EVALUATION OF POLYMERASE CHAIN REACTION OF MOUTHWASHES VERSUS SPUTUM CULTURE FOR DIAGNOSIS OF PULMONARY TUBERCULOSIS IN PATIENTS ATTENDING MBAGATHI COUNTY REFERRAL HOSPITAL, NAIROBI

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Evaluation of Polymerase Chain Reaction of Mouthwashes versus Sputum Culture for Diagnosis of Pulmonary Tuberculosis in Patients attending Mbagathi County Referral Hospital, Nairobi

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

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DECLARATION

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

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DEDICATION

To my family

ACKNOWLEDGEMENT

I thank the Almighty God for His love, benediction and spirit of hard work that He always reserves for us. My heartfelt gratitude goes to my respectable supervisors; Dr. Josephine Kimani, Dr. Jane Ngaira (DECEASED), and Dr. Jane Ong'ang'o for their overwhelming support, constructive advice, suggestions and comments in all phases of this thesis. I thank the KEMRI Director for the financial support. I say thanks to my family members for the love, encouragement, devotion and support. My appreciation goes also to all my workmates and classmates for their moral support which made my academic life pleasant. Last but not least, my special gratitude goes to all of you who, in one way or another, have contributed to the completion of this work.

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ABBRVIAIONS AND ACRONYMS

AFB	Acid-Fast bacilli
AIDS	Acquired Immunodeficiency Syndrome
AMTD	Amplified Mycobacterium Direct Test
ATPase	Enzyme Breaks ATP into ADP and Free Phosphate Group
BALF	Broncho Alveolar Lavage Fluid
CTAB	Acetyl Timthylammoniumbromide
CPC	Cetylpyridinium Chloride
CTM	Cobas Taqman
DNA	Deoxyribonucleic Acid
G	Grams
GyrA	*Gyrase Enzyme*
FDA	Food ad Dug Administration
HIV	Human Immunodeficiency Virus
HPTPP	High Priority Target Product Profile
HSP65	Heat Shock Protein 65
IS	Insertion Sequence
ITS	Intergenic Spacer
KatG	*Catalase Peroxidase*

KDa	Kilodalton
KEMRI	Kenya Medical Research Institute
LAM	Lateral Flow Urine Lipoarabinomannan Assay
LAMP	Loop Mediated Isothermal Amplification
LDT	Laboratory Developed Tests
LJ	Lowenstein-Jensen
LPA	Line Probe Assays
LTBI	Latent TB Infection
MDG	Millennium Development Goal
MDRTB	Multi - Drug - Resistant TB
MGIT	Mycobacteria Growth Indicator Tube
GC	Growth Control
MTBC/MTC	Mycobacterium Tuberculosis Complex
NAAT	Nucleic-Acid Amplification Tests
NCBI	National Centre for Biotechnology Information
NHP	Nucleic Hybridization Probes
NPV	Negative Predictive Value
NTM	Non-Tuberculosis Mycobacteria
PCR	Polymerase Chain Reaction

POC	Point of Care
PNB	p - Nitrobenzoic Acid
PPV	Positive Predictive Value
dN/dS	**R**atio of Nonsynonymous to Synonymous Substitutions
RNA	Ribonucleic Acid
rpoB	β Subunit of Bacterial RNA Polymerase
rRNA	Ribosomal Ribonucleic Acid
RRTB	Rifampicin-Resistant Tuberculosis
RTPCR	Real-Time Polymerase Chain Reaction
SEC PAHTWAY	Protein Secretory Pathway
SECA1	Part of the Sec Protein Translocase Complex
SECYEG	The Protein-Conducting Channel
SNPs	Single-Nucleotide Polymorphisms
TB	Tuberculosis
TrisEDTA	Hydroxymethyl- Ethylenediaminetetraacetic Acid
UV	Ultra Violet
UNG	Uracil N-Glycosylase
WHO	World Health Organization
ZN	Ziehl - Neelsen

ABSTRACT

Tuberculosis continues to pose a significant danger to human health, and current TB diagnostic techniques are not up to standard. Sputum, the most commonly used specimen for pulmonary tuberculosis diagnosis, is hazardous, challenging to manage, and frequently in short supply among TB patients. Therefore, invasive techniques such as bronchoscopy are used for obtaining high-quality sputum samples from HIV patients and children. Nucleic acid amplification tests are regarded more sensitive than sputum culture for detecting TB. The aim of this study, therefore, was to evaluate the reliability of Polymerase chain reaction of mouthwashes as an alternative sample compared to sputum culture for diagnosis of pulmonary tuberculosis. A sum of 300 individuals with suspected pulmonary TB were included for assessment. Novel reagents were used to homogenize fresh mouthwashes collected through gargling with normal saline, and DNA was extracted via the phenol-chloroform method. The sputum counterparts were decontaminated using a solution of N-acetyl cysteine, (NAlc-NaOH) technique and grown on Lowenstein - Jensen medium to isolate *M. tuberculosis.* The cultures were kept at 37 °C and checked for growth every week for a minimum of 8 weeks with the findings being documented. PCR targeting the secA1 gene from the mouthwashes was conducted between January 2016 to December 2018. and the accuracy, sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV of the PCR technique compared to that of sputum culture. PCR had an accuracy, sensitivity, specificity, PPV, and NPV of 129%, 100%, 65%, 67.6%, and 100% respectively. The extreme test accuracy may have been influenced by sample rejection in the laboratory, similar to the sensitivity. A substantial number of participants had false positive results as indicated by low specificity. The low PPV indicated that a positive test result was likely wrong. However, the high NPV indicated that no one with the negative test had the disease. Mouthwashes are an alternate specimen for sputum-based molecular diagnosis for MTB. The procedure is less invasive, easier, and non-aerosol producing efficient alternate approach with significant impact on clinical management and control of TB. More attention should be given to sample collection, and technique optimization to improve disease laboratory diagnostic accuracy using mouthwashes.

CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

Tuberculosis (TB) is the leading infectious cause of death globally and is responsible for more than 1.6 million deaths and about 10.6 million new cases annually (WHO, 2023). TB is caused by the *Mycobacterium tuberculosis* (MTB) complex consisting of *M. tuberculosis, M. africanum, M.bovis, M. canettii, M. microti, M. pinnipedii, M. orygis and M. caprae* (Chiner-Oms *et al.,* 2019). As primarily a lung disease, TB is acquired through inhalation of droplets containing the causative agent (WHO; 2013). Extra-pulmonary spread and multi-organ involvement or disseminated disease occur, particularly in immuno - compromised hosts (WHO; 2013).

According to the World Health Organization (WHO), early detection and treatment of active and latent TB are key factors in the control of the TB epidemic (WHO, 2013). About 64% of TB cases are detected globally, indicating that 36% are not identified (WHO, 2013). In 2016, 2.5 million people fell ill with TB in the African region (https://www.afro.who.int/health-topics/tuberculosis-tb). In the same year, Kenya had an estimated annual TB incidence rate of 348 per 100 000 people with about 169 falling ill with TB disease, translating to 0.42% prevalence against a population of 49 million (https://www.endtb.org/kenya). This makes Kenya among the 20 top countries contributing to the highest burden of TB, TB/Human - immunodeficiency virus (HIV) co-infection, and Drug-resistant tuberculosis (DR - TB) globally (WHO, 2019).

About half of the Kenyan population infected with TB did not however receive treatment in 2016 for lack of adequate diagnosis (Enos *et al.,* 2018). Strategies to find the missing TB cases are therefore needed to address the TB epidemic in Kenya and worldwide (Kirubi *et al.,* 2021).

The current CDC guidelines for prevention of MTB transmission in the healthcare setting recommend a 3-level hierarchy of control measures: administrative controls

aimed at reducing exposure to individuals with presumptive PTB; environmental controls by placing individuals with presumptive TB in airborne infection isolation (AII), ideally using single-patient, negative-pressure ventilation rooms; and respiratory protection controls through use of disposable N95 respirators by healthcare workers (Lippincott *et al*., 2014). Patients must produce 3 respiratory specimens 8–24 hours apart, with at least 1 early morning specimen, for smear microscopy for acid-fast bacilli (AFB) and MTB culture (Lippincott *et al*., 2014).

TB culture techniques generally have greater sensitivity than microscopy, and PCR has intermediate sensitivity between that of culture and microscopy (Datta *et al*., (2017). The positivity of laboratory tests often varies amongst samples from the same patients, so more than one sputum sample is usually tested from each person with suspected tuberculosis (Datta *et al*., (2017) .

The Xpert MTB/RIF assay (Xpert, Cepheid, Sunnyvale, California), an automated, rapid nucleic acid amplification test (NAAT) endorsed by the World Health Organization in 2010 (WHO; 2011) and authorized for marketing by the US Food and Drug Administration in 2013 (FDA; 2013) can expedite the diagnostic process given its sensitive and specific ability to diagnose tuberculosis within 2 hours with minimal operator training (Boehme *et al*., 2010; Steingart *et al*., 2013).

A definite diagnosis can only be established if MTB is isolated and identified from respiratory specimens, most frequently expectorated or induced sputum (Datta *et al*., 2017) . Therefore, when pulmonary TB (PTB) is suspected, standard guidelines recommend that clinical specimens should be collected and submitted for laboratory testing, such as smear microscopy, culture and nucleic acid amplification to increase the detection rate of MTB (WHO, 2013).

It has been stated that laboratory analysis should be performed on at least three sputum specimens, collected over consecutive days (Tavares *et al*., 2015). However, there has been some controversy attached to this methodology and some authors find examination of multiple specimens excessive in relation to the yield gain and hard to achieve especially in resource-limited settings (Tavares *et al*., 2015).

Culture identification using sputum is still the gold standard for diagnosis of pulmonary TB. For its improvement, liquid medium culture has emerged as a more sensitive and speedier technique to detect bacilli growth and simultaneously test for drug susceptibility (Tavares *et al*., 2015).

For patients with a poor response to therapy, sputum specimens are collected for culture and drug-susceptibility tests (DSTs), both molecular and conventional (WHO, 2011). Thus, performing monthly culture tests is the best strategy in identifying failures earlier (WHO, 2011).

Nucleic acid amplification tests (NAATs), such as Polymerase chain reaction (PCR), which was developed in 1983, are now a common tool for the rapid diagnosis of many infectious diseases, including TB (Wei *et al*., 2019). To date, some commercial tests, including COBAS TaqMan, Loop-mediated isothermal amplification (LAMP) and the Xpert MTB/RIF have been used for TB diagnosis (Wei *et al*., 2019).

Xpert MTB/RIF is useful for rapid diagnosis of TB disease and identification of drug resistance via amplification of the 81bp *rpoB* gene from patient samples within 2 h (Helb *et al.,* 2010), is used as baseline to ensure rapid diagnosis but not monitoring response to treatment (Wei *et al*., 2019). Despite significant advances in the development of novel tests, molecular tests cannot replace culture, but should be used in addition to conventional tests (smear microscopy, culture, and phenotypic drug susceptibility testing) and clinical data for TB diagnosis, as highlighted by other studies (Eddabra *et al*., 2018). The methods have shortened the time to diagnosis to a few days by allowing direct detection of low MTB genomic copy numbers in specimens (Kim *et al.,* 2014; Cho *et al.,* 2015; Huggett *et al.,* 2003;).

Gene Xpert was endorsed by the WHO in December 2010 for use in high-burden countries and is the first rapid near-point-f-care (POC) diagnostic to be widely implemented in TB-endemic settings (Dharan *et al.,* 2015). This and other NAAT systems offer alternatives to the limitations of conventional methods including rapid turn-around times, facilitate testing and treatment initiation in the same visit, and, therefore, loss to follow-up cases can be reduced (Niemz *et al.,* 2012).

There is a growing interest in search for alternative specimen samples with sampling of the upper airway offering an attractive option for PTB diagnosis (Rabie *et al*., 2024). Generally, the specimen type demonstrates accurate results with an acceptable sensitivity and are preferred in response to the enhanced processing and accessibility of oral specimens for tuberculosis diagnosis (Wei *et al.*, 2019). There is need, however, to address gaps in the pre - analytical stages of swab sampling, including the appropriate swab type and site (tongue vs buccal mucosa), storage, and laboratory optimization techniques. Ideally, feasibility should be assessed using commercially available automated TB - NAAT. Use of in-house tests have possibly contributed to the lower sensitivity and specificity of oral specimens compared with using Xpert MTB/RIF Ultra tests (Rabie *et al*., 2024; Church *et al*., 2024).

In people with high bacillary load, swabs are likely to perform as well as sputum. Use is less clear when bacillary load is low, such as in people who are unable to expectorate and people presenting for mass screening (Rabie *et al*., 2024; Lima *et al*., 2020). However, despite the lower sensitivity, the advantage of oral specimens is clear, as testing is not dependent on bowel movement and the health-care worker can collect the sample on the same day. This advantage is important as it prevents failure to collect the specimens as observed when using stool samples (Rabie *et al*., 2024; Marcy *et al*., 2023)

Despite holding promise, oral swabs have however had to report, in previous studies, sensitivity below the WHO cut - off for diagnostic tools (WHO, 2022; Church *et al*., 2024; Savage *et al*., 2023) Hopefully sensitivity can be improved though optimization specimen collection, including self-collection, and testing methods (Church *et al*., 2024; Andama *et al*., 2022).

Studies have indicated need to include patients with confirmed tuberculosis disease, ranging from non-severe to more severe disease, to measure the sensitivity of oral specimens on the basis of disease severity and therefore place in the diagnostic algorithm (Savage *et al*., 2023). Studies in children should include participants managed in primary care, who are less likely to have severe or confirmed

tuberculosis than hospitalized children. (WHO, 2022; Church *et al*., 2024; Savage *et al*., 2023).

The PCR targets widely used for diagnosing mycobacteria include; the 16S *rRNA* gene, 16S-23S internal transcribed spacer, 65-kDa heat shock protein, *recA*, *rpoB*, and *gyrB*. However, due to the small number of polymorphic sites in the 16S *rRNA* gene, some species cannot be differentiated. The *hsp65* gene-based PCR restriction pattern analysis (PRA) is also widely used for identification, but can sometimes result in species with similar patterns (McNabb *et al*., 2004), reported an overall agreement of 85.2% with other methods.

The Xpert MTB/RIF is a seminested real-time PCR (RT-PCR) technology can be used to detect MTB and *ropB* mutation of rifampicin resistance gene and is widely considered as a breakthrough in TB diagnosis (Zou *et al*., 2023). At present, several studies have shown that Xpert MTB/RIF technology has no cross with NTM, therefore can distinguish MTB from NTM in a short time, early rule out NTM infection, which guide the direction of clinical medication (Zou *et al*., 2023). However, studies show that some strains of MTB possess mutations at codon 513 and 516 in the *rpoB* region. This region of the *rpoB* gene is situated between two probes (WT 3 and 4) and, thus, can be missed due to their overlap leading to loss of some disputed isolates, whereas *some* susceptible isolates could be misclassified as resistant (Zou *et al*., 2023).

Protein export is essential in all bacteria and many bacterial pathogens depend on specialized protein export systems for virulence. In MTB, the etiological agent of the disease tuberculosis, the conserved general secretion (Sec) and twin-arginine translocation (Tat) pathways perform the bulk of protein export and are both essential (Feltcher *et al*., 2010). SecA1 shuttles evenly between the cytosol and the SecYEG membrane channel to continually deliver and translocate exported proteins (Feltcher *et al*., 2010). Sequence variability in the amplified segment of the *SecA1* gene allowed the differentiation of all species except for the members of the MTB complex, which had identical sequences. A range of 83.3 to 100% inter - species similarity was observed (Zelazny *et al*., 2005).

1.2 Statement of the Problem

Despite recent advances in tuberculosis diagnostics, most methods still require sputum for testing. However, many populations, including children and people living with HIV, have difficulty producing sputum samples. Obtaining sputum for diagnosis in people who cannot make it themselves can be resource-intensive and require trained staff. Production of an adequate amount of sputum (3 - 5ml) is often not achieved. Also, there is inconsistency in availing triplicate samples, contamination of samples, low sensitivity of technique, and requirement for level 3 labs which are not freely available locally. The sensitivity of oral swabs as alternate specimen samples in TB diagnosis is highly variable, with a requirement for sampling optimization.

1.3 Justification

To improve access to tuberculosis testing, mouthwashes have been proposed as a new clinical specimen for PTB diagnosis. Mouthwashes are safer, easier-to-collect, and more homogeneous alternative sample to sputum that would simplify TB diagnosis. Mouthwash is a cheaper and noninvasive sample collection method of producing high-quality specimens that would be more advantageous to provide access to a larger patient population. Mouthwashes are analyzed by PCR - based NAAT that amplify a specific gene in the the MTB DNA collected.

Multiple types of oral-based specimens and NAAT platforms are being studied with PCR, the most commonly used NAAT method. To date, there are no oral-based recommendations for tuberculosis detection by WHO.

1.4 Research Questions

1. Do sputum cultures from suspected TB-positive patients attending Mbagathi County Referral Hospital contain *M. tuberculosis?*

2. Can MTB be detected from mouthwashes of suspected TB positive cases through the amplification of *SecA1* gene from its DNA through PCR?

3. Does the PCR - based *SecA1* gene detection technique perform better compared to sputum culture for diagnosis of tuberculosis in patients attending Mbagathi County Referral Hospital?

1.5 Hypothesis

PCR - based *SecA1* gene detection from mouthwashes is a more reliable detection technique comparable to sputum culture for the diagnosis of tuberculosis in patients attending Mbagathi County Referral Hospital, Nairobi

1.6 Study Objectives

1.6.1 General Objective

To evaluate the use of PCR of mouthwashes versus sputum culture for detection of *M. tuberculosis* in patients attending Mbagathi County Referral Hospital, Nairobi

1.6.2 Specific Objectives

1. To determine presence of MTB from suspected TB-positive sputum samples of patients attending Mbagathi County Referral Hospital.

2. To determine the presence of *SecA1* gene from mouthwashes of suspected TB positive patients attending Mbagathi County Referral Hospital. for diagnosis of tuberculosis through PCR

3. To compare performance of the PCR - based *SecA1* gene detection technique against sputum culture for the diagnosis of tuberculosis in patients attending Mbagathi County Referral Hospital.

1.7 Thesis Scope

A good quality specimen is necessary for a successful diagnosis of TB. Yet, several patients fail to produce good-quality sputum or none at all. This study evaluated mouthwashes as a non-invasive sample source against sputum culture for the detection of MTB in patients attending Mbagathi County Referral Hospital, Nairobi.

1.8 Expected Impact

Prospective findings shall enrich the pipeline PCR-based molecular detection of tuberculosis to assist in scaling up active case detection in large populations.

CHAPTER TWO

LITERATURE REVIEW

2.1. Overview of the Literature

Roughly 15% of deaths globally are caused by TB, which continues to be a major infectious killer disease (WHO, 2023). A rise from 2021 to 2022 was observed in the predicted 7·5 million cases of TB diagnoses. About 167,000 fatalities from TB occurred in persons living with Human immunodeficiency virus (HIV) in 2022, out of a projected 1.3 million deaths from the disease. As of 2022, a microbiological test was only performed on 63% of patients receiving treatment for tuberculosis (Church *et al.,* 2024; WHO, 2023)

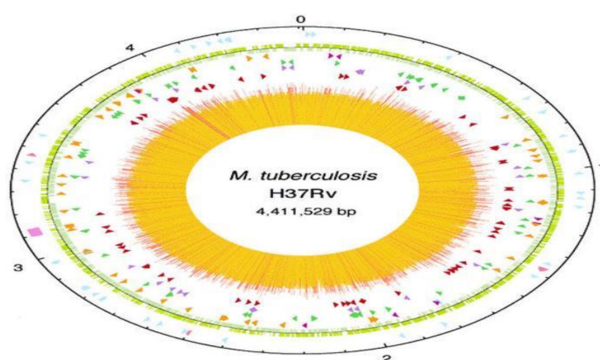
Aspirates from the stomach, nasopharynx, sputum, urine, and stool are specimens used in the diagnosis of pulmonary tuberculosis. For adults and children, when a specimen is available, Xpert MTB/RIF and XPERT MTB/RIF Ultra provide a high sensitivity molecular testing approach for diagnosing PTB using sputum (Church *et al.,* 2024; WHO, 2023).

HIV-positive patients, those with a mild cough, and children under five years old may have restricted sputum output. These restrictions frequently necessitate the use of alternative respiratory specimens (such as stomach aspirates in young children or induced sputum in adults), which call for specialized staff training and medical equipment (Church *et al.,* 2024; WHO, 2023).

2.2 Description of MTB Genome

The genome of MTB H37Rv, an integral and highly-valued reference strain for studying the biology of MTB, is composed of 4.4 × 106 bp and includes around 4,000 genes (Fig.1) (Cole *et al.,* 1998). Analysis of the MTB genetic material reveals distinct characteristics present in this microorganism. More than 200 genes are identified as coding for enzymes involved in the breakdown of fatty acids, making up 6% of the overall total (Table 1). Approximately 100 enzymes in *E. coli* are predicted to be involved in the β-oxidation of fatty acids, twice as many as the 50 enzymes in

E. coli that participate in fatty acid metabolism. The distantly related bacterium *Streptomyces coelicolor* has a total of 115 proteins, slightly over 1% of the total, with 59 of them identified as participating in fatty acid breakdown (Cole *et al.,* 1998). The high number of MTB enzymes that potentially utilize fatty acids could be linked to the pathogen's capability to thrive in the infected host's tissues, where fatty acids might serve as the main carbon source.



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC164219/

Figure 2.1: Circular Map of the Chromosome of MTB H37Rv.

The outer circle shows the scale in megabases, with 0 representing the origin of replication. The first ring from the exterior denotes the positions of stable RNA genes (tRNAs are blue, and others are pink) and the direct-repeat region (pink cube); the second ring shows the coding sequence by strand (clockwise, dark green; anticlockwise, light green); the third ring depicts repetitive DNA (insertion sequences, orange; 13E12 REP family, dark pink; pro-phage, blue); the fourth ring shows the positions of the PPE family members (green); the fifth ring shows the positions of the PE family members (purple, excluding PGRS); and the sixth ring shows the positions of the PGRS sequences (dark red). The histogram (center) represents the G+C content, with <65% G+C in yellow and >65% G+C in red (Cole *et al.,* 1998).

Table 2.1: General Classification of MTB Genes Showing the Gene Function, Number of Genes, the Percentage Total and Percentage Coding Capacity

Function	**No. of genes**	**% of total**	**% of Total coding capacity**
Lipid metabolism	225	5.7	9.3
Information pathways	207	5.2	6.1
Cell wall and cell processes	517	13.0	15.5
Stable RNAs	50	1.3	0.2
IS elements and bacteriophages	137	3.4	2.5
PE and PPE proteins	167	4.2	7.1
Intermediary metabolism and respiration	877	22.0	24.6
Regulatory proteins	188	4.7	4.0
Virulence, detoxification and adaptation	91	2.3	2.4
Conserved hypothetical function	911	22.9	18.4
Proteins of unknown function	607	15.3	9.9

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC164219/

Diagnostic advancements, such as the Xpert MTB/RIF and MTB/XDR assays, likewise hinge on the growing power of MTB genomics (Church *et al.,* 2024; WHO, 2023).

2.3. Protein Export System of Mycobacteria

Although little is known about the protein export pathway in mycobacteria, the general secretory (Sec) pathway has been extensively studied in other bacteria, *Escherichia coli* in particular (Zelazny *et al*., 2005). *SecA1* is the mycobacterial homologue of the *E. coli* SecA protein, an essential preprotein translocase ATPase that provides the driving force for the export of proteins across the cytoplasmic membrane. The mycobacterial Sec pathway is unusual in that it has two SecA proteins: *SecA1* is the essential housekeeping *SecA* protein, while *SecA2* is a nonessential accessory secretion factor (Zelazny *et al*., 2005).

Sequence variability in the amplified segment of the *SecA1* gene allows the differentiation of all species except for the members of the MTB complex, which has identical sequences with a range of 83.3 to 100% interspecies similarity observed (Zelazny *et al*., 2005).

2.4. PCR - Based Tools for Diagnosis of TB

PCR- based tools for TB detection have been reported, with various methods available for in-house tests and commercial kits (Mugenyi *et al.,* 2024). Traditional NAATs amplify a specific DNA fragment exclusive to the microbe, producing results in three to six hours. The FDA has approved specific commercial NAATs for respiratory specimens, but no technique has been approved for direct identification of MTB from extra-pulmonary specimens (Mugenyi *et al.,* 2024). Commercial NAATs have shown high specificity but modest sensitivity (55%) (Oreskovic *et al.,* 2021). The Loop-Mediated Isothermal Amplification (LAMP) technique, based on isothermal amplification of DNA, has emerged as a useful tool for TB diagnosis (Boehme *et al.,* 2007). The Amplicor MTB test (Roche Diagnostic Systems Inc., NJ), and the Gene - Probe Amplified MTB - Direct Test (AMTD) are the first two and oldest NAA products available. The COBAS Amplicor MTB test and the qualitative COBAS TaqMan MTB test are used for concentrated and decontaminated smear-positive respiratory samples from patients who have not received prior treatment (Mugenyi *et al.,* 2024). Semi-automated assays like Gene Xpert assay and kits with

standard formats and reagents make amplification technologies more feasible for use in clinical laboratories (Peralta *et al.,* 2016; Juan Carlos Palomino, 2009).

2.5. The Role of the Xpert MTB/RIF Assay in TB Diagnosis

Globally, 182 deaths from MDR-TB are estimated to have occurred in 2019. Numerous nations continue to record an increase in the percentage of MDR-TB cases (WHO, 2020). Additionally, there are more reports of extensive drug-resistant tuberculosis (XDR-TB) (Sharma *et al.,* 2017; Wilson *et al.*, 2016). In just a few hours, PCR-based NAATs with a quicker turnaround time can identify drug resistance-causing gene mutations directly on specimens (Gomathi *et al.,* 2020).

The WHO approved the Gene Xpert in 2010 for the diagnosis of TB and rifampicin resistance (WHO, 2019; Pai *et al.,* 2019). The assay, which has a two-hour turnaround time (TAT) and is based on nested real-time or quantitative PCR (nqPCR) on the *rpoB* gene, uses molecular beacon technology. According to Gomathi *et al,* (2020), it doesn't require many biosafety facilities and is not susceptible to cross-contamination. However, the Gene Xpert test's sensitivity for extra-pulmonary samples is frequently at a moderate level, declining particularly in samples that test negative for AFB (Hsin-YaoWang *et al.,* 2019). According to reports, the system's detection sensitivity is higher than that of solid culture or smear microscopy (Gomathi *et al.,* 2020).

Since 2017, the first-line molecular diagnostic tool has been advised to be the Gene (WHO, 2020; Michel *et al.,* 2019). Still, more precise diagnostic instruments are needed (Lange *et al.,* 2018). DR strains of MTB have made it more difficult to control TB, which has led to a large-scale adoption and escalation of molecular techniques for the quick detection of both tuberculosis and related drug resistance (Lange *et al.,* 2018).

Insertion elements (IS) 6110 and 1081 were introduced in 2017, when a new generation assay called Gene Xpert MTB/RIF Ultra was introduced. This assay incorporates two distinct multi-copy amplification targets, IS6110 and IS1081. The effectiveness of Gene Xpert MTB/RIF Ultra in children has not received much

attention in the literature to date (Zar *et al.,* 2019, Ssengooba *et al*., 2020; Sun *et al.,* 2020). Since false-negative results occur in about 25% of pediatric cases of PTB, there is a pressing need to increase the assay's sensitivity before using it as a rule-out test (Vaezipour *et al.,* 2022).

2.6. Oral - Based Specimens in TB Detection by PCR

The fight against TB is greatly hampered by the fact that patients who are unable to produce sputum for microbiological confirmation of MTB, such as children and certain HIV-positive people, are frequently overlooked (Wang, 2014; Floyd *et al.,* 2018). Simultaneously, processing sputum samples for NAAT can be hampered by their high viscosity (WHO, 2020). Due to their average textual matrix, the availability of more accessible and mechanically tractable samples, such as CBC, blood, saliva, stool, urine, and stool, is being assessed for prospective TB diagnosis (Mesman *et al.,* 2019).

Blood and urine samples from HIV-positive and negative patients with PTB have been found to contain MTB DNA in a number of reports, with varying degrees of success from individual studies (Wang, 2014;). Like most bacteria, mycobacterium cells have developed adherence mechanisms to cling to surfaces, including those of mammals (Wang, 2014). Whereas *M. avium* cells adhere to non-biological surfaces and the bronchial epithelium, *M. leprae* cells adhere to the nasal and alveolar epithelial cells (Song *et al.,* 2021; Mostowy *et al.,* 2008; Freeman *et al.,* 2006). As opposed to fluid matrices, surfaces are more frequently linked to numerous environmental mycobacterium species in nature (Song *et al.,* 2021; Falkinham *et al.,* 2001).

Because oral specimen collection is painless, noninvasive, and produces no aerosol, it is a very simple process (Luabeya *et al.,* 2019). According to Davies *et al.,* 2009; Wood *et al.* 2015), oral swabbing is quick and doesn't require isolation or privacy. It only takes a few seconds to complete. Oral swabs have been tested as substitute non-sputum samples (Eguchi *et al.,* 2003) and have proven effective in detecting tuberculosis in non-human primates (NHPs) who exhibit cutaneous lesions (Luabeya *et al.,* 2019). Research conducted in South Africa on oral swab samples from adults

and children revealed high sensitivity and specificity (Theron *et al.*, 2013 and Xu *et al.,* 2019). However, disparities in patient populations (varying disease burdens and levels of acuity) and different methods of collection and detection were linked to lower test sensitivity.

Due to their non-invasive nature and ease of collection via mail, buccal cells are a desirable source of DNA (Zayats *et al.,* 2009). In a convenience sample of 52 adult TB patients in Japan, the use of saliva for molecular diagnosis of TB was initially reported. The patients were assessed using a lab-developed, nested PCR assay, which demonstrated a sensitivity of 98% (Eguchi *et al.,* 2003).

In relation to sputum mycobacterial culture on previously frozen and thawed sputum, oropharyngeal wash specimens combined with a lab-developed PCR assay had a high sensitivity for TB diagnosis; however, a follow-up study was unable to corroborate these findings (Davis *et al.,* 2011). MTB PCR showed high sensitivity (90%) and specificity (100%) on buccal swabs of US controls and South African TB patients (Wood *et al.,* 2015).

2.7. The Genetics of MTB

MTBC is a genetically monomorphic group of bacteria whose members cause TB in humans and animals. The MTBC comprises both human - associated (L1, L2, L3, L4, L5, L6, L7, L8, and L9) and animal - associated (A1, A2, A3, and A4) clades (Chiner-Oms *et al*., 2022). Due to the absence of horizontal gene transfer, plasmids, and measurable recombination among strains and other species, chromosomal mutations represent the source of MTBC genetic diversity (Chiner-Oms *et al*., 2022)

The maximum genetic distance between any two MTBC strains is around 2,500 single-nucleotide polymorphisms (SNPs). Strikingly, studies have highlighted large phenotypic differences between strains involving traits like gene expression, drug resistance, transmissibility, and immune response, despite this limited variation (Chiner-Oms *et al*., 2022).

In some cases, the mutations driving phenotypic differences have been identified—for example, nonsynonymous variants in genes, such as *rpoB*, *katG*, or *gyrA*, cause drug-resistant phenotypes (Chiner-Oms *et al*., 2022). Furthermore, single mutations in regulatory elements can induce alterations to downstream gene expression, which can foster differential virulence characteristics (Chiner-Oms *et al*., 2022). Finally, specific gene mutations may affect transmission, host tropism within the complex, and the host immune response (Chiner-Oms *et al*., 2022). However, many of the genomic determinants of these phenotypes remain elusive, despite robust evidence that they are driven by genetic differences between strains (Chiner-Oms *et al*., 2022; Colangeli *et al.*, 2018).

Several types of evolutionary forces play crucial roles in the fixation of mutations in bacterial populations. Research studies have provided evidence for the ongoing positive selection of specific genes and regions (Pepperell *et al*., 2013), while other studies have reported ongoing purifying selection of specific genomic regions, especially in epitopes and essential genes (Chiner-Oms *et al*., 2022). Additionally, there exists some evidence that genetic drift may have significant functional and evolutionary consequences (Hershberg *et al*., 2008).

Detecting selection in MTBC at the genome-wide level remains a challenging task due to limited genetic diversity. The significant accumulation of nonsynonymous substitutions has been used to characterize patterns of mutation accumulation in large categories of genes (Chiner-Oms *et al*., 2022), however, with limited number of strains. Of note, the number of MTBC sequences has undergone a recent and rapid expansion, with studies involving hundreds to thousands of strains. The large number of available sequences has allowed, for example, the estimation of the ratio of nonsynonymous to synonymous substitutions (dN/dS) signatures in more than 10,000 strains (Wilson *et al*., 2020), thereby allowing the identification of targets of selection with some probably related to host–pathogen interactions.

Host–pathogen interaction signals are specially challenging as they are likely obscured by the force exerted by antimicrobial therapies. Weaker signals are also expected in genes related to second-line drugs related to the relative underuse of

related treatments and the low abundance of associated resistant strains in genome databases (WHO, 2021).

2.8. The basis for Alternative Genetic Targets for Diagnosis of TB

The ITS (intergenic spacer) from the 16S or 23S rRNA genes, MPB64, and insertion sequences 986 or 6110 (IS986 or IS6110) are among the most frequently chosen DNA regions that are NAAT PCR targets (Siguier *et al.,* 2006). The smallest and most common independently transposable mobile genetic elements that are extensively involved in modifying their host genomes, such as the bacterial and eukaryotic genomes, are called insertion sequences, or ISs (Siguier *et al.,* 2006; Varani *et al.,* 2011; Filee *et al.,* 2007). As a result, the transposase genes that the IS elements carry in their host sequence enhance the host's capacity to endure a range of environmental circumstances (Siguier *et al.,* 2006).

A number of target DNA sequences specific to MTB have proven helpful as a possible substitute for traditional methods in the diagnosis of tuberculosis (Garcia-Quintanilla *et al.,* 2008). According to Garcia-Quintanilla *et al.,* 2008, the targets include the gene encoding the 65 kDa Heat Shock Protein (HSP), IS6110, the gene encoding the 38 kDa only 85B antigen, and 16S rRNA. However, the most frequently used target region, IS6110, has been linked to having very few or sometimes no copies (Garcia-Quintanilla *et al.,* 2008). Certain strains of MTB have been found to have either no copy numbers of the IS6110 element at all or very low copy numbers—less than five—of the element (Huyen *et al.,* 2013). Because of this feature, the IS6110 is the most frequently used method for diagnosing tuberculosis in clinical specimens that has a valid detection rate (Lange and Mori *et al.,* 2010).

2.9. Targeting the Protein Export Pathway for Diagnosis of TB

Since most bacterial virulence factors are extra-cytoplasmic proteins, protein export plays a significant role in bacterial pathogenesis (Zelazny *et al.,* 2004; Forrellad *et al.,* 2013). The general secretory (Sec) pathway in other bacteria, particularly *E. coli,* has been thoroughly studied, despite the fact that little is known about the protein export pathway in mycobacteria (Alav *et al.,* 2021). *SecA1* is the mycobacterial homolog of

the SecA protein found in *E. coli*. It is a pre-protein translocase ATPase, also known as adenosine 5'-triphosphatase that is necessary for protein export across the cytoplasmic membrane. The unusual feature of the mycobacterial Sec pathway is the presence of two SecA proteins: The fundamental housekeeping is *SecA1* (Alav *et al.,* 2021)

It has also been attempted to amplify the *SecA1* gene, which codes for the vital protein *SecA1*, which is an important part of the main pathway of protein secretion across the cytoplasmic membrane (Shin *et al.,* 2010). It has been shown that the Mycobacterium *SecA1* gene can be utilized for species-level identification or, in the case of the MTB complex, complex-level identification (Shin *et al.,* 2010). Additionally, it was discovered that the moderate degree of inter-species variation for gene sequences among mycobacteria makes the *SecA1* gene a good target for diagnostics (Nema, 2012).

2.10. Research Gaps

(i). The diagnosis of TB is hampered by lack of sputum production or poor sample quality and therefore use of alternative specimens is increasing in the diagnosis of this infectious disease (Song *et al.,* 2021)

(ii). Manual PCR protocols need to be optimized to avoid contamination or be automated if costs can be met (Song *et al.,* 2021)

(iii). The ideal test for TB will be a true point-of-care assay that enables accurate diagnosis of tuberculosis and detection of drug resistance within the time of a clinic consultation, and one that can be implemented at all levels of the health system for adults and children, with and without HIV (McNerney *et al.,* 2011). Future advances in molecular diagnostics should build on this success and tackle these remaining challenges (McNerney *et al.,* 2012).

(iv). Despite the close relationship between secretion and pathogenesis, the *SecA* genes of Mycobacterium species have not been studied extensively (Zelazny *et al.*, 2005)

(v). Despite recent developments in nucleic acid amplification-based diagnostics and related technological platforms, the tuberculosis diagnostic pipeline is nevertheless weak and should be strengthened (McNerney *et al.,* 2012).

CHAPTER THREE

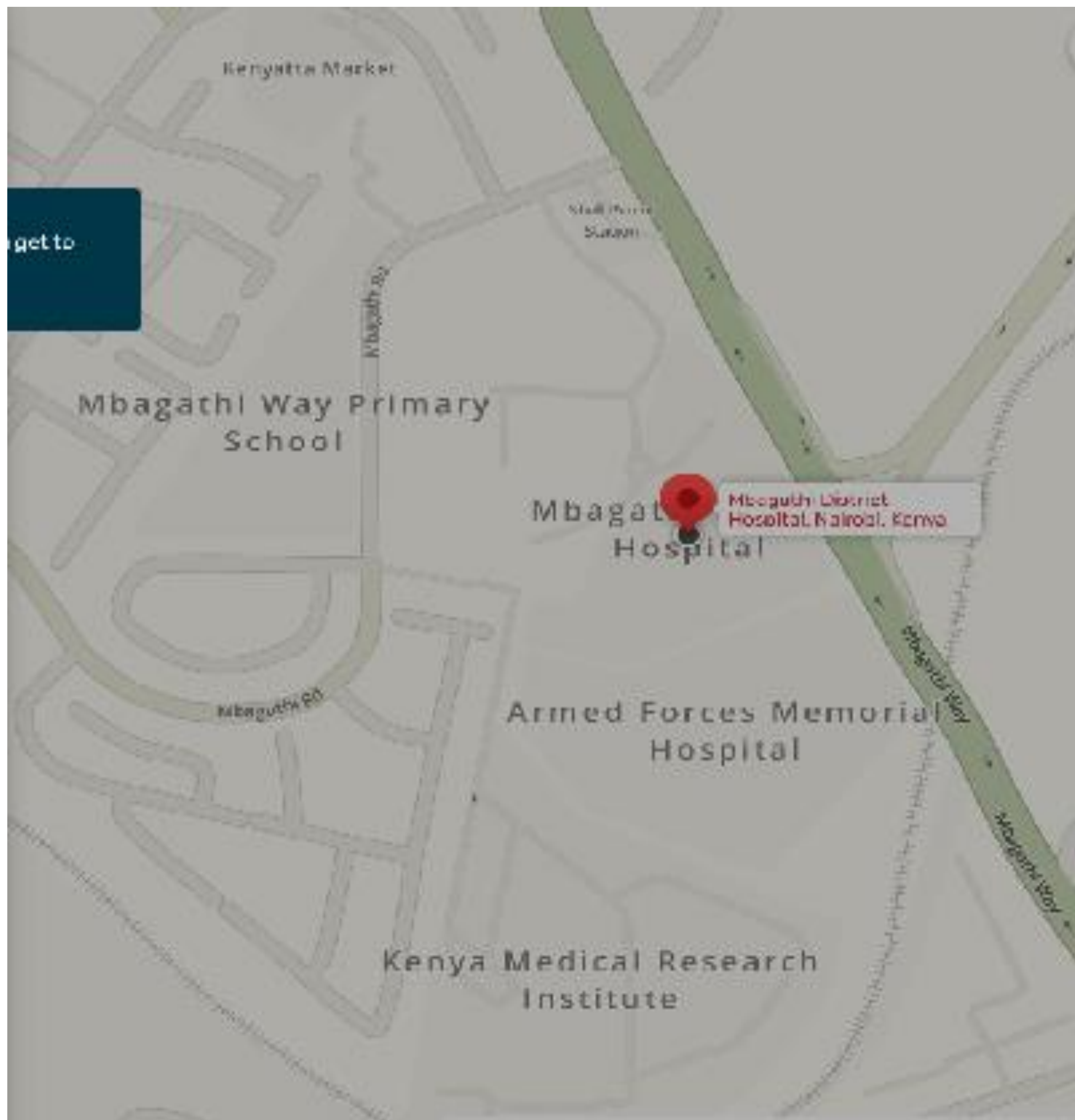
MATERIALS AND METHODS

3.1 Study Design

This was a cross - sectional study carried out on adult individuals who were seeking medical assistance at Mbagathi County Referral Hospital in Nairobi from January 2016 to December 2018, suspected to have TB. Elegible individuals supplied samples of sputum and mouthwash for tuberculosis testing. DNA was extracted from mouthwashes for MTB PCR analysis whereas sputum was used MTB culture.

3.2 Summary of the Research's Geographic Area

The study was conducted at Mbagathi County Referral Hospital in Nairobi, Kenya, located at GPS coordinates 1.3089° S, 36.8034° E. Mbagathi Hospital is conveniently located near Kibera, the biggest slum in Nairobi, as well as the largest urban slum in Africa. Kibera, located 6.6 kilometers from Nairobi's city centre in Kenya, is both a division and neighbourhood that offers a decentralized level of care, where a high volume of patients first engage with the healthcare system. The highest incidence of TB in the city is found in the slum.



https://www.waze.com/company

Figure 3.1: Mbagathi County Referral Hospital, Nairobi, Kenya

3.3. Criteria for Inclusion and Exclusion

All adult patients displaying symptoms resembling TB, such as a prolonged productive cough lasting at least 2 weeks, lack of appetite, fever, tiredness, headache, and night sweats, were included in the study after giving their consent. Our study did not include patients with other illnesses or those undergoing anti-TB therapy.

3.4 Determining the Size of the Sample

The Fisher *et al.,* 1998 equation was utilized to determine the appropriate sample size in the prevalence study; n = Z^2p (1-p) / d2

N represents the sample size,

Z is the statistic associated with the confidence level,

P is the anticipated prevalence, and d is the accuracy.

Where:

z = standard normal variable =1.96

p = prevalence proportion =0.34

d = Level of precision =0.05

$n = Z^2p\ (1\text{-}p) / d^2 = (1.96)^2\ 0.34(1\text{-}0.34) = 345$

3.5 Ethical Review

The study received ethical approval from the KEMRI - Scientific Steering Committee (Approval ref. number SSC 2792). The research followed the principles of the Helsinki declaration as all participants consented in writing. Research ethics were followed as all data were handled anonymously and kept confidential. The mycobacteriology research was conducted at the Bio-safety Level 3 (BSL-3) containment laboratory at KEMRI.

3.6 Gathering and Handling of Mouthwashes

Participants were given two 50 ml tubes with skirts, each holding 15-20 ml of sterile 0.9% NaCl. Subjects were told to vigorously swish the saline in their mouth for 20–30 seconds before spitting it back into the tube, repeating the process for each tube. In order to increase DNA output, participants were asked to do the mouthwash rinses

in the morning before brushing their teeth, eating, or drinking. In the lab, mouthwash was stored in tightly sealed containers, placed in Ziploc bags, and stored at 4 º C until ready to be transported to the KEMRI Research Laboratory. Upon arrival, mouthwash samples were cooled at 4ºC for a minimum of 40 minutes, spun at 3500 rpm for 5 minutes using a BOECO C-28 Centrifuge (Boeckel and Co, Hamburg, Germany), and the liquid above the sediment was removed. The pellet was utilized in the process of DNA extraction.

3.7 Decontamination of Sputum Samples for MTB Cultivation

In the KEMRI TB Research Lab, the sputum sample was mixed with a 4% NALC-NaOH solution in a centrifuge tube, stirred, and incubated for 15 minutes at 25°C–28°C. Afterwards, sterile PBS was added up to the 50-mL mark, and the mixture was centrifuged at 3500 r/min for 15 minutes. The top part of the combined substances was poured off gently, and the residue was mixed again with 0.3 mL of PBS. The McCartney bottles were filled with the deposit and LJ media, then marked with the patient identification number. The LJ media were incubated at a temperature of 37°C for a duration of 8 weeks and inspected on a weekly basis. The entire process took place within a biosafety cabinet class II. The LJ slopes had H37RV strain inoculated as the positive control, which is a recognized American Type Culture strain, and sterile PBS was inoculated as the negative control in random slants and incubated with every batch of tests (Rasool *et al.,* 2019). When bacterial growth were noticed colonies were exposed to ZN staining in order to detect AFB.

3.8. Staining Procedure for Bacterial Cultures with ZN

The stains were placed in consecutive sequence on a staining rack, with the smear facing upwards and then covered with filtered 0.1% Carbol - Fuchsin. The smudges were heated and left to set for 5 minutes, then washed with water and dried. They were treated with 25% Sulphuric acid for 5 minutes, washed with water, and then dried. Afterwards, they were stained again with a 0.1% solution of methylene blue for 1 minute and washed with water. The specimen was left to dry in the air and observed under a microscope using the oil immersion (100x) lens. Control slides

(both positive and negative) were used as procedural controls in every ZN staining run (Dzodanu *et al.,* 2019).

3.9. Verification of MTB through Microscopic Appearance

Serpentine cording in cultured smears may be a suitable quick way to identify MTB complex in the Mycobacteria Growth Indicator Tube (MGIT) positive cultures.

The MGIT consists of liquid broth medium that is known to yield better recovery and faster growth of mycobacteria. The MGIT contains 7.0 ml of modified Middlebrook 7H9 broth base.

In this study, slides from the findings and content of the culture containers were examined for AFB under a 100X microscope using ZN acid-fast staining. Cord morphology, categorized as cord-serpentine, cord-zoster, and cord-ladder, was utilized as the initial method of identification, distinguishing MTB clusters from other non-tuberculosis organisms. To further identify a potential mix of cultures, additional subculture in LJ or 7H11 media was utilized because the presence of both corded and non-cord morphology could suggest a combination of different cultures (e.g., MTBC and non-tuberculosis Mycobacteria (NTM) (Alva *et al.,* 2013).

3.10 DNA Extraction

Mouthwashes were processed as in section 3.6 above. The mycobacterial genomic DNA was extracted from the mouthwash pellet according to the method of (van Helden *et al.,* 2003), with few modifications using the Acetyl-N, N, N-trimethyl ammonium bromide CTAB (phenol-chloroform method). Briefly, the pellet was suspended in 500 µL of Tris-EDTA buffer, heated at 100°C for 10 minutes, and incubated with 20 µL of lysozyme (10 mg/mL) at 37°C for 2 hs. Then, 30 µL f10% SDS and 10 µL of proteinase K (20 mg/mL) were added, briefly vortexed, and incubated at 37°C for 1 hour, followed by incubation with 5M NaCl (100 µL) +10% CTAB (cetyltrimethylammonium bromide) (80 µL) at 65°C for 20 min (Chagas *et al.,* 2010).

Later, an equal volume of phenol- chloroform-isoamyl alcohol (25:24:1) mixture was added, mixed by inversion, and centrifuged at 14000× g for 10 minutes. This step was repeated twice for the removal of DNA contaminants. The supernatant was removed, followed by h addition of 3 M sodium acetate and isopropanol for DNA precipitation. The precipitate was washed with 70% ethanol and air dried. The DNA pellet was suspended in 100 µL of Tris-EDTA and stored at -20°C until further analysis (Chagas *et al.,* 2010).

3.11 Primer Details

Table 3.1: Primer Details for Amplification of Seca1 Gene

Gene	Primers (F/R)	Expected Product size	Reference
***secA*1**	Mtu.Forward1 (5-GAC AGY GAG TGG ATG GGYCGS GG CAC CG -3' and Mtu.Reverse3 (5'-ACC ACG CCC AGC TG TAG ATC TCG TGCAGC TC-3'	700 bp	Zelazny *et al.,* 2005; Davies *et al.,* 2009; Prabudiansyah *et al.,* 2015.

3.12 PCR Amplification

PCR amplifications of *SecA1* gene were performed in a Perkin-Elmer 9600 Thermocycler (Perkin Elmer, Foster City, California, USA) with a reaction mix containing 2.5 mM MgCl2, 1 pmol of forwarding primer, 1 pmol of reverse primer, (as detailed in Table 3.1), 1 µl of uracil N-glycosylase (UNG) (Roche Diagnostics),5 µl of extracted DNA, 0.125 µl (1.25 units/50 µl) of AmpliTaq Gold Polymerase (Perkin Elmer, Foster City, California, USA) and ultrapure water to a final volume of 25 µl (Zelazny *et al.,* 2005).

Amplification parameters consisted; of 30º C for 10min, 10 min at 95º C, and 49 cycles of 1 min at 95º C, 1 min at 65ºC, 1 min at 72ºC, and a final incubation step of 10 min at 72ºC (Zelazny *et al.,* 2005).

Non-template controls included ultrapure water while the H37Rv strain DNA was used as the positive control. PCR products were visualized by UV illumination of Ethidium bromidetained 2% agarose gel following electrophoresis (Lee, 2012).

The purification of the remaining PCR product was achieved with a Microcon-100 micro concentrator (Millipore, Bedford, Mass.), following the manufacturer's instructions (Zelazny *et al.,* 2005; Davies *et al.,* 2009).

3.13 Statistical Analysis

The sensitivity, specificity, positive predictive value, and negative predictive value for presence/absence of MTB morphologically among the participants' isolates were calculated according to the formula by Kabir *et al*., 2021);

Sensitivity = a/(a+c),

Specificity = d/(b+d)

PPV = a/(a+b)

NPV = d/(c+d)

Accuracy = (a+b)/(a+b+c+d)

where a = true positive, b = false positive, c = false negative and d = true negative.

CHAPTER FOUR

RESULTS

4.1 Samples

Between January 2016 and December 2018, 300 qualified participants provided sputum and mouthwashes for TB culture and DNA isolation for PCR. The two methods were compared for their reliability in TB diagnosis (Sensitivty and Specificity). All of the specimen samples were first received at the TB lab in Mbagthi, subjected to routine evaluation before dispatch to the TB Research Laboratory in KEMRI.

In October 2013, the CDC published interim practical considerations for incorporation of Xpert into diagnostic algorithms and infection control (Lippincott *et al*., 2014). They state that for active tuberculosis evaluation and all decision making, 3 sputum specimens should be collected 8–24 hours apart and tested by smear microscopy (Lippincott *et al*.,2014), a NAAT. It is in line with this requirement that sputum was sourced and apportioned at Mbagathi site for routine evaluation.

Although smear microscopy is considered redundant for all decision making, it remains necessary for NTM detection. AFB culture continues to be critical for drug susceptibility testing and public health surveillance (Lippincott *et al*., 2014).

4.2. Isolation and Morphological Identification of MTB Cultures

Culture, using both broth and solid media, remains the standard for establishing the laboratory-based diagnosis of TB (Bahar *et al*., 2019, Gill *et al*., 2022). Sputum specimens were cultured in the solid L-J and MGIT liquid media (BACTEC *Mycobacterium* Growth Indicator Tube (MGIT) 960 system) (Gill *et al*., 2022; WHO, 2022).

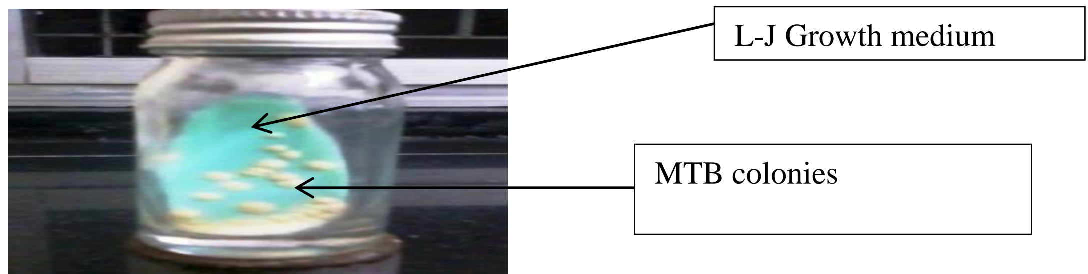


Figure 4.1 (i): Shows Visualization of Pigmented MTB Growth Colonies on L - J Medium

Figure 4.1(Ii): Shows *Mycobacterium* Colonies Under the Microscope at X40 Magnification.

The features demonstrate MTB isolate on L-J agar slants as “rough and buff,” with colonies that have a cauliflower-like appearance.

The MTB growth colonies on egg-based L-J medium are characteristic non-pigmented colonies, with a generally rough and dry appearance simulating breadcrumbs.

The description of the colonies do not sufficiently describe MTB and therefore it is important to carry out further identification using conventional biochemical reactions (Bahar *et al*., 2019).

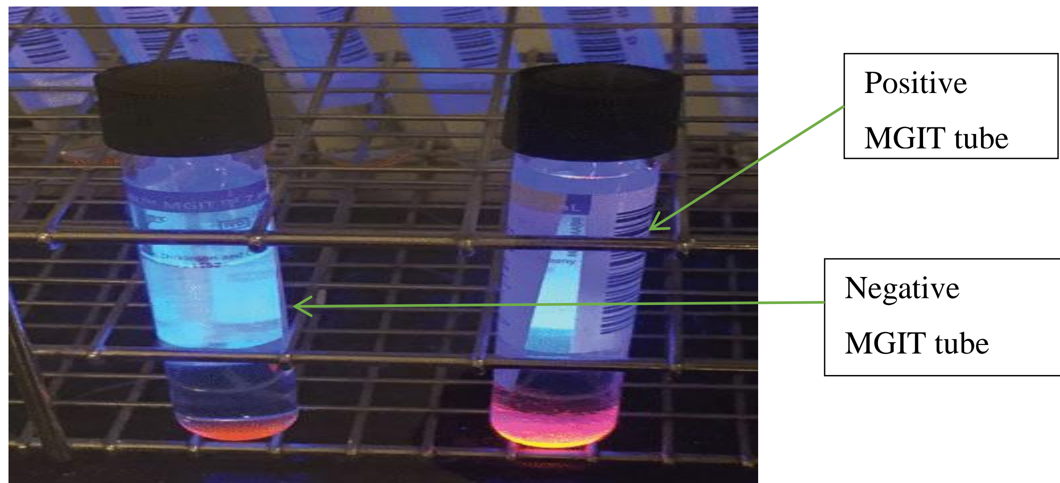


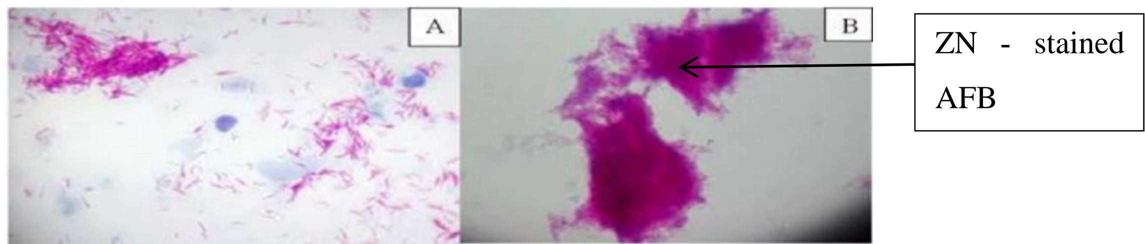
Figure 4.2: MGIT 960 Tubes.

On the right contains growing mycobacteria and is fluorescent when exposed to UV light. In contrast, the tube on the left contains no mycobacteria.

Tubes flagged positive by MGIT were evaluated for presence of serpentine cording. The cord formation was compared with isolates identified as MTBC based on p-nitrobenzoic acid (PNB) test (Singhal *et al.*, 2012). The sensitivity, specificity, contamination rates and time to detection are known to vary widely amongst both media, with WHO therefore advocating for dual use of systems where practical (WHO; 2022).

4.3. Staining of AFB using the Ziehl–Neelsen (ZN) Method

A acid-fast staining is usually performed on a positive culture to confirm the presence of acid-fast bacilli and to exclude the possibility of overgrowth of commensal microbiota.



Figures 4.3: A and B: Shows AFB Bacilli Stained with ZN as Observed Under a Microscope at a Magnification of 100 Times

Traditionally, the identification of MTB complex has relied on acid fastness, niacin production, nitrate reduction, and inactivation of catalase at 68°C.

These tests are rarely done in most laboratories, however, selected biochemical reactions may be necessary to distinguish MTB from other members of the MTB complex, namely, *Mycobacterium bovis*.

Generally, the recovery time for MTB complex varies; on solid media, colonies can be observed in as short a time as 12 days or as long as 4 to 6 weeks, with an average of about 3 to 4 weeks. The colonies are rough and colorless. Unlike most mycobacterial species, MTB lacks the enzyme to convert free niacin to niacin ribonucleotide, so niacin accumulates in the medium. This is the basis for a positive niacin test (Myneedu *et al.,* (2012). MTB possesses nitroreductase and yields a positive result using nitrate reduction testing. Among mycobacteria, the quantity of catalase produced and the stability of this enzyme at 68°C are species dependent (Myneedu *et al.* (2012). MTB complex produces a column measuring less than 50 mm in the quantitative catalase production test performed with L-J medium. This organism also produces a heat-labile catalase that is inactivated after 20 min of exposure to 68°C (Gill *et al*., 20240). Cord formation in acid - alcohol resistant bacilli.

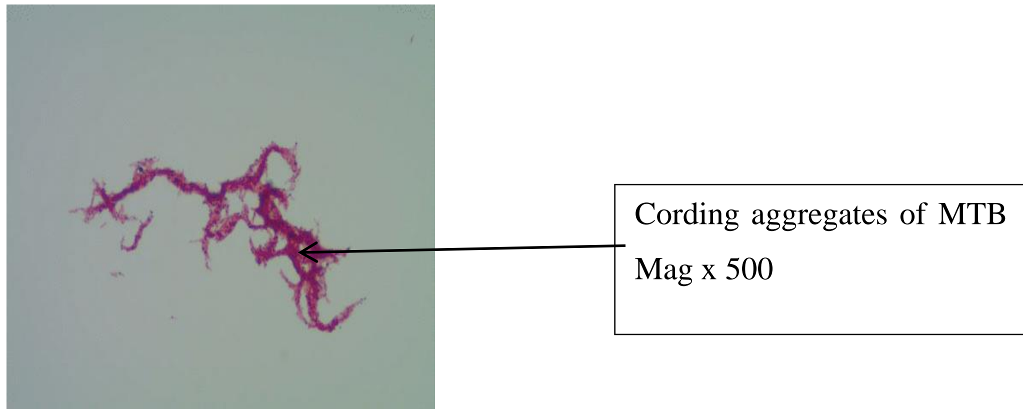
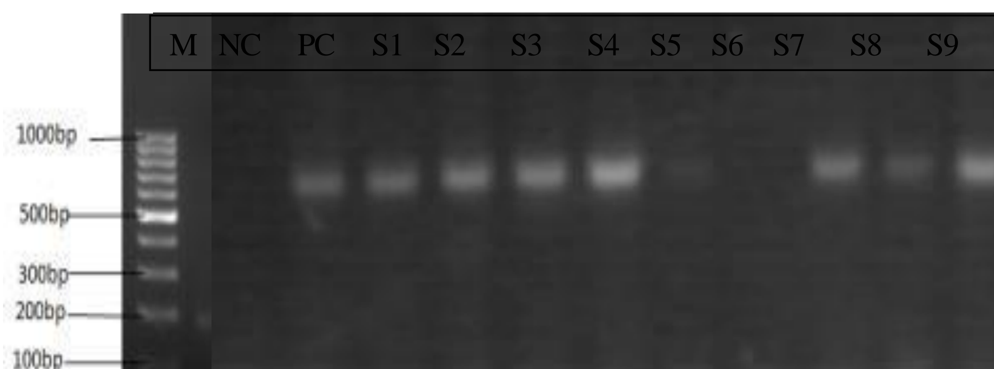


Figure 4.4: The Aggregation of Acid-Fast Bacilli into the Supra-Structure

The AFB features in Figure 4.4 are termed as cording. The culture from which this were derived contained MTB complex. The image is of a ZN stain at a magnification of ×500. Cords create compact, rope-shaped clusters where the bacterium's long axis aligns with the cord's long axis. The cords formed by the bacterial isolates in Figure 4.4 confirm the presence of MTB. Cording of AFB is highly predictive of MTB (Mishra *et al.*, 2023).

Culture is the reference standard in the TB diagnostic algorithm for evaluating patients with negative smears, as it increases sensitivity and enables earlier case detection (WHO, 2010; WHO, 2020). Culture also provides material for the identification of mycobacterial species and their drug susceptibility profiles, which is important in patients with suspected drug-resistant TB (WHO, 2010).

4.5. Detection of Amplified MTB S*eca1* Gene Products from Mouthwashes.



Key: M: DNA marker, NC -negative control, PC - positive control, S1 - S9 sample numbers

Figure 4.5: Agarose Electrophoresis of PCR Products

Detection of a 700bp DNA target sequence of *secA1* gene in clinical samples showed presence of MTB strain (Figure 4.5) by resolving in 2% agarose gel like the positive control and DNA ladder. The *secA1* gene was amplified in 102 out of 165 samples tested, representing 61.8% (Table 4.1).

Table 4.1: Showing Incidences of PCR Test and Culture Results

Patient no.	PCR test	Culture
272	P	p
273	P	p
192	P	p
268	P	p
271	P	p
269	P	p
283	P	p
245	P	p
244	P	p
238	N	N
270	N	N
236	N	N
291	P	N
216	P	N
253	N	N
258	N	N
218	P	N
250	P	N
262	P	N
252	P	N
260	P	N
215	P	N
246	N	N
258	P	N
264	P	N
257	P	P
242	P	P
284	P	P
282	P	P
240	P	P
241	P	P
213	P	P
221	P	P
256	N	N
261	N	N
248	N	N
257	N	N
223	P	P
166	P	P
165	P	P
164	P	p
163	P	N
161	P	p
162	P	N
285	p	P
184	P	N
183	P	N
182	P	N
181	P	p
180	P	p
178	P	p
177	P	N
176	P	p
168	N	N

Patient no.	PCR test	Culture
169	P	N
203	N	N
204	P	p
205	P	p
206	P	p
171	P	N
172	N	N
173	N	N
174	N	N
190	N	N
194	P	p
195	N	N
196	N	N
197	N	N
199	N	N
200	N	N
201	N	N
202	N	N
167	N	N
149	N	N
150	N	N
157	N	N
152	N	N
154	N	N
155	N	N
157	N	N
158	N	N
159	N	N
142	P	P
145	P	P
146	P	P
153	P	P
156	P	p
140	P	P
160	P	P
143	N	N
141	P	p
144	P	N
147	P	N
148	P	N
19	P	N
20	P	N
21	P	N
22	P	N
23	P	N
24	P	N
25	P	P
26	P	N
27	N	N
28	N	N
32	N	N
33	p	P
34	p	P
37	p	P
38	p	P
39	p	P
41	N	N

Patient no.	PCR test		Culture	
43	N		N	
44	N			P
51	N			P
53	N		N	
54	N			P
55	N		N	
56		P	N	
57		P	N	
58		P	N	
59		P	N	
98		P		P
97		P		P
62		P		P
69		P		P
66		P		P
68		P		P
122		P		P
134		P		P
128		P		P
109		P		P
95		P		P
16		P	N	
51	N		N	
60	N		N	
64	N		N	
63		P		P
61		P		P
65		P		P
113		P	N	
137		P		P
108		P	N	
105		P		P
67		P		P
114		P		P
138		P		P
133		P		P
124	N		N	
131	N		N	
118		P		P
120	N		N	
112	N		N	
130	N		N	
132	N		N	
106	N		N	
135	N		N	
116	N		N	
69	N		N	
2	N		N	
3	N		N	
15	N		N	
16		P	N	
17	N		N	
KJN	N		N	
KATUI	N		N	
Totals	63	102	96	69

Key: P - positive result and N - negative result

4.6. Comparison of Culture and PCR for Detection of MTB

Table 4.2: Shows MTB PCR versus Culture Results.

Result	Sputum Culture	ZN staining	MW PCR of *sec A1* gene	Morphology (cording)
Positive	69	69	102	69
Negative	96	96	63	96
Total	165	165	165	165

Culture positive results are confirmed by the characteristic morphology such as cord formation seen on AFB staining whereas PCR is shown by confirmation of *secA1* gene amplification.

PCR was rum directly from mouthwashes as a clinical sample. Cultured isolates are identified far less commonly by traditional biochemical profiling and more commonly by molecular methods such as PCR with as well as broad-range mycobacterial amplification assays are followed by some type of post-amplification analysis, such as melting-curve analysis, DNA sequencing, or microarray hybridization (Procop, 2016).

PCR is a useful method for identifying the origins of because it can identify genetic material from various MTB strains, is less impacted by prior antibiotic treatments, and produces results fast (Singh *et al*., 2012).

4.7. Calculation of Assay Parameters

Non - tallying results specimens were removed, valid results entered into excel spreadsheet, excluding those that were contaminated. Out of a grand total of 300 patients who were suspected of having TB and were registered, 165 samples were tested as 45 sputum cultures were contaminated and several samples were rejected

for various reasons according to the TB laboratory rejection criterion. Sample numbers without counterpart and those that were contaminated were also removed.

Using sputum culture as the benchmark, both clinical parameters (PPV and NPV) and assay performance parameters (accuracy, sensitivity, and specificity) were calculated based on the formula by Kabir *et al*., 2021.

Table 4.3: Assay Parameters Showing the Number of True and False Results of PCR against Culture

		TB Culture		**Total**
		Positive	**Negative**	
MW PCR	Positive	69 True Positive	33 false Positive	102
	Negative	0 False Negative	63 True Negative	63
Total		69	96	

MTB DNA fragments were compared with corresponding MTB isolates. Out of the 165 samples, culture testing showed that 69 samples (41.8%) were positive for MTB, while 96 samples (58.5%) were negative. Thus, among the 165 samples tested, 102 were found to be positive through Mouthwash PCR, with 69 of those also testing positive in culture. In the 33 remaining samples, PCR results were positive, but cultures came back negative. All 63 PCR samples were negative in both PCR and culture testing.

Mouthwash sensitivity was determined as the percentage of true positives that tested positive (n-69, Table 4.2). The true positives were samples that tested positive in both PCR and culture. The specificity refers to the percentage of negative tests that correctly identify as negative. The samples identified as true negatives (n=63) were PCR negative and showed no growth in culture. False positives (FP) (n=33) refer to samples that tested positive with PCR but negative with culture. Samples identified

as false negatives (n= 0) tested negative by PCR but tested positive in culture. The sensitivity and sensitivity calculated as sensitivity (Tp/[Tp + Fn]) ×100; specificity (Tn/[Tn + Fp]) ×100; Tp = total number of positives; Tn = total number of negatives; Fp = total number of false positive, Fn = total number of false negative; respectively.

CHAPTER FIVE

DISCUSSIONS AND SUMMARY

5.1 Discussions

A number of patients, children, the aged, and immuno-compromised may not expectorate quality sputum for testing PTB due to underlying medical conditions (Abi *et al*., 2023; Fauci *et al*., 2019; Lubeya *et al*., 2022). Oral samples provide several potential diagnostic benefits for most community settings where sputum collection for TB is unfeasible. This study aimed to identify the diagnostic accuracy of MTB in mouthwashes. Our data showed that adult participants' mouthwash yield of MTB DNA had a higher positivity rate than sputum culture. This finding is in tandem with previous reports (Abi *et al.*, 2023).

This was a cross-sectional laboratory-based whereby mouthwashes PCR outperformed sputum culture in terms of MTB confirmation. *Davis et al., 2009,* had earlier demonstrated sensitivity of 99%, 95 % CI (93 to 100%) and specific 88%, 95 % CI 77% - 965%) for PTB whereby oral-wash PCR had sensitivity (73%, 95% CI 62% - 83%) and specificity (88%, 95% CI 76% - 96%) for PTB, which was however less sensitive than sputum PCR (difference 25%, 955 CI 13% - 37%, $P < 0.001$), while utilizing a similar gene target; *that SecA1.*

In Uganda, high sensitivity and specificity of oral swabs using Xpert MTB/RIF Ultra were demonstrated, achieving a sensitivity of 72% and a specificity of 100% using a microbiological reference standard (Church *et al*., 2024). Two oral swabs per patient exhibited a combined sensitivity of 92.8% relative to sputum GeneXpert MTB/RIF assay by using a manual quantitative PCR targeting IS6110 in South Africa (Luabeya *et al.,* 2019). The GeneXpert MTB/RIF amplifies the MTB *rpoB* gene, however, some strains are missed due to the absence of this gene (Luabeya *et al.,* 2019).

A systematic review had 23 articles demonstrating pooled sensitivity, specificity, and area under the curve of NAATs in oral samples of 50% (95% CI, 37%–63%), 97% (95% CI, 93%–99%), and 0.89 (95% CI, 86%–92%; $I2$ = 99%; chi-square, 169.61; *P*

< .001), respectively for PTB diagnosis. The data demonstrated that NAATs using oral samples have less satisfactory sensitivity and high specificity for PTB diagnosis (Wang *et al*., 2023).

In another review, the sensitivity of oral swabs ranged from 36% to 91% for adults and between 5% and 42% for children with sensitivity-reducing in children that were more likely to have paucibacillary TB disease when compared to the reference standard (Church *et al*., 2024). Specificity was less variable than sensitivity, with most studies reporting specificity greater than 90% (Church *et al*., 2024).

Molecular methods that include MTBC-specific PCR assays and broad-range mycobacterial PCR assays are used to identify Mycobacterium species, assess resistance, and type organisms for epidemiologic purposes. Commercially available tests, such as rapid-cycle PCR systems, have shown good performance and are automated. The FDA-approved Xpert MTB/RIF detects MTBC and rifampin resistance, crucial for MDR-TB detection (Procop, 2016). Current evidence indicates that the impact of GeneXpert for diagnosing TB in low- and middle-income countries (LMICs) has not demonstrated equivalent outcomes when compared to Xpert MTB/RIF evaluations in upper-middle-income countries (Brown *et al*., 2021). Challenges associated with implementation are possible contributing factors preventing this innovative diagnostic technology from achieving more significant public health outcomes (Brown *et al*., 2021).

A study from a similar country had low sensitivity (39%) compared to sputum for MTB TB disease using GeneXpert MTB/RIF in saliva from adults, with sensitivity in children likely to be lower due to the paucibacillary nature of pediatric TB disease (Khambati *et al*., 2021). The fact that TB disease in young children is often more severe and more frequently disseminated than in adults, conceivably makes children more suited to host-based diagnostics (Khambati *et al*., 2021)

Clinical trials of TB patient identified 92.2% positive patients with a single Xpert MTB/RIF test and culture-proven TB (WHO; 2014). Although the sensitivity of the initial Xpert MTB/RIF was only 72.5% for smear-negative patients, this increased to just above 90% when three specimens were tested (WHO, 2014). Another powerful

proposal for the Xpert MTB/RIF system, which is reflected in the revised labeling of this product, is for the replacement of acid-fast smears (Sauzullo *et al*., 2016).

The slow growth, severity of disease, and communicability of MTB make this organism an ideal candidate for molecular detection and characterization (Alsayed *et al*., 2023). Molecular methods may be used directly on clinical specimens as in this study, or on cultured isolates to identify the *Mycobacterium* species present, to assess for genetic determinants of resistance, and/or to type to the organism for epidemiological purposes.

Culture sensitivity affects PCR's effectiveness, and sputum smear-negative samples can harbor further transmission (Courcoul *et al*., 2014). Combining PCR with other techniques is necessary to identify MTB-positive samples. The combination of culture and *secA1* PCR in mouthwashes was validated through MTB-positive detection.

Microbiological cultures are successful due to factors like decontamination and granuloma encapsulation (Gil *et al*., 2010). DNA amplification of MTBC can be faster with AFB, but microbiological culture remains the gold standard for MTB isolation and molecular epidemiology studies (Moyen *et al*., 2014). Impaired PCR sensitivity is caused by inhibitors of enzymatic amplification, carryover of target DNA from heavy MTB loads, and anti-tuberculous drug presence in treated patient samples (Greco *et al*., 2009).

In a program review, in 2011, up to 13– 18% of cultures were contaminated and attempts to culture MTB from 12% of smear-positive specimens also failed (Reddy *et al.,* 2014). PCR-based assays tend to be more sensitive for the detection of MTB through its DNA in TB-positive smears of respiratory samples (Gholoobi *et al.*, 2014; Ndugga *et al.,* 2004). Either, a 'clinical suspicion," demonstrated low positive predictive value, as well (Greco *et al.,* 2009). Both tests are therefore prone to overestimate the probability of pulmonary TB, leading to erroneous initiation of therapy, isolation, and contact investigation (Greco *et al.,* 2009).

Mouthwashes may have less bacilli than sputum, but sensitivity estimates are lower in paucibacillary forms. In-house PCR tests may have limited clinical applicability (Nahid *et al.*, 2006). The comparison of test results against culture allows the assessment and validation of new techniques (Barandiaran *et al.,* 2019). Comparison of mouthwash PCR with microbiological culture demonstrated a sensitivity of 100%; specificity of 65%; accuracy of 129% PPV of 67.6 % and NPV of 100%. Previously, PCR was useful in confirming TB, displaying sensitivity and specificity of 77.9 and 100%, respectively, for MTBC detection, with a much lower sensitivity but greater specificity, presumably owing to the sample type used (Sánchez-Carvajal *et al.,* 2021). Findings in this study support mouthwash PCR as a reliable tool for detection for rapid detection of MTB.

Recent reviews of TB diagnosis in Fiji identified issues affecting culture-positive rates, including culture degradation and contamination, which affects the accuracy of TB diagnosis and effective TB control (Reddy *et al.,* 2014).

The above issues have also been identified in other countries (Swai *et al.,* 2011). However, although microbiological culture is considered the gold standard for TB confirmation, this technique is time-consuming and imperfect, inducing false-negative results, and sensitivity and specificity will always be biased (Dunn *et al.*, 2016). However, contamination of TB culture samples is a common encounter in several studies as witnessed in the current scenario (Adam *et al.,* 2022).

In a program review, in 2011, up to 13– 18% of cultures were contaminated and attempts to culture MTB from 12% of smear-positive specimens also failed (Reddy *et al.,* 2014). PCR-based assays are more sensitive for MTB in TB-positive smears of respiratory samples (Gholoobi *et al.*, 2014; Ndugga *et al.,* 2004). Either, a 'clinical suspicion," demonstrated low positive predictive value, as well (Greco *et al.,* 2009). Both tests; culture and PCR are prone to overestimate the probability of PTB, leading to erroneous initiation of therapy, isolation, and contact investigation (Greco *et al.,* 2009).

Confirmation of TB in this study is in agreement with previous species-level identification or complex-level for the MTB complex, with a moderate degree of inter-species variation (Zelazny *et al.,* 2015). Whereas high sensitivity of PCR can prevent delayed or missed diagnoses, reducing the risk of progressive disease, low specificity may lead to unnecessary therapy, exposure, and transmission. PCR's affordability could improve TB management in low-resource settings (Zelazny *et al.,* 2015).

Among the limitations in this study was the detection sensitivity exceeding clinical significance and culture contamination, as well as individuals unable to provide a sputum sample or invalid sample might have been underrepresented in this study including the vast number of samples that got contaminated, amongst other rejection criteria.

5.2 Summary

The Mouthwash - PCR portrays the potential of PCR in rapid and accurate detection of TB in paucibacillary samples. It significantly impacts clinical management and control of TB. The highest sensitivities in this review approach the acceptable sensitivity range (as defined by WHO consolidated guidelines on tuberculosis) for an initial diagnostic test relative to performance of sputum-based diagnostics, suggesting that with continued optimization, mouthwashes could be a good sample type for tuberculosis detection.

Mouthwashes offer benefits like self-collection and suitability for populations struggling to produce sputum.

A high number of false-positive results was noted, thus affecting the generalization of the study. A high level of contamination was similarly noted in this study. The risk of false-positive results is a major problem facing all PCR assays. This is largely a consequence of the extreme sensitivity of PCR and may result from contamination by exogenous material or from detection of low levels of colonizing organisms in the sample. False-positive results may also be the result of amplification of microorganisms that have similar genome sequences to the target organism. False-

positive findings may be avoided by the use of appropriate controls, good laboratory practice to prevent contamination, and confirmation of all positive results by an independent method.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

1. Mouthwash PCR can become an alternative tool for the population with difficulties of obtaining sputum

2. Mouthwash PCR shows potential of a more easily accessible, safer and quicker screening tool for tuberculosis patients

3. Public health organizations can use mouthwash PCR as a point-of-care test to expedite diagnosis and lower MTB exposure, facilitating prompt decision-making,

6.2 Recommendations

1. More attention should be given to sample collection and handling to improve disease laboratory diagnostic accuracy using mouthwashes.

2. There is need to consider adopting mouthwashes as an alternative for rapid screening and contact screening of TB patients.

3. With further evaluation and optimization mouthwashes should be considered an option for early TB testing in congested facilities. Research going forward should compare specific aspects of mouthwashes, either through prospective cohort or clinical trials, so that optimal methods can be determined, such as direct comparisons of quantity or of time of collection; before or after sputum collection and processing methods.

4. Future studies should use a standardized protocol for comparing different aspects of mouthwash collection and processing, keeping all aspects of the protocol the same except one. To advance the potential of mouthwashes in a point-of-care diagnostic test, further study using existing point-of-care molecular WHO-recommended rapid diagnostics should be considered, as well as development of novel, low-complexity point-of-care platforms for use with mouthwashes.

5. Mouthwashes with molecular testing can provide accurate results for the diagnosis of pulmonary tuberculosis. The highest sensitivities in this review approach the acceptable sensitivity range (as defined by WHO consolidated guidelines on tuberculosis) for an initial diagnostic test relative to performance of sputum-based diagnostics, suggesting that with continued optimization, mouthwashes could be a good sample type for tuberculosis detection.

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APPENDICES

Appendix I: Informed Consent

Participating in the research protocol ' detection of pulmonary tuberculosis in oral

Washes using real-time PCR

Madam, Mister,

You are invited to take part in a study within the framework of new molecular diagnostics in TB. It is your right to be informed of any risks and benefits of this project so that you can make a well thought decision. This preliminary and common information procedure is what is referred to as a ‘consent form’.

You will be required to read the contained information carefully and if necessary, discuss any issues with the medical officer in charge of the project. If, after reading this information sheet and having had the opportunity to discuss the particular issues you decide to take part in the study, you will be asked to sign the ‘informed consent’ sheet jointly with the Medical Officer in charge of the study. There will be no interruption in your normal TB screening. You will receive one copy and we will keep one copy of the present document. You may wish not to participate in the study. If you are willing to take part in the study, you are free to interrupt your participation at any time without any effect on your treatment. Appending your signature does not mean a definite commitment from you.

Purpose

To evaluate the role of using real – time PCR on oral washes for detection of pulmonary tuberculosis

The normal initial diagnosis of mycobacterium disease is often based on clinical data; definitive diagnosis usually involves the isolation and identification of the infecting organism in the laboratory. The usual laboratory procedure for clinical specimens involves microscopic examination for the presence of acid-fast bacilli

(AFB), isolation of the organism by culture, and identification and drug susceptibility testing of the recovered organism. Because of the slow growth rate of mycobacterium, isolation, identification, and drug susceptibility testing can take several weeks or longer.

During the past several years, many molecular methods have been developed for direct detection, species identification, and drug susceptibility testing of mycobacterium. These methods can potentially reduce the diagnostic time from weeks to days. Oral wash is a much easier specimen to obtain than sputum and there is potential for this becoming useful for detection of TB by targeting a novel target in them.

Patient Procedures

All patients enrolled in the study will have their oral wash procedure performed within the first week of diagnosis and treatment initiation of TB. The participants will be fasting since midnight and asked to postpone brushing their teeth until after the oral wash, which include rinsing/gargling the mouth with 10 ml of sterile saline for 1min. The procedure will be monitored by a staff member. The samples will be collected in sterile tubes and centrifuged at 3000 × g for 30 min.

Subjects shall be instructed to cough vigorously 5 times, and then gargle 10 mL of sterile saline for 60 s. Samples will be refrigerated at 4˚C till processing. Upon transportation to the laboratory, acid-fast smear and mycobacterium culture will be performed on sputum samples, and PCR will be performed on sputum and oral wash specimens.

Confidentiality

All personal and medical information collected for this study will remain confidential and anonymous. If you consent to take part in this study the data recorded will be computerized.

The information is only meant for the project and will not be used by any other person except by those working with the study.

Certificate of Consent 1.

I, the undersigned, (Surname)………………………, (First name) ………………………, declare that I have been invited to take part in the project entitled: 'Detection of Pulmonary tuberculosis in Oral Washes using real – time Polymerase Chain Reaction(PCR)'.

I declare that I have been fully informed by

Prof/Dr/Mr./Mrs./Ms……………………. of the nature and process of the above mentioned study and that I have been given the possibility to address any query relating to this after enough reflection time.

I understand that I am absolutely free to interrupt my participation in this study without having to justify my decision, and my decision will have no consequence on the quality of treatment that I deserve. My consent does not spare the organizers of the present study of their responsibilities. I keep all my rights that are protected by law.

I understand that data concerning my person will be fully confidential. I do not authorize their exploitation to any other person except for purposes of this study. I allow data from this study to be computerized.

I accept to take part in this study according to the above written conditions. I have read and received a copy of the present document.

Date:/……. /………

Name and signature of the patient Name and signature of the

Medical Officer ………………………………… …………………….

Name and signature of witness (if patient illiterate)

……………………………………

Note. Original copy of this is to be filed whereas the patient carries one copy.

Certificate of Consent (2)

I, the undersigned, (Surname)………………………, (First name) ………………………, declare that I have been invited to take part in the project entitled: 'Detection of Pulmonary tuberculosis in Oral Washes using real – time Polymerase Chain Reaction(PCR)'.

I have been asked to be tested for HIV infection. I have understood that the result of the test will be kept fully confidential and anonymous.

I accept to be tested (tick one): Yes……...

No………

I want to know the result of the test: Yes………

No………

Date: ……/………/……………….

Name and signature of the patient Name and signature of the

Medical Officer

Time……………………………

Time………………………………………

Name and signature of witness (if patient illiterate) ……………………………………………………..

Time and Date…………………………………….

Kushiriki katika Itifaki ya utafiti: ' kugundua ya mapafu kifua kikuu katika mdomo Awaosha kutumia muda halisi PCR

Madam , Mheshimiwa

Mimi ni kufanya kazi kwa .... (Jina la taasisi yako) ....

Wewe ni umealikwa kuchukua sehemu katika utafiti ndani ya mfumo wa uchunguzi mpya Masi katika TB. Ni haki yako kuwa na taarifa ya hatari yoyote na manufaa ya mradi huu ili uweze kufanya uamuzi vizuri bila mawazo. Hii awali na kawaida habari utaratibu ni nini inajulikana kama ' ridhaa fomu .

Utahitajika kusoma habari zilizomo makini na kama ni lazima, kujadiliana masuala mbalimbali na afisa wa matibabu katika malipo ya mradi huo. Kama , baada ya kusoma karatasi hii na kuwa na fursa ya kujadili masuala fulani wewe unaamua kuchukua sehemu katika utafiti, utaulizwa kutia saini karatasi pamoja na Mganga Mkuu wa katika malipo ya utafiti. Hakutakuwa na usumbufu katika uchunguzi yako ya kawaida TB. Utapokea nakala moja na sisi kuendelea nakala moja ya hati hii ya sasa . Unaweza kuamua kutoshiriki katika utafiti . Kama wewe ni tayari kuchukua sehemu katika utafiti, unaweza kupinga ushiriki wako wakati wowote bila ya athari yoyote juu ya matibabu.Kutia sahihi yako haina maana ahadi uhakika kutoka kwako.

Kusudi

Kutathmini nafasi ya kutumia halisi - wakati PCR juu ya washes mdomo kwa ajili ya kugundua kifua kikuu cha mapafu

Kawaida uchunguzi wa awali ya ugonjwa wa kifua kikuu mara nyingi ulingana na data kliniki; utambuzi yakinifu unahusu kutengwa na utambulisho wa viumbe kuwaambukiza katika maabara. Kawaida maabara utaratibu kwa ajili ya vielelezo kliniki inahusisha uchunguzi microscopic kwa uwepo wa asidi -haraka bacilli (AFB) , kutengwa ya viumbe na utamaduni , na kitambulisho na madawa ya kulevya kuhisi upimaji wa viumbe zinalipwa. Sababu ya kiwango cha ukuaji wa polepole wa viini vya kifua kikuu, kutengwa, kitambulisho, na madawa ya kulevya kuhisi kupima inaweza kuchukua wiki kadhaa au zaidi.

Katika kipindi cha miaka kadhaa, njia nyingi Masi kuwa maendeleo kwa ajili ya kuchunguza moja kwa moja, aina ya utambulisho, na madawa ya kulevya kuhisi upimaji wa viini vya kifua kikuu. Mbinu hizi ziko na uwezekano wa kupunguza muda uchunguzi kutoka wiki kwa siku . Mdomo safisha ni kielelezo rahisi kupata kuliko kikihozi na kuna uwezekano kwa hili kuwa muhimu kwa ajili ya kugundua TB kwa kulenga lengo riwaya katika yao.

Taratibu

Wagonjwa wote waliojiunga na utafiti watakuwa na osha yao ya mdomo utaratibu kazi ndani ya wiki ya kwanza ya utambuzi na matibabu ya uanzishwaji wa TB. Washiriki watakuwa wanafunga tangu usiku wa manane na kuulizwa kuahirisha kusukua meno yao mpaka baada ya mdomo safisha, ambayo ni pamoja na kusafisha kinywa na 10 ml ya kuzaa chumvi kwa 1min . Utaratibu utakuwa unafuatiliwa na wafanyakazi . Sampuli kukusanywa katika zilizopo kuzaa na kuzungushwa kwa 3000 × g kwa dakika 30.

Masomo atakuwa maelekezo ya kikohozi nguvu mara 5, na kisha safisha na 10 mililita ya kuzaa chumvi kwa 60 s . Sampuli itakuwa jokofu saa 4 ° C mpaka usindikaji. Juu ya usafiri wa maabara, asidi- haraka kupaka na utamaduni wa viini vya kifua kikuu itakuwa kazi kwenye sampuli makohozi, na PCR itakuwa kazi juu ya kikohozi na mdomo safisha sampuli.

Siri

Taarifa za kibinafsi na matibabu zilizokusanywa kwa ajili ya utafiti huu kubaki siri na majina. Kama wewe kukubaliana na kuchukua sehemu katika utafiti huu data kumbukumbu itakuwa kompyuta.

Habari ni maana tu kwa ajili ya mradi na si kutumika na mtu mwingine yeyote ila kwa wale wanaofanya kazi na utafiti.

Hati ya Ridhaa 1.

I, aliyetia, (jina) .... (jina la kwanza) .... , kutangaza kwamba mimi wamealikwa kushiriki katika mradi haki: ' kugundua ya mapafu kifua kikuu katika mdomo Awaosha kutumia halisi - wakati Polimeresi Reaction (PCR) . Mimi kutangaza kwamba mimi wamekuwa dully taarifa na Prof / Dk / Mr . / Bi. /

Bi .... ya asili na mchakato wa utafiti zilizotajwa hapo juu na kwamba mimi wamepewa uwezekano wa kushughulikia swala lolote linalohusiana na hii baada ya kutosha kutafakari wakati .

Mimi kuelewa kwamba mimi niko huru kabisa kwa kupinga ushiriki wangu katika utafiti huu bila ya kuwa na kuhalalisha uamuzi wangu, na uamuzi wangu hawana matokeo juu ya ubora wa matibabu ambayo mimi stahili. Idhini yangu haina vipuri waandaaji wa utafiti wa sasa wa majukumu yao. Mimi kuweka haki yangu yote ni kulindwa na sheria.

Naelewa kwamba data kuhusu mtu wangu itakuwa kikamilifu siri. Mimi si kuidhinisha matumizi yake kwa mtu mwingine yeyote ila kwa madhumuni ya utafiti huu . Mimi kuruhusu data kutoka utafiti huu kuwa kompyuta.

Mimi kukubali kuchukua sehemu katika utafiti huu kwa mujibu wa hali ya juu kwa maandishi. Nimesoma na kupokea nakala ya waraka huu .

Tarehe: ...... / .... / ....

Jina na saini ya Jina mgonjwa na sahihi ya Mganga Mkuu wa

....

Jina na saini ya ushahidi (kama mgonjwa hawajui kusoma na kuandika)

....

Kumbuka. Original nakala ya hii ni kuwa filed ambapo mgonjwa hubeba nakala moja.

Hati ya Ridhaa (2)

I, aliyetia, (jina) .... (jina la kwanza) .... , kutangaza kwamba mimi wamealikwa kushiriki katika mradi haki: ' kugundua ya mapafu kifua kikuu katika mdomo Awaosha kutumia halisi - wakati Polimeresi Reaction (PCR) . Mimi wametakiwa kuwa kipimo kwa maambukizi ya VVU. Mimi kuelewa kuwa matokeo ya mtihani kutimilika siri na majina.

Mimi kukubali kuwa kipimo (Jibu moja): Ndiyo .... No ....

Mimi nataka kujua matokeo ya mtihani : Ndiyo .... No ....

Tarehe: ...... / .... / ....

Jina na saini ya Jina mgonjwa na sahihi ya Mganga Mkuu wa
Wakati .... Muda ....

Jina na saini ya ushahidi (kama mgonjwa hawajui kusoma na kuandika)

....

Wakati na tarehe ....

Back translation

Participating in the research protocol: Evaluation of Real -Time Polymerase Chain

Reaction on Oral Wash samples for diagnosis of Pulmonary Tuberculosis at Mbagathi District Hospital, Nairobi.

Madam, Sir

I am working for................................ (Name of your organization)................... You are invited to take part in research into new diagnostic molecular system in TB. It is your right to be informed of any risks and benefits of this project so that you can

make good decisions without thinking. This original and unusual about what the procedure is referred to as ' the consent form.

You will need to read the information contained carefully and if necessary, discuss issues and medical officer in charge of the project. If, after reading this paper and have the opportunity to discuss some issues you decided to take part in the survey, you will be asked to sign a paper with the Medical Officer in charge of the study. There will be no interruption in your regular screening for TB. You will receive one copy and we will keep one copy of the present document. You can decide not to participate in the study. If you are willing to take part in the survey, you can resist your participation at any time without any effect on treatment. Appending your signature does not mean guaranteed commitment from you.

Purpose

Evaluate the opportunity to use real - time PCR on oral washes for detecting pulmonary tuberculosis usually the initial screening of tuberculosis often banded and clinical data, a definitive diagnosis involves isolation and identification of the infecting organism in the laboratory. Common laboratory procedure for microscopic examination involves clinical specimens for the presence of acid -fast bacilli (AFB), isolation of the organism and culture, and identity and feels drug testing of organisms recovered. Because of the slow growth rate of tuberculosis bacteria, isolation, identification, and drug testing sensing can take several weeks or more.

In the past several years, many methods have been developed for molecular examined one by one, a kind of identity, and feel the drug testing of tuberculosis germs. These methods are potentially reducing diagnostic time from weeks to days. Mouth wash is easier to find than sputum and this is likely to be important for the detection of TB.

Procedures

All patients enrolled in the study will have their oral wash procedure work within the first week of diagnosis and treatment of the establishment of TB. Participants will be

fasting since midnight and asked to postpone brushing their teeth until after the oral rinse, which includes rinsing the mouth with 10 ml of sterile saline for 1min. The process will be monitored by staff. Samples collected in sterile tubes and rotated for $3000 \times g$ for 30 min.

Subjects will be instructed to cough strength 5 times, and then rinse with 10 mL of sterile saline for 60 s. Samples will be refrigerated at 4 ° C until processing. On the transport of laboratory, acid -fast paint and culture TB germs will work on sputum samples and PCR will be performed on cough and oral rinse samples.

Confidentiality

Personal and medical information collected for this study will remain confidential and anonymous. If you agree to take part in this study data records will be computerized.

Information is meant only for the project and not used by any other person except for those who work and study.

Certificate of Consent 1.

I, the undersigned, (name)........................... (First name) ...., Declare that I have been invited to participate in a project entitled: ' detection of Mycobacterium tuberculosis in oral Washes using real - time Polymerase chain Reaction (PCR).

I declare that I have been fully informed Prof / Dr / Mr. . . . . / Mrs. /.......................... the nature and process of the study mentioned above and that I have been given the possibility to handle any matter relating to this after sufficient time to reflect.

I understand that I am completely free to interrupt my participation in this study without having to justify my decision, and my decision to have no impact on the quality of treatment that I deserved. My consent does not spare the organizers of the current study of their functions. I put all my rights are protected by law.

I understand that the data on my person will be fully confidential. I do not endorse its use by any other person except for the purposes of this study. I allow the data from this study have a computer.

I agree to take part in this study in accordance with the above conditions in writing. I have read and received a copy of this document.

Date: ...... / .... /.........

Name and signature of the patient's name and signature of Medical Officer ....

Name and signature of witness (if the patient cannot read and write)

....

Remember.

Original copies of this are to be filed where the patient carries one copy.

Certificate of Consent (2)

me, the undersigned, and (name).......................... (First name) ...., Declare that I have been invited to participate in a project entitled: ' detection of pulmonary tuberculosis in oral Washes using real - time Polymerase chain Reaction (PCR).

I have been asked to be tested for HIV infection. I understand that the test results including my names and will remain confidential.

I agree to be tested (tick one): Yes......... No.........

I want to know the results of the test: Yes......... No.........

Date: ...... / .... /..................

Name and signature of the patient's name and signature of Medical Officer

When................................ Time..

Name and signature of witness (if the patient cannot read and write)

....

Time and date.......................................…

Appendix II: Publication

patients were culture negative but positive for GeneXpert and lastly,7 (4.3%) of the patients were culture-positive but GeneXpert negative. 45(27%) of the patients had their cultures contaminated. The test performances were as follows:100%,94%,92% and 94% for culture,90.1%,99%,90% and 91% for ZN smear, 83%,97%,81% and 83% for PCR and 97.1%,92%,88% and 90.1% for the GeneXpert respectively.

CONCLUSIONS

PCR test accurately and rapidly detected *M. tuberculosis* - specific DNA sequences of small numbers of mycobacteria in mouthwashes and was easily manipulable. Further refinements of the test may improve the diagnosis of tuberculosis in resource-constrained countries.

Keywords: *Polymerase chain reaction, tuberculosis, Ziehl–Neelsen, Mouthwashes, GeneXpert, Mycobacterium tuberculosis*

[Afr. J. Health Sci. 2021 34(5): 551 - 565]

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Appendix III: Raw Laboratory Data

Lab No.	Pat No.	PCR test results		Ref culture results	
1	272		P		p
2	273		P		p
3	192		P		p
4	268		P		p
5	271		P		p
6	269		P		p
7	283		P		p
8	245		P		p
9	244		P		p
10	238	N		N	
11	270	N		N	
12	236	N		N	
13	291		P	N	
14	216		P	N	
15	253	N		N	
16	258	N		N	
17	218		P	N	
18	250		P	N	
19	262		P	N	
20	252		P	N	
21	260		P	N	
22	215		P	N	
23	246	N		N	
24	258		P	N	
25	264		P	N	
26	257		P		P
27	242		P		P
28	284		P		P
29	282		P		P
30	240		P		P
31	241		P		P
32	213		P		P
33	221		P		P
34	256	N		N	
35	261	N		N	
36	248	N		N	
37	257	N		N	
38	223		P		P
39	166		P		P
40	165		P		P
41	164		P		p
42	163		P	N	
43	161		P		p
44	162		P	N	
45	285		p		P
46	184		P	N	
47	183		P	N	
48	182		P	N	
49	181		P		p

50	180		P		p
51	178		P		p
52	177		P	N	
53	176		P		p
54	168	N		N	
55	169		P	N	
56	203	N		N	
57	204		P		p
58	205		P		p
59	206		P		p
60	171		P	N	
61	172	N		N	
62	173	N		N	
63	174	N		N	
64	190	N		N	
65	194		P		p
66	195	N		N	
67	196	N		N	
68	197	N		N	
69	199	N		N	
70	200	N		N	
71	201	N		N	
72	202	N		N	
73	167	N		N	
74	149	N		N	
75	150	N		N	
76	157	N		N	
77	152	N		N	
78	154	N		N	
79	155	N		N	
80	157	N		N	
81	158	N		N	
82	159	N		N	
83	142		P		P
84	145		P		P
85	146		P		P
86	153		P		P
87	156		P		p
88	140		P		P
89	160		P		P
90	143	N		N	
91	141		P		p
92	144		P	N	
93	147		P	N	
94	148		P	N	
95	19		P	N	
96	20		P	N	
97	21		P	N	
98	22		P	N	
99	23		P	N	
100	24		P	N	
101	25		P		P
102	26		P	N	

103	27	N		N	
104	28	N		N	
105	32	N		N	
106	33		p		P
107	34		p		P
108	37		p		P
109	38		p		P
110	39		p		P
111	41	N		N	
112	43	N		N	
113	44	N			P
114	51	N			P
115	53	N		N	
116	54	N			P
117	55	N		N	
118	56		P	N	
119	57		P	N	
120	58		P	N	
121	59		P	N	
122	98		P		P
123	97		P		P
124	62		P		P
125	69		P		P
126	66		P		P
127	68		P		P
128	122		P		P
129	134		P		P
130	128		P		P
131	109		P		P
132	95		P		P
133	16		P	N	
134	51	N		N	
135	60	N		N	
136	64	N		N	
137	63		P		P
138	61		P		P
139	65		P		P
140	113		P	N	
141	137		P		P
142	108		P	N	
143	105		P		P
144	67		P		P
145	114		P		P
146	138		P		P
147	133		P		P
148	124	N		N	
149	131	N		N	
150	118		P		P
151	120	N		N	
152	112	N		N	
153	130	N		N	
154	132	N		N	
155	106	N		N	

156	135	N		N	
157	116	N		N	
158	69	N		N	
159	2	N		N	
160	3	N		N	
161	15	N		N	
162	16		P	N	
163	17	N		N	
164	KJN	N		N	
165	KATUI	N		N	
Totals 165		63	102	96	69