostic evaluation. Samples were transported same day in cool boxes from the

hospital to the Mycology Reference Laboratory at KEMRI.

3.3 Study Population and Clinical Parameters

The study population consisted of patients (≥ 18 years) confirmed to have smear-negative tuberculosis (TB) but presenting with or suspected of having a pulmonary TB infection.

3.3.1 Target Population

The target population was the sputum samples of 100 TB patients who were confirmed to be TB smear negative and/or retreatment cases.

3.3.1.1 Smear Negative Patients

A patient was classified as having smear-negative pulmonary tuberculosis if they possess a minimum of two sputum specimens that test negative for acid-fast bacilli (AFB). The subjects were tuberculosis patients at study locations, either as inpatients or outpatients, who were smear-negative, relapsed, or retreated.

3.3.1.2 Retreatment Cases

The patients being treated as TB smear negative patients and were on retreatment for TB. These cases can include patients who relapsed after initial treatment, failed initial treatment, and were recruited into the study irrespective of their HIV status also included radiological abnormalities that are consistent with active PTB.

3.4 Selection Criteria

3.4.1 Inclusion Criteria

- i. Patients with confirmed TB smear negative or retreatment and agreed to have their sputum sampled.
- ii. Those who provided informed consent to participate in the study.

3.4.2 Exclusion Criteria

- i. Those with confirmed TB smear positive and have known respiratory tract disease or TB and on therapy.
- ii. Patients who were unable to expectorate sputum

3.5 Sample Size Determination

The sample size was determined based on the 37% prevalence of PJP in an urban hospital study carried out at Mbagathi District Hospital, Nairobi (Chakaya *et al.*, 2004). Using Fischer's exact test (Jung, 2013) with 95% confidence interval;

$$n = \frac{z^2 p(1-p)}{d^2}$$

Where:

n= Sample size, z = 1.96

$$p = \text{Prevalence of PJP (37\%)}, d = \text{Absolute precision (0.05)}. \text{ Therefore } N = \frac{1.96^2 \times 0.37(1-0.37)}{0.05^2} = 96.8$$

We therefore recruited 100 patients from the study site. Each patient was required to give two specimens.

3.6 Sampling Technique

A consecutive sampling technique was used to recruit study participants. All smear-negative and retreatment tuberculosis patients attending Coast General Teaching and Referral Hospital (CGTRH) who met the inclusion criteria and provided informed consent were enrolled consecutively until the required sample size was reached. Consecutive sampling was suitable for this hospital-based cross-sectional study. It allowed for the inclusion of all eligible participants who showed up during the study

period. This approach helped reduce selection bias and ensured proper representation of the target population. Recruitment continued until the set sample size was met.

3.7 Recruitment of Patients

The participants (Adults) were recruited when they came for the clinic mainly those that had been referred for further treatment or referrals including retreatment cases to the Coast General Hospital. The patients were informed about the study by the clinician and provided with informed consent after agreeing to participate. Their clinical data were then assessed, following approval from the hospital administrator. The patients being treated as TB smear negative patients and were on retreatment for TB were recruited into the study irrespective of their HIV status.

3.7.1 Study Participants

The study population included patients with smear-negative pulmonary tuberculosis who visited the TB clinic at Coast General Teaching and Referral Hospital during the study period. Eligible participants were those who had at least two sputum smear tests that were negative for acid-fast bacilli (AFB), had radiological signs of active pulmonary TB, and showed ongoing clinical symptoms despite being treated with broad-spectrum antibiotics. Both newly diagnosed patients and those requiring retreatment (due to relapse, treatment failure, or loss to follow-up) were included regardless of their HIV status.

Participants were recruited in the order they attended the TB clinic and met the study criteria. The focus was on patients referred for further evaluation and management of smear-negative pulmonary TB, including those needing retreatment. The attending clinician explained the study to eligible patients, and those who agreed were asked to provide written consent.

After giving consent, participants filled out a semi-structured questionnaire to gather socio-demographic information (Quittner *et al.*, 2014). We obtained relevant clinical data from patient records after receiving the necessary approvals from the hospital

administration. Sputum samples were then collected from all enrolled participants for laboratory analysis.

3.8 Data Collection Tools

3.8.1 Evaluation of Socio-Demographic and Risk Factors

Socio-demographic feature and risk factors were evaluated using a standardized questionnaire given to all participants who provided consent. The questionnaire included variables chosen based on findings from prior research indicating factors linked to respiratory infections and *Pneumocystis jirovecii* infection, as well as their significance to the goals of the study. The socio-demographic factors evaluated comprised age, gender, employment status, and housing type. Environmental and behavioral risk factors encompassed household flooding, the presence of mold or fungal growth indoors, the type of cooking fuel used, smoking habits, exposure to second-hand smoke, and the status of tuberculosis treatment. Clinical features, such as fever, night sweats, and symptoms related to diminished quality of life, were also recorded (Quittner *et al.*, 2014).

3.9 Laboratory Methods

3.9.1 Sample Collection

Clinical officers on duty conducted initial screenings to exclude ineligible candidates. After receiving a clear explanation of the study objectives, participants who met the inclusion criteria provided informed consent or assent. Emphasis was placed on ensuring proper sputum collection procedures. (Bateman, Oladele, and Kolls, 2020).

Participants were instructed to brush their teeth and rinse their mouths thoroughly before collecting sputum samples. The study specifically required first morning deep-cough sputum, collected within 15 to 30 minutes of waking and before breakfast. Each participant provided at least 2 mL of sputum. Specimens contaminated with saliva or lacking adequate diagnostic quality were discarded following quantitative quality

assessment.

3.9.2 Toluidine Blue O Staining Procedure and Microscopic Evaluation

Each expectorated sputum specimen collected was mixed with an equal volume of dithiothreitol (sputolysin), (Sigma-Aldrich Co., USA) vortexed briefly, and incubated at 37°C for 15 minutes. After incubation, equal amount of phosphate-buffered saline (PBS) (Thermo Fisher Scientific, USA) was added before being briefly vortexed and centrifuged at 13000 g for 5 minutes. Ten microliters of the resultant pellets from each sample were smeared onto clean microscope glass slides, air-dried and heat-fixed. The slides were soaked in a sulphating solution for 10 minutes, washed with cold water for 5 minutes to remove extra acid, and stained with Toluidine Blue O solution for 3 minutes. Next, the slides were washed with 95% ethanol(Thermo Fisher Scientific, USA) then absolute ethanol, and xylene, then left to dry in the air and examined under a microscope at 40× and 100× magnification for *P. jirovecii* cysts (Bateman, Oladele, and Kolls, 2020).

The Toluidine Blue O-stained slides were examined under 100 × magnifications for *P. jirovecii* cysts and trophozoites. The *P. jirovecii* cysts were identified for their typical round or oval shape, double walls, and stained blue or purple with TBO which differentiated it from common artifacts, air bubbles and debris (Cartagena *et al.*, 2025).

A specimen was considered a positive if at least one cyst cluster was observed for quality control, each slide was examined by two laboratory personnel for confirmation, accuracy and consistency.

3.9.3 DNA Extraction

Due to the resilient nature of *Pneumocystis jirovecii* cell structures, multiple extraction approaches are commonly used to improve DNA yield. In this study, sputum samples were first centrifuged to concentrate *Pneumocystis jirovecii* organisms into a pellet.

The pellet was then suspended in 200 µL of phosphate-buffered saline (PBS) (Thermo

Fisher Scientific, USA). Thereafter, the suspension was transferred into a bead-beating tube containing lysis buffer, bead solution, and Inhibitor Removal Solution (IRS) (Thermo Fisher Scientific, USA). Microbial cell disruption was achieved through a combination of chemical lysis by detergents and mechanical agitation using vortex mixing, thereby facilitating the release of pneumocystis DNA.

The DNA was extracted from the samples using the MycXtra fungal DNA extraction kit (Myconostica Ltd., Manchester, United Kingdom), according to the manufacturer's instructions. Briefly, the samples were centrifuged at 7000xg for 10 minutes at 4 °C, the supernatant discarded, and the pellet resuspended in 1 mL of sterile 1x phosphate-buffered saline (PBS) (Thermo Fisher Scientific, USA) before a second centrifugation at 10,000 x g for 2 minutes at 4°C. Subsequently, the pellet was suspended in 50 µL PBS(Thermo Fisher Scientific, USA), and the entire solution was extracted with CE-marked MycXtra Fungal DNA Extraction Kit (Myconostica, UK, now Novacyt), and the extraction process was carried out per the manufacturer's instructions (Guegan and Robert-Gangneux, 2019).

The released DNA bound to a silica spin filter; was washed eluted aliquoted into 3 aliquots and stored at –80°C for PCR.

3.9.4 PCR Amplification

One of the attractive gene targets for diagnostic PCR is the mitochondrial large subunit ribosomal RNA locus (*mtLSU rRNA*) of *P. jirovecii*. The mt LSU rRNA target was selected due to its high specificity and sensitivity in the detection of *P. jirovecii*. Amplification products were analyzed by agarose gel electrophoresis device (UVP Benchtop Transilluminator, Analytik Jena, USA), and positive samples were identified by the presence of a 346 bp band.

PCR amplification was performed using primers targeting a 346 base pair (bp) region of the mitochondrial large subunit ribosomal RNA (mt LSU rRNA) gene of *Pneumocystis jirovecii*, as previously described in (Khehra, Padda, and Swift, 2023). PCR targeting mt LSU rRNA of *P. jirovecii* was performed using primers pAZ102-H

and pAZ102-E (Thermo Fisher Scientific, USA) as shown in table 3.1 below.

Table 3.1: Primers Used for Amplification of the Mitochondrial LSU rRNA Gene

Primer	Sequence	Position in Mitochondrial Genome
pA102E (Forward)	5'-TCAAGGAGTGAGAAAGCCG-3'	102–121
pA102Z (Reverse)	5'-GGAGGAGAGGTTAGAGCAG-3'	221–240

Briefly, the PCR master mix consisted of; one µl of extracted DNA, 2 µl of nuclease free water 0.5 µl of both forward and reverse primers as shown in table 3.1, and 20 µl of a ready-to-use master mix that included; a reaction buffer, Mg Cl₂, dNTPs, and a DNA polymerase (Thermo Fisher Scientific, USA), making the total volume 25 µl. The thermal cycling conditions for the primary PCR included; a pre- amplification denaturation at 94°C for 5 min; 35 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min; and a final extension at 72°C for 5 min in an Applied Biosystem Gene Amp[®] PCR System 9700 thermocycler (PE Biosystems, Foster City, CA) as shown below in table 3.2 below.

Table 3.2: Amplification Conditions for *Pneumocystis jirovecii* Gene

Step	Time	Temperature (°C)
Denaturation	30 s	94
Annealing	30 s	56
Extension	30 s	72
Final extension	5 min	72
PCR cycles	35	—

Nested PCR was performed using the same thermal cycling profile except for annealing at 56°C.

3.9.5 Electrophoresis

The PCR amplification products were electrophoresed resolved on a 2% agarose gel (Sigma-Aldrich Co., USA) and assessed using the UV Trans illuminator device (UVP Benchtop Transilluminator, Analytik Jena, USA). For quality control, each PCR run was performed with a positive and negative control against a standard molecular base pair (100bp) ladder (Thermo Fisher Scientific, USA) and photographed (Li *et al.*, 2024).

3.9.6 Prevalence Assessment

The study employed both Toluidine Blue O (TBO) staining and nested polymerase chain reaction (PCR) to detect *P. jirovecii* in the collected samples. TBO staining enabled identification of cystic forms under light microscopy, while PCR targeted the mitochondrial large subunit rRNA gene for DNA amplification. The number of positive detections was used to calculate the prevalence by dividing it by the total number of samples analyzed (Nkosi, Hoog, and Lowe, 2025). This dual diagnostic strategy enhanced the accuracy of prevalence estimation within the study population (Babic-Erceg *et al.*, 2014). The proportion of positive PJP tests was calculated using the following formula:

$$\text{Prevalence} = \frac{\text{Number of existing cases}}{\text{Total population at that time}} \times 100$$

3.9.7 Sensitivity and Specificity Assessment

The study assessed the sensitivity and specificity of *Pneumocystis jirovecii* detection using Toluidine Blue O staining and nested PCR on respiratory samples from patients suspected of PCP. A composite reference standard was used to determine PCP infection status. A 2×2 contingency tables were used for sensitivity, specificity, positive predictive value, and negative predictive value (Iturrieta-González *et al.*, 2024).

$$\text{Sensitivity} = \frac{\text{True Positive (TP)}}{\text{True Positive (TP)} + \text{False Negative (FN)}} \times 100$$

$$\text{Specificity} = \frac{\text{True Negative (TN)}}{\text{True Negative (TN)} + \text{False Positive (FP)}} \times 100$$

3.10 Statistical Data Analysis

Data obtained from structured questionnaires and laboratory analyses were verified for completeness, coded, and entered into Microsoft Excel. The data were subsequently exported to the Statistical Package for the Social Sciences (SPSS) version 27.0 (IBM Corp., Armonk, NY, USA) for analysis. Both descriptive and inferential statistical methods were applied. Statistical significance was defined as $p < 0.05$.

3.10.1 Social Demographic Data Analysis

Socio-demographic variables, including age, gender, employment status, and housing type, were summarised using frequencies and percentages. Associations between socio-demographic factors and PJP positivity were assessed using the Chi-square test or Fisher's exact test where appropriate. Results were presented in tables, and variables with p-values less than 0.05 were considered statistically significant in association with PJP infection. To assess the relationship between the study variables, a bivariate logistic regression model was applied using R statistical software. The strength of these associations was quantified using unadjusted odds ratios, along with their corresponding 95% confidence intervals and p-values (Alison Morris *et al.*, 2004).

3.10.2 Prevalence Analysis

The prevalence *Pneumocystis jirovecii* pneumonia was calculated by the percentage of participants who tested positive for *Pneumocystis jirovecii* using Nested PCR. These results were presented as percentages in frequency tables (Guto, Bii, and Denning, 2016).

3.10.3 Diagnostic Performance of Polymerase Chain Reaction and Microscopy Analysis

A 2×2 contingency table was constructed to compare the results obtained by Nested PCR and Toluidine Blue O staining. Nested PCR was considered the reference method. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and diagnostic accuracy of Toluidine Blue O staining were calculated and presented in tabular form (Iturrieta-González *et al.*, 2024).

3.11 Ethical Consideration

The KEMRI Scientific Ethical Review Unit granted ethical approval for this study under reference number KEMRI/SERU/CMR/P00041/3339. Eligible patients were briefed on the research by healthcare providers, granted informed permission upon deciding to participate, and had their clinical data evaluated following approval from hospital administrator. The patient's demographic and clinical information, including medical history, examination details, and investigation reports.

CHAPTER FOUR

RESULTS

4.1 Socio-Demographic Characteristics

Table 4.1 presents the association between sociodemographic characteristics and infection status. Participants aged 18–29 years and 30–39 years had higher odds of infection compared to those aged 60 years and above. The study included 100 patients, consisting of 63 males and 37 females, with a median age of 42 years. Among them, 41 patients (14 women and 27 men) were diagnosed with *Pneumocystis jirovecii* pneumonia. Male participants had slightly higher odds of infection than females (OR= 1.23, 95% CI: 0.54–2.87), and employed participants also had marginally higher odds compared to unemployed participants (OR=1.19, 95% CI: 0.40–3.78). Employment status revealed that 84% (n= 84) were employed, while 16% (n = 16) were unemployed. However, none of the sociodemographic variables were significantly associated with infection status ($p > 0.05$).

Table 4.1: Association between Social Demographic Factors and *Pneumocystis jirovecii* Status

Variable	Category	Positive n (%)	Negative n (%)	Overall n (%)	Unadj usted OR	95% CI	P-value
Age (years)	18–29	11 (26.8)	10 (16.9)	21 (21.0)	1.57	0.43–5.69	0.497
	30–39	10 (24.4)	9 (15.3)	19 (19.0)	1.59	0.43–6.13	0.493
	40–49	6 (14.6)	17 (28.8)	23 (23.0)	0.50	0.13–1.92	0.317
	50–59	7 (17.1)	13 (22.0)	20 (20.0)	0.77	0.20–2.94	0.700
	≥60	7 (17.1)	10 (16.9)	17 (17.0)	Ref		
Gender	Female	14 (34.1)	23 (39.0)	37 (37.0)	Ref		
	Male	27 (65.9)	36 (61.0)	63 (63.0)	1.23	0.54–2.87	0.622
Employment status	Employed	35 (85.4)	49 (83.1)	84 (84.0)	1.19	0.40–3.78	0.756
	Unemployed	6 (14.6)	10 (16.9)	16 (16.0)	Ref		

4.1.1 Environmental Factors

Table 4.2 summarizes the frequencies and percentages of positive and negative participants according to house wall type, flood exposure, and smoking habit, together with the corresponding unadjusted odds ratios, 95% confidence intervals, and p-values.

Table 4.2: Environmental Factors Associated with *Pneumocystis jirovecii* Status

	Category	Positive n(%)	Negative n(%)	Overall n (%)	Unadjusted OR	95%CI	P- value
House Wall Type	Brick wall	21(51.2)	33 (55.9)	54 (54.0)	Ref		
	Concrete	8 (19.5)	10 (16.9)	18 (18.0)	1.26	0.42-3.70	0.678
	Makuti/Thatched/Wood	2 (4.9)	2 (3.4)	4 (4.0)	1.57	0.18-13.93	0.663
	Mud	10 (24.4)	14 (23.7)	24 (24.0)	1.12	0.41-2.98	0.817
Flood Exposure	No	29 (70.7)	45 (76.3)	74 (74.0)	Ref		
	Yes	12 (29.3)	14 (23.7)	26 (26.0)	1.33	0.53-3.28	0.535
Smoking Habit	No	39 (95.1)	52 (88.1)	91 (91.0)	Ref		
	Yes	2 (4.9)	7 (11.9)	9 (9.0)	0.38	0.07-1.94	0.244

Participants living in concrete, mud, and makuti/thatched houses had slightly higher odds of infection compared to those living in brick houses, although these associations were not statistically significant. Participants exposed to flooding had higher odds of infection (OR = 1.33, 95% CI: 0.53–3.28), while smokers had lower odds of infection (OR = 0.38, 95% CI: 0.07–1.94). However, none of the environmental or behavioral factors showed a statistically significant association with infection status ($p > 0.05$).

The majority of participants (35.8%) utilized charcoal as cooking fuel ($n=29$), while 17.3% ($n=14$) combined using charcoal with firewood, kerosene, or gas. Additionally, 17.3% ($n = 14$) relied on kerosene, 16% ($n= 13$) on firewood, and 13.6% ($n = 11$) on gas. Twenty-eight per cent of the participants reported exposure to fungal spores due to leaking residences, while 26% attributed it to moldy living conditions. Only 9% had a history of smoking or passive smoking (Table 4.2).

Environmental factors including house wall type and flood exposure were also not significantly associated with *P. jirovecii* positivity. Participants exposed to flooding had 1.33 times higher odds of infection compared to those not exposed, though this finding was not statistically significant ($p = 0.535$).

4.1.2 Clinical Presentation of *Pneumocystis jirovecii*

Participants currently on TB treatment, those with fungal growth, those reporting night sweats, and those with a positive EuroQol quality-of-life status had lower odds of infection compared to their reference groups. In contrast, participants reporting fever had higher odds of infection (OR = 1.63, 95% CI: 0.72–3.77). However, none of the clinical factors were significantly associated with infection status ($p > 0.05$).

The Table 4.3 shows the relationship between several clinical factors (Fever, Night Sweat, and EuroQol) and an outcome using unadjusted odds ratios (OR), 95% confidence intervals (CI), and p-values. For each variable, the reference group (Ref) represents the absence of the symptom or condition.

Clinical and health status factors such as TB treatment status, fungal growth, fever, night sweats, and EuroQol quality of life scores did not demonstrate statistically significant associations with *P. jirovecii* positivity. Participants with fever had increased odds of infection (OR = 1.63, 95% CI: 0.72–3.77), while those currently on TB treatment had lower odds of infection (OR = 0.61, 95% CI: 0.20–1.70).

Among patients with *Pneumocystis jirovecii* infection, the most common symptoms were productive cough (87%), night sweats (71.7%), fever, and chest pain (59%). Less frequent symptoms included bloody cough (19.6%), shortness of breath (54.3%), and exhaustion (43.5%). Most of the patients were classified as TB smear-negative or retreatment cases (on either treatment or having completed treatment). The results indicated that 41 % of patients with pulmonary tuberculosis also had *P. jirovecii* infections.

The findings in table below indicate that individuals reporting fever had 1.63 times the

odds of having *P. jirovecii* pneumonia compared to those without fever. However, this relationship was not statistically significant (95% CI: 0.72–3.77; $p = 0.2468$), suggesting that fever may not be a reliable independent predictor in this context.

In addition, participants with night sweats showed lower odds (OR = 0.71) of having *P. jirovecii* pneumonia compared to those without night sweats. Nonetheless, this relationship was also not statistically significant (95% CI: 0.31–1.60; $p = 0.403$), indicating limited evidence of a protective or risk association.

Whereas, participants with Euroqol-reported issues had slightly reduced odds (OR = 0.93) of having the pneumonia, but the result was not statistically significant (95% CI: 0.38–2.34; $p = 0.875$). This suggests there was no meaningful relationship between self-reported health status and *P. jirovecii* infection.

In summary, none of the clinical factors (fever, night sweat, Euroqol health status) showed a statistically significant association with *P. jirovecii* pneumonia in unadjusted analysis, as all p-values were above the 0.05 threshold and confidence intervals included as presented below in the Table 4.3.

Table 4.3: Association between Clinical and Health Status Factors and *Pneumocystis jirovecii* Status

Variable	Category	Unadjusted OR	95% CI	P-value
TB Treatment	Not on treatment	Ref		
	Currently on treatment	0.61	0.20–1.70	0.356
Fungal growth	No	Ref		
	Yes	0.73	0.30–1.77	0.486
Fever	No	Ref		
	Yes	1.63	0.72–3.77	0.247
Night sweats	No	Ref		
	Yes	0.71	0.31–1.60	0.403
Euroqol (quality of life)	No	Ref		
	Yes	0.93	0.38–2.34	0.875

4.2 Prevalence, Detection and Diagnostic of *Pneumocystis jirovecii*

The prevalence of infection was determined using both Nested PCR and Toluidine Blue O staining. Nested PCR identified 41 participants as positive, giving a prevalence of 41.0%, while 59 participants (59.0%) tested negative. In comparison, Toluidine Blue O staining detected 29 positive cases (29.0%) and 71 negative cases (71.0%). These findings indicate that Toluidine Blue O detected fewer positive cases compared to Nested PCR.

Using a sample size of 100, the Table 4.4 below, compares the results of the prevalence and diagnostic performance of Toluidine Blue O (TBO) staining to nested polymerase chain reaction (PCR) in detecting *Pneumocystis jirovecii* among the study participants. Microscopy found *P. jirovecii* cysts in 29% of cases, while nested PCR identified 41 positive samples, which is 12 more than what the toluidine blue O (TBO) staining method found.

The prevalence of *Pneumocystis jirovecii* was assessed using two diagnostic methods: Toluidine Blue O (TBO) staining and nested polymerase chain reaction (PCR). Out of the 100 sputum samples analyzed, TBO staining detected *P. jirovecii* in 29 samples, representing a prevalence of 29.0%. The remaining 71 samples (71.0%) tested negative using this method. In contrast, nested PCR identified 41 positive cases, indicating a higher prevalence of 41.0%, with 59 (59.0%) samples testing negative.

To further evaluate the diagnostic performance of TBO staining, its results were compared with those of nested PCR, which served as the gold standard. Cross-tabulation revealed that 29 samples were positive by both methods, indicating agreement. However, 12 cases that were PCR-positive tested negative by TBO staining, representing false negatives. Only one case was TBO-positive but PCR-negative, suggesting a rare false positive. From this comparison, the sensitivity of TBO staining was calculated at 68.29%, indicating that the method correctly identified approximately 68% of the true positive cases. The specificity was found to be 98.31%, demonstrating that TBO is highly effective in correctly ruling out negative cases.

4.3 Diagnostic Performance of PCR vs. Microscopy

The nested PCR demonstrated higher sensitivity (4.27%) but lower specificity (20.6%) compared to TBO staining, which had moderate sensitivity but very high specificity. Using Nested PCR as the reference standard, the diagnostic performance of Toluidine Blue O staining is summarized below. Table 4.4 Toluidine Blue O correctly identified 29 true positive cases and 59 true negative cases. However, 12 cases were missed (false negatives), and 1 case was incorrectly classified as positive (false positive).

Table 4.4: Detection and Diagnostic Performance of Toluidine Blue O Staining Compared to Nested PCR for *P. jirovecii* (N = 100)

Type of test	Result	Frequency
Toluidine Blue O	Positive	29 (29.0%)
	Negative	71 (71.0%)
Nested PCR	Positive	41 (41.0%)
	Negative	59 (51.0%)
Toluidine Blue O VS Nested PCR	Nested PCR	Nested PCR Total
	Positive	Negative
Toluidine Blue O Positive	29	1 29
Toluidine Blue O Negative	12	59 71
Diagnostic parameter	Point Estimate	95% Confidence Interval
Sensitivity	68.29%	54.08% - 82.50%
Specificity	98.31%	95.04% - 100%

4.4 Microscopy Results

Microscopic detection of *Pneumocystis jirovecii* cysts using Toluidine Blue O (TBO) staining revealed a prevalence of 29.0%, with 29 out of 100 samples testing positive. Toluidine Blue O staining selectively highlighted the cystic forms of the organism, providing clear contrast under light microscopy. Photomicrographs of *P. jirovecii* stained with TBO are presented in Figures 4.1 and 4.2. The cysts appear lavender in color with a grainy appearance, indicating successful staining of cyst structures. Figure 4.2, captured at 100× magnification, shows cysts with a spherical to cup-shaped

morphology measuring 5 μm in diameter. The cyst outlines are distinct, and the internal regions stain uniformly, which facilitates identification, in both figures are faint but recognizable, and which is a characteristic of Toluidine Blue O staining. The cysts were found in clusters without budding. Background staining was minimal, most likely due to the sulfation reagent, which helps eliminate extraneous material and improves contrast, allowing for better observation of cyst structures.

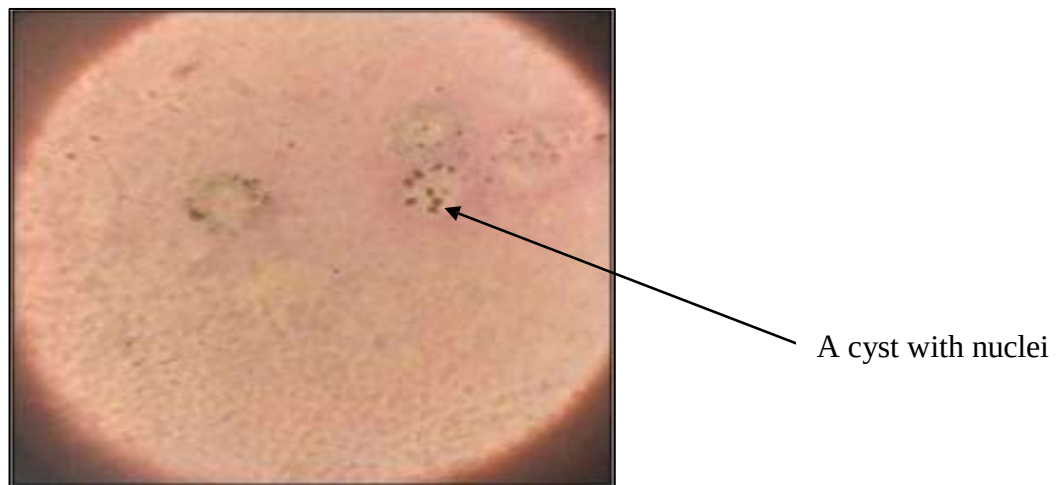


Figure 4.1: Microscopic Appearance of *Pneumocystis jirovecii* Cysts in Sputum following Toluidine Blue O Staining (100 $\times$ Magnification)



Figure 4.2: Characteristic Cystic Structures of *Pneumocystis jirovecii* at 100 $\times$ Magnification

In both plates, the cysts appeared in clusters without budding. Minimal background was visible due to the sulfation reagent. Toluidine Blue O preferentially stained the cyst walls of *Pneumocystis jirovecii*, allowing differentiation of the organisms from surrounding cellular debris and background material as illustrated above in Figures 4.1 and Figure 4.2.

4.5 Molecular Results

Nested PCR confirmed 29 samples that were previously identified as positive by microscopy and detected an additional 12 positive cases that were missed by Toluidine Blue O staining, bringing the total number of PCR-positive samples to 41 out of 100 (41.0%). Representative gel electrophoresis (UVP Benchtop Trans illuminator, Analytik Jena, USA) images are shown in Figures 4.3 and 4.4.

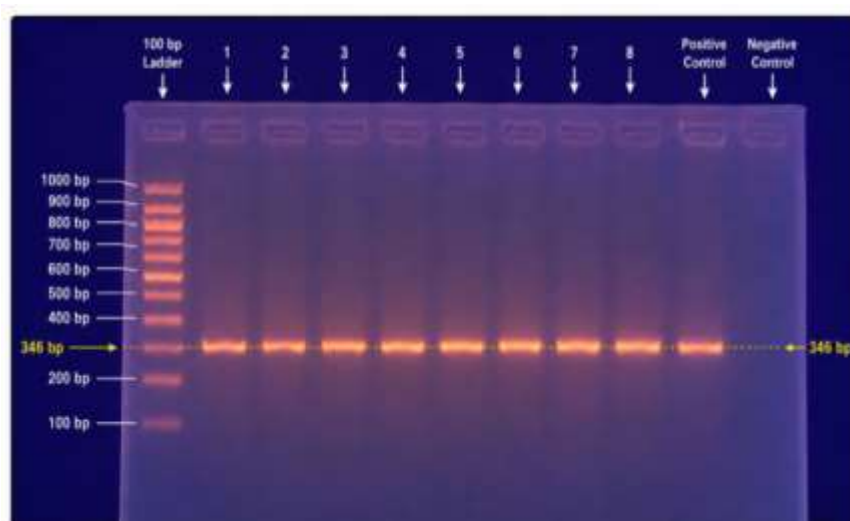
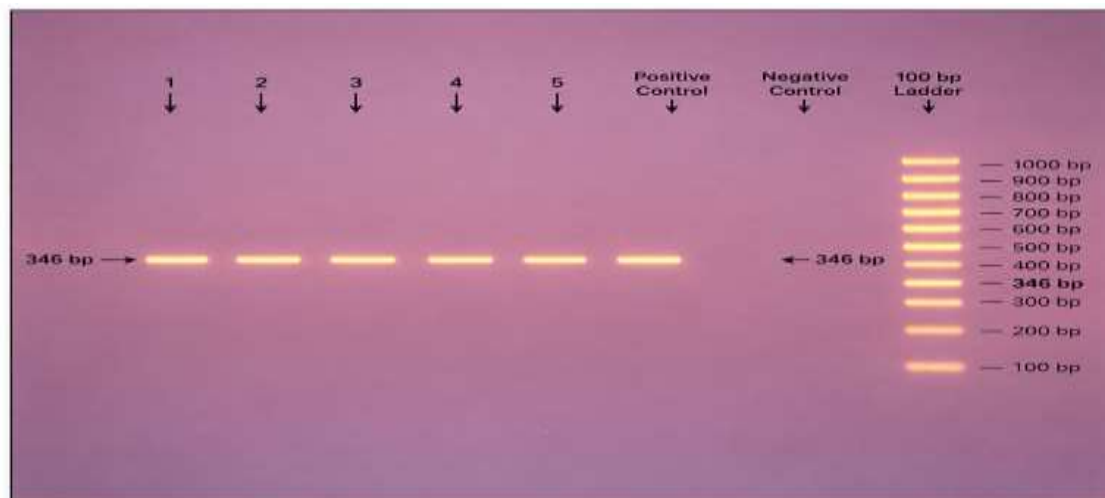


Figure 4.3: Gel Showing PCR Amplification of different Sputum Samples



KEY
 100 bp DNA Ladder – 100 bp
 Molecular size marker
 Lane 1–8 – sample lanes containing
 extracted DNA
 Positive Control – Target DNA; at 346 bp
 Negative Control – Contains no template
 DNA
 Expected Amplicon Size – 346 base pairs

KEY
 100bp ladder DNA size marker
 Lanes 1-5 sample lanes numbered
 Positive control target DNA amplicon size 346bp
 Negative control No template DNA

Figure 4.4: Representative Image Showing Positive Results under Nested PCR

The 100 bp DNA ladder (Thermo Fisher Scientific, USA) served as a molecular size marker with bands ranging from 100 bp to 1000 bp in 100 bp increments, used to estimate fragment sizes.

Clinical samples were loaded in lanes 1–8. The positive control contained known target DNA and produced the expected amplicon band at 346 bp, while the negative control (no template DNA) showed no amplification, confirming the absence of contamination. Positive samples were identified by clear bands at the expected product size of 346 bp, corresponding to the *Pneumocystis jirovecii* target gene.

In the positive samples, the expected 346 bp amplicon was obtained by nested PCR amplification. From the clinical samples analyzed, positive specimens showed clear

bands corresponding to the expected product size of the *Pneumocystis jirovecii* target gene, while negative specimens showed no detectable amplification. A total of 41 samples were identified as positive using nested PCR, including 29 samples that were also positive by Toluidine Blue O microscopy and 12 additional samples that were negative by microscopy. Results of representative gel electrophoresis showing amplification of the 346 bp target fragment are presented.

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATION

5.1 Discussion

5.1.1 Socio-Demographic Characteristics of *P. jirovecii*-Positive Patients

This study sought to establish the socio-demographic characteristics of *P. jirovecii*-positive patients among tuberculosis smear-negative and retreatment cases attending Coast General Teaching and Referral Hospital. The findings showed that the highest proportion of *P. jirovecii*-positive participants was observed among individuals aged 18–29 years (26.8%) and 30–39 years (24.4%). However, age was not significantly associated with *P. jirovecii* infection ($p > 0.05$). Participants aged 18–29 years and 30–39 years had higher odds of infection compared to those aged 60 years and above, although these associations were not statistically significant.

The predominance of infection among younger adults is consistent with studies conducted among HIV-infected and immunocompromised populations, where *P. jirovecii* pneumonia is commonly reported among economically productive age groups. The present findings are consistent with those of a Kenyan study conducted among HIV-infected adults with chronic cough and negative tuberculosis (TB) smears at Kenyatta National Hospital, which identified *Pneumocystis jirovecii* as a common cause of respiratory illness among adults in the productive age groups. The authors reported that *P. jirovecii* pneumonia (PCP) was diagnosed in 35.5% of patients, underscoring the importance of considering PCP in smear-negative patients presenting with chronic respiratory symptoms (Siika *et al.*, 2006).

A study by (Alison Morris *et al.*, 2004) found that PJP frequently affects adults between 20 and 40 years of age due to higher exposure to risk factors such as HIV infection and occupational mobility. Similarly, research conducted in Ethiopia by (Dereje *et al.*, 2019), reported a higher burden of PJP among younger adults attending referral hospitals. The lack of a statistically significant association between age and

infection in the current study may be attributed to the relatively small sample size and the widespread distribution of risk factors across all age groups.

Individuals in the 31-to-40-year age group may be more vulnerable than others due to various factors. Firstly, individuals in this age group may be at a crossroads of several risk factors, including the incidence of HIV/AIDS, comorbidities, and exposure to medical environments. Further extensive epidemiological and clinical investigations are necessary to explore the particular causes of PJP in this age group (García-Moreno *et al.*, 2020). Age was usually chosen as a predictor of risk evaluated rules for community-acquired pneumonia, and older age was associated with worse PJP prognosis among immunosuppressed patients (Ricciardi *et al.*, 2017).

Regarding gender, males constituted the majority of *P. jirovecii*-positive cases (65.9%) compared to females (34.1%). Nevertheless, gender was not significantly associated with infection (OR = 1.23, 95% CI: 0.54–2.87, $p = 0.622$). This finding agrees with several studies conducted in sub-Saharan Africa, which have reported a higher proportion of PJP among males but without significant sex-related differences in infection risk. The distribution by sex showed a predominance of males ($n = 26$, 81.3%) compared to females ($n = 6$, 18.7%). In general, our data are similar to those in the literature for people at risk for developing PCP pneumonia (Ide *et al.*, 2019). The observed male predominance may reflect healthcare-seeking patterns, occupational exposures, and the generally higher burden of tuberculosis among men (Brown *et al.*, 2024). The higher prevalence observed among males may reflect differences in environmental and immunological factors rather than an independent effect of sex itself. The absence of statistical significance however, suggests that both sexes were similarly susceptible to infection within the study population. Employment status was also not significantly associated with *P. jirovecii* positivity (OR = 1.19, 95% CI: 0.40–3.78, $p = 0.756$). Most participants who tested positive were employed (85.4%), reflecting the overall distribution of employment status in the study population. Similar findings have been reported in studies investigating PJP among tuberculosis suspects, where socioeconomic indicators such as employment status did not independently predict infection after accounting for other clinical risk factors

(Navin *et al.*, 2000). This suggests that biological and immunological determinants may play a more important role in *P. jirovecii* acquisition and disease development than employment status alone. Environmental reservoirs of *P. jirovecii* include water and soil; people with HIV who contract PCP are more likely to participate in activities such as gardening, camping, and hiking (McDonald *et al.*, 2024).

Overall, none of the socio-demographic variables examined in this study demonstrated a statistically significant association with *P. jirovecii* infection. These findings indicate that PJP may affect tuberculosis smear-negative and retreatment patients across different age groups, sexes, and employment categories, highlighting the importance of considering all high-risk patients for diagnostic evaluation regardless of socio-demographic background.

5.1.1.1 Environmental Factors Associated with *Pneumocystis jirovecii* Infection

This study evaluated selected environmental factors, including house wall type, flood exposure, and smoking habits, to determine their association with *P. jirovecii* infection among tuberculosis smear-negative and retreatment patients. None of the environmental variables assessed demonstrated a statistically significant association with *P. jirovecii* positivity ($p > 0.05$).

Regarding housing characteristics, participants living in brick-walled houses constituted the largest proportion of both positive (51.2%) and negative (55.9%) cases and were therefore used as the reference category. Compared with brick-walled houses, living in concrete houses (OR = 1.26, 95% CI: 0.42–3.70, $p = 0.678$), makuti/thatched/wooden houses (OR = 1.57, 95% CI: 0.18–13.93, $p = 0.663$), and mud houses (OR = 1.12, 95% CI: 0.41–2.98, $p = 0.817$) was not significantly associated with *P. jirovecii* infection. These findings suggest that housing wall type did not independently influence the occurrence of *P. jirovecii* infection within the study population (MORRIS *et al.*, 2000). Urban infrastructure has also been shown to play a role, with transmission rates and genotypes of PCP varying by location (DOHN *et al.*, 2000).

The high environmental associations observed in this study suggest that housing improvements and clean cooking initiatives could considerably lessen *Pneumocystis jirovecii* pneumonia disease burden, as illustrated in Malawi's effective program for reducing indoor air pollution (Mortimer *et al.*, 2017).

The lack of association between housing type and infection is consistent with previous studies that have identified immunosuppression, particularly HIV infection, corticosteroid use, and underlying pulmonary disease, as the primary determinants of PJP rather than structural housing characteristics (Deribew *et al.*, 2018).

Studies conducted in sub-Saharan Africa have similarly reported limited evidence linking housing materials directly to *P. jirovecii* transmission. The organism is believed to spread primarily through airborne person-to-person transmission, making host immune status more important than housing construction in determining infection risk (Azoulay *et al.*, 2020).

Flood exposure was also not significantly associated with *P. jirovecii* infection (OR = 1.33, 95% CI: 0.53–3.28, $p = 0.535$). Participants reporting previous exposure to flooding had slightly higher odds of infection than those without such exposure; however, the difference was not statistically significant. Although flooding may contribute to damp indoor environments that favor the growth of various respiratory pathogens and fungi, the current findings suggest that flood exposure alone was not a major determinant of *P. jirovecii* infection in this study population. Similar observations have been reported in studies investigating environmental risk factors for opportunistic respiratory infections, where no clear relationship between flooding and PJP occurrence was demonstrated (Vallabhaneni and Chiller, 2016).

Smoking habits were likewise not significantly associated with *P. jirovecii* infection (OR = 0.38, 95% CI: 0.07–1.94, $p = 0.244$). Only a small proportion of participants reported smoking, with 4.9% of positive cases and 11.9% of negative cases identified as smokers. Although smoking has been associated with impaired mucociliary clearance and increased susceptibility to respiratory infections in several studies, the

present findings did not demonstrate a significant relationship (Babic-Erceg *et al.*, 2014). The wide confidence interval and small number of smokers in the study suggest that the analysis may have lacked sufficient statistical power to detect a true association if one exists.

In literature, the smoking and mortality rates were higher in the group of patients with chronic lung disease (Brooks, Kaplan, and Fitzpatrick, 2016). The toxic chemicals in smoke can damage the mucosal barriers of the throat and airway. The lack of protection of the airway mucosa can easily result in bacterial or fungal colonization (Salvi, 2014). The colonization of *Pneumocystis* then aggravated the damage to the anatomical structure of the airway. These processes might be the initial phase in the development of pneumocystis pneumonia. The above results indicate that smoking cessation may reduce the colonization of pneumocystis, prevent airway damage and prevent *Pneumocystis pneumonia* which is beneficial to the prognosis of the disease (Takeuchi & Yakushijin, 2021).

Overall, the environmental factors examined in this study were not significantly associated with *P. jirovecii* infection among tuberculosis smear-negative and retreatment patients. These findings support evidence from previous studies indicating that host-related factors, particularly immune status and underlying comorbidities, are more important determinants of PJP than environmental exposures alone. Further studies involving larger sample sizes and a broader range of environmental variables may be necessary to better understand the potential contribution of environmental conditions to *P. jirovecii* transmission and infection.

5.1.1.2 Clinical Factors Associated with *Pneumocystis jirovecii* Infection

The present study assessed selected clinical factors, including tuberculosis treatment status, fungal growth, fever, night sweats, and quality of life, to determine their association with *Pneumocystis jirovecii* infection among tuberculosis smear-negative and retreatment patients. None of the clinical variables examined showed a statistically significant association with *P. jirovecii* infection ($p > 0.05$). A cohort study

demonstrated that diagnosing concurrent pulmonary tuberculosis (PTB) and *Pneumocystis jirovecii* pneumonia (PJP) is particularly challenging. The clinical presentation is characterized by non-specific respiratory symptoms, such as cough, fever, and dyspnea, which are common to both PTB and PJP (Pan *et al.*, 2025). This overlap in clinical features complicates differential diagnosis and may result in delays in the identification and appropriate management of patients. Nevertheless, the direction and magnitude of the observed associations provide insights into potential clinical patterns that warrant further investigation.

Participants who were receiving anti-tuberculosis treatment had lower odds of *P. jirovecii* infection compared with those who were not on treatment (OR = 0.61, 95% CI: 0.20–1.70, $p = 0.356$). Although the association was not statistically significant and the confidence interval included the null value, the observed trend may suggest a possible protective effect of ongoing tuberculosis treatment. Similar findings have been reported by (Arefaine *et al.*, 2020), who found that tuberculosis treatment status was not independently associated with *Pneumocystis* colonization after accounting for other clinical and immunological factors. The current findings therefore suggest that tuberculosis treatment status alone may not be a key predictor of *P. jirovecii* infection.

Fungal growth was also not significantly associated with *P. jirovecii* positivity (OR = 0.73, 95% CI: 0.30–1.77, $p = 0.486$). Participants with fungal growth demonstrated slightly lower odds of infection than those without fungal growth, although the association was weak and not statistically significant. This finding may reflect the heterogeneous nature of respiratory fungal colonization, which does not necessarily influence the occurrence of *P. jirovecii*. Similar observations were reported by (Gutiérrez *et al.*, 2011) who demonstrated that respiratory fungal colonization and *Pneumocystis jirovecii* colonization may occur independently among patients with chronic pulmonary diseases, suggesting that the presence of other fungi is not necessarily predictive of *P. jirovecii* infection.

Fever was associated with increased odds of *P. jirovecii* infection (OR = 1.63, 95% CI: 0.72–3.77, $p = 0.247$), although the association did not reach statistical

significance. The direction of the association is biologically plausible, as fever is a common clinical manifestation of *Pneumocystis pneumonia* and has been widely reported among affected patients, particularly in immunocompromised populations. According to (Hosseinikargar *et al.*, 2024), fever, dyspnea, and non-productive cough are among the most frequently reported symptoms of *Pneumocystis pneumonia* in both HIV-infected and non-HIV immunocompromised patients. However, fever is also a common symptom of tuberculosis and other respiratory infections, reducing its specificity as a clinical indicator of *Pneumocystis* infection (Zhong *et al.*, 2023). This overlap in symptom presentation may explain the lack of a significant association observed in the present study.

Night sweats were associated with lower odds of *P. jirovecii* infection (OR = 0.71, 95% CI: 0.31–1.60, $p = 0.403$), although the relationship was not statistically significant. Night sweats are frequently reported among patients with active tuberculosis and other chronic infectious diseases. The observed inverse association may indicate that this symptom is more strongly related to tuberculosis disease activity than to *P. jirovecii* infection. Similar findings were reported by (Arefaine *et al.*, 2020), who found that classical tuberculosis symptoms, including night sweats, were not reliable predictors of *Pneumocystis* colonization among patients undergoing investigation for pulmonary tuberculosis. However, given the absence of statistical significance, this finding should be interpreted with caution.

Quality of life, as assessed using the EuroQol instrument, was not significantly associated with *P. jirovecii* infection (OR = 0.93, 95% CI: 0.38–2.34, $p = 0.875$). Participants reporting poorer quality of life did not have substantially different odds of infection compared with those reporting better quality of life. Similar observations have been reported in studies of respiratory infections, where quality-of-life measures are influenced by multiple physical, psychological, and social factors rather than by a single infectious condition (Cañas-Arboleda *et al.*, 2019). Consequently, quality of life may not be a sensitive indicator of *P. jirovecii* infection in this patient population.

Overall, none of the clinical factors examined in this study demonstrated a statistically

significant association with *P. jirovecii* infection. The findings suggest that clinical symptoms and patient-reported health status alone may have limited value in predicting infection among tuberculosis smear-negative and retreatment patients. Instead, the occurrence of *P. jirovecii* infection may be more strongly influenced by underlying immunological factors, including HIV infection, CD4 cell count, and other causes of immunosuppression (Zhang *et al.*, 2024). This observation is consistent with the findings of (A. Morris and Norris, 2012), who reported that impaired immune function remains the principal determinant of susceptibility to Pneumocystis infection. Further studies involving larger sample sizes and comprehensive assessment of immune status are recommended to better elucidate the determinants of *P. jirovecii* infection in this population.

5.1.2 Prevalence of *Pneumocystis jirovecii* Pneumonia among Tuberculosis Smear-Negative and Retreatment Patients

The present study determined the prevalence of *Pneumocystis jirovecii* pneumonia among tuberculosis smear-negative and retreatment patients attending Coast General Teaching and Referral Hospital using nested PCR and Toluidine Blue O staining. Nested PCR detected *P. jirovecii* in 41.0% (41/100) of sputum samples, while Toluidine Blue O staining identified 29.0% (29/100) of samples as positive. The higher prevalence detected by nested PCR demonstrates the high sensitivity of molecular methods in identifying *P. jirovecii* infections that may be missed by conventional microscopic techniques.

The prevalence of 41.0% observed using nested PCR indicates that *P. jirovecii* is a common pathogen among tuberculosis smear-negative and retreatment patients in the study setting. The ability of Nested PCR to detect 12 additional positive cases beyond those identified by microscopy suggests that molecular methods are more sensitive, particularly in cases with low organism burden that may be difficult to detect using conventional staining techniques. This finding is comparable to that reported by (Dini *et al.*, 2010), who found a prevalence of 24% among patients with suspected Pneumocystis pneumonia using conventional diagnostic methods and identified an

additional 17% of cases through PCR among patients who had tested negative by microscopy. Their findings underscore the enhanced diagnostic yield of molecular techniques and support the higher detection rate observed in the present study.

The prevalence observed in this study is also consistent with reports from sub-Saharan Africa, where molecular diagnostic methods have revealed substantial rates of *Pneumocystis* infection among patients presenting with respiratory symptoms (Guto, Bii, and Denning, 2016). Research carried out in Uganda, Tanzania, and Ethiopia has indicated prevalence rates between about 20% and 50%, which vary based on the study group, HIV prevalence, type of specimen, and the diagnostic techniques used (Deribew *et al.*, 2018). A thorough review and meta-analysis aimed to estimate the prevalence of *Pneumocystis pneumonia* (PCP) in HIV-infected individuals in Ethiopia. The pooled prevalence of PCP was found to be 5.65% (95% confidence interval: 3.74%-7.56%). These findings highlight the continued burden of PCP among HIV-infected people. They also emphasize the need for better screening, diagnosis, and management options in high-risk groups (Yared Mulu Gelaw *et al.*, 2023). These findings suggest that *P. jirovecii* remains an important opportunistic respiratory pathogen.

The prevalence detected in the current study is higher than that reported in studies relying exclusively on microscopic staining techniques. Microscopy-based methods such as Toluidine Blue O, Gomori methenamine silver, and Giemsa staining generally have lower sensitivity because they require a relatively high organism burden for visualization (Cartagena *et al.*, 2025). Consequently, studies using these methods often report lower prevalence estimates than those utilizing PCR-based assays. This difference is reflected in the current study, where nested PCR detected substantially more positive cases than Toluidine Blue O staining.

The relatively high prevalence observed may be explained by the characteristics of the study population. Tuberculosis smear-negative and retreatment patients often present with persistent respiratory symptoms despite negative sputum smear results or previous tuberculosis treatment, increasing the likelihood that alternative pulmonary pathogens contribute to disease. (Harizanov *et al.*, 2023).

Opportunistic infections such as *P. jirovecii* may therefore be overlooked if only routine tuberculosis investigations are performed. Furthermore, underlying immunosuppression, particularly HIV infection and reduced CD4 cell counts, may increase susceptibility to Pneumocystis infection and contribute to the high prevalence observed (Buonomo *et al.*, 2023).

However, the findings differ from those of a Tanzanian study that reported a very low prevalence of *P. jirovecii* among TB patients and healthy controls using polymerase chain reaction (PCR) analysis of oral wash specimens. In that study, *P. jirovecii* was detected in only 0.3% of participants (Jensen *et al.*, 2010). This discrepancy may be explained by differences in specimen type, diagnostic methods, HIV prevalence, patient selection criteria, and geographical factors. Furthermore, the use of oral wash samples in the Tanzanian study may have resulted in lower detection rates compared with the sputum-based PCR approach employed in the present study.

The findings of the present study also fall within the range reported by (Khodavaisy *et al.*, 2015), in their systematic review, which documented a prevalence of proven Pneumocystis pneumonia ranging from 1.1% to 81.8% across different studies, with a pooled mean prevalence of 20.7%. The higher prevalence observed in the current study compared with the overall mean may be attributable to differences in study population, geographical setting, burden of immunosuppressive conditions, and the use of highly sensitive nested PCR techniques.

Previous research, (Julien Senécal *et al.*, 2022) has indicated that PCR conducted on induced sputum samples demonstrates high sensitivity for identifying *P. jirovecii* and is especially useful in ruling out PCP when the results are negative. Nonetheless, since PCR can identify both colonization and active infection, positive outcomes must be evaluated alongside clinical observations and additional diagnostic assessments. The integration of PCR with microscopy techniques, immunofluorescence tests, or serum β -D-glucan analysis has been suggested as a means to enhance diagnostic specificity and minimize false-positive results. These results advocate for the application of molecular methods as supplementary instruments in conjunction with traditional

staining techniques for the diagnosis of PCP.

Differences between the present findings and those reported elsewhere may also be attributed to variations in study design, sample size, specimen type, HIV prevalence, and diagnostic approaches. Nevertheless, the results highlight the importance of considering *P. jirovecii* pneumonia as a differential diagnosis among tuberculosis smear-negative and retreatment patients presenting with persistent respiratory symptoms. The findings further emphasize the value of incorporating molecular diagnostic methods into routine clinical investigations to improve detection and management of Pneumocystis infection in high-risk populations.

5.1.3 Diagnostic Performance of Nested PCR Compared with Toluidine Blue O Staining

This study compared the diagnostic performance of nested PCR and Toluidine Blue O staining for the detection of *Pneumocystis jirovecii* in sputum samples from tuberculosis smear-negative and retreatment patients. Using nested PCR as the reference method, Toluidine Blue O staining demonstrated a sensitivity of 68.29% and a specificity of 98.31%. This indicates that Toluidine Blue O staining correctly identified approximately two-thirds of patients who were positive by nested PCR. However, 12 PCR-positive cases were not detected by microscopy, suggesting that Toluidine Blue O staining may fail to identify infections with low organism burdens. This finding is consistent with previous studies demonstrating that microscopic staining techniques have lower sensitivity than molecular methods, particularly among patients with early infection, mild disease, or partial immune competence (Li *et al.*, 2024).

In a study by (Rouhi *et al.*, 2024), the probe-based qPCR targeting mtLSU-rRNA gene exhibited good diagnostic performance with a sensitivity of 92.5% and specificity of 98.7%. Nested PCR targeting the *Pneumocystis* mitochondrial large subunit (mtLSU)-rRNA gene or the multicopy major surface glycoprotein (msg) gene remains the most extensively used technique to identify *P. jirovecii*. According to, (Phoompoung *et al.*,

2025) , estimating the amount of *Pneumocystis jirovecii* in broncho alveolar lavage fluid (BALF) is highly accurate for diagnosing Pneumocystis pneumonia (PCP). Non-invasive qPCR tests have moderate sensitivity but high specificity and negative predictive value, making them helpful for ruling out PCP when clinical suspicion is low.

In contrast, the specificity of 98.31% demonstrates that Toluidine Blue O staining was highly accurate in identifying patients who did not have *P. jirovecii* infection. Only one false-positive result was observed among PCR-negative samples. The high specificity indicates that positive Toluidine Blue O staining results are highly reliable and supports the continued utility of this method in settings where molecular diagnostic techniques are unavailable or unaffordable (Ibrahim *et al.*, 2022).

The high detection rate of nested PCR observed in this study is consistent with findings from numerous investigations worldwide, (Guegan and Robert-Gangneux, 2019), reported that molecular diagnosis has become the reference standard for the diagnosis of Pneumocystis pneumonia because of its ability to detect very low concentrations of *P. jirovecii* DNA in respiratory specimens. PCR-based methods amplify specific genetic targets of the organism, enabling detection even when only a small number of organisms are present. Consequently, PCR assays have consistently demonstrated substantially higher sensitivity than conventional staining techniques.

The findings of the present study are further supported by a systematic review and meta-analysis conducted by (Senécal *et al.*, 2022), which evaluated the diagnostic accuracy of PCR for Pneumocystis pneumonia. The authors reported that induced sputum PCR had a pooled sensitivity of 99% and specificity of 96%, values comparable to those obtained using broncho alveolar lavage specimens, which demonstrated a sensitivity of 98% and specificity of 91%. These findings highlight the excellent diagnostic performance of PCR-based methods even when non-invasive respiratory specimens are used.

The *P. jirovecii* Mt LSU-Rna is one of the highly sensitive and specific genetic

regions because of multiple copies of genomes (Huang *et al.*, 2023). The Mt LSU rRNA gene is involved in basic metabolic functions and is considered a highly informative locus (Khehra *et al.*, 2023). This is also the locus used most widely for PCR-based detection of *P. jirovecii*, as it is present in multiple copies (Elst *et al.*, 2020). To date, seven different Mt LSU rRNA sequences have been identified for *P. jirovecii*, meaning that those sequences are useful for investigating intra specific differences among populations.

The discrepancy between nested PCR and Toluidine Blue O staining observed in the current study may be explained by differences in analytical sensitivity. Microscopic methods rely on direct visualization of cysts or trophic forms and therefore require an adequate organism burden as well as considerable technical expertise for accurate interpretation. In contrast, nested PCR can detect minute quantities of *P. jirovecii* DNA and is therefore particularly valuable for diagnosing infection in patients with low *P. jirovecii* loads, early disease, or partial treatment (Senécal *et al.*, 2022).

PJP is a serious opportunistic illness that affects PTB patients in coastal Kenya, requiring an integrated approach. The findings of the present study support the use of nested PCR as a more sensitive diagnostic method for the detection of *P. jirovecii* among tuberculosis smear-negative and retreatment patients. However, because of its high specificity, Toluidine Blue O staining remains a valuable diagnostic and screening tool in resource-limited settings where molecular diagnostic facilities may not be readily available. The combined use of microscopy and molecular techniques may improve diagnostic accuracy, facilitate early diagnosis, and contribute to improved management of affected patients (Brown *et al.*, 2024).

5.2 Conclusion

1. The study identified *Pneumocystis jirovecii* infection across all socio-demographic groups, with higher rates in individuals aged 18–29 and 30–39, particularly among males. However, no significant correlations were found between infection rates and demographic factors like age, gender, or

employment status, suggesting these alone are unreliable indicators. Exposure-related factors such as biomass fuel use, charcoal, and smoking habits may play a role.

2. A significant prevalence of *P. jirovecii* infection was found in tuberculosis patients who were smear-negative and that undergoing retreatment, with Nested PCR detecting infection in 41.0% of sputum samples. This indicates a substantial burden of undiagnosed *P. jirovecii* infection, potentially worsening respiratory symptoms and complicating diagnoses in smear-negative tuberculosis cases.
3. In terms of diagnostic efficacy, Nested PCR showed higher sensitivity than Toluidine Blue O (TBO) staining and detected more positive cases. While TBO had high specificity, its lower sensitivity meant some infections were missed. This makes Nested PCR the most reliable way to diagnose *P. jirovecii*, but TBO can still be a cost-effective screening option where resources are limited.

5.3 Recommendations

1. *Pneumocystis jirovecii* infection was detected across all socio-demographic groups, despite the absence of statistically significant associations with age, sex, and employment status. Therefore, screening and preventive strategies should focus on exposure-related risk factors rather than demographic characteristics alone. Particular attention should be given to reducing exposure to biomass fuel smoke, charcoal smoke, and tobacco smoke through public health interventions that promote improved household ventilation, the adoption of cleaner cooking fuels, and smoking cessation programs.
2. The notable prevalence of *P. jirovecii* infection (41.0%) among patients with smear-negative tuberculosis and those undergoing retreatment underscores the necessity for routine evaluation of this infection within these clinical populations. It is advisable to incorporate *P. jirovecii* into the differential diagnosis for patients suspected of having smear-negative TB, particularly

those exhibiting persistent respiratory symptoms. Early screening in this demographic may mitigate misdiagnosis, enhance treatment outcomes, and address the concealed burden of opportunistic infections.

3. Nested PCR detected more *Pneumocystis jirovecii* infections than Toluidine Blue O staining in this study. This suggests that Nested PCR might be a better method for diagnosis. When possible, labs should include molecular methods like Nested PCR in their routine practices to better detect *P. jirovecii* infections. While Toluidine Blue O staining is still a helpful and affordable first screening method in settings with limited resources, building capacity for molecular diagnostics could improve accuracy and lower the number of missed infections.

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APPENDICES

Appendix I: Informed Consent (English Version)

TITLE: Prevalence of *Pneumocystis jirovecii* in TB smear negative sputum samples at the Coast General Teaching and Referral Hospital.

Researcher Statement

My name is Anne Sein Saitoti, a postgraduate student at the Jomo Kenyatta University of Agriculture and Technology (JKUAT). I wish to request that you participate in the study at your will. This consent form is to help you decide whether to join the study. You are free to ask questions about the purpose of the research, your role in the study, the possible risks and benefits you will encounter, your rights as a volunteer, and anything else about it for clarification. Without any coercion, you have the freedom to choose whether to participate in the study. You have a copy of this consent for your records.

Background

This study is set to determine the cause of your chest infection, which may be caused by a germ called *Pneumocystis jirovecii*, which can cause a severe chest infection or pneumonia (PCP).

This study aims to do several tests for PCP to investigate whether you have PCP. Should the germ be the cause of the chest infection, your child will receive PCP treatment?

Purpose and benefits

The purpose of this research study is to investigate these opportunistic pneumonia infections in sputum among TB smear-negative patients presenting with pulmonary conditions. The finding will aid in the proper clinical management of PCP.

Risk, Stress or discomfort

The interview may be awkward due to sensitive questions, but we'll try to make it nice.

We will discuss the questions with you in a language you are comfortable with. You may skip any questions that you are uncomfortable with. You may skip any questions that you don't want to answer and may terminate the interview at any time without consequence.

Confidentiality

All records will be identified by serial numbers only; to maintain your confidentiality, we will not record your names or personal identifiers. We will store completed study forms in locked cabinets within an access-limited area.

Voluntary participation

You may refuse to participate and withdraw from the study at any time without consequences.

If you choose not to participate in the study, then you will get regular treatment for the chest infection, including PCP. Being in the study will not affect any other treatment that you will receive.

Other information

You are also free to ask the investigator questions both before consenting to participate and anytime thereafter.

Do you have any questions?

If any specify

.....

.....
.....

Do you agree to participate in the study?

Name of researcher..... Signature of researcher.....

Date.....

Subject statement

This study described above has been explained to me, and I volunteer to take part. I have read and understood this form. My questions have been answered; I voluntarily consent to participate. If I have future questions about the research, I can contact the following:

Coast General Teaching and Referral Hospital

REC Mombasa Coast General Teaching and Referral Hospital Chairperson

P.O. Box 90231, Mombasa, Kenya 254-11-222867, 254-11-220161

254-11-225405, 254-11-230303

chiefadmin@cphg.co.ke.

Participants name.....Signature.....Date.....

Doctor Name.....Signature..... Date.....

Appendix II: Informed Consent (Kiswahili Version)

FOMU YA IDHINI

KICHWA CHA UTAFITI: UTAFITI KUHUSU MARADHI YA MAPAFU YANAYOTOKANA NA KUATHIRIKA

Kwa majina naitwa **Anne Sein Saitoti**, mwanafunzi kutoka **Chuo Kikuu cha Jomo Kenyatta**. Ningependa kukujulisha kuhusu utafiti ninaokusudia kufanya, unaolenga kuchunguza maradhi ya mapafu yanayoweza kutokana na kuathirika, hasa magonjwa kama **kifua kikuu (TB)**.

Lengo kuu la utafiti huu ni kuchunguza iwapo umeathirika na maradhi ya kifua kikuu au mengine yanayofanana. Endapo umegunduliwa kuathirika, utapewa matibabu yanayofaa haraka iwezekanavyo.

Ushiriki wako ni wa hiari kabisa. Kabla ya kushiriki, utapewa nafasi ya kuuliza maswali kuhusu utafiti huu, faida zake, na hatari zinazoweza kujitokeza. Ukijiridhisha na maelezo haya, unaweza kuamua kushiriki au kutoshiriki. Hakuna mtu atakayekulazimisha kushiriki ikiwa hutaki.

Ikiwa utakubali kushiriki:

- Utajibu maswali machache yanayohusiana na afya yako.
- Muuguzi atakuelekeza jinsi ya kutoa **kikohozi cha asubuhi** kabla ya kula kifungua kinywa, kwa kutumia chombo maalum utakachopewa.

Tafadhali fahamu:

- Baadhi ya maswali au vipimo vinaweza kukukumbusha mambo ya kibinafsi au kukuathiri kihisia. Ikiwa hivyo itatokea, uko huru kuacha kushiriki wakati wowote.
- Maelezo yako yote yatatunzwa kwa usiri mkubwa na hayatasambazwa kwa mtu yeyote bila ruhusa yako.

Ikiwa una maswali yoyote kuhusu utafiti huu, tafadhali jisikie huru kuniuliza. Ushiriki wako utasaidia sana katika kubaini na kuinga maradhi haya kwa jamii nzima.

Asante kwa kuchukua muda kusoma barua hii.

Una maswali yeyote.....

Uameamua kushiriki kwa utafiti huu.....

Jina la mtafitiSahihi..... Tarehe.....

Uamuzi wa mshiriki

Nimeleezwa vyema juu ya huu utafiti, mimi mwenyewe nimeamua bila kushurutiwa na yeyote. Nikiwa na maswali juu ya huu utafiti nitayawasilisha kwa;

Mwenyekiti wa kamati ya maadili bodi kamati,

Coast General Teaching and Referral Hospital

P.O.Box 90231, Mombasa, Kenya

254-11-222867, 254-11-220161

254-11-225405, 254-11-230303

chiefadmin@cphg.co.ke

Jina la mhusika Sahihi..... Tarehe.....

Jina la Daktari.....Sahihi Tarehe.....

Appendix III: Structured Questionnaire

STRUCTURED QUESTIONNAIRE

Study Title: Detection of *Pneumocystis jirovecii* among TB Smear-Negative and Retreatment Cases at Coast General Teaching and Referral Hospital.

SECTION A: SOCIAL DEMOGRAPHIC CHARACTERISTICS

1. Participant ID: _____
2. Date of Interview: ____ / ____ / _____
3. Age (Years): _____
4. Gender:
 - ☐ Male
 - ☐ Female
5. Employment Status:
 - ☐ Employed
 - ☐ Self-employed
 - ☐ Unemployed
 - ☐ Student
6. Type of House:
 - ☐ Brick Wall
 - ☐ Concrete
 - ☐ Makuti/Thatched
 - ☐ Wooden
7. Is the Home Prone to Flooding?
 - ☐ Yes ☐ No
8. TB Treatment Status:
 - ☐ Currently on Treatment
 - ☐ No Treatment

9. Presence of Fungal Growth/Mold in Home:

☐ Yes ☐ No

10. Main Fuel Used for Cooking:

☐ Charcoal

☐ Kerosene

☐ Gas

11. Smoking Habit:

☐ Yes ☐ No

12. If No, Does the Participant Live with Someone Who Smokes?

☐ Yes ☐ No

SECTION B: CLINICAL FEATURES

13. Fever:

☐ Yes ☐ No

14. Night Sweats:

☐ Yes ☐ No

15. EuroQol Symptoms/Reduced Quality of Life:

☐ Yes ☐ No

SECTION C: ADDITIONAL COMMENTS

Appendix IV: SOPs of Sputum Collection and Processing

- Patients suspected to have pulmonary tuberculosis were involved to give out the sputum samples.
- The patients were instructed to brush and rinse mouths thoroughly, inhale and exhale deeply forcing air from the lungs using the diaphragm.
- This was repeated until the patient coughed and was able to produce a sputum specimen.
- At least one teaspoon of sputum was collected within 15 to 30 minutes after waking and before taking breakfast.
- The specimens were collected in clearly labeled leak proof laboratory containers. The specimens were also placed inside a biohazard transport bag.
- The sputum specimens were transported to the laboratory in a cool box as soon as possible.

Appendix V: SOPs for Microscopy Technique and Stain

Toluidine Blue O stain preparation

This was used for identification of *Pneumocystis jirovecii* and was prepared according to manufacturer's instruction (Nissui, Japan).

Glycial acetic acid	45ml
Concentrated sulfuric acid	15ml
Toluidine powder	0.3g
Distilled Water	60 ml

- Mix 45ml of glycial acetic acid (Nissui, Japan) with 15ml of concentrated sulfuric acid and dissolve 0.3g of dye in 60ml of water
- (i) For sulfation reagent, 45 ml of glacial acetic acid was poured into a Coplin jar which had been placed into a plastic tub filled with cool tap water (not below 10°C). A 15-ml portion of concentrated sulfuric acid (Sigma, USA) was slowly added with a glass pipette, being careful not to produce splashing. The solution was gently mixed with a glass rod. The Coplin jar was then sealed with petroleum jelly. The sulfation reagent was kept at room temperature and could be used for 1 week.
- (ii) For Toluidine blue 0 solution (Sigma, USA), 0.3 g of toluidine blue 0 (52% dyecontent-0.16 g of dye) was dissolved in 60 ml of distilled water to which was added 2.0 ml of concentrated hydrochloric acid (Sigma, USA), followed by 140 ml of absolute ethyl Alcohol (Sigma, USA). The sulfation solution was stored at room temperature and could be used for 1 year. Staining was performed with all solutions in Coplin jars.

For other reagents, two Coplin jars each were needed for 95% ethyl alcohol (Sigma, USA), absolute ethyl alcohol (Sigma, USA), and xylene (Sigma, USA). The 95% ethyl alcohol solutions were changed when they were no longer clear or darker than a pale

blue. The absolute alcohol and xylene solutions were changed monthly or sooner when they became cloudy or dark blue.

The TBO staining procedure was as follows.

- (i) With forceps, slides were placed in the sulfation reagent for 10min.
- (ii) The reagent was mixed with a glass stirring rod immediately after insertion of the slides and again after 5 min.
- (iii) The slides were removed from the sulfation reagent with forceps, placed in a glass slide holder, and washed gently under cold running tap water for 5 min. The excess water was then drained.
- (iv) The slides were placed in toluidine blue 0 for 3 min.
- (v) The slides were dipped in and out of 95% ethyl alcohol (in two Coplin jars) for approximately 10s until clean, with most of the blue dye being removed in the first jar.
- (vi) The slides were dipped in and out of absolute ethyl alcohol (in two Coplin jars) for approximately 10 s for further decolorizing.
- (vii) The slides were dipped in and out of xylene (in two Coplin jars) for approximately 10 s until clean.
- (viii) The back and frosted areas of the slides were wiped dry with a paper towel. With a sterile glass Pasteur pipette, a small amount of per mount (Fisher Scientific Co., Fair Lawn, N.J.) was placed along a long edge of a large cover slip (20 by 50 mm) which was then placed on the slide while the specimen area was still moist with xylene.
- (ix) The slides were examined with 20x and 40x objectives; examination under oil was not necessary but could be done once the cover-slipped slides had dried. The staining procedure itself took approximately 20 min. Up to 4 slides could be stained readily simultaneously.

Appendix VI: Approval Letters



**JOMO KENYATTA UNIVERSITY
OF
AGRICULTURE AND TECHNOLOGY
DIRECTOR, BOARD OF POSTGRADUATE STUDIES**

P.O. BOX 62000
NAIROBI – 00200
KENYA
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TEL: 254-067-5211/52181-4
FAX: 254-067-52164/52030

REF: JKU/2/11/TM305-2784/2015

31ST OCTOBER, 2018

SAITOTI ANN SEIN
C/o SOBMS
JKUAT

Dear Ms. Sein,

RE: APPROVAL OF RESEARCH PROPOSAL AND OF SUPERVISORS

Kindly note that your MSc. research proposal entitled: *"PREVALENCE OF PNEUMOCYSTIS JIROVECI IN SPUTUM FROM TB SMEAR-NEGATIVE PATIENTS AND RETREATMENT CASES AT THE COAST GENERAL HOSPITAL"* has been approved. The following are your approved supervisors:

1. Prof. Fred Wamunyokoli
2. Dr. Christine Bii

Yours sincerely,

PROF. MATHEW KINYANJUI
DIRECTOR, BOARD OF POSTGRADUATE STUDIES

Copy to: Dean, SOBMS
/cm



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KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
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KEMRI/RES/7/3/1

November 03, 2016

TO: ANNE SEIN SAITOTI,
PRINCIPAL INVESTIGATOR

THROUGH: PROF. SAM KARIUKI,
THE DIRECTOR, CMR,
NAIROBI

forwarded
SK 04/11/16

Dear Madam,

RE: KEMRI/SERU/CMR/P00041/3339 (RESUBMISSION OF INITIAL
SUBMISSION): PREVALENCE OF PNEUMOCYSTIS JIROVECI IN SPUTUM FROM
TB SMEAR-NEGATIVE PATIENTS AND RETREATMENT CASES AT THE COAST
GENERAL HOSPITAL

Reference is made to your letter dated 24th October 2016. The KEMRI/Scientific and Ethics Review Unit (SERU) acknowledges receipt of the revised study documents on the 26th October 2016.

This is to inform you that the Committee determined that the issues raised at the 255th Committee B meeting of the KEMRI/SERU held on 21st September 2016 are adequately addressed.

Consequently, the study is granted approval for implementation effective from **3rd November 2016** for a period of one year. Please note that authorization to conduct this study will automatically expire on **November 2, 2017**. If you plan to continue with data collection or analysis beyond this date, please submit an application for continuation approval to the SERU by **September 22, 2017**.

You are required to submit any proposed changes to this study to SERU for review and the changes should not be initiated until written approval from SERU is received. Please note that any unanticipated problems resulting from the implementation of this study should be brought to the attention of the SERU and you should advise the SERU when the study is completed or discontinued.

You may embark on the study.

Yours faithfully,

Fok: DR. EVANS AMUKOYE,
ACTING HEAD,
KEMRI/SCIENTIFIC AND ETHICS REVIEW UNIT

In Search of Better Health



KENYA MEDICAL RESEARCH INSTITUTE

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KEMRI/RES/7/3/1

October 10, 2017

TO: ANNE SEIN SAITOTI,
PRINCIPAL INVESTIGATOR

THROUGH: THE DIRECTOR, CMR,
NAIROBI

*Forwarded
SK*

Dear Madam,

RE: **SSC PROTOCOL NO. 3339 (REQUEST FOR ANNUAL RENEWAL): PREVALENCE OF PNEUMOCYTIS JIROVEII IN SPUTUM FROM TB SMEAR NEGATIVE PATIENTS AND RETREATMENT CASES AT THE COAST GENERAL HOSPITAL.**

Thank you for the continuing review report for the period **03 November 2016- 5th September 2017**

This is to inform you that the expedited review team of the KEMRI Scientific and Ethics Review Unit (SERU) conducted the annual review of the above referenced application and was of the informed opinion that the progress made during the reported period is satisfactory. The study has therefore been granted **approval**.

This approval is valid from **3rd November, 2017** through to **2nd November, 2018**. Please note that authorization to conduct this study will automatically expire on **2nd November, 2018**. If you plan to continue with data collection or analysis beyond this date please submit an application for continuing approval to the SERU by **September 22, 2018**.

You are required to submit any amendments to this protocol and any other information pertinent to human participation in this study to the SERU for review prior to initiation.

You may continue with the study.

Yours faithfully,

SK

for

DR. MERCY KARIMI NJERU,
ACTING HEAD,
KEMRI/SCIENTIFIC AND ETHICS REVIEW UNIT



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KEMRI/RES/7/3/1

February 15, 2021

**TO: ANNE SEIN SAITOTI,
PRINCIPAL INVESTIGATOR.**

Forwarded 18/3/2021

**THROUGH: THE DEPUTY DIRECTOR, CMR,
NAIROBI.**

Dear Madam,

RE: SERU PROTOCOL NO. 3339 (RESUBMISSION II OF REQUEST FOR ANNUAL RENEWAL): PREVALENCE OF PNEUMOCYTIS JIROVEII IN SPUTUM FROM TB SMEAR NEGATIVE PATIENTS AND RETREATMENT CASES AT THE COAST GENERAL HOSPITAL.

Reference is made to your letter dated February 4, 2021. The KEMRI Scientific and Ethics Review Unit (SERU) acknowledges receipt of the revised application on February 05, 2021.

The Expedited Review Team notes that the issues it raised on the letter dated **January 26, 2021** have been adequately addressed.

Consequently, the study is granted approval for continuation effective **February 15, 2021** through to **February 14, 2022**. Please note that authorization to conduct this study will automatically expire on **February 14, 2022**. If you plan to continue with data collection or analysis beyond this date, please submit an application for continuing approval to the SERU by **January 3, 2022**.

You are required to submit any amendments to this protocol and any other information pertinent to human participation in this study to the SERU for review prior to initiation.

You may continue with the study.

Yours faithfully,

**ENOCK KEBENEI,
THE ACTING HEAD,
KEMRI SCIENTIFIC AND ETHICS REVIEW UNIT.**

In Search of Better Health

Appendix VII: Publication



Journal of Tuberculosis Research, 2024, 12, 215-231

<https://www.scirp.org/journal/jtr>

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Prevalence of *Pneumocystis jirovecii* in Smear Negative Sputum Samples at the Coast General Teaching and Referral Hospital in Kenya

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How to cite this paper: Saitoti, A., Wamunyokoli, F. and Bii, C. (2024) Prevalence of *Pneumocystis jirovecii* in Smear Negative Sputum Samples at the Coast General Teaching and Referral Hospital in Kenya. *Journal of Tuberculosis Research*, 12, 215-231. <https://doi.org/10.4236/jtr.2024.124016>

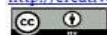
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Open Access

Abstract

Pneumocystis jirovecii is a pathogen that causes *Pneumocystis pneumonia* (PCP), an infection in (HIV/AIDS) and other immunocompromised patients. The rare reports of *P. jirovecii* pneumonia in sub-Saharan Africa are controversial due to the high HIV/AIDS seropositivity. The study determined the significance of *P. jirovecii* in TB smear negative retreatment patients at the Coast General Teaching Referral Hospital-Mombasa. Sputum samples were subjected to toluidine blue O (TBO) stain for microscopy and polymerase chain reaction (PCR). A total of 100 TB smear negative participants were enrolled in the study and expectorated sputum was collected. Out of the 100 patients, 63 men and 37 women. Patients aged 31 to 53 made up 62% of the patients. The patients aged 31 to 53 made up 75% of the patients (Min = 11y, Max = 85y). The median age of the patients was 42 years. Nested PCR has a prevalence of 41% (41 instances). TBO staining has a prevalence of 29% (29 instances). Detecting an additional 13 more patients than toluidine O staining technique. The sensitivity of toluidine blue O staining was 33.82%, which indicates that it correctly identified 33.82% of true positive cases compared to PCR. The specificity of toluidine blue O staining was 97.82%, indicating that it had a high ability to correctly identify true negative cases compared to PCR, suggesting that it was good at ruling out non-*P. jirovecii* cases. The study confirms that *P. jirovecii* is as a significant cause of persistent symptoms in TB patients that could be responsible for persistent symptoms despite TB treatment. We recommend fungal diagnostics in such patients before retreatment.

Keywords

Pneumocystis jirovecii, Nested PCR, *Pulmonary tuberculosis*, Smear Negative