STUDIES ON EVALUATION OF PERFORMANCE OF TRAINED SNIFFER RATS IN DETECTION OF *MYCOBACTERIA SPECIES* FROM CLINICAL CASES OF HUMAN TUBERCULOSIS

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A DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN APPLIED MICROBIOLOGY OF SOKOINE UNIVERSITY OF AGRICULTURE.

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ABSTRACT

The Belgian humanitarian research organisation Anti-Persoonmijnen Ontmjinende Product Ontwikelling (APOPO), in collaboration with the Sokoine University of Agriculture (SUA), has pioneered a project that involves the training of African Giant Pouched rats (*Cricetomys gambianus*) as a potentially faster tool for the detection of mycobacteria species by sniffing out positive sputum samples. This study has evaluated the performance of these rats in detection of *Mycobacteria species* from human sputum samples. A total of 910 sputum specimens from patients with suspected pulmonary tuberculosis (PTB) were collected and tested. All specimens were tested by the sniffer rats, microscopy and culture followed by PCR for all culture positive samples. The Culture results were used as the reference for assessment. Out of 910 specimen tested, 161 (17.7%) were culture positive, 90 (9.9%) were positive by microscopy and 120 (13.2%) were positive by trained sniffer rats technique. A total of 481 samples (52.9%) were declared as negative by all three techniques. Sensitivity of trained sniffer rats technique and microscopy was 74% and 56% (P <0. 0076) respectively, whereas specificity of trained sniffer rats and microscopy was 64% and 98% respectively (P< 0.0001). The sniffer rats technique is significantly more sensitive and less specific than microscopy, hence recommended to be used as screening tool for human cases of PTB. The 161 specimens which were culture positive and also confirmed to have acid-fast-bacilli by microscopy were subjected to mycobacterium genus PCR typing. Out of 161 isolates, 127 isolates were identified to belong to the group of *Mycobacterium tuberculosis complex.* Using RD9 PCR which further characterizes *mycobacterium tuberculosis complex* into their specific species, it was confirmed that 127 (78.4%) of the isolates belonged to the specific species of the *Mycobacterium tuberculosis*. Out of 161 culture positive isolates, 108 isolates were also detected by PCR and sniffer rats.

DECLARATION

I, George Makingi do declare to the Senate of Sokoine University of Agriculture that this dissertation is my own original work. It has been done within the registration and that it has neither been submitted nor being concurrently submitted in any other institution.

_____ _____

George Makingi Date

(MSc. Candidate)

The declaration above is confirmed by;

_____ _____

Professor.R.R.Kazwala (PhD) Date

(Supervisor)

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABREVIATIONS

A	Adenine
AFB	Acid-fast bacilli
AIDS	Acquired immunodeficiency syndrome
APOPO	Anti-Persoonsmijnen Ontmijnende Product Ontwikkeling
Bp	Base pair
BD	Bacton, Dickinson *and Company*
C	Cytosine
Conc.	Concentration
DNA	Deoxyribonucleic acid
DOTS	Directly Observed Treatment Short-course
FM	Fluorescent microscope
FW	Forward
HIV	Human immunodeficiency virus
ICMR	Indian Council of Medical Research
IUATLD	Union against Tuberculosis and Lung Disease
Kb	Kilobase
LJ	Lowstein-Jension
MDR	TB Multidrug-resistant tuberculosis
Micros	Microscopy
MTB	Mycobacterium tuberculosis
MTC	Mycobacterium tuberculosis complex
Neg	Negative
NPV	Negative predictive value
NTM	Non-tuberculous mycobacteria

PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
Pos	Positive
PTB	Pulmonary tuberculosis
PPV	Positive predictive value
RD	Region of difference
Rev	Reverse
RNA	Ribonucleic acid
rRNA	ribosomal Ribonucleic acid
SDA	Strand displacement amplification
SUA	Sokoine University of Agriculture
T	Thymine
TB	Tuberculosis
TBE	Tris Boric EDTA
WHO	World Health Organization
ZN	Ziehl-Neelsen

CHAPTER ONE

1.0 INTRODUCTION

Tuberculosis (TB) is an infectious disease caused by various strains of *mycobacteria* (Kumar *et al.,* 2007). TB in humans can be caused by species belonging to the *Mycobacterium tuberculosis complex* (MTC) which includes the species *M. tuberculosis, M. bovis, M. africanum, M. canetii, and M. microti* and some nontuberculous mycobacteria (NTM)(Murray *et al.,* 2003). A study on TB and human immunodeficiency virus (HIV) has shown that a substantial proportion of the increase in TB cases is attributable to HIV infection (Mfinanga *et al.,* 2003). It was also reported that mycobacteria other than *M. tuberculosis* can frequently cause pulmonary and extrapulmonary mycobacteriosis in HIV infected patients as well as in immunocompetent subjects (Parmelo *et al.,* 2009) .

The magnitude of the global TB problem is immense, with 8.8 million new cases annually and 2 million deaths (WHO, 2010). HIV is fuelling the epidemic in many developing countries and multidrug-resistance is a growing threat (Richard *et al.,* 2000). Death from TB comprises 25% of all avoidable deaths in developing countries (WHO, 2009a). Nearly 95% of all TB cases and 98% of death due to TB are in developing countries and 75% of cases are in the economically productive age group (Pai *et al.,* 2002). In 2009, the National Tuberculosis and Leprosy Programme (NTLP) reported 64, 267 cases of tuberculosis of all forms (new and re-treatment) from Tanzania. This report shows an increase of 903 (1.4%) cases compared to those reported in 2008 (NTLP, 2009).

Since disease control depends on appropriate antimicrobial therapy, the precise identification of these species of clinical importance has become a major public health concern (WHO, 2009a). Identification of mycobacteria has been hampered because of the lack of specific, rapid, and inexpensive methods which leave millions undiagnosed and

untreated (Martinez *et al.,* 2008). World leaders, public health officials and international donors have taken action to control TB, and financial resources for control and research on finding rapid, specific and inexpensive diagnosis have increased dramatically in recent years (Dag *et al,*. 2008).

Fast, cheap and accurate diagnosis of the TB and initiating optimal treatment would not only enable a cure of an individual patients but will also curb the transmission of infection and disease to others in the community (WHO, 2006). Of the several distinct components of the TB control programme, case finding remains the corner stone for the effective control (Mohammed *et al.,* 2000). In the hope of developing an alternative fast, cheap and accurate diagnostic technique, the Anti-Persoonsmijnen Ontmijnende Product Ontwikkeling (APOPO), a non-profit humanitarian organization located in Tanzania, has begun to explore the use of African giant pouched rats (*Cricetomys gambianus*) to detect the presence of TB (Weetjens *et al.,* 2009b).

1.1 Objectives of the study.

1.1.1 General objective

The aim of this study is to evaluate the performance of trained sniffer rats in the detection of *Mycobacteria species* in clinical specimens of human cases of TB.

1.1.2 Specific objectives.

- To detect *mycobacteria species* in sputum samples using microscopy, trained sniffer rats and culture techniques to selected sputum samples.
- To compare the results of culture (gold standard) technique with sniffer rats and microscopy techniques.

- To amplify DNA fragments by PCR for identification of mycobacterium tuberculosis complex specific species isolated from culture.

CHAPTER TWO

2.0 LITERATURE REVIEW

Rapid and accurate diagnosis is critical to the care of tuberculosis (TB) patients and to arrest disease transmission (WHO, 2009b). Despite of the global strategy for diagnosing and treating of TB which extend to over 182 countries worldwide, a minority of the nearly 9 million new TB sufferers each year receives a laboratory-confirmed diagnosis (Mishra *et al.,* 2005). Since the discovery of the TB bacillus in 1882, microscopic examination of stained sputum has remained the cornerstone of PTB diagnosis throughout most of the world (Mark *et al.*, 2007).

Despite substantial success in implementing standardized care and improving rates of cure in recent years, the global burden of TB remains enormous. Lack of rapid, inexpensive and accurate diagnosis and case detection are major obstacles to TB control. Diagnostic test manufacturers, in large part, have not invested in the development of new tests targeting the needs of developing countries, where 90% of all TB patients live (WHO, 2009b).

In developing countries, most of the bacteriological diagnosis of TB is carried out in peripheral or local laboratories, whose major responsibility is to provide diagnostic microscopy based on sputum smear examination by Ziehl-Neelsen **(**ZN**)** staining. These laboratories, located in health centres, health posts, hospitals, etc., usually have qualified technical personnel capable of performing among other duties sputum smear microscopy (Hans *et al.*, 2000).

Microscopy is the simplest and most rapid procedure currently available to detect acid-fast bacilli (AFB) in clinical specimen by zienhl-Neelsen staining method or its modifications (Cheesbrough, 2000). Microscopic examination of sputum smears for AFB forms the basis of TB diagnosis in developing countries. Microscopy is cheap to perform,

specific enough to indicate treatment in countries where TB is prevalent, and can be completed within hours if necessary (WHO, 2006).

The limitation of microscopy for detection of TB is that it requires a large number of bacilli to be present in order for the result to be positive (5000–10,000 bacilli per ml of sputum) (Khagi *et al.*, 2009). However, this renders the test unable to detect less advanced cases. The results of microscopy can be influenced by the type of specimens, thickness of the smear, extent of decolarization, type of counterstain used, training and experience of the person examining the smear (Medappa *et al.*, 2002). Smear microscopy has been in use for more than 100 years and detects only half of patients tested and is particularly ineffective for diagnosis of TB in children and in patients co-infected with HIV (WHO, 2006). A recent review, expressed concern that immunosuppression resulting from HIV type 1 has reduced the sensitivity of sputum smear by reducing caseation necrosis and thus the number of acid fast bacilli in the airway (Colebunders *et al.*, 2000). The review also indicated that immunosuppression may also have affected the specificity of the sputum smear by increasing the proportion of patients with nontuberculous mycobacteria. Furthermore, it was stated that the delay in accessing proper diagnosis has costed patients and their families' valuable time and money with consequent delay in start of treatment and leading to continued transmission of infections to close contacts (Gates Foundation, 2009).

Isolation of mycobacteria from sputum samples by culture still represents the corner stone on which definitive diagnosis of TB relies and is currently the gold standard for the primary isolation of mycobacteria (WHO, 2006). At present, mycobacterial culture is performed on Lowenstein Jensen medium (LJ medium) (Pai *et al.*, 2009).

The major constraint of culturing mycobacteria in conventional media is its slow growth which necessitates a mean incubation period of at least 4 weeks (Medappa *et al.*, 2002). Also, mycobacterial culture method is feasible only if > 100 *M. tuberculosis* bacilli are present in one ml of specimen. The specificity of the culture method is close to 100% and it is used for final diagnosis in most of developing countries. Despite the acceptance of culture as the definitive tool for the diagnosis of TB, some microscopy-positive specimens fail to yield mycobacteria on culture. This may be due to harsh chemical treatment which is used to decontaminate specimens from other bacteria (Debra *et al.*, 1991).

Molecular technique constitutes a rapidly evolving improvement in the detection and identification of *M. tuberculosis.* Bacterial DNA is enzymatically amplified and detected with appropriate reading system via a signal-generating probe. Several enzymatic amplification processes have been developed and the most widely used are PCR (polymerase chain reaction) and SDA (strand displacement amplification) (Araj *et al.*, 2000). Tests based on nucleic acid amplification are usually highly specific for *M. Tuberculosis,* close to 100% (WHO, 2006), although some tests require a two-step diagnostic procedure (initial test for mycobacteria genus, followed by tests which differentiate *M. tuberculosis* from non-tuberculous mycobacteria). Positive results can be obtained with less than 10 bacteria/ ml, therefore sensitivity is much better than microscopy and culture (Dwivedi *et al.*, 2003).

Other methods such as high-performance gas-liquid and thin-layer chromatographies are labor-intensive, difficult, and expensive for routine use. Because of their price and complexity the use of these methods is still limited to developed countries especially for research purpose (Pai *et al.*, 2009).

The diagnosis of TB in developing countries is mainly based on smear microscopy stained by the Ziehl Neelsen (ZN) method (Perkins *et al.*, 2009). Other rarely used methods include fluorescent microscopy (FM) and culture (gold standard) that requires laboratory facilities that are not readily available in resource-limited areas. For example, Tanzania has only three TB culture laboratories serving nearly 40 million people, which makes the smear microscopy the most accessible diagnostic tool for TB control. The case detection rate in developing countries is around 47%, far below the global target of 70%. This is attributed to the poor sensitivity of the method; the huge workload, which causes fatigue to the microscopist and may result in significant number of false-negatives (misdiagnosis), and too difficult in diagnosing TB in patients co-infected with HIV because most of them produce negative smear results which delay the diagnosis and treatment in the absence of culture facilities. Therefore, a fast, simple and more efficient diagnostic system is highly required (Weetjens *et al.*, 2009c).

In the hope of developing a viable alternative to or adjunct for microscopy, APOPO has begun to explore the use of African giant pouched rats (*Cricetomys gambianus*) to detect the presence of TB in human sputum sample. The rats, which are native to much of African countries including Tanzania have an excellent sense of smell. The training of these rats as a potentially faster tool for the detection of TB by sniffing out positive sputum samples is based on the fact that mycobacteria emit specific volatile organic compounds that are likely to be detected by the rats through olfactory perception (Weetjens and Mgode, 2009). The rats' single most important exteroceptive sense is smell. They detect TB by sniffing sputum samples. They are trained to respond consistently in one way (pause) if the sample contains the TB bacillus (i.e., positive) and respond in another way (not pause) if the sample does not contain the bacillus (i.e., is negative). Initial research using *C. gambianus* to detect vapours released from buried

landmines by sniffing has shown that these rats have a highly developed olfactory capacity. It was on this basis that the same species of rats were tested as bio-detectors for pulmonary TB in sputum samples ((Weetjens *et al.*, 2009b).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study area

This study involved a population of suspected PTB patients who visited selected Directly Observed Treatment Short-course (DOTS) centres in Dar es salaam City and Morogoro Municipality. Dar es salaam is located in Tanzania between latitudes 6.36 degrees and 7.0 degrees south of Equator and longitudes 39.0 and 33.33 east of Greenwich. It is bounded by the Indian Ocean on the east and by the Coast Region on the other sides. The DOTS centres in Dar es salaam were from Mwananyamala Hospital, Magomeni Hospital, Amana hospital, Temeke Hospital and Tandale Health cente. In Morogoro Municipality, the DOTS centre selected was at Morogoro Regional Hospital. The laboratory work of this study was undertaken at the Faculty of Veterinary Medicine laboratory and at Apopo TB research laboratory of the Sokoine University of Agriculture. The Morogoro Municipality is located in Tanzania at 6°51 degrees south and 37°41 degrees to the east of Equator. It is extended on slopes of Uluguru Mountains at an altitude of 500-600 metres above sea level. This study was conducted in the period from January and July 2011.

3.2 Study design and Sample size

Cross-sectional study design was used to evaluate the results of trained sniffer rats in the examination of sputum specimens for the direct detection of Mycobacteria species. Sample size was estimated by the formula:

$N= Z^2PQ/L^2$ (Martin *et al.,* 1987).

Where; N= sample size, Z=1.96 at 95% Confidence level, P= the proportion of finding a positive case=50% (0.5), Q= (1-P), L= allowable error= 0.03.

$N= (1.96)^2 (0.5) (0.5)/ (0.032)^2$

N= 902

3.3 Ethical clearance

This was part of the ongoing study of APOPO tuberculosis research project, the ethical clearance to conduct this research project was obtained from the Tanzanian National Institute for Medical Research (**Appendix**).

3.4 Sample collection.

World Health Organization guidelines for sputum sample collection was followed during this study(WHO, 2009a). The samples were accompanied by data on the history of the sputum donor, including patient number, sample number, sex, age and acid-fast bacilli result. Samples were transported to Sokoine University of Agriculture at APOPO TB research laboratory using special project car. Upon arrival at the laboratory, all samples were assessed by visual observation, whereby specimen with less than 2ml of sputum, and those containing saliva instead of sputum were considered as unsatisfactory, hence omitted for this study. Selected specimens were brought into the Biosafety Cabinet level II for aliquoting, whereby one ml of sputum was aliquoted into special cryovials and shifted to TB culture laboratory at the Faculty of Veterinary Medicine and stored in the deep freezer for culture. The remaining part was processed for sniffer rat test. This involved additional of sterile phosphate buffered saline (5 ml) into each sputum sample and inactivating by heating at 90^{o}C in a water bath for 30 minutes. The samples were then frozen (at -20^{o}C) until the day of sniffer rat evaluation. This procedure was undertaken to protect the staff from exposure to live bacteria.

3.5 Trained sniffer rats technique

Figure 1. Cricetomys gambianus at work.

Standard Operating Procedure for African pouched rats for the detection of pulmonary tuberculosis in sputum samples was used (Weetjens *et al*., 2009b).

Layout of the evaluation set-up

In this study, ten adult rats (*Cricetomys*) obtained from breeding colony, 5 males and 5 females, evaluated all sputum samples. The animals were housed and maintained as detailed by the project (Weetjens *et al.,* 2009a). Some of the rats had been used in previous studies and all of the rats had been evaluating sputum samples for TB for at least one year. Testing was conducted in a chamber of 205 cm long, 55 cm wide, and 55 cm high with clear plastic walls and ceiling and a stainless steel floor. Ten holes with sliding lids 2.5 cm in diameter were spaced equidistance apart along the centreline of the chamber floor's long axis **(Fig. 1).** Pots containing sputum were placed beneath the holes for the rats to evaluate. Edible reinforcers (rewards), consisting of a mixture of mashed banana with ground rodent diet pellets, were delivered through a plastic syringe through feeding holes.

Each rat evaluated each tested sample twice, in a different order, and seven known positive samples were also presented to the rats. The seven known positive samples served as reinforcement opportunities to maintain the rats' indications while the remaining samples were categorized as "unknown". During the sessions, the trainer opened each hole in the cage as the rat passed over and sniffed. When the rat paused for 5sec. (i.e., emitted an indicator response), the trainer informed an observer who then stated whether the sample was the known positive or a suspect positive detection. If the rat made an indication response to a known positive sample, the trainer sounded a click and delivered food, after which the rat then moved to the next hole to continue evaluations. If the rat emitted an indicator response at the "unknown" sample, the experimenter closed the hole but did not sound a click or present food and the observer recorded it as new positive detection. A cut-off of 3 rats was used, i.e. Sample was deemed rat positive if three or more rats indicated it, samples indicated by only 2, 1, or 0 rats were deemed as negative.

This experiment was performed by a group of three people, a trainer (responsible for everything related to the rats), a handler (responsible for sample handling) and an observer (responsible for data recording) (**Fig.2**).

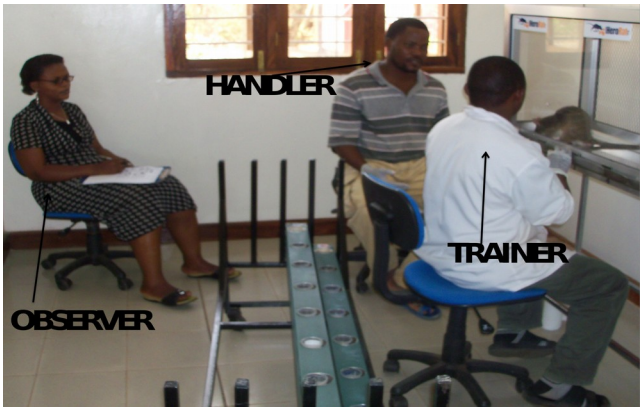

Figure 2. Trained sniffer rats technique**.**

This technique was a blind testing, whereby only the observer knew the position of the positive sample intended to reinforce the desired behaviour of the trained rats, but he didn't know the position of the blind positive samples.

3.6 Mycobacterial culture

Standard Operating procedure used in this study for culturing of sputum specimen was from Becton, Dickinson and Company (BD) (BD, 2011).

Preparation of Lowenstein-Jensen (LJ) media

Lowenstein-Jensen egg-based solid media is specific for TB bacterial culture only. The Media was prepared in the clean, media preparation room at the Faculty of Veterinary Medicine TB laboratory. Care was taken to ensure that the room was absolutely kept clean, free from dirt or dust and thus, the benches were swabbed with 70% alcohol. During and at all stages of media preparation, the door was kept closed, to avoid contamination of the media. Equipments and glasswares were sterilized prior to the day of media preparation.

Materials and chemicals used for media preparation were; Warring Blender (mixer) sterile jar of 1000ml, sterile funnel , one litre round flat bottomed flask, 28 ml sterile universal containers, sterile mineral salt solution, malachite green, Fresh hen's eggs and Inspissator.

The two types of LJ media were prepared; LJ media with glycerine and LJ media with pyruvate.

LJ Egg Media with Glycerine

The complete medium (measured for 400ml of the media**).**

- 150.0ml of Mineral salt solution
- 0.6 g of KH_2PO_4 anhydrous (potassium dihydrogen orthophosphate)
- 0.06 g of MgSO4.7H2O (Magnesium sulphate)
- 0.15 g of Magnesium citrate
- 0.06 g of $Na_2HPO_4.2H_2O$
- 0.9 g of L-asparagine
- 3.0 ml of Glycerine
- 150 ml of distilled water.
- 5.0 ml of 2% Malachite green
- 250.0 ml of Whole eggs solution homogenized in a 1000ml conical flask. All chemicals and ingredients were dissolved aseptically and autoclaved at 121^{o}C for 15 minutes to sterilize. Then, 6ml of the complete media was aseptically

distributed into universal bottles, the bottle caps were closed tightly and inspissated for 60 minutes at temperature of 80^{o}c in a slanted position to form a slope.

LJ Egg Media with Pyruvate

Similar ingredients used for making LJ Egg Media with glycerine were used for this media except that 2.0g of Sodium pyruvate was added instead of glycerine and also Trypan blue 0.6 % (V/V) or 1 % (W/V) (0.0125g of Trypan blue and 1.25ml of sterile distilled water, then dissolved aseptically and autoclaved at 121^{o} C for 15 minutes to sterilize.

Processing and inoculation of the sputum samples (culturing)

One ml of 4% sodium hydroxide was added to one ml of sputum samples in the universal container to make the sputum homogenous, and mixed well and left to stand for 45 minutes at room temperature with occasional shaking. The sputum homogenate was then centrifuged at 3000 rpm for 15 minutes. The supernatant was decanted into a jar containing disinfectant. The sediments were neutralized with one ml of 14% KH_2PO_4 solution to which had been added 3-4 drops of 0.1% phenol red indicator. The colour change which may extend from yellow (acid) to orange/red (neutral) to red (alkali) was noted. The sediments were again mixed and two loopful of the sample was inoculated on each of LJ media (i.e. glycerine and pyruvate containing media) (**Fig. 3**). The inoculated LJ slants were then incubated at 37^{o}C for 6 to 12 weeks.

Figure 3. Samples inoculation in LJ media

The culture was observed for colonies on the 7th day for rapid growers and on fourth week for slow growers. If the colonies did not appear at the time mentioned above, observation was done weekly until 8 weeks, and for 8 to 12 week for slow growers before giving decision as negative or positive (**Fig. 4**). Recording and reporting was done in register book. All the positive growth were examined by microscopy to confirm the acid fast bacilli.

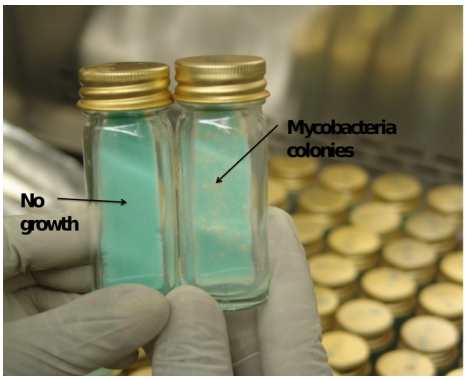

Figure 4. Appearance of Mycobacteria in LJ media

3.7 Microscopy

Smear preparation

Direct smears were prepared from each culture positive container and stained using ZN stain as previously described (Cheesbrough, 2000). The identity number of the culture positive container was written on a new slide, this number corresponded with the number on the culture positive container. Labelling was done by lead pencil. Using a disposable plastic loop (one mm in diameter), a portion of growth that appeared clearly thick was selected and picked up and spreaded at the middle of the slide 2 x 1 cm and allowed to dry. The cap of growth container was closed firmly and the container returned in the incubator, the loops were disposed in a plastic container containing disinfectant and then autoclaved. The smeared slides were then stained using ZN stain.

ZN staining

The slides with smear were placed upwards on a staining rack over a sink about one cm apart and flooded with filtered carbofuchsin staining solution. The slides were then heated by keeping the burning spirit a little below the slides and moving it continuously forth and back along the line until steam arose. The process was repeated twice at intervals of 3-5 minutes. The slides were then tilted using forceps to drain off the staining solution. Then, slides were rinsed well with running tap water. The decolorizing solution (i.e. 20% sulphuric acid) was poured over the smear covering it completely for 2 minutes. The slides were then tilted with forceps to drain off the decolouring solution, and then the slides were gently rinsed The slides were then flooded with 0.3% methylene blue solution for one minute, then tilted with forceps to drain off the Methylene blue and washed with clean water. The slides were taken from racks, drained off water and left to stand on the edge to air dry on the drying rack then observed under light microscope at x100 and results recorded as Acid Fast Bacilli (AFB) positive for mycobacteria or AFB negative for non mycobacteria.

3.8 Polymerase Chain Reaction (PCR)

The Polymerase chain reaction (PCR) is a scientific technique in molecular biology which amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. Primers (short DNA fragments) containing sequences complementary to the target region along with a DNA polymerase (after which the method is named) are key components to enable selective and repeated amplification.

In this study, standard Operating Procedure for Mycobacterium genus typing and deletion typing PCR was used (Berg, 2008).

Mycobacterium genus typing PCR

The genus typing procedure identified species from Mycobacterium genus, but also differentiated species of the M. Tuberculosis complex from *M.avium, M. intracellulare* and other *Mycobacterium species*. This method was applied to heat killed Mycobacterial suspensions.

In this multiplex PCR protocol, six different *Mycobacterium* genus typing Primers were used;

Oligonucleotide primer's sequences:

100µM MYCGEN-F 5'-AGA GTT TGA TCC TGG CTC AG-3'

100µM MYCGEN-R 5'-TGC ACA CAG GCC ACA AGG GA-3'

100µM MYCAV-R 5'-ACC AGA AGA CAT GCG TCT TG-3'

100µM MYCINT-F 5'-CCT TTA GGC GCA TGT CTT TA-3'

100µM TB1-F 5'-GAA CAA TCC GGA GTT GAC AA-3'

100µM TB1-R 5'-AGC ACG CTG TCA ATC ATG TA-3'

Firstly, it targeted a sequence region within the 16S rRNA gene specific for the Mycobacterium genus. The two primers MYCGEN-F and MYCGEN-R are designed to amplify a specific PCR product from genomic DNA of all known mycobacteria. Secondly, the PCR mix also included primers that were specific for a hyper variable region of the 16S rRNA gene of *M.intracellulare* (MYCINT-F) and *M.aviam* (MYCAV-R) respectively, giving one additional PCR product if the DNA template was any of these two species. Thirdly, species from the *M. tuberculosis complex* could also be identified

due to the two primers (TB-F, TB-R) that targeted the MPB70 gene, specific for mycobacteria from the complex.

Preparation of the master mix and the set up of PCR amplification

The room for preparing mastermix was cleaned and disinfected with alcohol for at least 15 minutes before starting the work. The master mix was prepared on the day of use. Water, HotStarMaster mix, and primers were mixed in a sterile eppendorf tube by using filter tips.

Table 1.The mastermix for 20 samples:

PCR mix	**Number of samples 20**
6.2ul H20 Qiagen	124
10ul Mastermix	200
0.3ul MYCGEN-F 100uM 3	6
0.3ul MYCGEN-R 100uM 3	6
0.3ul TB1-F 100uM	6
0.3ul TB1-R 100uM	6
0.3ul MYCAV-R 100uM	6
0.3ul MYCINT-F 100uM	6
18ul/tube	
+2ul DNA template	
20ul total volume/reaction	

Then, 18µl of the mix was aliquoted into PCR tubes. Each tube labelled with a number. The tubes were closed and moved to a different area in the laboratory that was designated for addition of the DNA template. 2µl of mycobacterial DNA template were added to respective PCR tube **(Table. 1).** The tubes were then placed in the PCR machine (Thermocycler**)** and started the correct PCR program.

Amplification

The reaction mixture was cycled through the following temperature profile in a Mastercycler (Eppendorf®, Germany): an initial denaturation step at 95°C for 10 min, 35 cycles of 95°C for 1 min, 61°C for 0.5 min, and 72°C for 2 min; and a final elongation step at 72°C for 10 min. The holding temperature was 4^{o}c.

Deletion typing

This protocol presented a tool which showed how to differentiate *Mycobacterium* isolates that were known to be from the *Mycobacterium tuberculosis complex*, it further characterized them into their specific species. This was made by PCR amplification of species-specific DNA fragments.

This procedure included protocols for deletion typing of three different regions: RD4, RD9, and RD10. Each protocol aimed to find out if a specific strain had the RD-region present or deleted. A strain that had RD4 deleted was typed as *M. bovis*, while a strain with RD9 present was most likely *M. tuberculosis.* Similarly, an isolate that had the RD9 deleted but the RD10 present was classified as *M. Africanum.* Deletion typing was designed as a multiplex PCR using three different primers. RD9-FlankFW and RD9-InternalRev which amplified a PCR product while RD9-FlankRev did not contribute to any amplification since the elongation time in the PCR set up was too short. RD9 (with a size of about 2.5kb) was deleted in *e.g. M. africanum* and *M. bovis* making the primer

“RD9-InternalRev” redundant in such PCR amplifications. RD4 and RD10 typing were designed in the same way but showing different sizes of the expected PCR products. This protocol was applied to heat-killed bacterial suspensions or extracted genomic DNA.

Mycobacterium tuberculosis complex Deletion typing primers used were;

Oligonucleotide primer’s sequences:

100µM RD4-FlankFW 5’- CTC GTC GAA GGC CAC TAA AG -3’

100µM RD4-FlankRev 5’- AAG GCG AAC AGA TTC AGC AT -3’

100µM RD4-InternalFW 5’- ACA CGC TGG CGA AGT ATA GC -3’

100µM RD9-FlankFW 5’- AAC ACG GTC ACG TTG TCG TG -3’

100µM RD9-FlankRev 5’- CAA ACC AGC AGC TGT CGT TG -3’

100µM RD9-InternalRev 5’- TTG CTT CCC CGG TTC GTC TG -3’

100µM RD10-FlankFW 5’- CTG CAA CCA TCC GGT ACA C -3’

100µM RD10-FlankRev 5’- GAA GCG CTA CAT CGC CAA G -3’

100µM RD10-InternalRev 5’- GAA GTC GTA ACT CAC CGG GA -3’

Preparation of the master mix and the set up of PCR amplification

The master mix room was cleaned and disinfected with 70% ethanol alcohol for at least 15 minutes before starting the work. The master mix was prepared on the day of use. Water, HotStarMaster mix, and primers were mixed in a sterile eppendorf tube by using filter tips.

Table 2. The mastermix for 20 samples:

PCR mix:	**Number of samples: 20**
7.1ul H20 Qiagen	142
10ul Mastermix	200
0.3ul RDx_FlankRev 100uM	6
0.3ul RDx_FlankFW 100uM	6
0.3ul RDx_Internal 100uM	6
18ul/tube	
+2ul DNA template	
20ul total volume/reaction	

Then, 18µl of the mix was aliquoted into PCR tubes. Each tube labelled with a number. The tubes were closed and moved to a different area in the laboratory that was designated for addition of the DNA template. 2µl of mycobacterial DNA template were added to respective PCR tube to make a total volume of 20µl **(Table. 2).** The tubes were then placed in the PCR machine (Thermocycler**)** and started the PCR program:

Amplification

The reaction mixture was cycled through the following temperature profile in a Mastercycler (Eppendorf®, Germany): an initial denaturation step at 95°C for 15 min, 35 cycles of 95°C for 1 min, 55°C for 0.5 min, and 72°C for 1 min; and a final elongation step at 72°C for 10 min. The holding temperature was 4^0c.

Agarose gel electrophoresis

Materials that were used for gel electrophoresis are DNA Ladder, loading Dye, Agarose and 10×TBE Running Buffer and Ethidium Bromide.

To prepare 1.5% agarose gel, 2.25g of agarose powder was added to 150mls of 1 x TBE buffer and stirred. The agarose was the heated in the heating block until all crystals were dissolved. About 5-10µl Ethidium Bromide solution was added per 150ml agarose gel solution (final conc. ~0.3µg EtBr/ml). The agarose was left to cool to 50-60°C before casting the gel. The gel was then placed in the tank. The samples and the DNA ladder were then loaded into the gel. The electrophoresis was run at about 100V. The photos of the agarose gel were saved for further reference.

3.9 Statistical analysis

Culture results were used as the reference method (gold standard) in this evaluation. Sensitivity was defined as the proportion of positive TB cases correctly identified by microscopy or trained sniffer rats to the total number of positive cases by reference method (culture method), and specificity as the proportion of TB negative cases correctively identified by the microscopy or trained sniffer rats to the total number of TB negative cases correctly identified by the reference method (culture). The MS EXCEL (2007), Statview and the Epi Info package for statistics were used for comparison of differences between the microscopy and sniffer rats techniques using culture as gold standard technique, the value of $P < 0.05$ was considered significant. Cohen's Kappa test was used to measure the agreement between sniffer rats and microscopy. The formular of Kappa was as follows:

$$\kappa = \frac{\Pr(a) - \Pr(e)}{1 - \Pr(e)},$$

Where Pr (a) is the agreement between compared techniques and Pr (e) the likelihood of random agreement. If techniques were in complete agreement $\kappa = 1$, If they disagree (or agree due to chance alone) $\kappa \leq 0$. (Cohen, 1960)

CHAPTER FOUR

4.0 RESULTS

This study involved a total of 910 sputum samples from PTB suspected patients, from DOTS centres in Dar es salaam and Morogoro. The samples were accompanied by microscopy acid-fast bacilli results from respective centres. And each test was conducted without prior knowledge of results from the other tests.

Table 3. **Summary of confirmed positive cases according to different diagnostic methods**

Method	Positive case	Negative case
Sniffer rats	120 (13.2%)	790 (86.8%)
Smear microscopy	90 (9.9%)	820 (90.1%)
Culture	161 (17.7%)	748 (82.2%)

Out of 910 samples, 161 (17.7%) were culture positive, 90 (9.9%) were positive by microscopy and 120 (13.2%) were positive by sniffer rats as confirmed by culture (**Table. 3**). A total of 86 (9.5%) samples were TB positive by all three methods. Also, 34 (3.7%) were positive by trained sniffer rats and culture but negative by microscopy, while 4 (0.4%) were positive by microscopy and culture but negative by trained sniffer rats, and 37 (4%) were only culture positive samples but negative by microscopy and trained sniffer rats. A total of samples 481(52.9%) samples tested negative by all three techniques **(Table. 6)**.

Table 4. Agreement between sniffer rats and culture for 910 samples tested.

Technique	Culture Pos	Culture Neg	Total
Sniffer rat Pos	120	266	386
Sniffer rat Neg	41	483	524
Total	161	749	**910**

Table 5. Agreement between microscopy and culture for 910 samples tested.

	Culture Pos	Culture Neg	Total
Microscopy Pos	90	12	102
Microscopy Neg	71	737	808
Total	161	749	**910**

Table 6. Agreement of microscopy and sniffer rats compared to culture for 910 Samples tested.

	Culture Pos	Culture Neg	Total
Micros & Rats Pos	86	10	96
Micros & Rats Neg	37	481	518
Total	123	491	**614**

Table 7. Agreement of microscopy and sniffer rats for 910 Samples tested.

	Rat Pos	Rat Neg	Total
Microscopy Pos	96	6	106
Microscopy Neg	290	518	808
Total	386	524	**910**

Table 8. Sniffer rats versus microscopy technique

	Sensitivity	Specificity	PPV	NPV
Sniffer rats	75%	64%	31%	92.5%
Microscopy	56%	98%	88%	91.2%

The calculated Kappa value *(K)* (agreement) when Sniffer rats and microscopy were used together was 0.6.

Table 9. Agreement of PCR and sniffer rats for 161 culture positive Samples tested.

	Rat Pos	Rat Neg	Total
PCR Pos	108	19	127
PCR Neg	12	22	34
Total	120	41	**161**

Sensitivity of trained sniffer rats technique was significantly higher (75%) compared to microscopy (56%) (P < 0.0076). And specificity of sniffer rats was significantly lower (64%) compared to microscopy (98%) (P< 0.0001). The positive predictive value (PPV) of trained sniffer rats and microscope was 31% and 88% and the negative predictive values (NPV) were 92.5% and 91.2% respectively (**Table. 8).**

The PCR was used to amplify species specific DNA fragments for identification of *mycobacterium species* isolated from culture. A total of 162 specimens which were culture positive were subjected to mycobacterium genus typing, where by 127 isolates **(Table. 9)** produced two bands of 1030bp and 372bp size (**Fig. 5**) which are specific for mycobacterium *genus* and also specific group for the *M. tuberculosis complex* respectively

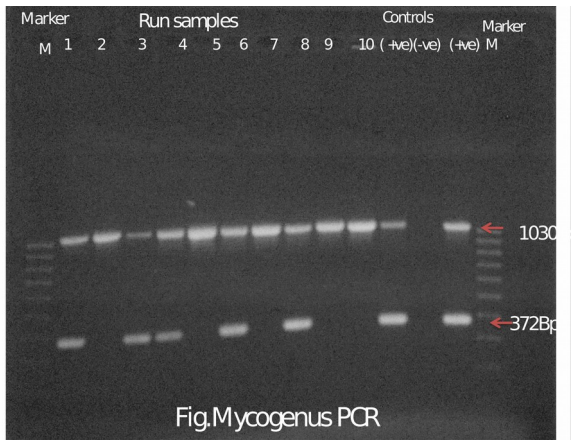

Figure 5. **Mycogenus PCR product of 1030bp and 372bp.**

The remaining 34 were unknown microganisms and were not further characterized. The mycobacterium isolates that were known to be from the group of the *M. tuberculosis complex* were further characterized by deletion typing PCR into their specific species, where by all 127 produced a single band of 396bp size and belong to specific species *mycobacteria tuberculosis* **(Fig.6).** Out 161 culture positive, 127 (78.8%) isolates of

mycobacteria tuberculosis were detected by PCR and 108(67%) isolates were detected by both sniffer rats and PCR **(Table. 9).**

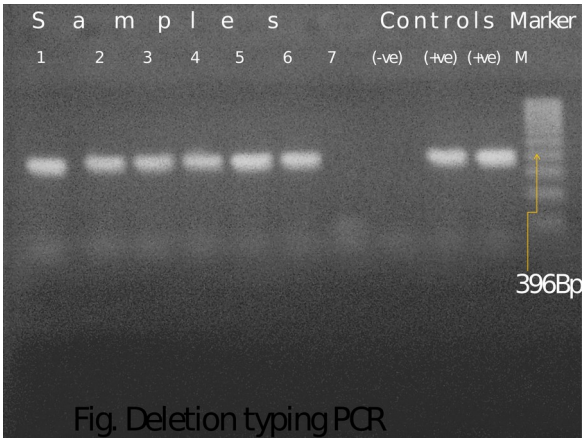

Figure 6. **Deletion typing PCR products of 396bp.**

CHAPTER FIVE

5.0 DISCUSSION

This study of evaluating the performance of sniffer rats in detection of human cases of TB was important based on the fact that, the method can be used as a bio- sensor or screening tool for the detection of PTB in humans. The rats consistently differentiated *M. tuberculosis* infected sputum from non-infected sputum samples (Weetjens *et al.*, 2009).

A case study conducted on 15,041 sputum samples collected from four selected DOTS centres in Dar es Salaam, Tanzania from January 2008 to May 2009, ZN microscopy detected a total of 1,838 (12.2%) samples whereas sniffer rats detected 2,415 sample (16.1%) (Weetjens *et al.*, 2009b). In this study, 10 adult *Cricetomys* evaluated 910 sputum samples collected from patients suspected for PTB. Out of 910 samples, 161 (17.7%) were culture positive, 90 (9.9%) were positive by microscopy and 120 (13.2%) were positive by sniffer rats as confirmed by culture. These two studies shows similar increased case detection by sniffer rats method compared to microscopy. Also in the study which was comparing culture and the use of multiple sniffer rats (18 rats in the case) the results showed that, the mean sensitivity increased to 86.6%, while the specificity decreased to 89.1%. The positive predictive value (PPV) was 41.4% and the negative predictive value (NPV) was 98.7% (Weetjens *et al.*, 2009b). The sensitivity of trained sniffer rats in this study was significantly higher (75%) than that of microscopy (56%) ($P< 0.0076$). Also, the specificity of the sniffer rats was 64% and that of microscopy was 98% ($P< 0.0001$). The positive predictive value (PPV) of the sniffer rats and microscopy were 31% and 88% respectively and the negative predictive value (NPV) was 92.5% and 91.2% respectively (**Table. 8**). This study has shown that the use of multiple rats significantly improves the sensitivity, but reduces the specificity. Also, there

was moderate agreement when the Microscopy and sniffer rats method were used together (*K*= 0.6). The majority of TB positive samples were detected by trained sniffer rats and, also detected on culture (120 specimens). Out 161 culture positive, 127 (78.8%) isolates of *mycobacteria tuberculosis* were detected by PCR and 108(67%) isolates were detected by both sniffer rats and PCR **(Table. 9).** This confirms the high ability (67%) of sniffer rats in consistently differentiating *M. tuberculosis* from other microorganisms (Weetjens *et al.,* 2009). However in the case of samples which were positive on trained sniffer rats but negative culture (266 specimen), the possibility of dealing with a population of dead bacilli from patients who could have already been on treatment, cannot be excluded On the other hand the possibility of false negative (41 specimens) from trained sniffer rats couldn't also be excluded (Poling *et al.,* 2010).

These rats have a long lifespan (up to 8 years in captivity) and are widely distributed in sub-Saharan Africa. They are docile and can easily be domesticated and trained to do repetitive works and have shown to be resistant to TB. The use of trained sniffer rat technique to detect TB appears to be reliable, potentially cheaper, faster and more sensitive than sputum smear microscopy. One rat can screen 140 samples in 40 minutes. The evaluation setup can therefore process up to 1500 samples per day (weetjens *et al.,* 2009b), while microscopist can process up to only a maximum of 40 samples per day whereas WHO recommends an average of 20 samples per day (WHO, 2009a).

The use of sniffer rats greatly improves the diagnosis value of the sputum especially in patients with a low density of bacilli that are likely to be missed on stained smear microscopy technique. The method is economical in both time and expense and is recommended for laboratories handling large numbers of sputum specimens. When dealing with trained sniffer rats, skill is essential for carefully handling of the samples,

the failure of which could lead to a high false positivity rate. It is therefore advisable to have all doubtful samples be smeared and counterchecked microscopically by more experienced senior technicians, this is an additional advantage of trained sniffer rats as the same sample can be applicable to other techniques including microscopy technique. However the smear for microscopy must be prepared from the fresh samples as the attempt to prepare the smear from inactivated samples has shown failure due to heating effect that destroys the nature of the organisms, and hence causing poor smear and staining.

The study also suggests that, there is a need for trained sniffer rats technique and microscopy technique to be used together simultaneously**.** In fact, this approach with two different techniques will indirectly demonstrate the reliability of the techniques in the laboratory. The high consistency of the two techniques will resolve a pre-eminent question of quality assurance, using a system of internal quality control without resources to culture or external proficiency testing. Moreover, this technique will need to be tested by operational research in the country like Tanzania whereby 64, 267 cases of tuberculosis all forms have been notified (NTLP, 2009) hence meeting the WHO stop TB strategy of developing new diagnostic tools and enable their timely and effective use (WHO, 2006). Designing and conducting locally relevant operational research in the field can help in identifying challenges and workable solutions of these new tools. For this purpose, collaboration between National Tuberculosis Programme managers and researchers is essential (WHO, 2009b).

In conclusion, the advantages and limitations of each available TB diagnostic method are evident and no test is yet available that meets target specification. Therefore, the sniffer rats technique is recommended be used as screening tool in high TB incidence

regions that also have a high incidence of HIV co-infection. Detection of TB using rats can significantly increase the current levels of TB detection, the effectiveness of public health control and the reduction of TB transmission in developing countries in general, and sub-Saharan Africa in particular.

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APPENDIX: Ethical Clearance

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28th October 2011

Bart Weetjens
Founder APOPO vzw
C/O Dr Amos Kahwa
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APPROVAL FOR EXTENSION OF ETHICAL CLEARANCE

This letter is to confirm that your application for extension on the already approved proposal: Training African giant pouched rats as a cheap tool for early diagnosis of Tuberculosis (*Weetjens B et al*), has been granted ethics clearance to be conducted in Tanzania.

The extension approval is based on the progress report dated 29th September, 2011 on the project, Ref NIMR/HQ/R.8c/ Vol. I /89. Extension approval is valid until 26th August, 2012.

The Principal Investigator must ensure that other conditions of approval remain as per ethical clearance letter. The PI should ensure that progress and final reports are submitted in a timely manner.

Name: Dr Mwelecele Malecela

Signature

CHAIRPERSON
MEDICAL RESEARCH
COORDNATING COMMITTEE

Name: Dr Deo Ntasiwa

Signature

CHIEF MEDICAL OFFICER
MINISTRY OF HEALTH
AND SOCIAL WELFARE

CC: RMO
DMO