Determination of *Mycobacterium tuberculosis* odour compounds detected by *Cricetomys gambianus* rats for diagnosis of pulmonary tuberculosis in low-income settings

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For my beloved parents, wife and children

DECLARATION

I declare that the work presented in this thesis is my own work and that it has not been submitted anywhere for any award. Where information from other sources and collaboration was used, it has been indicated with references and acknowledgement.

Georgies Mgode **Berlin, 14 November 2011**

ABSTRACT

Novel methods for rapid diagnosis of tuberculosis (TB) are urgently needed to complement the widely used smear microscopy in low income settings. Trained TB detection rats offer a promising tool for rapid diagnosis of TB in resource limited settings. The *Mycobacterium tuberculosis* (*Mtb*) specific volatile compounds detected by trained *Cricetomys gambianus* rats in sputum of TB patients are unknown. It is also not known whether rats detect odour signals from other mycobacteria and microorganisms related to *Mtb*, which cause pulmonary infections resembling TB. In this thesis I investigated *Mtb*-specific volatile compounds detected by trained rats and whether the target compounds are found in other mycobacterial species and related pulmonary pathogens. The ability of rats to discriminate cultures of different microorganisms, clinical sputa with different *Mycobacterium* spp., *Mtb* genotypes and other respiratory tract microorganisms was investigated. Finally, the ability of the rats to discriminate *Mtb*-specific odour compounds from shared compounds found in *Mtb* and other microorganisms was determined. Results show that *Mtb* produce specific volatile compounds which are not produced by other mycobacterial species, mycobacteria-related *Nocardia* spp. and *Rhodococcus* spp. and other microorganisms. Trained rats use these *Mtb*-specific compounds to distinguish TB-positive sputa from TB-negative sputa. Volatile compounds shared by *Mtb* and other mycobacteria and non-mycobacterial species are not detected by trained rats. A blend of *Mtb*-specific compounds is detected by rats better than individual compounds. Rats can discriminate cultures of *Mtb* from those of other microorganisms. Detection of *Mtb* cultures is influenced by growth stage. Rats detect more frequently TB-positive sputa than negative sputa spiked with *Mtb* or specific volatiles. Clinical sputa containing *Mtb* are well discriminated by rats from sputa containing other microorganisms. Sputa containing different *Mtb* genotypes are also detected. It is concluded that trained rats can reliably diagnose TB. Further studies are needed to determine the optimal combinations/ratios of *Mtb*-specific volatile compounds to yield similarly higher responses of rats as those of detection of typical TB-positive sputa. Future studies should assess detection of sputa with frequent nontuberculous

mycobacterial species. Further investigations including recovery of dormant *Mtb* in sputum may give insights on actual cause of detection of false positive sputa currently judged by microscopy and conventional *Mtb* culture in which dormant *Mtb* cannot grow.

ZUSAMMENFASSUNG

In Regionen mit geringem Einkommen sind neue Methoden zur Schnelldiagnose der Tuberkulose (TB) dringend erforderlich, auch in Ergänzung zur hauptsächlich genutzten Mikroskopie. Ratten, die speziell auf die Erkennung von TB trainiert wurden, bieten eine vielversprechende Möglichkeit zur schnellen TB-Diagnose gerade in Regionen mit limitierten Ressourcen. Die spezifischen flüchtigen Verbindungen von *Mycobacterium tuberculosis* (*Mtb*), die im Sputum von TB Patienten von *Cricetomys gambianus* Ratten erkannt werden, sind bislang unbekannt. Ebenso ist nicht bekannt, ob diese Ratten Geruchssignale von anderen Mykobakterien und *Mtb*-ähnlichen Mikroorganismen, welche ebenfalls Lungenerkrankungen auslösen, erkennen. In dieser Arbeit wurden die *Mtb*-spezifischen flüchtigen Verbindungen untersucht, welche von Ratten erkannt werden. Desweiteren wurde geprüft, ob diese Verbindungen auch von anderen Mykobakterien-Arten abgegeben werden. Dabei wurden klinische Sputen mit verschiedenen *Mycobacterium* spp, *Mtb* Genotypen und anderen Mikroorganismen der Atemwege verwendet. Schließlich wurde die Fähigkeit der Ratten untersucht, *Mtb*-spezifische Verbindungen von anderen verwandten Verbindungen aus *Mtb* und anderen Mikroorganismen zu unterscheiden. Die Ergebnisse zeigen, dass *Mtb* spezifisch flüchtige Substanzen bildet, die in anderen Mykobakterien sowie den mykobakterien-ähnlichen Bakterieren *Nocardia* spp. und *Rhodococcus* spp. und weiteren Mikroorganismen nicht vorkommen. Die trainierten Ratten nutzen diese flüchtigen Substanzen, um TB-positive Sputen von TB-negativen zu unterscheiden. Flüchtige Verbindungen, die sowohl von *Mtb* als auch von anderen Mykobakterien produziert werden, ignorieren die Ratten. Ein Gemisch dieser Substanzen wird von den Ratten deutlich besser erkannt, als einzelne Verbindungen. Die Ratten können weiterhin *Mtb* von anderen Mikroorganismen unterscheiden. Die Detektionsrate wird dabei von der Wachstumphase der Kultur beeinflusst. Die Ratten erkennen schliesslich viel häufiger TB-positive Sputen als negative Sputen, die mit *Mtb* beimpft bzw. mit den flüchtigen Substanzen versetzt wurden. Klinische Sputen, die *Mtb* enthalten, werden von den Raten gegenüber Sputen mit anderen Mikroorganismen unterschieden. Sputen, die verschiedene *Mtb* Genotypen

beinhalten, werden ebenso detektiert. Daher ist die Schlussfolgerung zulässig, dass die trainierten Ratten zuverlässig TB erkennen. Weitere Studien sind erforderlich, um die optimalen Bedingungen und Anteile der *Mtb*-spezifischen flüchtigen Substanzen zu bestimmen, um ähnlich starke Reaktion der Ratten im Vergleich zur Reaktion auf typische TB-positive Sputen zu erhalten. Weitere Untersuchungen sollten die Detektionsrate für Sputen mit häufig vorkommenden nichttuberkulösen Mykobakterien feststellen. Zudem können weitere Untersuchungen, die auch dormante *Mtb* einschließen, Einblicke in die Ursachen für die Detektion falsch-positiver Sputen geben, die durch Mikroskopie und konventionelle *Mtb*-Kultur ermittelt wurden, in denen sich dormante *Mtb*-Erreger nicht anziehen lassen.

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LIST OF ABBREVIATIONS AND TERMS

AFB…….	acid-fast bacilli
BCG ……	Bacillus Calmette-Guérin
CFU ……	viable colony forming unit
DOTS ……	directly observed treatment,short-course
DNA …….	deoxyribonucleic acid
ESAT-6 …….	early secretory antigen target-6
GC/MS ……..	gas chromatography/mass spectrometry
HIV ……	human immunodeficiency virus
MDR …….	multidrug-resistant
MAC …….	*Mycobacterium avium* complex
MST ……..	multispacer sequence typing
MTB ……...	*Mycobacterium tuberculosis*
MTC ……...	*Mycobacterium tuberculosis* complex
NTM ……..	nontuberculous mycobacteria
PBSGG ……...	phosphate buffered saline glucose glycerol
PCR ……	polymerase chain reaction
rRNA ……..	ribosomal ribonucleic acid
16S rRNA ……..	gene encoding RNA component of the smaller subunit of RNA (16S is the rate of sedimentation of RNA in centrifugation (Svedberg units)
rpoB ……...	ribonucleic acid polymerase beta subunit

SUA-APOPO ……	Sokoine University of Agriculture, and Anti-Persoonmijnen Ontmijnende Product Ontwikelling
Sp ……..	species (singular, one species of organism)
Spp ……	species (plural, more than one species)
TB ……	tuberculosis disease

Term	**Meaning**
Rat-positive……..	sputum sample designated as TB-positive by at least two trained *Cricetomys gambianus* rats for TB diagnosis.
Rat-negative…….	sputum sample designated as TB-negative by at least two trained *Cricetomys gambianus* rats for TB diagnosis.
Detected sputum/sputa…….	sputum sample identified (pinpointed) by at least two trained *Cricetomys gambianus* rats in a set consisting of other sputa not identified by the rat. This is the same as rat-positive.
Culture-positive……..…	sputum sample which yield mycobacterial isolates after culturing on Lowenstein Jensen medium.
Culture-negative……..…...	sputum sample which does not yield mycobacterial isolates after culturing on Lowenstein Jensen medium.
Smear-positive……..……..	sputum sample which contain acid-fast bacilli microorganisms in its stained smear examined under microscope.
Smear-negative……..…….	sputum sample which does not contain acid-fast bacilli microorganisms in its stained smear examined under microscope.
Rat……	"rat" in this thesis refers only to trained *Cricetomys gambianus* rat for *Mycobacterium tuberculosis* detection and/or TB diagnosis.

1 INTRODUCTION

Tuberculosis (TB) remains a public health threat, killing more than 1.7 million people per year worldwide (WHO 2010). TB is caused by *Mycobacterium tuberculosis* (*Mtb*) which is the deadliest bacterial pathogen of humans (Snider et al. 1994). The disease is more prevalent in low income countries particularly in Africa, Asia and Eastern Europe (Fig. 1) (http://www.stoptb.org/countries/tbdata.asp). TB disease has major socioeconomic impact and is a drawback in achieving the millennium development goal (MDG) 6 of reducing TB prevalence and deaths by 50% in 2015 compared to 1990 (http://www.who.int/mediacentre/factsheets/fs104/en/).

1.1 Drug resistant tuberculosis

Treatment of TB is to date the most complicated, costly and lengthy among bacterial diseases of humans. The lengthy treatment duration of 6 to 9 months consisting of combination of expensive drugs is due to slow generation time of *Mtb* (20–24hrs) (Cole et al. 1998) and resistance of *Mtb* to many antibiotics (Cole and Telenti 1995). The cell envelope of *Mtb* is highly hydrophobic and acts as an impermeable barrier for most antibiotics. *Mtb* is also equipped with highly conserved genome for resistance which include genes encoding hydrolytic/drug modifying enzymes (Cole et al. 1998). Mismanagement of first-line TB drugs and incompliance to treatment regime is also a major cause of multidrug-resistance (MDR) TB (WHO 2011). MDR TB is estimated to be around 10–20% of all TB cases with only 7% of MDR TB cases detected (WHO 2011). The extensively drug-resistant (XDR) TB is resistant to

first and second-line drugs. The totally drug-resistant (TDR) TB is the worst form of TB (Velayati et al. 2009). TDR TB consists of MDR strains which are resistant to all second-line drug classes (Velayati et al. 2009). The cost of treating MDR TB is 100 times that of normal TB case (Kaufmann 2004). Most of the MDR TB cases are caused by Beijing/w strains of *Mtb*, which is also the most widely spread TB causing genotype family (Glynn et al. 2002). This shows the need for inclusion of the Beijing strains of *Mtb* in any evaluation studies of new TB diagnostic tools.

1.2 Tuberculosis in Africa

Most of the TB cases and deaths occur in developing countries (Dye et al. 1999), where people have limited or no access to quality health services. The prevalence of TB and HIV co-infections is also high in these areas and TB kills more than half of the human immunodeficiency virus (HIV) patients (http://www.who.int/tb/hiv/faq/en/). There are 22 countries worldwide which have highest burden of TB contributing to 80% of global TB cases. Nine of the 22 countries are in Africa (Fig. 1) (http://www.stoptb.org/countries/tbdata.asp).

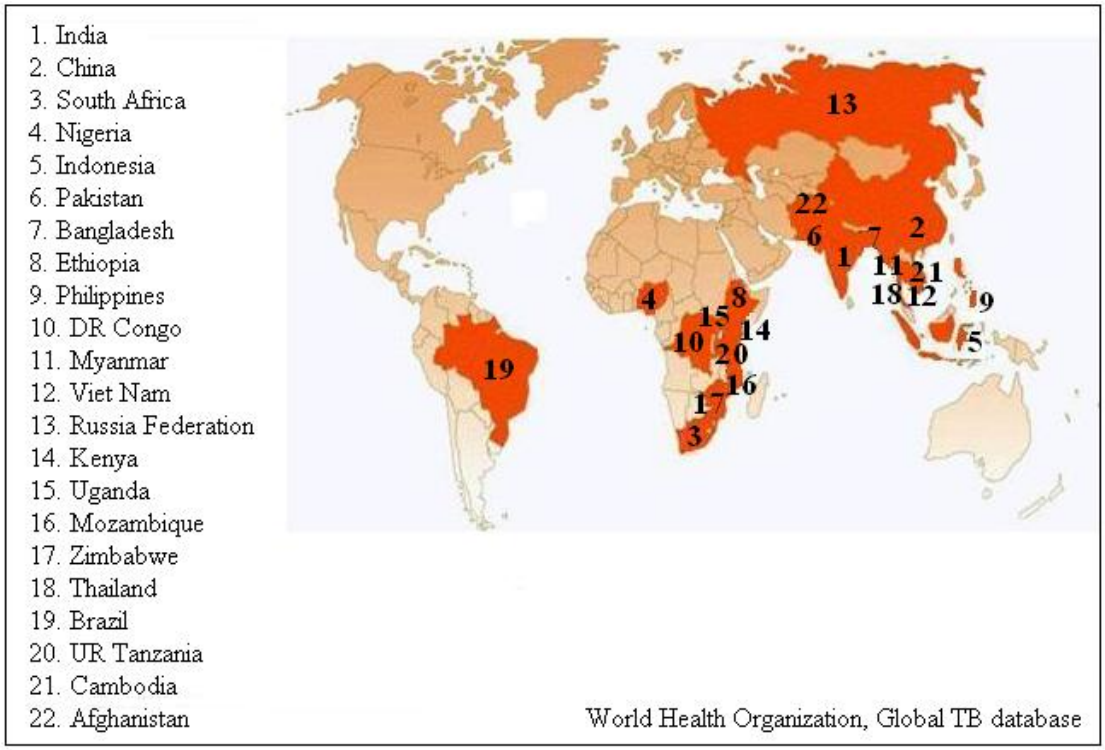

Figure 1 Countries with high TB burden in the world. The 22 countries are ranked based on TB incidences for 2009, adapted from WHO 2010 report.

1.3 Tuberculosis in Tanzania

Tanzania is among the 22 countries with high burden of TB in the world (Fig. 1). A prevalence of 8.5% is reported in TB/HIV co-infected patients in rural areas (Ngowi et al. 2008). The prevalence of invasive disease caused by nontuberculous mycobacteria (NTM) reported from northern Tanzania is also high (9%) (Crump et al. 2009). Diagnosis of TB in Tanzania is by direct microscopy, with recently recorded sensitivity of 62%, detecting only 55% of TB culture-positive HIV-infected patients (Matee et al. 2008). This sensitivity is higher than that of 37% reported in the same country one year before (Mfinanga et al. 2007). This indicates the variability of sensitivity of microscopy and the need for new diagnostic tests which

can increase the case detection. The use of African giant pouched rats (*Cricetomys gambianus*) in TB detection (Weetjens et al. 2009) showed profound increase in case detection in Dar es Salaam, Tanzania (Weetjens et al. 2009b, Poling et al. 2010, Mahoney et al. 2011). Despite the limitation of microscopy, few laboratories in Tanzania culture specimens for mycobacteria identification and drug-susceptibility testing. This limits the knowledge of the circulating mycobacteria (Pfyffer 2007) and the knowledge of prevalent *Mtb* genotypes in pulmonary TB. This knowledge apart from contributing to better management of TB is also required for evaluation studies of performance of novel TB diagnostic tools, such as the TB detection *Cricetomys* rats (Weetjens et al. 2009).

1.4 *Mycobacterium tuberculosis* pathophysiobiology

Mtb is characterized by complex hydrophobic cell envelope which protects it against many antibiotics and harsh environments (acidic, alkaline and oxidative) in phagocytes (Brennan and Draper 1994, Kaufmann 2001). Other characteristics of *Mtb* include intracellular pathogenesis, slow growth and ability to persist in dormant state and reactivate (resuscitation) when the immunity diminishes (Wheeler and Ratledge 1994, Chan and Kaufmann 1994, Wayne and Sohaskey 2001). *Mtb* copes with immune system and manages to survive life-long in host tissues (Kaufmann 2001, Russell 2001) through many mechanisms including arrest of normal maturation of phagosomes (Russell 2001) to favour its survival in macrophages. Other mechanisms include successful competition with host cells in acquiring iron (Collins and Kaufmann 2001, Kaufmann 2001) and switching of dormant *Mtb* to lipid catabolism and nitrate respiration to utilize lipids found in caeseous granulomas

(McKinney et al. 2000). Anti-TB drugs are only effective against metabolically active *Mtb* (Parida and Kaufmann 2010), whereas dormant *Mtb* are not affected by the drugs. Dormant *Mtb* resuscitating into active pulmonary TB involving the lung, contributes to 80% of TB cases (Kaufmann 2008), whereas those entering the blood circulation affect other parts of the body (extrapulmonary) including the brain (meningeal TB). Meningeal TB contributes up to 30% of extrapulmonary TB (Kaufmann 2008). The pathophysiobiology of TB or the infection process of TB and body response to this infection (Fig. 2) provides potential avenues for developing tools for rapid diagnosis of TB based on different markers.

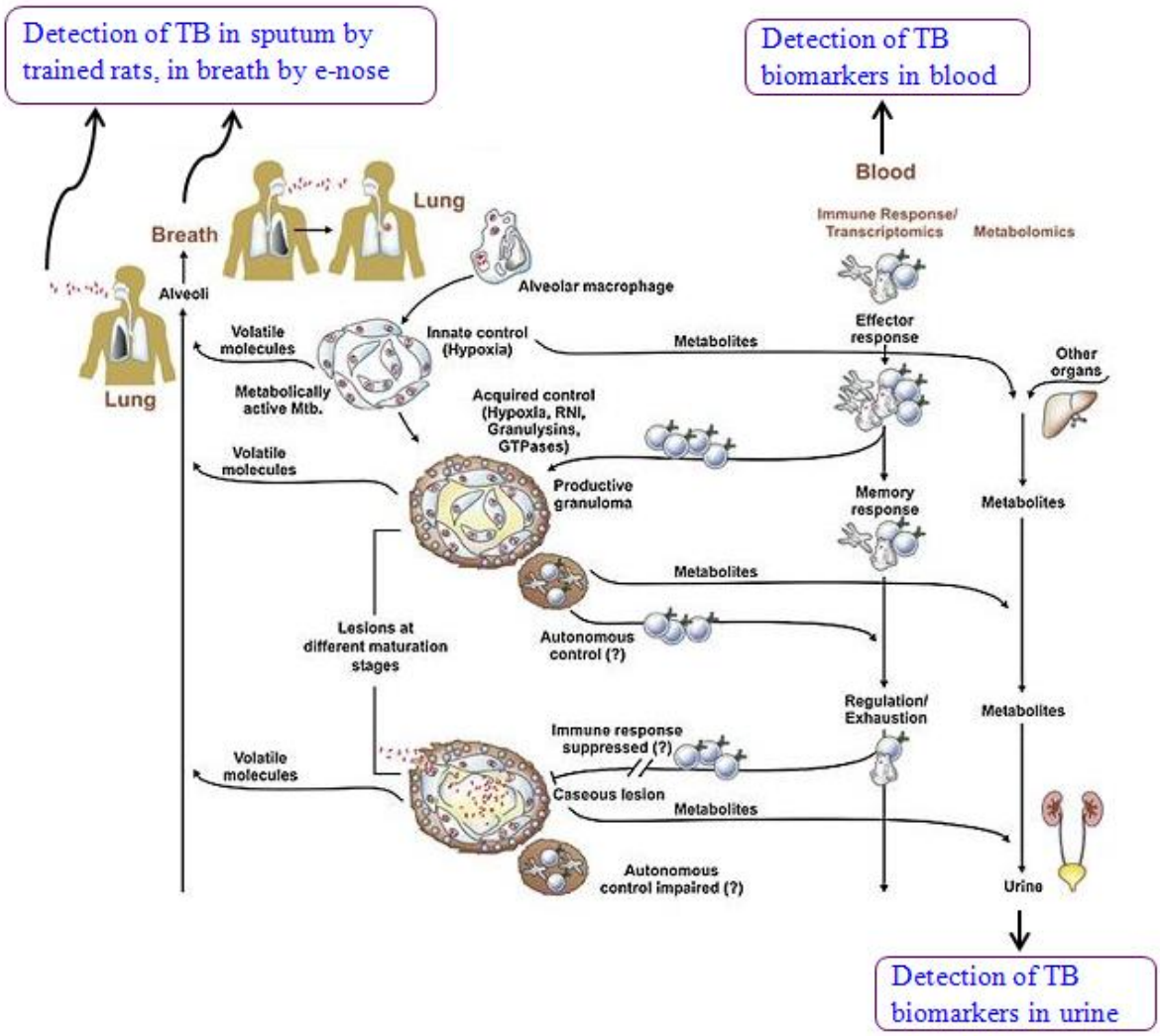

Figure 2 Pathogenesis and defense mechanisms in the lung during latent *Mtb* infection and active TB disease. Potential biomarker avenues for noninvasive diagnosis of TB include breath and sputum samples. Transcriptomic and immunologic biomarkers can be detected in blood. Metabolites in urine can also be targeted for noninvasive TB diagnosis (adapted from Kaufmann and Parida 2008).

1.5 Mycobacteria-related pathogens (*Nocardia* and *Rhodococcus*)

Members of the genera *Mycobacterium*, *Nocardia* and *Rhodococcus* are closely related mycolic acid bearing bacilli which can be misdiagnosed for *Mtb* by acid-fast

staining (microscopy). *Mycobacterium* and *Nocardia* possess extracellular and intracellular siderophores (mycobactin and nocobactin) which are iron carriers enabling them to cope with iron deficiency (Ratledge and Dover 2000). *Nocardia* spp. and *Rhodococcus* spp. are important pulmonary pathogens in immunocompromised populations (Prescott 1991, De La Iglesia et al. 2002, Wadhwa et al. 2006, Martinaud et al. 2011). Cases involving *Nocardia* spp. in pulmonary and central nervous system infections are reported worldwide (Osoagbaka and Njoku-Obi 1985, Jones et al. 2000, Hamid et al. 2001, Martinaud et al. 2011). This relationship shows the need for considering these pulmonary pathogens when evaluating new tools for diagnosis of TB.

1.6 Diagnosis of tuberculosis

1.6.1 Classical microscopy

The classical diagnosis of tuberculosis from the past 129 years is by microscopy. Sputum smear is examined for acid-fast bacilli (AFB) under the microscope after Ziehl-Neelsen (ZN) staining. Microscopy remains the most widely used method for diagnosing TB in low-income countries (Steingart et al. 2006), with the highest burden of TB in the world (http://www.stoptb.org/countries/tbdata.asp). Microscopy method is simple, of low cost and specific for *Mtb* in high burden areas. However, it has relatively low sensitivity varying from 20–60% (Urbanczik 1985, Mfinanga et al. 2007). Performance of microscopy is poorer with sensitivity less than 60% in areas with high prevalence of HIV infections and immunocompromised populations (Perkins and Cunningham 2007). The proportion of smear-negative patients in

TB/HIV endemic areas is usually higher (Elliott et al. 1993, Johnson et al. 1998) indicating that most of the smear negative TB patients are not detected. This is due to increase in extrapulmonary TB in these areas. Sub Saharan Africa contributes to two-third of the global HIV burden (Perkins and Cunningham 2007) and patients co-infected with HIV and TB have few *Mtb* in sputum which cannot be detected by microscopy (Colebunders and Bastian 2000). This indicates that majority of TB patients in this region are misdiagnosed due to smear negativity. It is estimated that one-third of TB cases remain undetected, and in 2009 the case detection rate was only 63% worldwide (WHO 2010). This emphasizes the need for rapid diagnostic tests to increase case detection rate. Fluorescence microscopy (FM) and light emitting diode (LED) microscopes with improved visualization of *Mtb* bacilli in sputum are also used (Marais et al. 2008), but the prices of these microscopes can be an issue in resource-limited settings.

1.6.2 *Mycobacterium tuberculosis* culture

Mtb culture is the gold standard method for confirming TB. However, the slow growth "generation time" of *Mtb* makes diagnosis of TB by culture very lengthy, with up to 8 weeks of incubation. This delay may cause death and further transmission of the disease. Liquid culture system such as the Mycobacteria Growth Indicator Tube (MGIT) and other more sophisticated commercial culture tools which can produce results quicker than the conventional cultures are rarely affordable and sustainable in most resource-limited settings (Perkins and Cunningham 2007). Additionally, conventional cultures have limitation in recovering dormant *Mtb* which are not culturable in absence of resuscitation promoting factors (*rpfs*) in the medium

which induces growth (Mukamolova et al. 2010). Cross-contamination is also a major problem in TB laboratories (Ruddy et al. 2002, de Boer et al. 2002, Djelouadji et al. 2009). Determination of cross-contamination in laboratories in low-income areas can be difficult leading to false diagnosis and treatment (Djelouadji et al. 2009). This underlines the challenges of diagnosing TB in resource-limited settings with the current methods and the need for simpler but accurate diagnostic tools that can improve active case detection in relatively short periods of time.

1.6.3 Xpert MTB/RIF

The Xpert$^{(R)}$ MTB/RIF (Cepheid Inc) is the newest rapid diagnostic test recently endorsed by the World Health Organization (WHO) for use in diagnosis of TB. This nucleic acid based method can detect TB and rifampin resistance in 2 hr (Boehme et al. 2010) and is capable of detecting a high proportion of extrapulmonary TB (Vadwai et al. 2011) which is difficult to diagnose with microscopy. However, the high cost of the Xpert MTB/RIF instrument (USD 17,000) and cartridges (USD 16.8) excluding maintenance costs which may include charges for shipping the equipment from the end user makes this tool not feasible for routine diagnosis of TB in most resource-limited settings (McNerney and Daley 2011).

1.6.4 Chest radiography (chest x-ray)

Chest radiography (chest x-ray) is another widely used TB diagnostic tool. However, chest x-ray cannot distinguish pathological manifestation of pulmonary TB from that of other pulmonary infections such as nocardiosis which is closely related to TB (WHO 1996). Misdiagnosis of pulmonary nocardiosis and TB may lead to

unnecessary treatment with anti-TB drugs. X-ray can also not distinguish previous (treated) TB from active TB and detect early stages of this disease. Unavailability of x-ray in most peripheral health centres and ambiguous interpretation of results is another limitation of this diagnostic tool (WHO 2007).

1.6.5 Tuberculin skin test (TST)

Tuberculin skin test (TST) is widely used to screen *Mtb* infection in large populations. However, this test cannot distinguish between active TB and latent TB (Lalvani 2007); it is also not specific due to cross-reaction with antibodies against *M. bovis* including previous BCG vaccination, and antibodies against nontuberculous mycobacteria spp. (Paul et al. 1975, Farhat et al. 2006).

1.7 Potential biomarker based tuberculosis detection

1.7.1 Tuberculosis odour markers

Successful treatment and control of TB depends on accuracy and capacity to diagnose TB (http://www.WHO.int/tb/laboratory/en). Biomarkers, defined as measurable characteristic features that can indicate normal or abnormal biological processes (Biomarker Definition Working Group 2001), show potential for TB diagnosis and may enhance achieving the global goal of reducing TB prevalence and deaths by 50% in 2015 compared to 1990. (http://www.who.int/mediacentre/factsheets/fs104/en/). Recent studies show that TB can be detected in sputum from TB patients by trained *Cricetomys gambianus* rats in relatively short period of time (Weetjens et al. 2009). Similar studies show that

cancers can also be detected by trained dogs targeting volatile compounds specific for prostate cancer in urine (Cornu et al. 2011), lung and breast cancer (McCulloch et al. 2006) and colorectal cancer in breath and faeces (Sonoda et al. 2011). A number of tools for rapid detection of TB in breath samples are also being developed (Perkins and Cunningham 2007). These include electronic devices (e-nose) for detection of TB odour markers in breath samples (Fend et al. 2006, Phillips et al. 2007, Syhre et al. 2009, Phillips et al. 2010, Kolk et al. 2010). However, lack of robustness and reproducibility, and low sensitivity and specificity of these devices (Knobloch et al. 2009 and 2009b, Kolk et al. 2010; Phillips et al. 2010) remains obstacles for application of these tests. The higher price of breath analysis equipments may also limit application of these electronic tools in resource-limited settings (McNerney and Daley 2011).

1.7.2 Metabolomic TB biomarkers

Metabolomics, which can be defined as the systematic study of metabolites of specific cellular processes (http://en.wikipedia.org/wiki/Metabolomics), can provide useful information about TB infection and open up new avenues for control of disease (Kaufmann and Parida 2008). Shin and co-workers (2011) reported metabolic changes in lung and serum of mice infected with *Mtb* as compared to uninfected mice. There was a distinct increase of 16 metabolites and decrease of 5 metabolites, with *Mtb* infection in mice. Remarkable changes in metabolites occurred in lungs, which is the target organ of *Mtb* (Shin et al. 2011). The study of TB biomarker (metabolites) in human urine shows that TB patients can be differentiated from healthy individuals by increased levels of isopropyl acetate and *o*-xylene and

decreased levels of cymol, 2,6-dimethylstyrene and 3-pentanol in TB patients (Banday et al. 2011). Profiles of these metabolites can also distinguish TB from lung cancer and chronic obstructive pulmonary disease. However, the requirement for expensive GC/MS apparatus for analysis of headspace samples of urine limits the application of this diagnostic method in resource-limited settings.

1.7.3 Immunologic TB biomarkers

Immunological markers such as immune responses to *Mtb* antigens (ESAT-6, CFP-10 and TB7.7) are also used to detect TB (Parida and Kaufmann 2010). Quantiferon (QFT)-TB-gold and T-spot assays can diagnose latent TB by measuring the amount of interferon gamma (IFN-γ) produced in the reaction of white blood cells against *Mtb*-specific antigens (Streeton et al. 1998, Meier et al. 2005, Menzies et al. 2007). However, certain nontuberculous mycobacteria with genes encoding ESAT-6 antigen such as *M. kansasii*, *M. marinum*, *M. flavescens*, *M. gastrii* and *M. szulgai* (Harboe et al. 1996) cross-react with T-spot test (Menzies et al. 2007). This indicates further the challenges of diagnosing TB.

1.7.4 Transcriptomic TB biomarkers

Transcriptomic refers to the study of the RNA transcripts of a cell, tissue, or organism (http://www.encyclopedia.com/doc/1O6-transcriptomics.html). Host transcriptional biomarkers have potential for diagnosis of TB. Rachman and co-workers (2006) demonstrated that *Mtb* found in different sites of the lung express different as well as overlapping gene profiles. This shows that transcriptomics can also provide useful information about biological processes of pathogens in different

host environments. The transcript profiles of an immune response to *Mtb* infection are unique and differ from the response to other antigens such as *M. bovis* BCG vaccine (Mollenkopf et al. 2006). For examples, unlike BCG, *Mtb* infection can activate genes encoding IFN-γ (Mollenkopf et al. 2006). Three differentially expressed genes, namely, CD64, lactoferrin (LTF) and Rab33A were recently reported to discriminate active TB-patients from *Mtb*-infected healthy donors and uninfected healthy ones (Jacobsen et al. 2007). Maertzdorf et al. (2011a, 2011b) also reported successful discrimination of patients with active TB, latent TB and uninfected healthy donors using transcriptional profiles. Transcriptomic patterns of human blood can also show different profiles between patients with active TB and those with other diseases (Berry et al. 2010). Mistry et al (2007) reported nine genes which can differentiate patients with recurrent TB from cured or patients with latent TB, with sensitivity (90%) and specificity (83%). These studies indicate that transcriptomic profiles can aid in the diagnosis of TB in future.

1.8 State of the art

1.8.1 Diagnosis of TB using *Cricetomys gambianus* rats

1.8.1.1 Olfactory detection of tuberculosis using *Cricetomys* rats

The olfactory detection of *Mtb* using trained *Cricetomys gambianus* rats (Weetjens et al. 2009) is a novel rapid diagnostic test for TB currently under evaluation (Perkins and Cunningham 2007, McNerney and Daley 2011). This novel technology enables rapid detection of TB with higher sensitivity and specificity of more than 86% and

91%, respectively (Weetjens et al. 2009 and 2009b, Poling et al. 2010). Active TB case detection was increased by 43–44% when *Cricetomys* rats were used as second-line screening tool for TB in Dar es Salaam, Tanzania, after smear microscopy in DOTS centres (Poling et al. 2010, Mahoney et al. 2011). This increase has been reported in two studies conducted in 2009 and 2010 whereby in 2009 the rats increased the case detection by 44% after finding 620 additional TB patients previously missed by microscopy in DOTS centres (Poling et al. 2010), and in 2010 the rats increased the case detection by 43% after detecting 716 additional TB cases not detected by microscopy in DOTS centres (Mahoney et al. 2011). This indicates that TB detection by rats is a promising rapid screening test. Major criteria of the good screening test are higher sensitivity and specificity such that the test can detect individuals with disease such as TB and rule-out individuals without the disease. TB detection rats detect sputum samples with broad range of acid-fast bacilli (AFB) counts ranging from 1–9 AFB, 1+, 2+ to 3+. One rat can screen 140 sputum samples in 40 min (Weetjens et al. 2009), which is much faster than the smear microscopy in which one microscopist can analyze an average of 20 samples per day based on recommendations of the WHO, which aim to maintain the quality of smear microscopy results (WHO 2005). This indicates that TB detection rats can be used to screen TB in high burden areas in a short period of time and enhance early case detection and reduce transmission.

1.8.2 African giant pouched rats (Genus *Cricetomys*)

Cricetomys gambianus Waterhouse, 1840, also known as Gambian rats, and other *Cricetomys* spp. are rodents of the Order: Rodentia; Family: Nesomyidae, found in

diverse habitats in many parts of sub Saharan Africa (van der Straeten et al. 2008). To date, *Cricetomys* spp. have been reported in 32 African countries on three geographical sides of the continent except the northern part which has a desert. This indicates the wider distribution and adaptation of *Cricetomys* rats to different habitats. Outside Africa, *Cricetomys* rats are kept as pets in Europe and North America where they even became invasive species in some parts (Perry et al. 2006, Peterson et al. 2006, Engeman et al. 2006 and 2007). This indicates further the ability of *Cricetomys* rats to adapt to different climates, which shows that they can be used as TB detectors in many countries with different climatic condition. These rats can live longer in captivity. Currently, trained *Cricetomys* rats have been performing TB detection work for 8 years now (APOPO TB detection laboratory, *unpublished data*). Thus, once trained, *Cricetomys* rats can serve as rapid TB detectors for a prolonged period of time. Training of the rats starts with 4 weeks old young ones and it takes 6 to 9 months for the rat to qualify to detect TB (Weetjens et al. 2009). The average cost of training one rat is 6000 euro. This includes all aspects of training, evaluation and care. The monthly feeding and healthcare costs for one *Cricetomys gambianus* rat is 5 euro (http://www.apopo.org/cms.php?cmsid=44&lang=en#18).

1.8.3 Olfactory capacity of *Cricetomys* rats

Rodents, to which *Cricetomys* rats belong, have the highest number of olfactory receptor (OR) genes among mammalian species (Gilad et al. 2004, Niimura and Nei 2007, Nei et al. 2008, Hayden et al. 2010). The capacity of rats' olfactory detection and discrimination can remain intact even after removal of 80% of the glomerular layer in the olfactory bulb (Bisulco and Slotnick 2003). This highly developed sense

of smell is certainly what enables successful conditioning of *Cricetomys gambianus* to accurately detect TB in human sputum with higher sensitivity and specificity.

1.9 Problem statement

Novel methods for rapid diagnosis of TB are urgently needed to complement the widely used smear microscopy in low-income settings. TB detection rats' technology is the most promising diagnostic tool for rapid detection of TB in resource-limited settings. The *Mtb*-specific volatile compounds detected by trained *Cricetomys gambianus* rats in sputum of TB patients are not yet known. The knowledge of odour compounds detected by these rats is important for evaluation and understanding the reliability of this technology. It is not known whether the rats detect odour signals from other sources such as other mycobacteria and microorganisms related to *Mtb*, which can cause pulmonary infections resembling TB. This knowledge is important because the diagnosis of TB in HIV endemic areas has become difficult due to smear negativity, and there is increasing occurrence of NTM and other related microorganisms which may also produce target odour in sputum.

1.10 Hypotheses

The underlying hypotheses of this thesis are the following:

- *Mtb* produces specific volatile organic compounds (odour) in a unique composition.
- Trained *Cricetomys gambianus* rats utilize the unique *Mtb* odour to discriminate TB-positive sputum from TB-negative sputum.

1.11 Objectives of this thesis

The purpose of this thesis was to identify the target volatile compounds of *Mtb* detected by trained *Cricetomys gambianus* for TB diagnosis and to determine whether other microorganisms related to *Mtb* such as the NTM, *Nocardia* spp., *Rhodococcus* sp., *Streptomyces* spp. and many other respiratory tract microorganisms, which are also associated with pulmonary infections in humans, confound *Mtb* detection in sputum samples by rats.

1.11.1 Specific objectives

The specific objectives of this thesis were:

- To determine *Mtb*-specific volatile organic compounds detected by trained TB detection *Cricetomys* rats; in reference *Mtb* and other microorganisms under different conditions.
- To determine whether the target volatile compounds are specific to *Mtb* and are found in other mycobacteria species and related pulmonary pathogens.
- To determine the extent and ability of *Cricetomys* rats to discriminate clinical sputum samples with different *Mycobacterium* spp. (*Mtb* and NTM) and other microbes of the upper and lower respiratory tract.
- To determine *Mtb* genotypes in clinical sputum samples and evaluate the performance of *Cricetomys* rats in samples containing different *Mtb* genotypes.
- To evaluate the ability of rats to discriminate cultures of reference mycobacterial species and related microorganisms.

- To determine whether trained rats can discriminate *Mtb*-specific odour compounds from shared compounds found in *Mtb*, other mycobacteria and respiratory tract microorganisms spiked into negative TB sputum.

1.12 Thesis outline

The introduction part of this thesis provides information about TB problem in the world, in Africa and Tanzania. The study of the novel method of diagnosing TB using *Cricetomys gambianus* rats is conducted in Tanzania. An outline of approaches for TB diagnosis currently in use and under development is provided. The pathogenicity and survival mechanisms of *Mtb* in host tissue are briefly introduced and literature on potential application of biomarkers in diagnosis of TB is provided. The material and methods part gives an account of methods used to address the research questions. Well established methods elaborated elsewhere such as polymerase chain reactions (PCR) are mentioned with provision of oligonucleotides (primer) sequences and references. The assessment of genetic diversity of mycobacteria in the study area, and evaluation of detection of sputa containing different mycobacteria and genotypes of *Mtb* by rats is described. Subsequent methods depict determination of respiratory tract microbes in sputa, and evaluation of the ability of rats to discriminate sputa with non-mycobacterial species. Furthermore, evaluation of the rats' ability to discriminate sputa spiked with pure cultures of different microbes is also recounted. Methods for identification of volatile compounds from different microbes and testing of candidate compounds by rats to determine *Mtb*-specific odour compounds detected by rats are also stated.

Interpretations and discussion of findings of this thesis with emphasis on implication in the diagnosis of TB using trained rats is provided as well.

2 MATERIAL AND METHODS

2.1 Population and study area

A total of 289 individuals attending six selected TB clinics (i.e. Dar es Salaam = 5; Morogoro = 1) donated 514 sputum samples analyzed in different parts of this study. Individuals donated two to three samples at TB clinics of which one to two samples were enrolled in the study. The age of sputum donors (150 males and 139 females) varied from < 1 year to 86 years (mean ± standard deviation, 32 ± 3 years). A gender ratio (male: female) of 1.08 which is closer to Tanzania's national gender ratio (male/female = 0.98) was obtained. The specimens were part of a large sample size collected for training TB detection rats at Sokoine University of Agriculture, Morogoro, Tanzania (SUA-APOPO). The TB clinics in Dar es Salaam, namely Amana, Magomeni, Mwananyamala and Tandale, are located in different catchment areas, and hence likely serve diverse populations and provide sufficient geographic coverage of Dar es Salaam city (Fig. 4). Morogoro TB clinic at Morogoro hospital is located 200 km west from Dar es Salaam. This study is part of SUA-APOPO study on the application of trained *Cricetomys gambianus* rats in the diagnosis of pulmonary TB approved by the Ethics Committee of the National Institute for Medical Research (NIMR, Tanzania).

2.2 *Mycobacterium* species diversity

2.2.1 Specimens

Over the March–June 2009 study period, a total of 252 sputum specimens were collected from 161 individuals attending four selected DOTS centres in Dar es Salaam, from a larger sample size of sputa for routine training of the TB detection rats at the SUA-APOPO TB laboratory. The proportions of patients recruited per DOTS centre were: Magomeni (11.3%); Tandale (15%), Amana (18.1%) and Mwananyamala (55.6%). The DOTS centres, which provided large numbers of sputum samples for TB detection by rats, had higher proportion of patients included in this study.

Qualitative (sputum not saliva) and quantitative (volume) criteria were used for sample selection, whereby only samples with sufficient volume to provide aliquot for culture ($\geq$ 1 ml) and adequate amount ($\geq$ 3 ml) for TB detection by rats were included in this study. This enabled determination of rats' performance on sputa containing different mycobacteria. Sputa with insufficient volumes for the two tests were excluded in this study but not for routine experiments of TB detection by rats. Sputum aliquots for cultures were aseptically transferred using sterile disposable transfer pipettes into sterile screw-capped microtubes and the remaining volume was processed for routine training of the TB detection rats as described by Weetjens et al. (2009). Briefly, during training sessions, rats were rewarded with food (mashed banana mixed with crushed commercial rat food) when they paused for 5 s at known TB-positive sputum samples (positive control). The rats did not receive food for pausing at known TB-negative samples. With extensive training the rats learned to

consistently pause at TB-positive samples but not at TB-negative samples. Similarly, during the reward condition in the present study, identification responses to the TB-positive sputa (reward samples) were followed by food delivery, and food was not presented on indication of any other samples because there was no prior knowledge of mycobacterial presence in these samples.

2.2.2 Processing of sputum for TB detection by rats

Sputum aliquots (≥ 3 ml) in autoclavable polypropylene sputum containers (55 ml capacity) with lid were processed for TB detection rats by adding 5 ml of phosphate buffered saline (PBS) to increase the volume of sputum and avoid drying of the sputum sample during heat inactivation. The sputum sample (≥ 8 ml including PBS) was heat inactivated at 90 °C for 30 min. Samples were cooled to room temperature and stored at –20 °C until later use in routine TB testing by trained rats.

2.2.3 Mycobacterial culture

Sputum samples were cultured on Lowenstein Jensen medium with pyruvate and Lowenstein Jensen with glycerine after standard decontamination with 4% sodium hydroxide (NaOH) added to sputum in a 1:1 ratio, mixing well and leaving to stand for 45 min for sodium hydroxide to act. The mixture was centrifuged at 3000 *g* for 20 min, supernatant decanted and neutralization of the sediment was performed with 14% potassium dihydrogen phosphate (KH_2PO_4). Cultures were incubated at 37 °C for a minimum of eight weeks with weekly observation for growth (WHO 1998).

2.2.4 Identification of isolates

Isolates were stained by standard Ziehl Neelsen (ZN) method to determine the acid-fastness which is characteristic of mycobacteria and related *Nocardia* spp.

2.2.4.1 Molecular analyses – specific PCR and *rpo*B gene sequencing

DNA was extracted from all acid-fast bacilli by the bead beating method (Tell et al. 2003) and subjected to multiplex real-time PCR for preliminary affiliation to the genus *Mycobacterium*, the *Mtb* complex (MTC) and the *M. avium* complex (MAC) according to Shrestha and co-workers (2003) and Richardson et al. (2009). MAC isolates were analysed by *rpo*B gene sequencing to determine their specific identification using the procedures described by Adekambi and co-workers (2003) and Cayrou et al. (2010). An additional conventional PCR for all *Mycobacterium* spp., MTC, MAC and *M. intracellulare* was also performed according to Wilton and Cousins (1992).

2.2.4.2 Multispacer sequence typing (MST) for *M. tuberculosis* genotypes

MTC isolates were further analyzed by multispacer sequence typing (MST) by PCR-sequencing the MST 4, MST 11, MST 12 and MST 13 spacers as described by Djelouadji and co-workers (2008). This analysis was undertaken in collaboration with M. Drancourt (URMITE UMR, Marseille, France).

2.2.5 Rats detection of sputa with different *Mycobacteria* and *M. tuberculosis* genotypes

The performance of rats on mycobacteria culture-positive sputum specimen was determined by examining results of all rats which analysed the sputum samples cultured for mycobacteria. These results were obtained from the SUA-APOPO TB detection laboratory database in which all information about specimens, number of rats which tested the specimens, and numbers of correct hits (positive detection) and misses (no detection) are kept. A cut-off point of two rats was used to classify sputum samples as detected (rat-positive) or not detected (rat-negative). This cut-off point was also applied on clinical samples routinely tested by these rats. A minimum of 6 rats from a group of 22 rats which were already trained to detect TB participated in analysis of sputum samples in this study.

The procedure for training *Cricetomys* rats to detect TB in sputum and judging of positive and negative results of the rats has been described in detail elsewhere (Weetjens et al. 2009, Poling et al. 2010). The performance of the rats was expressed as proportion of detected sputum with species or genotypes divided by total number of sputum with that species or genotype.

2.2.6 Data validation and analysis

All PCR-based experiments were validated by inclusion of positive control DNAs of MTC and negative controls consisting of distilled water, at a ratio of one negative control per three specimens. Numerical data were compared using Chi-squared (χ^2) test and a P value < 0.05 was used to establish the statistical significance of different comparisons.

2.3 Isolation of other microorganisms in detected sputum

I determined whether the respiratory tract microorganisms other than *Mtb* such as NTM, *Nocardia* spp. and *Rhodococcus* spp. which are related to *Mycobacterium* spp. cause the detection of sputum. Different microorganisms were isolated from sputum samples and those from smear-and *Mtb* culture-negative sputa detected by rats were further investigated by analyzing their odour compounds and comparing with those of *Mtb*.

2.3.1 Study population characteristics

Two hundred eighty-nine subjects were included in this study. These were classified into three TB diagnostic categories: (i) confirmed TB: individuals with two smear-positive (AFB+) sputum samples in smear microscopy, and/or -positive mycobacterial (*Mtb*) culture; (ii) suspected TB: individuals with only one smear-positive culture-negative sample (*Mtb*); (iii) non-TB (negative): individuals with smear-negative and *Mtb* culture-negative sputum. Individuals with NTM isolates were classified in the non-TB category.

2.3.2 Specimens

A total of 514 sputa from six selected Tanzanian TB clinics (i.e. Dar es Salaam = 5; Morogoro = 1) were selected for isolation of mycobacteria and other microorganisms and TB detection by a group of 4–10 trained rats as described by Weetjens et al. (2009). Sputum aliquots (1 ml) were used for isolation of microorganisms and the remaining volume ($\geq$ 3 ml) was processed and used for TB detection by rats. The performance of the rats on these samples was matched with isolates found in

respective samples. A sample was considered positive if it was detected by at least two rats. Isolates from smear-negative and *Mtb* culture-negative sputum detected by at least two rats (rat-positive) were subjected to further microbiological identification and comparison of volatile organic compounds of the representative isolates with volatile compounds of *Mtb*.

2.3.3 Isolation and identification of mycobacteria

A total of 380 sputa (collected April–June 2009 and July 2010) from 289 donors consisting of 252 sputa (161 donors) which were cultured on Lowenstein Jensen medium (LJ) with pyruvate and LJ with glycerine, and 128 sputa (128 donors) cultured on LJ with glycerine only. Isolates were subjected to ZN staining and molecular identification.

2.3.4 Isolation of non-mycobacterial microorganisms

Four types of media, namely, chocolate agar, sabouraud dextrose agar, buffered charcoal yeast extract agar and paraffin agar (Shawar et al. 1990) were used to isolate *Nocardia* sp., *Rhodococcus* sp., *Streptomyces* sp., *Moraxella* sp., *Streptococcus* sp., and yeasts. *Staphylococcus* sp. and *Enterococcus* sp. from smear-negative sputum samples detected by rats were also collected.

2.3.5 Medium for isolation of non-mycobacterial microorganisms

The media for isolation of non-mycobacterial species were prepared according to manufacturers' instructions or standard procedures for preparation of medium from

individual ingredients. The list of chemical reagents used to prepare the medium for isolation of microorganisms and biochemical reactions is presented in Table 1.

2.3.5.1 Chocolate agar

Chocolate agar with 3.5% haemolysed blood, for isolation of fastidious or microorganisms with high nutritional requirements was prepared by dissolving 40 g of blood agar base (Becton, Dickinson & Co., Sparks, USA) in 1000 ml distilled water and sterilizing at 121 °C for 15 min. After sterilization, the medium was cooled down to 70–80 °C and 35 ml of horse blood was added aseptically. The medium was dispensed into sterile disposable plates (20 ml) and allowed to solidify at room temperature.

2.3.5.2 Sabouraud dextrose agar

Sabouraud dextrose agar for isolation of yeasts was prepared by dissolving 10 g of peptone, 40 g of D(+) glucose monohydrate and 12 g of agar in 1000 ml distilled water. The medium was sterilized by autoclaving at 121 °C for 15 min. The medium was cooled down to 50–55 °C and dispensed into sterile disposable plates (20 ml) in which agar was allowed to solidify at room temperature.

2.3.5.3 Paraffin agar

This carbon free medium for isolation of *Nocardia* spp. was prepared according to Shawar et al. (1990). The medium contained 9 parts of carbon free agar and 1 part of paraffin oil. Carbon free agar was prepared by dissolving the following chemical reagents into 1000 ml distilled water: potassium dihydrogen phosphate (3 g),

dipotassium phosphate (1 g), ammonium chloride (5 g), ammonium nitrate (1 g), ferrous sulphate (0.05 g), magnesium sulphate heptahydrate (0.05 g), manganese sulphate (0.05 g), zinc sulphate (0.05 g) and agar (17 g). The pH was adjusted to 7.2. The carbon free agar (900 ml) was mixed with 100 ml liquid paraffin and the 9:1 mixture was sterilized by autoclaving at 121 °C for 15 min. The medium was cooled down to 50–55 °C and dispensed into sterile disposable plates (20 ml) in which agar was allowed to solidify at room temperature.

2.3.5.4 Buffered charcoal yeast extract agar

Buffered charcoal yeast extract agar was prepared by dissolving 40 g of buffered charcoal yeast extract agar base (Sigma-Aldrich Chemie GmbH) in 1000 ml of distilled water and autoclaving at 121 °C for 15 min. The medium was cooled down to 50–55 °C, dispensed into sterile disposable plates (20 ml) and allowed to solidify at room temperature. The media were incubated at 37 °C overnight to check sterility before inoculating test samples. Sterile disposable plastic loops were used to inoculate the medium with sputum samples and parafilm was used to wrap the culture plates to prevent loss of moisture during the prolonged incubation at 37 °C for 6 weeks. Sterile (un-inoculated) medium was incubated in parallel with inoculated plates as controls and identification of contaminants.

2.3.6 Identification of non-mycobacterial isolates

Non-mycobacterial isolates were identified by colony morphology, Gram stain, biochemical tests and a few isolates by 16S rRNA sequencing. *Nocardia* sp., *Rhodococcus* sp. and *Streptomyces* sp. were preliminarily identified by growth

characteristics in different media including opacification of the Mycobacteria 7H11 medium (Flores and Desmond 1993) and characteristic formation of chalky white colonies in selective medium. The Gram stained microorganisms were viewed under the microscope (Leica DMLB, Leica Microsystems, Wetzlar, Germany) equipped with Jenoptik, ProgRes CT5 USB C camera for capturing images (Jenoptik Laser Optik Systeme GmbH, Jena, Germany).

2.3.7 Biochemical identification of *Nocardia*, *Rhodococcus* and *Streptomyces* isolates

The biochemical tests used to identify *Nocardia* sp., *Rhodococcus* sp., and *Streptomyces* sp. from sputum samples detected by rats included xanthine, tyrosine, sorbitol, mannitol, casein hydrolysis, aesculine, uric acid, starch, urea and gelatin liquefaction tests recommended by Isik et al. (1999).

The media for biochemical identification of isolates were sterilized by autoclaving at 121 °C for 15 min. Heat degradable medium was filter sterilized using vacuum driven disposable bottle top filter with 0.22 µm membrane filter (Millipore Corp., USA). All media were dispensed into 20 ml Petri dish plates, except urea and gelatin that was dispensed into 15 ml screw capped vials (Sarstedt, Nümbrecht, Germany).

Table 1 Chemicals/reagents used for preparation of culture medium and medium for biochemical tests.

Chemical name	Supplier/manufacturer
Gelatin peptone	Sigma-Aldrich Chemie GmbH
Potato starch	Sigma-Aldrich Chemie GmbH
Christensen's urea agar	Sigma-Aldrich Chemie GmbH
Urea	Biorad
Peptone	Becton, Dickinson & Co
Beef extract	Becton, Dickinson & Co
Xanthine	Sigma-Aldrich Chemie GmbH
L-tyrosine	Sigma-Aldrich Chemie GmbH
Casein	Becton, Dickinson & Co
Sodium chloride	Sigma
Disodium phosphate	Merck
Evans blue	Fluka Chemika
Tryptone	Oxoid
Yeast extract	Becton, Dickinson & Co
Sodium citrate	Sigma-Aldrich Chemie GmbH
Aesculin	Merck
Ferric ammonium citrate	Sigma
D-sorbitol	Sigma-Aldrich Chemie GmbH
Potassium dihydrogen phosphate	Sigma-Aldrich Chemie GmbH
Dipotassium phosphate	Merck
Ammonium chloride	Merck
Ammonium nitrate	Acros Organics
Ferrous sulphate	Sigma-Aldrich Chemie GmbH
Magnesium sulphate heptahydrate	Sigma-Aldrich Chemie GmbH
Manganese sulphate	Sigma-Aldrich Chemie GmbH
Zinc sulphate	Sigma-Aldrich Chemie GmbH
Paraffin, liquid (pure)	Acros Organics
D(+) glucose monohydrate	Merck KGaA
Glycerol (99%)	Sigma-Aldrich Chemie GmbH
Phenol red	n/a
Uric acid	Sigma-Aldrich Chemie GmbH
Dipotassium phosphate	Merck
Magnesium sulphate	Merck
Agar	Becton, Dickinson & Co
D-mannitol	Sigma-Aldrich Chemie GmbH
Neutral red	Merck

2.3.7.1 Xanthine medium

The medium for determining the ability of bacteria to degrade xanthine shown by clear halo around the colony, consisted of gelatin peptone (5 g), beef extract (3 g), xanthine (4 g) and agar (15 g) in 1000 ml distilled water.

2.3.7.2 Tyrosine medium

Medium for determining the ability of bacteria to degrade tyrosine indicated by clear halo around the colony, consisted of gelatin peptone (5 g), beef extract (3 g), tyrosine (5 g) and agar (15 g) in 1000 ml distilled water.

2.3.7.3 Sorbitol medium

The medium for determining the ability of bacteria to ferment sorbitol indicated by pink/red pigmented colonies, consisted of nutrient broth (8 g), D-sorbitol (10 g), neutral red (0.03 g) and agar (12 g) in 1000 ml distilled water.

2.3.7.4 Mannitol medium

Medium for determining fermentation of mannitol indicated by yellow colonies with yellow zones, consisted of nutrient broth (8 g), D-mannitol (10 g), phenol red (0.025 g) and agar (12 g) in 1000 ml distilled water.

2.3.7.5 Casein hydrolysis

Hydrolysis of casein was determined in medium containing casein (10 g), beef extract (3 g), sodium chloride (5 g), disodium phosphate (2 g), Evans blue stain (0.05 g) and agar (15 g) in 1000 ml distilled water.

2.3.7.6 Aesculine hydrolysis

The medium for determining the ability of bacteria to hydrolyze aesculine into aesculetin and glucose indicated by black zones around colonies consisted of tryptone (20 g), yeast extract (5 g), sodium chloride (5 g), aesculine (1 g), ferric ammonium citrate (0.5 g) and agar (10 g).

2.3.7.7 Uric acid medium

Degradation of uric acid (0.4%) indicated by clear zones around colonies was determined in medium containing uric acid (4 g), nutrient broth (8 g) and agar (15 g) in 1000 ml distilled water.

2.3.7.8 Starch hydrolysis

Starch medium for determining starch hydrolysis indicated by colourless or clear zones around colonies after flooding with Gram's iodine was prepared by dissolving 3 g of beef extract, 10 g of potato starch and 12 g of agar in 1000 ml distilled water.

2.3.7.9 Urea medium

Christensen's urea agar was used to prepare urea medium for determining metabolization of urea indicated by change of colour of medium from colourless to

pink red. The medium was prepared according to manufacturer's instruction. Briefly, it consisted of urea agar (21 g) in 950 ml distilled water, sterilized at 121 °C x 15 min and cooled down to 50 °C before adding 50 ml of filter sterilized 40% urea. Urea was filter sterilized using vacuum driven disposable bottle top filter with 0.22 µm membrane filter (Millipore Corp., USA). The mixture was dispensed into 15 ml Sarstedt screw capped vials (Nümbrecht, Germany) and allowed to set in slants.

2.3.7.10 Gelatin liquefaction

Medium for gelatin liquefaction test was prepared according to manufacturer's instructions. Briefly, 38.4 g of nutrient gelatin was dissolved in 300 ml distilled and boiled to dissolve completely. The medium was sterilized at 121 °C x 15 min, cooled down to 60 °C and dispensed into 15 ml Sarstedt screw capped vials (Nümbrecht, Germany) while mixing well. After cooling to room temperature, the medium was allowed to set at 4 °C. Inoculated gelatin medium was incubated at 35 °C for 2 weeks with un-inoculated controls.

2.3.8 Specific PCRs for identification of non-mycobacterial microorganisms

Moraxella sp. and *Streptococcus* sp. from smear-negative sputum samples detected by rats were subjected to species-specific PCR according to Hendolin and co-workers (1997). *Nocardia* spp. isolates were further subjected to specific PCR for *N. asteroides* complex according to Brown et al. (2004) and Hasegawa et al. (2007). These PCRs were selected because *N. asteroides* complex is considered to be responsible for more than 70% of pulmonary nocardiosis (Martinaud et al. 2011). Positive control strains for these PCRs were reference *M. catarrhalis* (DSM 9143),

Haemophilus influenzae (DSM 4690), *S. pneumoniae* (DSM 20566), *N. africana* (DSM 44491), *N. asteroides* (LMG 4062), *Mtb* H37RV, *M. avium* subsp. avium, *M. intracellulare*. HotStar Taq Master Mix kit (Qiagen GmbH) was used in these PCRs. The PCR was run in the PTC-100 Programmable thermal controller (MJ Research, Inc. Watertown, Mass. USA). PCR products were separated by electrophoresis in 1.5% agarose gel stained with ethidium bromide and visualized on UV-transilluminator attached with gel documentation unit.

Table 2 Primer sequences for specific PCRs for identification of *Nocardia* sp., *Moraxella catarrhalis,* and *Streptococcus pneumoniae,* isolates from sputum samples detected by rats.

Primer name	Sequence (5'→3')	Supplier	Reference
H. influenzae	CGT ATT ATC GGA AGA TGA AAG TGC	TIB MOLBIOL	Hendolin et al. 1997
M. catarrhalis	CCC ATA AGC CCT GAC GTT AC	TIB MOLBIOL	
S. pneumoniae	AAG GTG CAC TTG CAT CAC TAC C	TIB MOLBIOL	
Universal-R	CTA CGC ATT TCA CCG CTA CAC	TIB MOLBIOL	
Nocardia Nf1	CCGCAGACCACGCAAC	TIB MOLBIOL	Brown et al. 2004
Nf2	ACGAGGTGACGGCTGC	TIB MOLBIOL	
Nfar-T-F Nfar-T-R	GGCGAGCCCAGTACCGATTAA AAGCCACGCACCTGTTTC	TIB MOLBIOL	Hasegawa et al. 2007

2.3.8.1 16S rRNA sequencing

Sequencing of the 16S rRNA gene was performed on isolates from detected sputum which were difficult to characterize by other approaches. These included isolates subsequently identified as *Enterococcus* sp., *Staphylococcus* sp. and *Rhodococcus* sp. The DNA was isolated by bead beating method (Tell et al. 2003), material was

subjected to PCR using two universal primers (fD1 and rP2) described by Weisburg et al. 1991. The PCR products were purified with QIAamp DNA purification kit (Qiagen, Hilden, Germany). The quantity of purified DNA for sequencing was measured using NanoDrop spectrophotometer (ND 1000 v3.5.2) (NanoDrop Technologies, USA).

Table 3 Primers for 16S rRNA gene sequencing for identification of isolates from sputum samples detected by rats

Primer name	Sequence (5'→3')	Supplier	Reference
fD1	CCG AAT TCG TCG ACA ACA GAT TTT GAT CCT GGC TCA G	TIB MOLBIOL	Weisburg et al. 1991
rP2	CCC GGG ATC CAA GCT TAC GGC TAC CTT GTT ACG ACT T		

The sequences were aligned manually and the regions of similarity were searched in the GenBank using the basic local alignment search tool (BLAST) (http://blast.ncbi.nlm.nih.gov/). For each sequence, multiple homologies were obtained but only the highest similarities were recorded. Sequence homology data together with morphological data were used to assign isolates to genus or species especially where the sequences allocated the isolates to a morphologically different but genetically related species such as *Nocardia* sp. and *Rhodococcus* sp.

2.3.9 Sensitivity, specificity and accuracy of the rats

The sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of *Mtb* detection by rats were determined using confirmed TB and non-TB subjects. Suspected TB cases were excluded in the subsequent evaluation. *Sensitivity*

= TP/TP+FN; *Specificity* = TN/TN+FP; *Positive predictive value* (PPV) = TP/TP+FP; *Negative predictive value* (NPV) = TN/TN+FN; *Accuracy of the TB detection rats* = TP/TP+TN+FP+FN, whereby TP = true TB positives detected by rats; FP = false positives (TB negatives detected as positive by rats); TN = true TB negatives not detected by rats; and FN = false TB negative (missed positives).

2.3.10 Odour compounds of non-mycobacterial isolates from sputum detected by rats

Non-mycobacterial isolates from smear-and *Mtb* culture-negative sputum samples detected by rats were cultured on brain heart infusion agar and headspace samples (volatile compounds in a space above liquid or solid culture) were collected from these cultures and analysed by gas chromatography and mass spectrometry (GC/MS). The GC/MS analyses were performed in collaboration with T. Nawrath and S. Schulz of the Institute for Organic Chemistry, Technical University of Braunschweig, Germany. The details of headspace sampling and GC/MS analysis is provided in the section on volatile compounds of mycobacteria and related species of this thesis (section 2.5).

2.3.11 Statistical analysis

A Fisher's exact test was used to determine whether the distribution of detected (rat-positive) and undetected (rat-negative) samples with *Mtb* was different from that of sputa with nontuberculous mycobacteria (NTM) and non-mycobacterial species. A P value < 0.05 was used to establish the statistical significance of comparisons.

2.4 Discrimination of cultures of *Mycobacterium* spp. and other microorganisms by rats

2.4.1 Microorganisms

To determine whether the TB detection rats detected *Mtb* exclusively, 38 bacterial and yeast strains from the genera *Mycobacterium*, *Nocardia*, *Rhodococcus*, *Streptomyces*, *Bacillus*, *Candida* and *Saccharomyces* were cultured in liquid medium and presented to the rats. These strains originated from the Belgian Coordinated Collections of Microorganisms (BCCMtm/LMG), Gent, Belgium (http://bccm.belspo.be); the German Collection of Microorganisms and Cell Cultures (DSMZ) (http://www.dsmz.de); the Max Planck Institute for Infection Biology, Berlin, Germany, and Sokoine University of Agriculture, Morogoro, Tanzania (Table 4). Mycobacterial clinical isolates (no. 25–33, Table 4) were obtained from the National Institute for Medical Research (NIMR), Muhimbili Medical Research Centre, Dar es Salaam, Tanzania.

Table 4 Microorganisms (20 bacterial and 2 yeast species) cultured and tested by trained rats.

No.	*Species*	*Strain*	Source	Samples tested (n)*
1	*Mycobacterium tuberculosis(Mtb)*	H37Rv	Lab. strain	9
2	*Mtb*	Beijing 2	Netherlands	14
3	*Mtb*	Beijing 3	South Korea	4
4	*Mtb*	4	Netherlands	3
5	*Mtb*	Beijing 5	South Africa	39
6	*Mtb*	Beijing 6	Mongolia	8
7	*M. smegmatis*	MC^2155	n/a	25
8	*M. avium* subspecies avium	n/a	n/a	5
9	*M. scrofulaceum*	n/a	n/a	5
10	*M. vaccae*	n/a	n/a	5
11	*M. aichiense*	LMG 19259	Soil	15
12	*M. alvei*	LMG 19260	Water	2
13	*M. aurum*	LMG 19255	Soil	19
14	*M. neoaurum*	LMG 19258	Soil	20
15	*M. peregrinum*	LMG 19256	Human	7
16	*M. bovis*	BCG – Pasteur	n/a	7
17	*M. bovis*	BCG-Copenhagen	n/a	7
18	*Streptomyces antibioticus*	LMG 5966	Soil	11
19	*S. griseoflavus*	LMG 19344	Soil	20
20	*S. griseoluteus*	LMG 19356	Soil	6
21	*S. coelicolor/ S. albidoflavus*	DSM 40233	n/a	8
22	*Nocardia lutea*	LMG 4066	Soil	4
23	*N. uniformis*	LMG 4082	Soil	3
24	*N. asteroides*	LMG 4062	n/a	7
25	*Mtb* complex, clinical isolate	N 185/08	Human-Tanzania	12
26	*Mtb* complex, clinical isolate	RT 1340	Human-Tanzania	5
27	*Mtb* complex, clinical isolate	N 1283/08	Human-Tanzania	3
28	*Mtb* complex, clinical isolate	RT 1284/08	Human-Tanzania	9
29	*Mtb* complex, clinical isolate	N 1080/08	Human-Tanzania	9
30	*Mtb* complex, clinical isolate	BR 30	Human-Tanzania	9
31	*Mtb* complex, clinical isolate	RT 1104	Human-Tanzania	2
32	*Mtb* complex, clinical isolate	N 194/08	Human-Tanzania	4
33	*M. bovis* clinical isolate	KP 20	Human-Tanzania	6
34	*Mtb*	H37Ra	Lab. strain	4
35	*Rhodococcus equi*	n/a	Lab. strain	4
36	*Bacillus subtilis*	n/a	Local isolate	4
37	*Candida albicans*	n/a	Human-Tanzania	5
38	*Saccharomyces cerevisiae*	n/a	Baker's yeast	5
Total number of microorganism samples tested				334

* Different age-based cultures of same species/strain, including same-age cultures, tested repeatedly on different days (technical replicates)

2.4.2 Culture and inactivation of microorganisms

Lyophilized bacterial strains were reconstituted according to supplier's instructions and inoculated into 14–20 ml of Middlebrook 7H9 liquid medium containing albumin dextrose catalase (ADC) enrichment without Tween and glycerol. Cultures were incubated at temperature ranges of 28–30 ºC and at 37 ºC for a period of 4 days

to 9 weeks for slow-growing species, under appropriate biosafety conditions. A loopful of culture was then inoculated on Luria/Miller (LB agar) (Carl Roth GmbH & Co., Germany) and incubated at 37 ºC for purity check. Viable colony forming units (CFUs) of liquid cultures were assessed by plating on Mycobacteria 7H11 gar and LB agar. Growth was also determined by measuring optical density (OD_{580nm} or OD_{600nm}) using a UV/Visible spectrophotometer (Amersham Biosciences, Uppsala, Sweden). For *Mtb*, an OD_{580nm} of 0.1 was equal to 5×10^7 bacterial cells/ml. All test organisms, except *Mtb*, were heat-inactivated in a 90 ºC water bath for 30 min and left to cool at room temperature before being stored at –20 ºC until later use. Aliquots of 4 ml of *Mtb* culture in 15 ml Sarstedt secured screw-capped plastic vials (Nümbrecht, Germany) were inactivated on a dry heating block at 100 ºC for 1 h, in a biosafety level 3 facility. The level of *Mtb* culture in vials did not exceed the part of vial that was inside the holes of dry heating block to ensure contact with heated area. The efficiency of dry heat inactivation of *Mtb* was assessed by culturing aliquots of inactivated cultures in 7H9 and 7H11 media incubated at 37 ºC for 3 months while checking eventual growth at 7-day intervals.

Table 5 List of equipments used in different analyses

Equipment name	Supplier
Vortex – MS1 minishaker	IKA works Inc, Wilmington USA
Heraeus biofuge - centrifuge	Thermo Electron Corp, Osterode, Germany
Heraeus megafuge centrifuge	Thermo Electron Corp, Osterode, Germany
Hotplate	Liebisch, Bielefeld, Germany
Eppendorf thermomixer	Ependorf Ag, Hamburg, Germany
Water bath	PD Industriegesellschaft, Germany
UV/Visible spectrophotometer	Amersham Biosciences, Uppsala, Sweden)
NanoDrop spectrophotometer	NanoDrop technologies, USA
Leica DMLB microscope	Leica Microsystems, Wetzlar, Germany
Jenoptik ProgRes CT5 USB C camera	Jenoptik Laser Optik Systeme GmbH, Jena, Germany
pH Meter – 761 Calimatic	Knick, Germany

2.4.3 Spiking of sputum samples with inactivated microorganisms

Negative sputum samples from TB clinics in Dar es Salaam, Tanzania, were used for spiking with test microorganisms. The negative status of these samples was confirmed by smear microscopy [Ziehl Neelsen (ZN)], fluorescent microscopy (FM), mycobacterial culture and by TB detection rats (*C. gambianus*). About 10 ml of heat-inactivated negative sputum with PBS were spiked with 100 µl, 500 µl and or 1000 µl of bacterial culture. A minimum of two replicate samples of each test microorganism were assessed by a minimum of six rats, performing two test sessions each per day. The positive controls consisted of confirmed TB-positive sputum samples (n=7), which were mixed with sterile medium. These consisted of various acid-fast bacilli (AFB) counts: 1–9AFB, 1+, 2+ to 3+, whereby 1–9 AFB refers to observation of 1–9 acid-fast bacilli per 100 microscopy fields; 1+ is 10–99 acid-fast bacilli per 100 fields; 2+ is 1–10 acid-fast bacilli observed per field, and 3+ refers to > 10 acid-fast bacilli per field (CDC 2000). For negative controls, confirmed TB-

negative sputum mixed with sterile medium was used. One day was skipped between subsequent tests to allow the rats to perform routine TB detection. The experiments were conducted over a period of 94 days (January–April 2009). Microorganisms detected in initial tests were further presented to rats to confirm findings. Further tests included cultures of different age (growth phase) to determine the most detectable phase since different volatiles can be produced by a given microorganism depending on growth stage and culture conditions.

The rats' training procedure and judging of positive detection has been described elsewhere (Weetjens et al. 2009, Poling et al. 2010). Briefly, detection of the 7 TB-positive sputa (reward samples) was followed by food reward and food was not presented on indication of any of the spiked samples.

2.4.4 Mycobacterial growth phases detected by rats

Reference species *Mtb* and *M. smegmatis* (representing pathogenic and NTM species) were grown in Middlebrook 7H9 broth and incubated at 37 ºC with shaking. Culture samples (4 ml) were heat-inactivated after 10, 21, 30 and 41 days, whereas *M. smegmatis* cultures were further sampled at 65 days of incubation. CFUs were measured by spectrophotometer and colony counting on 7H11 plates inoculated with serial dilutions of the cultures. Three replicates were collected at each of the four and five growth intervals of *Mtb* and *M. smegmatis*, respectively, and were tested by six trained rats without rewarding them upon detection of the test microorganisms.

2.4.5 Sample layout and presentation

The test layout involved 70 sputum samples of which 9 (12.9%) were test microorganisms; 7 (10%) were TB-positive controls for rewarding the rats; and 54 (77.1%) were negative controls mixed with sterile 7H9 medium. Negative sputum samples spiked with test microorganisms were spread across the sample panels arbitrarily to avoid introducing a pattern that could be easily learned by the rats. In the first sessions, the rats analyzed samples in an $A_{1–10}$ to $G_{1–10}$ series, whereby A–G refers to codes of 7 sample panels each with 10 holes for holding 10 sputum sample pots (Fig. 3), and $A_{1–10}$ is position 1–10 in the metal panel coded A. In the second sessions the A–G series were randomized again to avoid eventual memorization of the arrangement of samples. Samples detected by at least two rats (cut-off level) were considered significantly detected. These criteria were used during routine TB detection by the rats.

Figure 3 Sputum samples in sample panels (A-G) ready for spiking with test microorganisms and presentation to the TB detection rats.

2.4.6 Statistical analysis

A Fisher's exact test was used to determine significance between rats' detection of cultures of *Mtb* and NTM species. Odds ratio (OR) was used to determine whether the detection of cultures was better than random chance. A P value < 0.05 was used to establish the statistical significance of comparisons. Sensitivity, specificity and accuracy of the trained rats to detect cultures of *Mtb* and NTM (*M. smegmatis*) were determined using detection scores of different growth phases and combined detection of all growth phases of each species.

2.5 Identification of volatile odour compounds detected by rats

2.5.1 Microorganisms

The target TB volatile compounds detected by rats were investigated using reference *Mtb* strains from Africa, Asia and Europe, selected nontuberculous mycobacteria (NTM), *N. africana* and *N. asteroides*; a species complex representing *N. farcinica* isolated in this study, from clinical sputum detected by rats; reference *Streptomyces* spp. (representing clinical *Streptomyces* spp. from sputa detected by rats), clinical *Rhodococcus* sp., *Staphylococcus* sp. and *Candida* sp. from smear-negative sputum samples detected by rats.

Table 6 Microorganisms used for studying volatile compounds by GC/MS included reference strains and clinical isolates from smear-negative *Mtb* culture-negative sputa detected by rats.

No.	Species	Strain	Source	Medium	Culture (headspace samples) analysed (n)
1	*Mtb*	H37Rv	Lab. strain	[b]	3
2	*Mtb*	H37Ra	Lab. strain	[b]	1
3	*Mtb*	Beijing 2	Netherlands	[a, b]	5
4	*Mtb*	Beijing 5	South Africa	[a, b, c, d]	23
5	*Mtb*	Beijing 6	Mongolia	[a, b, c, d]	9
6	*M. smegmatis*	MC^2155	n/a	[a, b, c, d]	12
7	*M. avium* subspecies avium	n/a	n/a	[a, b, c]	4
8	*M. scrofulaceum*	n/a	n/a	[a, b]	3
9	*M. vaccae*	n/a	n/a	[a, b]	3
10	*M. aichiense*	LMG 19259	Soil	[a, b, c]	8
11	*M. aurum*	LMG 19255	Soil	[a, b, c]	9
12	*M. neoaurum*	LMG 19258	Soil	[a, b, c]	9
13	*Streptomyces antibioticus*	LMG 5966	Soil	[a, b, c]	8
14	*S. griseoflavus*	LMG 19344	Soil	[a, b, c]	10
15	*S. coelicolor/ S. albidoflavus*	DSM 40233	n/a	[a, b, c]	7
16	*Nocardia asteroides*	LMG 4062	n/a	[a, b, d]	8
17	*N. africana*	DSM 44499	Human	[a, b]	6
18	*Rhodococcus* sp.	isolate 61	Human-Tanzania	[e]	2
19	*Candida albicans*	isolate 25	Human-Tanzania	[e]	2
20	*Staphylococcus* sp.	Isolate 102	Human-Tanzania	[e]	2

[a] Middlebrook 7H9 broth, [b] Mycobacteria 7H11 agar, [c] Sauton liquid medium, [d] Minimal nutrient PBSGG medium, [e] Brain heart infusion (BHI) agar

2.5.2 Culture of microorganisms for headspace samples

Reference strains of *Mtb*, NTM, *Nocardia* spp. and *Streptomyces* spp., and clinical isolates (Table 6) were cultured in different media that included Middlebrook 7H9 broth with ADC enrichment only (without Tween and glycerol), Mycobacteria 7H11 agar with oleic acid albumin dextrose catalase (OADC) enrichment only, Sauton liquid medium (without glycerol), minimal nutrient medium consisting of phosphate buffered saline (PBS) glucose and glycerol (PBSGG). *Rhodococcus* sp., *Candida* sp., and *Staphylococcus* sp. from detected sputa were cultured on Brain heart infusion agar (BHI agar).

2.5.2.1 Middlebrook liquid and solid medium

Middlebrook 7H9 broth (Becton, Dickinson & Co., Sparks, USA) was prepared according to manufacturer's instructions. 100 ml of Middlebrook ADC enrichment

was added to 900 ml of sterilized 7H9 medium base. The Mycobacteria 7H11 agar (Becton, Dickinson & Co., Sparks, USA) was also prepared according to manufacturer's instructions and 100 ml of Middlebrook OADC enrichment was added to 900 ml of sterilized 7H11 agar base.

2.5.2.2 Sauton medium

This medium was prepared by dissolving the following ingredients into total volume of 1000 ml distilled water: asparagine (4 g) in 250 ml distilled water at 80 °C, magnesium sulphate (0.5 g), dipotassium phosphate (0.5 g), citric acid (1.83 g), ferric ammonium citrate (0.05 g), D(+) glucose monohydrate (4.82 g) and pyruvic acid (sodium salt) (4.82 g). The pH was adjusted to 6.8 and the medium was filter sterilized using vacuum driven disposable bottle top filters with 0.22 µm membrane filter (Millipore Corp., USA). Sterile medium was dispensed into 20 ml sterile glass universal bottles.

2.5.2.3 Minimal nutrient medium (PBSGG)

This minimal nutrient medium was prepared by dissolving 1 g of glucose monohydrate and 2 ml of pure glycerol into a total volume of 1000 ml distilled water. The medium was filter sterilized using vacuum driven disposable bottle top filter with 0.22 µm membrane filter (Millipore Corp., USA) and thereafter dispensed into 20 ml sterile glass universal bottles.

2.5.2.4 Brain heart infusion medium

This medium was prepared by dissolving 37 g of brain heart infusion medium base (Becton, Dickinson & Co., Sparks, USA) and 15 g of agar in a total volume of 1000 ml distilled water. After dissolving the medium was sterilized by autoclaving at 121 °C x 15 min.

All microorganisms were grown on medium in glass plates or in glass universal bottles to avoid contamination of the microorganisms' odour by odours from plastic materials. The glass wares were thoroughly cleaned using acetone (Suprasolv, Merck, Germany) and distilled water. The charcoal filter was cleaned using non-polar and polar solvents that included *n*-pentane (Unisolv grade, Merck, Germany), methanol (Suprasolv, Merck, Germany) and dichlomethane (Suprasolv, Merck, Germany). Cultures were incubated at 28 °C and 37 °C and checked for purity by plating a loopful of culture on LB agar incubated at 37 °C before collection of headspace samples for GC/MS analysis.

Table 7 Glassware and apparatus for culturing, handling microorganisms' cultures and collection of headspace samples for GC/MS analyses.

Apparatus	Supplier
Pyrex glass petri dishes (100x15 mm)	Fisher Scientific
Soda glass petri dishes (80x15 mm)	Fisher Scientific
Glass petri dish plates, Duran (60 x20 mm)	VWR International GmbH
Serological glass pipette (10 ml)	Fisher Scientific
Universal bottles (glass) (28 ml) with aluminium cap	Fisher Scientific
Erlenmeyer conical flasks (250 ml)	VWR International GmbH
Hamilton syringe (glass) and needle (25, 50µl)	VWR International GmbH
GC sample collection glass vials (32x11.6 mm) with caps	IVA Analysentechnik
Activated Precision charcoal filter, 5 mg	Chromtech

2.5.3 Collection of headspace volatile compounds from microorganisms

Headspace samples from microorganisms in different media and at different growth-phases (age) were collected for 18–24 h using a closed loop stripping apparatus (CLSA) as described by Schulz et al. (2004), fitted with an activated charcoal filter (Chromtech; Precision Charcoal Filter, 5 mg). The collected volatiles were eluted from the filter for GC/MS analysis using 30 µl of dichloromethane (Suprasolv, Merck, Germany). Glass vials with samples for GC analysis were protected from light by wrapping with aluminium foil and kept frozen at –20 °C until used.

2.5.4 Volatile compound analysis by gas chromatography and mass spectrometry (GC/MS)

The GC/MS analysis was carried out by T. Nawrath and S. Schulz of the Institute of Organic Chemistry, Technical University of Braunschweig, Braunschweig, Germany. Briefly, analyses were carried out on an Agilent 7890A GC system connected to an Agilent 5975C inert mass detector fitted with an HP-5MS fused silica capillary column (30 m, 0.25 mm i.d., 0.25 µm film; J&W Scientific, USA). Conditions were as follows: inlet pressure: 77.1 kPa, He 23.3 mL min^{-1}; injection volume: 2 µl; transfer line: 300 °C; electron energy: 70 eV. The following GC program was applied: 5 min at 50 °C, increasing at 5 °C min^{-1} to 320 °C, operated in the splitless mode (60 s valve time); He carrier gas flow was 1.2 ml min^{-1}. Compounds were identified by comparison of GC/MS retention indices with those of mass spectral libraries and comparison with synthetic reference compounds. Retention indices *I* were determined from a homologous series of *n*-alkanes (C_8-C_{35}) (van den Dool and Kratz 1963).

2.5.5 Specificity of volatile compounds

Volatile compounds produced by microorganisms in different media and growth phases were used to establish patterns and clusters of microorganisms and to determine specificity of compounds. The specificity of compounds was assessed by determining occurrence (presence and absence) in different cultures ($n \geq 2$), different media and age (in days) of the microorganism. Volatile compounds identical to those found in blank medium were excluded in the analysis. Compounds recovered in at least two cultures were considered significant for the given microorganism.

2.5.6 Statistical analysis of volatile compound data

Random Forest's cluster analysis was performed in R-statistical software (http://www.r-project.org/) to construct clusters of microorganisms based on specific and shared volatile compounds. The significance level or *alpha* (α) of the cluster analysis was 0.05, and the clusters with AU (approximately unbiased) *p*-value greater than 95% were considered strongly supported by the data (http://www.is.titech.ac.jp/~shimo/prog/pvclust/). In this cluster analysis, the probability values (*p*-values) for each cluster are obtained by bootstrap resampling method (Suzuki and Shimodaira 2006).

2.5.7 Detection of volatile compounds by rats

The specific volatile compounds responsible for clustering of *Mtb* strains only, *Mtb* plus NTM, and *Mtb* plus all other microorganisms were selected for determination of the TB target volatiles (odour) detected by rats in TB sputum samples. Selected compounds were tested individually, in pairs, triplicates and combinations of several

compounds spiked into TB-negative sputum samples and presented to 5 trained rats (Table 8).

2.5.7.1 Volatile compound test categories

The volatiles compounds for testing by rats were assigned to 5 test categories which were created to reflect and mimic the possible occurrence and composition of the odour compounds in TB sputum. The compounds were tested individually and in combination series: (i) *Mtb*-specific volatiles (n=8); (ii) overlapping/shared volatiles from *Mtb*, NTM and others (n=7); (iii) combinations (blends) of 7 *Mtb*-specific compounds; (iv) blends of 7 shared volatile compounds; (v) blend of 7 *Mtb*-specific volatile compounds and 7 shared volatiles (Table 8).

Table 8 Odour compounds from *Mtb*, other mycobacteria, *Nocardia* spp., *Rhodococcus* sp. and other microorganisms tested by rats.

Code	Compound name	Supplier	Species specificity	Presentation to rats
1	Methyl nicotinate (99%)	Sigma-Aldrich Chemie	*Mtb*-specific	Presented individually in different concentration
2	Methyl 4-anisate (99+ %)	Sigma-Aldrich Chemie		
3	2-Phenylanisol (2-methoxybiphenyl, 98+ %)	Merck		
4	4-Methylanisol	Merck		
5	Ethyl 4-anisate (97%)	Sigma-Aldrich Chemie		
6	Chalcogran	Sigma-Aldrich Chemie		
7	Benzothiazole (96%)	Sigma-Aldrich Chemie		
8	2-Phenylethanol	Sigma-Aldrich Chemie	Overlapping compounds /found in *Mtb*, NTM and other microorganisms	Presented individually in different concentration
9	Methyl benzoate (99%)	Sigma-Aldrich Chemie		
10	4-Pentanolide (γ-valerolactone) (99%)	Sigma-Aldrich Chemie		
11	Methylphenylacetate (99+ %)	Sigma-Aldrich Chemie		
12	Methyl 2-furoate (98%)	Acros Organics		
13	Methyl salicylate (99%)	Acros Organics		
14	Camphor (96%)	Sigma-Aldrich Chemie		
15	Proline + glycine	Alfa Aesar, Acros Organics	*Mtb*-specific	Presented in pair
16	Methyl nicotinate + Methyl 4-anisate		*Mtb*-specific	Presented in pair
17	Methyl nicotinate + 2-Phenylanisol (2-methoxybiphenyl)			
18	Methyl nicotinate + 4-Methylanisol			
19	Methyl nicotinate + Ethyl 4-anisate			
20	Methyl nicotinate + Chalcogran			
21	Methyl nicotinate + Benzothiazole			
22	Methyl nicotinate Methyl 4-anisate 2-Phenylanisol (2-methoxybiphenyl) 4-Methylanisol Ethyl 4-anisate Chalcogran Benzothiazole		*Mtb*-specific	Presented in combination (blend) of 7 compounds
23	2-Phenylethanol + Methyl benzoate		Overlapping compounds /found in *Mtb*, NTM and other microorganisms	Presented in pair
24	2-Phenylethanol + Pentanolide (γ-valerolactone)			
25	2-Phenylethanol + Methylphenylacetate			
26	2-Phenylethanol + Methyl 2-furoate			
27	2-Phenylethanol + Methyl salicylate			
28	2-Phenylethanol + Camphor			
29	2-Phenylethanol Methyl benzoate 4-Pentanolide (γ-valerolactone) Methylphenylacetate Methyl 2-furoate Methyl salicylate Camphor		Overlapping compounds/found in *Mtb*, NTM and other microorganisms	Presented in combination (blend) of 7 compounds
30	Methyl nicotinate Methyl 4-anisate 2-Phenylanisol (2-methoxybiphenyl)		*Mtb*-specific	Presented in combination of 3 most abundant compounds
31	Methyl nicotinate Methyl 4-anisate 2-Phenylanisol (2-methoxybiphenyl) 4-Methylanisol Ethyl 4-anisate Chalcogran Benzothiazole 2-Phenylethanol Methyl benzoate 4-Pentanolide (γ-valerolactone) Methylphenylacetate Methyl 2-furoate Methyl salicylate Camphor		*Mtb*-specific and Overlapping compounds /found in *Mtb*, NTM and other microorganisms	Presented in combination (blend) of 7 *Mtb*-specific and 7 overlapping compounds

2.5.7.2 Spiking of sputum with volatile compounds

Confirmed TB-negative sputum samples (checked by smear microscopy in TB-clinics) not detected by trained rats in previous routine TB detection sessions, were spiked with different concentrations of candidate volatile compounds. A minimum of two and maximum of five different concentrations of candidate volatile compound were tested by a group of 5 trained rats, performing two tests per day. The average sensitivity and specificity of these rats determined from detection of TB sputum examined by microscopy in DOTS centres and SUA-APOPO laboratory was 81.6% and 88%, respectively (Table 9). Each volatile sample presented to the rats per test (day) was hence tested 10 times on that particular day. A one to two-days interval was kept between tests for rats to undergo routine TB detection training. The cut-off point of two rats used in routine TB detection by rats was employed in this study whereby the detection of a sample was considered significant when at least two rats detected that sample. A maximum of 10 correct detections (scores) were expected for each TB-positive control sputum samples and potential target volatile compounds. Non-target volatile compounds were not expected to be detected by rats. The positive control consisted of known smear positive (TB) sputum samples with varying number of acid-fast bacilli count ranging from 1–9 AFB, 1+, 2+ and 3+. Rats were rewarded with food upon correct detection of positive control samples. Food was not provided when rats detected any of negative sputa spiked with test volatile compounds and the negative control sputum samples which consisted of confirmed TB-negative sputa not spiked with volatile compounds. The overall setup consisted of 70 samples of which 14–18 were various concentrations of test volatiles spiked

into negative sputa; 7 TB-positive control sputa and 42–49 confirmed TB-negative control sputum samples.

Table 9 Sensitivity and specificity of the 5 rats used for determination of candidate TB volatile compounds.

Rat	Sensitivity [#]	Specificity [#]
1	75	91
2	95	85
3	75	93
4	79	90
5	84	81
Average	81.6	88

Determined from smear microscopy results from DOTS and SUA-APOPO TB laboratory.

One rat analyzed the set of 70 samples at an average rate of 8 min per session (thus two sessions = 16 min). Therefore, the consensus results with the cut-off point of two rats (two rats × two sessions each) were obtained in 32 min. Fresh smears were made from all detected spiked and negative control sputa to re-assess possible presence of acid-fast bacilli.

2.5.8 Statistical analysis of volatile compounds detection by rats

Differences between the detection of *Mtb*-specific volatiles and shared volatiles by the rats, and differences between detection of different concentrations of the volatiles were analysed using Fisher's exact test. The P value < 0.05 was considered statistical significant difference. Analysis was run in R-statistical software (http://www.r-project.org/).

3 RESULTS

3.1 *Mycobacterium* species diversity and detection by rats

3.1.1 Mycobacterial isolation and molecular identification

Isolates of the genus *Mycobacterium* were cultured from sputa of 34 out of 161subjects (21.1%) with a prevalence of 23.3% in males (21/90) and 18.6% in females (13/70). This difference in prevalence in males and females is not statistically significant. Of the 28 mycobacterial isolates available for further molecular study, 26 isolates (93%) were identified as *Mtb*, one as *M. avium* subsp. *hominissuis* and one as *M. intracellulare* (Table 10). The multiplex real-time PCR and conventional PCR for genus *Mycobacterium*, *Mtb* complex, *M. avium* and *M. intracellulare* species were able to preliminarily identify these isolates. The *rpoB* sequence analysis identified further the *M. avium* isolate as *M. avium* subsp. *hominissuis*.

3.1.2 *M. tuberculosis* genotypes

Multispacer sequence typing (MST) analysis of the 26 *Mtb* isolates yielded 10 distinct genotypic patterns (mean molecular diversity, 0.38): (1) MST 67 comprised of 10/26 (38.5%); (2, 3) MST 4 and MST 16 were found in 3 out of 24 isolates each (11.5%); (4-6) MST 53, MST 59 and MST 68 were found in 2 isolates each; and (7-10) MST 3, MST 21, MST 52 and MST 69 were found once each. Sequence

clustering analysis revealed two clusters of related patterns for the four groups (52, 67, 59 and 4, respectively), which were only differentiated by a single sequence repeat or a single nucleotide polymorphism. The five remaining genotypes exhibited a higher degree of variability; genotypes 16 and 69 being the most distant patterns. New genotypes were found in the course of this study: MST 67 and MST 68 were new genotypes due to a new combination of previously known spacer sequences, whereas MST 69 was a new genotype due to a new sequence of the spacer 12, combining six modules of a 77-bp repeat unit.

Table 10 Results of the multiplex real-time PCR, multispacer sequence typing (MST) of the acid-fast bacilli and testing of the respective sputum samples by TB detection rats.

No.	Specimen ID	DOTS centre	Age	Gender	RT-PCR	MST type	Identification	TB detection rats
1	257837	Mwananyamala	24	M	MTC	67	*Mtb*	–
2	258362	Mwananyamala	17	M	MTC	67	*Mtb*	–
3	258429	Mwananyamala	25	F	MTC	67	*Mtb*	+
4	266786	Mwananyamala	40	F	MTC	67	*Mtb*	–
5	267265	Mwananyamala	35	F	MTC	67	*Mtb*	+
6	257729	Mwananyamala	22	M	MTC	67	*Mtb*	+
7	257763	Mwananyamala	32	F	MTC	67	*Mtb*	+
8	258471	Mwananyamala	28	M	MTC	67	*Mtb*	+
9	258473	Mwananyamala	27	F	MTC	67	*Mtb*	+
10	265826	Amana	33	F	MTC	4	*Mtb*	+
11	265903	Amana	< 1	M	MTC	53	*Mtb*	+
12	265916	Amana	24	F	MTC	53	*Mtb*	–
13	266889	Tandale	< 1	M	MTC	59	*Mtb*	–
14	266022	Amana	24	M	MTC	68	*Mtb*	+
15	265962	Amana	45	M	MTC	67	*Mtb*	+
16	266934	Tandale	29	M	MTC	52	*Mtb*	+
17	266865	Tandale	< 1	F	MTC	59	*Mtb*	–
18	267017	Tandale	14	M	MTC	68	*Mtb*	+
19	267021	Tandale	50	M	MTC	16	*Mtb*	+
20	267005	Tandale	27	M	MTC	4	*Mtb*	+
21	267905	Magomeni	85	M	MTC	16	*Mtb*	–
22	267922	Magomeni	18	M	MTC	69	*Mtb*	+
23	267893	Magomeni	50	M	MTC	3	*Mtb*	–
24	263702	Magomeni	30	M	MTC	16	*Mtb*	+
25	267871	Magomeni	21	F	MTC	4	*Mtb*	+
26	263679	Magomeni	35	M	NTM	–	*M. intracellulare*	–
27	267841	Magomeni	31	M	MAC	–	*M. avium* hominissuis	–
28	267922	Magomeni	18	M	MTC	21	*Mtb*	+

Table notes: *Mycobacterium tuberculosis* (*Mtb*) complex (MTC); nontuberculous mycobacteria (NTM); *M. avium* complex (MAC); multispacer sequence typing (MST); real-time (RT) PCR; tuberculosis (TB).

The map (Fig. 4) displaying the distribution of genotypes according to sample location revealed that related patterns were not significantly grouped together in geographical areas. However, the genotype MST 67 was significantly associated with the Mwananyamala clinic (P<0.05).

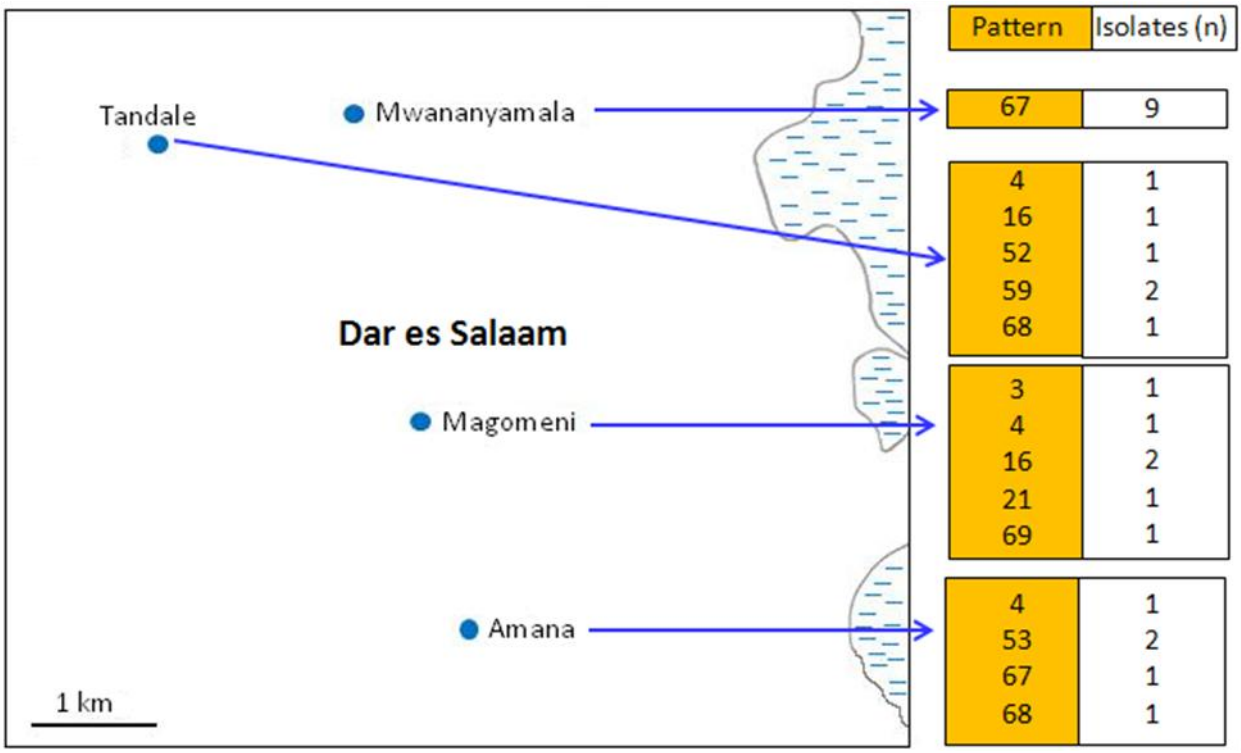

Figure 4 Map of Dar es Salaam (modified from Google map) showing the location of TB clinics where sputum samples were collected, with respective *Mtb* genotypes based on multispacer sequence typing (MST). Number (n).

3.1.3 Rat performance on sputa with different *M. tuberculosis* genotypes and mycobacterial species

The results of TB detection rats show that sputum specimens containing different *Mtb* genotypes are detected by rats (Table 10, Fig. 5). Rats detected 8 of the 10 MST genotypes (80%). Two MST genotypes (MST 59 and MST 3) with two isolates and

one isolate, respectively, were from sputum samples not detected by the rats. MST 59 and MST 3 genotypes cluster together with genotypes from sputa detected by rats (Fig. 5B). The nontuberculous *M. intracellulare* and *M. avium* subspecies *hominissuis* were from sputum samples not detected by rats (rat-negative). The performance of rats on sputa containing the MST 67 genotype, which was present in a high proportion of isolates (n = 10), shows that 7 (70%) of the 10 sputa with this genotype were detected by rats. Six of the 7 samples were TB smear-positive and one smear-negative from a different locality. The remaining three specimens containing *Mtb* MST 67 genotypes, which were not detected by the rats, were smear-negative sputa.

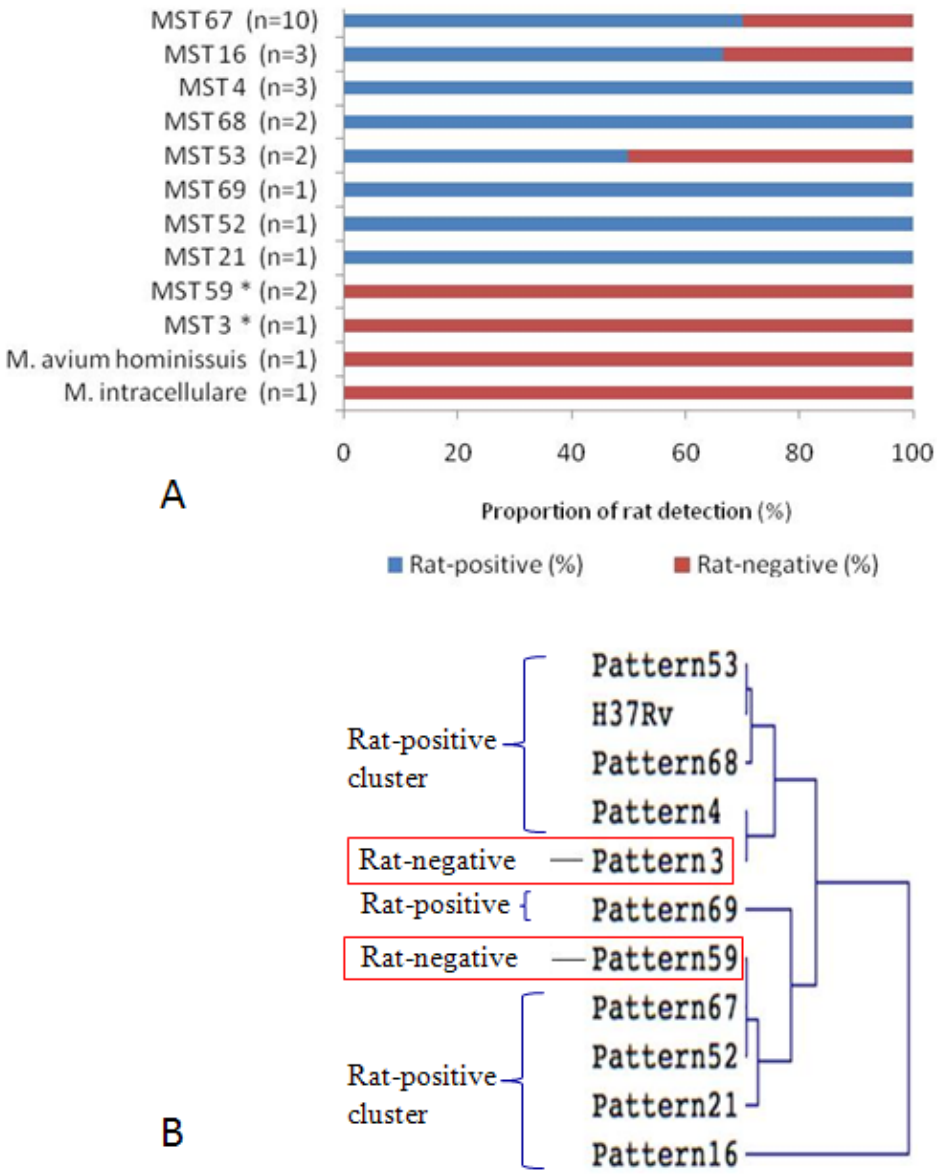

Figure 5 Detection proportions of different multispacer sequence typing (MST) genotypes of *Mtb* by rats (A). Overall, rats detected a majority of MST genotypes (8/10; 80%). Two MST genotypes (MST 59 and MST 3) (with asterisks) represented by two isolates and one isolate, respectively, were the only genotypes from sputa not detected by rats. However, the two genotypes belong to rat-positive clusters (B). The nontuberculous *M. intracellulare* and *M. avium* subspecies *hominissuis* were from sputum samples not detected by rats (rat-negative).

3.2 Respiratory tract microorganisms from sputum detected by rats

3.2.1 Population characteristics of sputa assessed by rats

In this experiment, 514 sputum samples from 289 subjects were examined. Of these 289 subjects, there were 56 confirmed TB cases based on smear microscopy and culture, 228 TB-negative and 5 suspected TB cases. Rats correctly detected 45 (true positive) of the 56 confirmed TB cases. The rats detected 63 (false positives) of the 228 negative subjects. Four (80%) of the five suspected TB cases with one AFB-positive sputum were detected by rats (rat-positive). Sensitivity and specificity were 80.4% and 72.4%, respectively. The positive predictive value (PPV) was 41.7% and the negative predictive value (NPV) was 93.8%. Test efficiency/accuracy of TB diagnosis by rats was 73.9%. The present sensitivity (80.4%) and specificity (72.4%) of TB diagnosis by rats is high but lower than the previously reported sensitivity and specificity (86.6% and 93.8%, respectively) by Weetjens and co-workers (2009). These lower levels could be attributed to modifications in sample treatment before conducting the first part of this study from April to June 2009. Indeed, the sensitivity and specificity varied between the two study periods: first study period sensitivity was 72.2% and specificity was 80.6% whilst the sensitivity and specificity found during the second study period (July 2010) was 95% and 62.5%. Although positive predictive values (PPV) and negative predictive values (NPV) are largely affected by the prevalence of a given disease in given population and the need for longitudinal studies of a particular population over a long time period to determine the PPV and NPV, this study indicates that harnessing rats for early TB diagnosis could have a

significant impact on control of TB. This is supported by the higher NPV (93.8%), which indicates that individuals with rat-negative sputum have a 93.8% likelihood of not having active TB disease.

3.2.2 *Mycobacterium* species and detection of sputa with different mycobacteria by rats

Mycobacterium spp. were isolated from 47 patients out of 289 subjects (16.3%). Thirty-seven of these isolates were MTC (78.7%) based on specific multiplex PCRs for the *Mycobacterium* genus and MST analyses. The majority of the MTC (75.7%) were from sputum samples detected by rats (Table 11, Fig. 6).

Table 11 *Mycobacterium* spp. from sputum samples (n=47) tested by trained *Cricetomys gambianus*.

Mycobacterial designation	Number (n)			Distribution (%)	Rat-positive			Rat-negative			Detection (%)
	(n)	Smear $^{+}$	Smear $^{-}$		(n)	Smear^{+}	Smear $^{-}$	(n)	Smear^{+}	Smear $^{-}$	
Mtb	37	25	12	78.7	28	25	3	9	0	9	75.7
NTM	8	1	7	17.0	4	1	3	4	0	4	50.0
M. avium subsp. *hominissuis*	1	0	1	2.1	0	0	0	0	0	1	0.0
M. intracellulare	1	0	1	2.1	0	0	0	0	0	1	0.0

Smear positive, smear +; smear negative, smear–. Combined rat-positive (detection) of sputa with NTM and *M. avium* subsp. *hominissuis* and *M. intracellulare* is 40%.

Ten mycobacterial isolates were NTM species of which two were *M. intracellulare* and *M. avium* subsp. *hominissuis*. Eight NTM (17%) of 47 mycobacterial isolates were not identified to species level. Four of the eight NTM were from rat-positive sputum samples, of which one was smear-positive. The isolates identified as *M. avium* subsp. *hominissuis* and *M. intracellulare* were both from rat-negative sputum samples (Table 11). Nine (90%) of the 10 NTM were from smear-negative sputum

samples. Overall, 21 (44.7%) of all mycobacterial isolates (n=47) were from smear-negative sputum samples, revealing that a significant proportion of smear-negative sputum contained mycobacterial species, which were probably the cause of detection of these sputa by rats. Six (28.6%) of the 21 mycobacterial isolates from smear-negative sputum samples were rat-positive indicating that rats increased detection rate of smear-negative TB by > 28%.

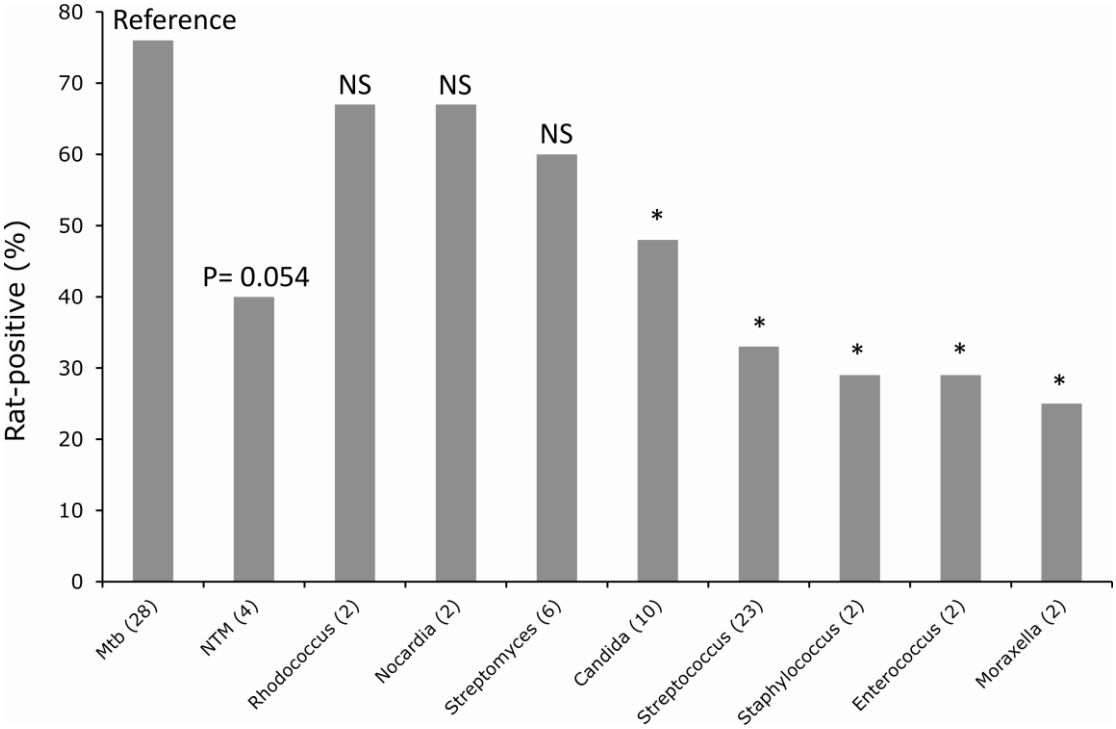

Figure 6 Rat-positive (%) sputum samples with different individual microorganisms. Number of isolates of each species in detected sputum is indicated in brackets. Statistically significant difference (*P* < 0.05, Fisher's exact test) between rat-positive sputa with *Mtb* (reference) and rat-positive sputa with non-mycobacterial species is shown by an asterisk. Rat-positive not significantly different from sputa with *Mtb* is shown by NS.

The detection trend for sputa with NTM (including *M. avium* subsp. *hominissuis* and *M. intracellulare*) was marginally different from detection of sputa with *Mtb* (P = 0.054, Fisher's exact test) (Fig. 6).

3.2.3 Non-mycobacterial respiratory tract microorganisms and detection by rats

3.2.3.1 Isolation, colony and cell morphology

Among the four media used to isolate different respiratory tract microorganisms, paraffin agar improved isolation of pulmonary pathogens including *Nocardia* sp., *Streptomyces* sp., *Candida* sp., and one NTM. These microorganisms were preliminarily identified by their characteristic colony morphology and pigmentation (Fig. 7). *Rhodococcus* spp. characterized by smooth mucoid and creamy pigmented colonies (Fig. 7) were isolated on chocolate agar, buffered charcoal yeast extract agar and paraffin agar. *Moraxella* spp., *Streptococcus pneumoniae* and *Enterococcus* spp. were isolated on chocolate agar. Yeast species were isolated on all four media used.

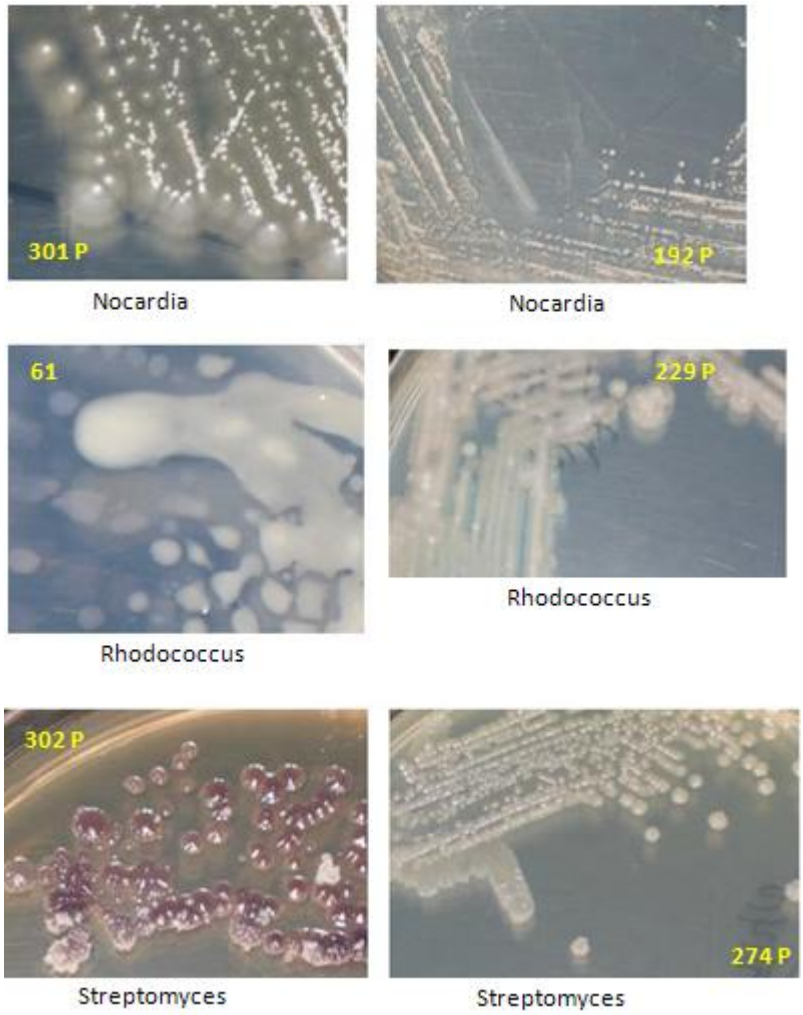

Figure 7 Colony morphology of non-mycobacterial species from sputum samples detected by rats. *Nocardia* species show characteristic chalky white and creamy colonies (301 P and 192 P) on Mycobacteria 7H11 agar. *Rhodococcus* sp. has smooth mucoid colonies on BHI medium (61 and 229 P) whereas *Streptomyces* spp. show pigmented (302 P) and un-pigmented large dry colonies (274 P) on 7H11 medium.

Gram stain discriminated *Nocardia* spp. and *Streptomyces* spp. isolates (Fig. 8) which has closely related colony morphology and both may form characteristic chalky white colonies on solid medium. The cell morphology of microorganisms grown in suitable artificial medium normally resembles that of microorganisms in host tissue such that the morphology of cultured microorganisms shown here

represents those of same microorganisms in sputum samples. *Nocardia* spp. and *Streptomyces* spp. have branched filaments whereby *Nocardia* is granulated/beaded (unevenly stained) unlike *Streptomyces* sp. which is evenly stained and not beaded. Also, *Nocardia* filaments are fragmented whereas *Streptomyces* filaments are long un-fragmented (Fig. 8).

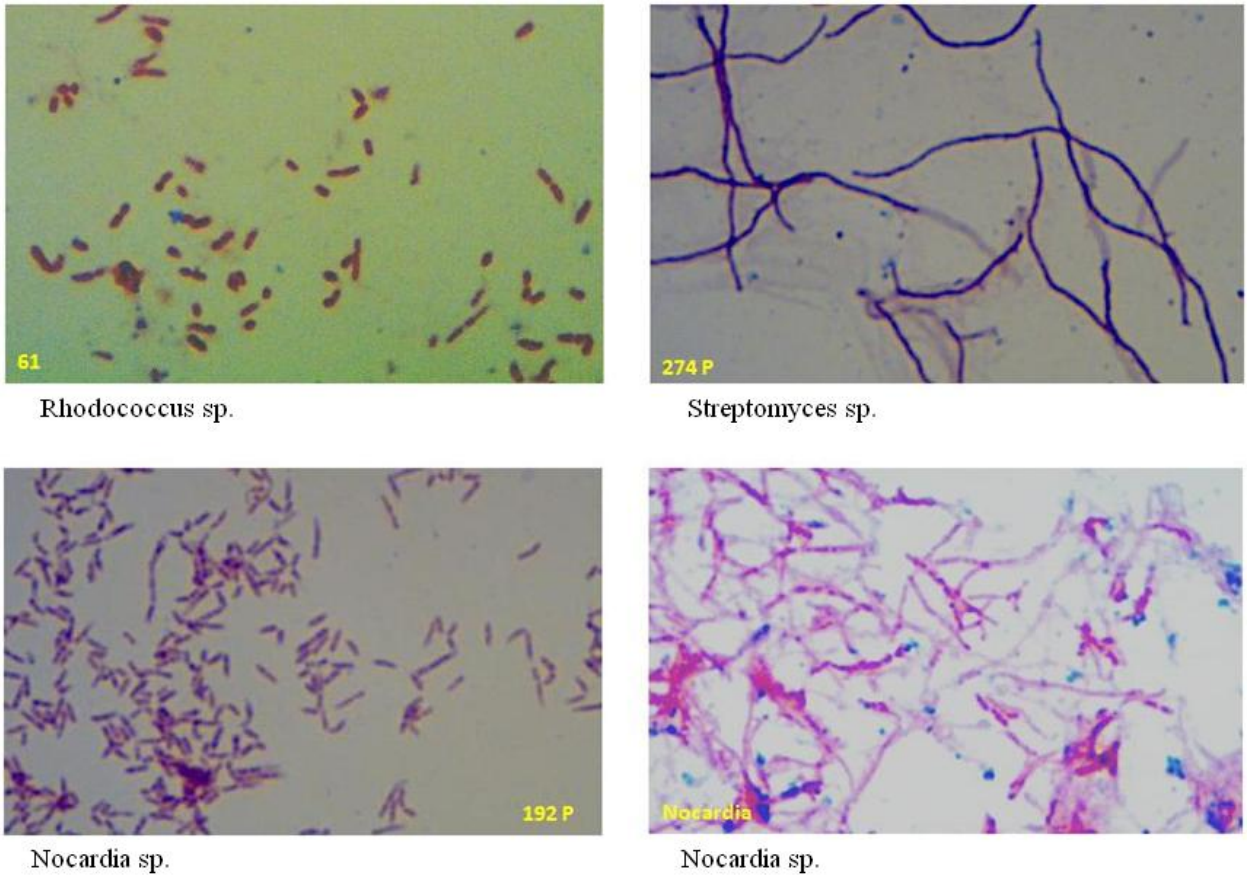

Figure 8 Cell morphology of selected non-mycobacterial microorganisms from sputum samples detected by rats. Gram stained smears were made from cultures grown on different media including Mycobacteria 7H11 agar, LB agar and BHI agar. *Rhodococcus* sp. (61) shows unbranched pleomorphic (variable shape and size) rods or coccobacilli which may resemble the unbranched *Nocardia* (192 P). The branched *Nocardia* filaments is distinguished from the related branched *Streptomyces* (274 P) filaments by fragmentation and beading whereby *Streptomyces* spp. filaments are long and un-fragmented.

3.2.3.2 Biochemical reactions of non-mycobacterial isolates

The biochemical reactions which included degradation of xanthine, tyrosine, sorbitol, mannitol, casein, aesculine, uric acid, starch and urea as well as gelatin liquefaction were not conclusive due to variability even within isolates identified as *Nocardia* spp. by specific PCRs. There were also variable reactions within *Rhodococcus* spp. and *Streptomyces* spp. isolates which indicates plasticity of biochemical reactions. However, urea degradation test discriminated *Nocardia* spp. from *Streptomyces* spp, whereby *Nocardia* isolates degraded urea (positive) indicated by change of colour of urea medium from colourless to pink red unlike *Streptomyces* isolates which were negative.

Opacification of the Mycobacteria 7H11 medium (Flores and Desmond 1993) was useful in identification of *Nocardia* spp. by the characteristic formation of chalky white colonies on this medium being remarkable for *Nocardia* spp. than *Streptomyces* spp. (Fig. 9).

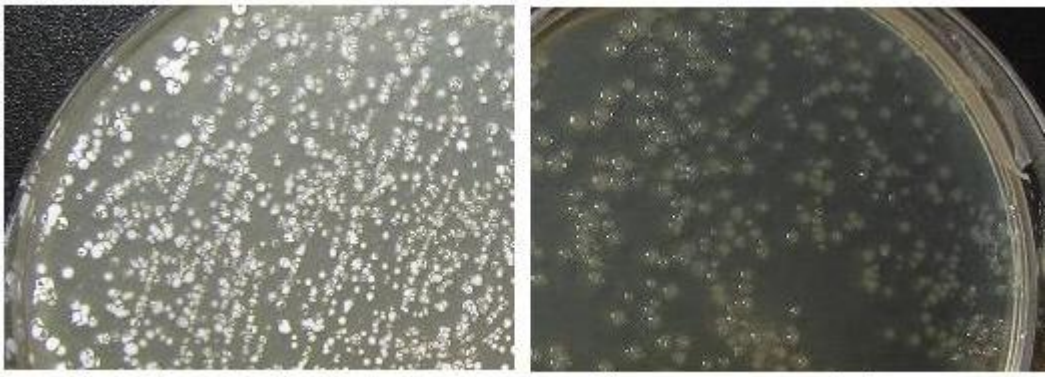

Figure 9 Opacification of the Mycobacteria 7H11 medium by *Nocardia* sp. isolates from sputum samples detected by rats, unlike *Streptomyces* spp. *Nocardia* isolates render the medium opaque and have characteristic chalky white colonies.

3.2.3.3 Distribution of non-mycobacterial microorganisms in sputum and detection by rats

Streptococcus spp. were the most abundant among the respiratory tract bacterial isolates (n=69). Thirteen isolates from rat-positive sputum samples were identified as *S. pneumoniae* (Hendolin et al 1997). Other streptococcal isolates were assigned to *S. pneumoniae* based on colony and cell morphology which were similar to 13 isolates identified by PCR. Majority of *S. pneumoniae* (56.5%) from rat-positive sputa also occurred with mycobacteria (Table 12). Other respiratory tract microorganisms obtained were *Streptomyces* spp. (n=10); *Nocardia* sp. (n=3) with two isolates identified as *N. farcinica* belonging to the *N. asteroides* complex according to Brown et al. (2004) and Hasegawa et al. (2007). *Rhodococcus* spp. (n=3); *Moraxella catarrhalis* (n=8) confirmed by specific PCR for *M. catarrhalis* according to Hendolin et al. (1997); *Candida* spp. (n=21); *Enterococcus* spp. (n=7); *S. succinus*

and other *Staphylococcus* spp. Rat-positive sputum samples with *Staphylococcus* spp. and *Enterococcus* spp. were also either TB smear-positive or *Mtb* culture-positive. Abundant *Streptococcus* spp. co-occurred with other microorganisms including *Candida* spp. (n=5); *M. catarrhalis* (n=3); *Streptomyces* spp. (n=3); *Rhodococcus* spp. (n=1), and *Nocardia* spp. (n=1). Co-occurrence was also observed for *Nocardia* spp. and *Streptomyces* spp. (n=2), *Candida* spp., and *M. catarrhalis* (n=4). The rat-positive sputum samples with co-occurring microorganisms are shown in Table 12 and Fig. 10. A different detection pattern was found for some species, which were detected by the rats more frequently when they occurred together with *Mtb* (Fig. 10).

Comparison of distribution of rat-positive and rat-negative sputa with non-mycobacterial species (Table 12) shows that the detection of *M. catarrhalis*, *S. pneumoniae*, *Candida* spp., *Enterococcus* spp., *S. succinus* and other *Staphylococcus* spp. is significantly different from the detection of sputa with *Mtb* ($P < 0.05$) (Table 12, Fig. 6). This indicates that these non-mycobacterial species do not cause detection of sputa by rats compared to *Mtb*. The distribution of rat-positive and rat-negative sputa with *Rhodococcus* spp., *Nocardia* spp., *Streptomyces* spp., and few unknown microorganisms was not significantly different from that of *Mtb*. However, these species were not as abundant in detected sputa as *Mtb* (Tables 11 and 12) and have low prevalence.

Table 12 A. Respiratory tract microbes from smear-positive and -negative sputum samples tested by rats.

Species	Number (n)			Rat-positive			Rat-negative			Smear negative detection (%)	Significance^
	(n)	Smear $^{+}$	Smear $^{-}$	(n)	Smear^{+}	Smear $^{-}$	(n)	Smear^{+}	Smear $^{-}$		P-value
Moraxella catarrhalis	8	1	7	2	1	1	6	0	6	12.5	0.011
Rhodococcus	3	0	3	2	0	2	1	0	1	66.7*	NS
Nocardia (*N. farcinica*)	3	0	3	2	0	2	1	0	1	66.7*	NS
Streptomyces	10	2	8	6	2	4	4	0	4	40.0*	NS
Candida	21	3	18	10	3	7	11	0	11	33.3*	0.045
Streptococcus	69	14	55	23	13	10	46	1	45	14.5	3.85e-5
Enterococcus	7	2	5	2	2	0	5	0	5	0.0	0.025
Staphylococcus	7	2	5	2	2	0	5	0	5	0.0	0.025
Unidentified	11	4	7	6	4	2	5	0	5	18.2	NS
Total	139	28 (20.1%)	111 (79.9%)	55 (39.6%)	27 (49.1%)	28 (50.9%)	84 (60.4%)	1 (1.2%)	83 (98.8%)		

Two *Streptococcus pneumoniae* isolates from smear-negative *Mtb* culture-positive sputum not detected by rats are not presented in this Table.

* Frequently detected microorganisms from TB-negative, rat-positive sputum had their volatile compounds analyzed by GC/MS and compared with volatiles of *Mtb*.

^ Significance in Fisher's exact test as compared to distribution of rat-positive/rat-negative *Mtb* data (Table 12 B). Each row in Table 12 A is compared with Table 12 B. Rat-positive not significantly different from sputa with *Mtb* is shown by NS.

Table 12 B. Distribution of rat-positive and rat-negative in sputa with *M. tuberculosis*.

Mycobacterial designation	Number (n)			Distribution (%)	Rat-positive			Rat-negative			Detection (%)
	(n)	Smear $^{+}$	Smear $^{-}$		(n)	Smear^{+}	Smear $^{-}$	(n)	Smear^{+}	Smear $^{-}$	
Mtb	37	25	12	78.7	28	25	3	9	0	9	75.7

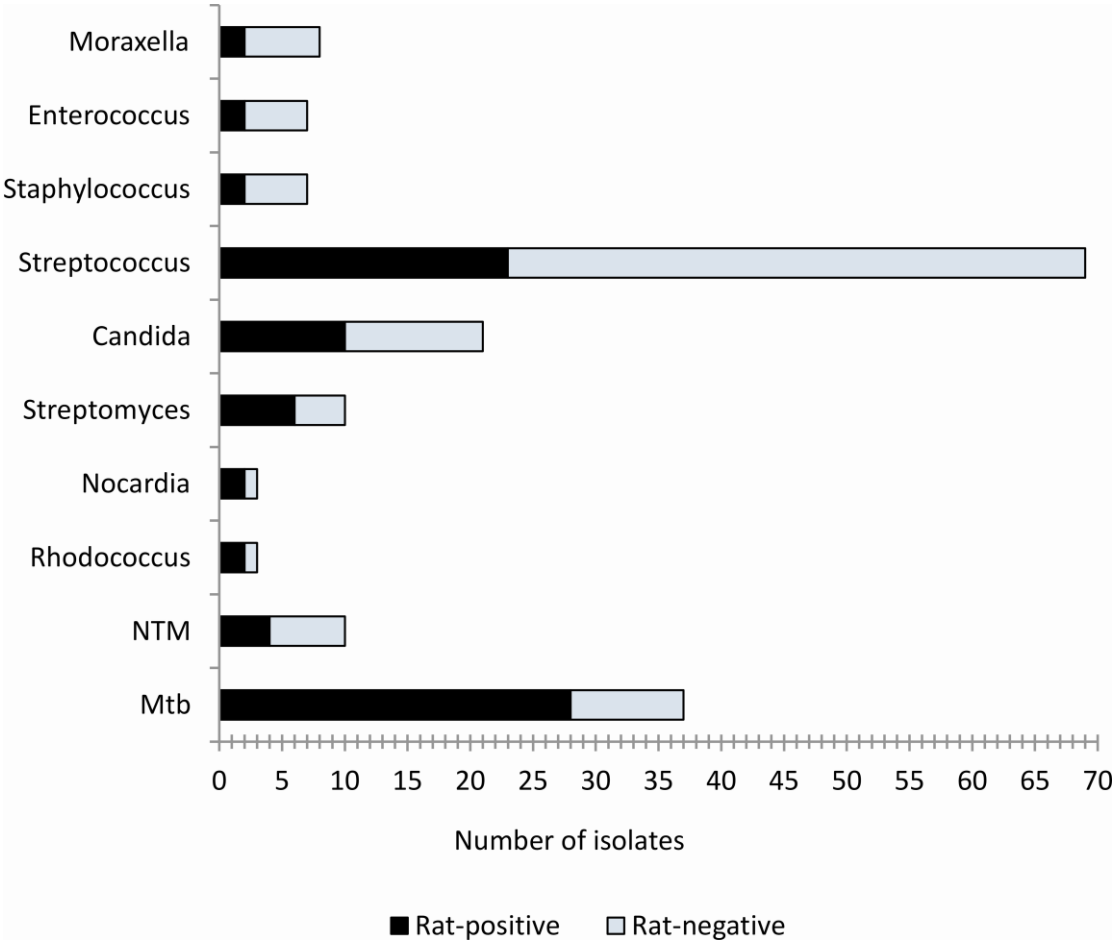

Figure 10 Microorganisms isolated from rat-positive sputa and respective proportions of rat-positive and rat-negative of these microorganisms. With exception of *Nocardia* sp. and *Rhodococcus* sp., other microorganisms co-occurred with *Mtb* (confirmed by culture) or mycobacteria sp. (AFB+) confirmed by microscopy.

3.2.4 Odour compounds of non-mycobacterial isolates

Analysis of odour compounds of selected isolates from *Mtb* smear-negative, culture-negative but rat-positive sputum samples, namely, *Rhodococcus* sp., *Candida* sp., and *Staphylococcus* sp. isolates as well as representative reference strains of

Nocardia spp. (*N. asteroides* and *N. africana*) and *Streptomyces* spp. (*S. coelicolor, S. griseoflavus,* and *S. antibioticus*) revealed that these microorganisms do not produce the *Mtb*-specific volatile compounds. However, they shared with *Mtb* a number of volatile compounds which are of no value for TB diagnosis (Table 13). The complete list of *Mtb*-specific and shared or overlapping volatile compounds found in *Mtb* and other microorganisms is provided in other section of this thesis covering odour compounds of *Mtb*, NTM and related microorganisms. Candidate volatile odour markers of TB also reported by Syhre and Chambers (2008), namely methyl nicotinate, methyl *para*-anisate, *ortho*-phenylanisole, and methyl phenylacetate were predominant in *Mtb* but not in any of these opportunistic pulmonary tract pathogens. However, methyl phenylacetate was not specific to *Mtb* as it was found in other mycobacteria. Volatile compounds shared by *Mtb*, *Nocardia* spp., *Streptomyces* spp., and *Rhodococcus* sp., which cannot be regarded as specific markers for *Mtb* are shown in Table 13. Many of these compounds are also produced by other microbial species (Schulz et al. 2004, Schulz and Dickschat 2007, Verhulst et al. 2009, Wilkins and Schöller 2009). For example, aciphyllene, a known sesquiterpene from the endophytic fungus *Muscodor albus* (Atmosukarto et al. 2005) is a more specific compound occurring in *Nocardia* spp.

Table 13 Volatile compounds of isolates from sputum samples and reference *Mtb, Nocardia* spp. and *Streptomyces* spp.

Compounds	Microbial species tested (n)								
	Mtb	*Rhodoccus* isolate	*Staphylococcus* isolate	*Candida* isolate	*Nocardia asteroides*	*Nocardia africana*	*Streptomyces coelicolor*	*Streptomyces antibioticus*	*Streptomyces griseoflavus*
	35	2	2	2	4	6	3	3	4
Dimethyl disulfide			X						
Dimethyl trisulfide		X	X	X			X	X	X
Dimethyl tetrasulfide		X					X	X	
Methyl methanethiosulfonate		X							
2,3-Dimethyl-5-isopentylpyrazine		X	X	X					
Unknown pyrazine		X	X	X					
Camphor	X						X		
Linalyl acetate							X		
Isobornyl acetate			X						
Aciphyllene					X	X			
Unknown diterpenoid					X	X			
2-Hydroxy-3-butanone							X	X	X
2-Hydroxy-3-pentanone	X						X	X	X
2,5-Dimethylthiopene								X	X
1-Hexanol	X								X
1-Octanol									X
4-Methyl-2-pentanone							X		X
4-Methylpent-3-en-2-one							X		
Methylbutyrolactone	X				X				
2-Phenylethanol	X						X	X	
Ethyl phenylacetate							X		X
Methyl phenylacetate [a]	X								
Methyl nicotinate [a]	X								
Methyl *para*-anisate [a]	X								
ortho-Phenylanisol [a]	X								

a Syhre and Chambers. 2008

3.3 Detection of cultures of *Mycobacterium* and related species

3.3.1 Screening (initial detection) of microorganisms by rats

A total of 334 samples from 38 strains (20 bacterial and 2 yeast species) were tested by TB detection rats (Table 4). Initial testing of different culture batches of the same

strain, but using different incubation periods showed variable and inconsistent detection of certain batches. For example, some cultures of the reference species *Mtb* and the clinical isolate of this species coded N185/8 were detected more often than others. The rats occasionally detected *M. smegmatis*, *M. neoaurum*, *M. aurum* and other *Mtb* clinical isolates. Rats did not detect mycobacteria-related *Nocardia* spp., *Rhodococcus* sp. and the other microbes. This led to another experiment with cultures of well defined growth interval (age) that included *Mtb* and the nontuberculous *M. smegmatis* grown in parallel in order to understand whether the detection was associated with a certain age of cultures.

3.3.2 Detection of different growth phases of *M. tuberculosis* and *M. smegmatis* by rats

Testing of various growth intervals of *Mtb* and *M. smegmatis* revealed that *Mtb* is more frequently detected in exponential (log phase) and early stationary phase cultures (21–30 days) than in early log phase (≤10 days) and late stationary phase cultures (≥41 days) ($P < 0.001$, Fisher's exact test) (Fig. 11). The detection of nontuberculous *M. smegmatis* was random with no growth-related pattern. Four of the five interval samples of this species were each detected only once unlike *Mtb*. Comparison of the detections of the two species showed a significant difference with *Mtb* detected by more than two rats repeatedly as compared to *M. smegmatis* ($P < 0.008$, Fisher's exact test), which was detected only once in each of the four intervals (Table 14, Fig. 11). The detection of *Mtb* corresponded with the standard growth

curve of this species constructed from CFU values obtained at each interval before inactivation of the culture (Fig. 12).

The overall analyses revealed that the TB detection rats have a better detection rate than random (Odds ratio) for *Mtb* [OR = 3.77 (95% CI: 2.34–5.94), $P < 10^{-5}$] but not for the NTM, *M. smegmatis* [OR = 1.42 (95% CI: 0.72–2.61), $P = 0.23$].

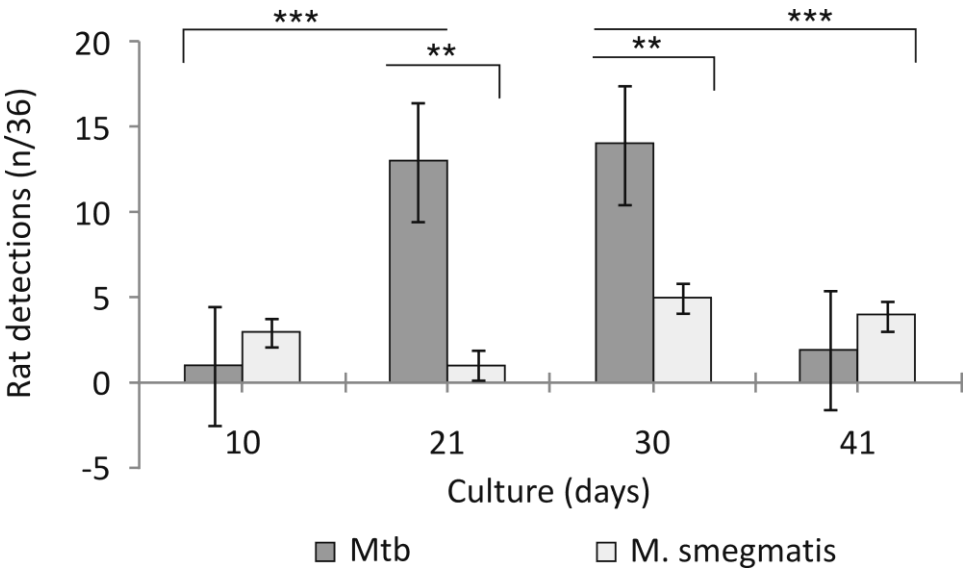

Figure 11 Detection of different growth phases of *Mtb* and *M. smegmatis* cultures by rats. *Mtb* was more frequently detected in exponential (log phase) and early stationary phase cultures (21–30 days) than in early log phase (≤10 days) and late stationary phase cultures (≥41 days) (three asterisks, $P < 0.001$, Fisher's exact test). *Mtb* detection was significantly different compared to *M. smegmatis* in exponential (log phase) and early stationary phase cultures (21–30 days) (two asterisks, $P < 0.008$, Fisher's exact test). Error bars represent standard error (SE) of detections (n/36 expected detections).

The sensitivity, specificity, and accuracy of detecting *Mtb* cultures in exponential and stationary phases were higher than for NTM (83.3%, 94.4% and 94%, respectively) (Table 14). Sensitivity value dropped to 50% when all growth phases,

including the less-detected early and late cultures, were included in the analysis (Table 14). The specificity of the rats was unchanged by the inclusion of all *Mtb* cultures (growth phases).

Table 14 Detection of *Mtb* and *M. smegmatis* and sensitivity, specificity and accuracy of TB detection rats.

Species	Samples (n)	Rats positive response (n)	Rats negative response (n)	Sensitivity (%)	Specificity (%)	Rats accuracy (%)
Mtb: exponential- and early stationary-phase (21–30 days)	6	5	1	83.3	94.4	94
Mtb: all growth phases (10–41 days)	12	6/12	6	50	94.4	91.3
M. smegmatis: all growth phases (10–65 days)	14	4*/14	10	28.5	94.4	88.6
Negative sputum	161	9/161	152	n/a	n/a	n/a

* The detection of *M. smegmatis* was random (not growth phase-related) whereas the four detections are sum of unrepeated single detection of four different growth phases.

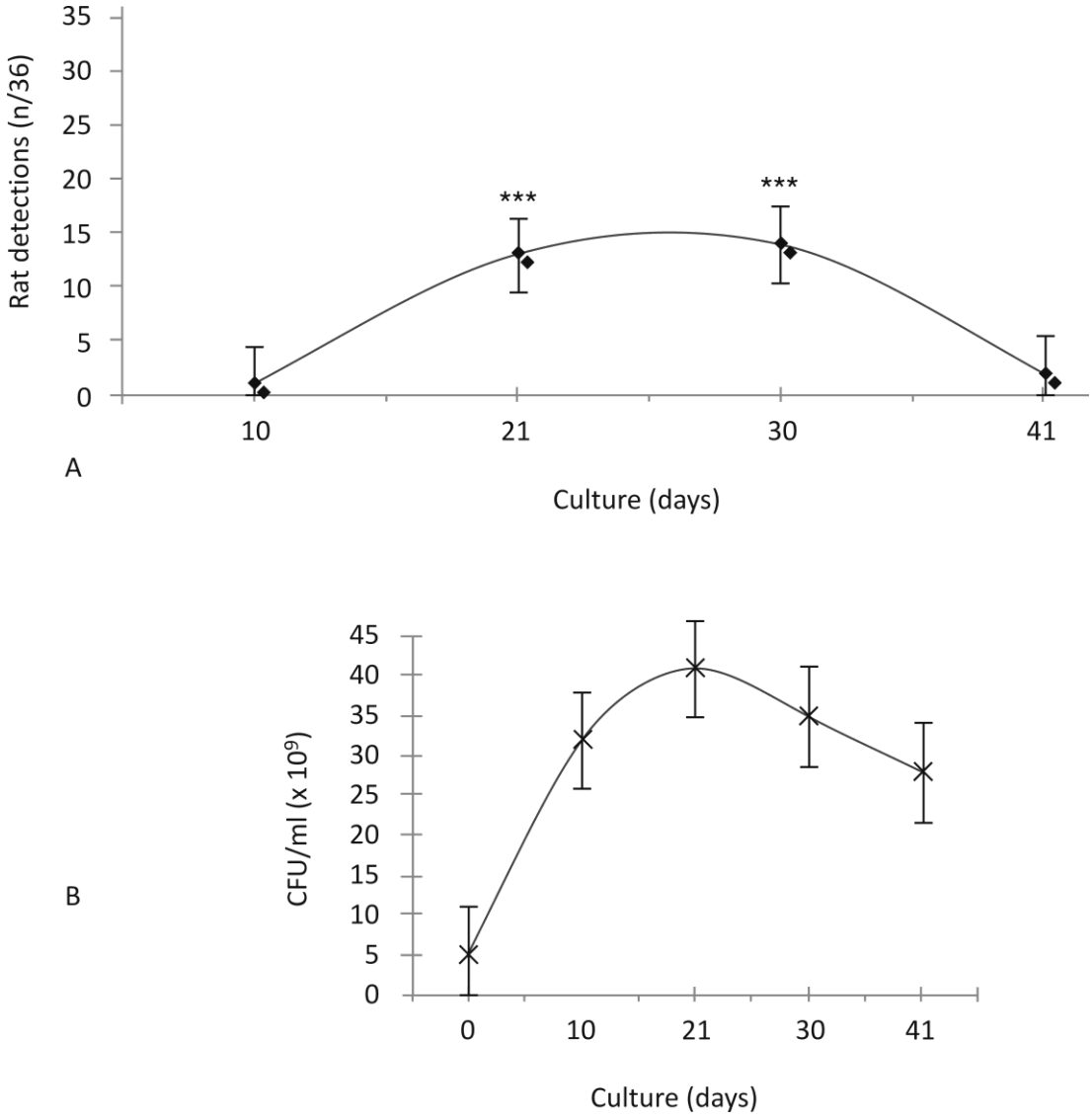

Figure 12 Detection of different growth phases of *Mtb* in 7H9 medium by rats and growth curve of respective *Mtb* culture. Upper curve (A) shows numbers of positive rat scores and lower curve (B) shows *Mtb* bacteria counts established from OD_{580nm} values whereby an OD of 0.1 was equal to 5×10^7 mycobacteria cells/ml. Exponential and early stationary phase cultures (21–30 days) were detected more frequently than early log phase (≤10 days) and late stationary phase cultures (≥41 days) (three asterisks, $P < 0.001$, Fisher's exact test). Error bars represent standard error (SE) of rats' detections and CFU counts.

These results show that rats can discriminate cultures of *Mtb* from NTM species and the related *Nocardia* spp. and *Rhodococcus* spp. which are also acid-fast bacilli and

can often be misdiagnosed as *Mtb* in smear-microscopy. The members of these genera were isolated in human sputum including smear-negative sputum detected by rats (Table 12). The fact that detection of *Mtb* cultures was age-related, with exponential and early stationary phase detected more frequently than early log phase and late stationary phase ($P < 0.001$, Fisher's test) (sensitivity=83.3%, specificity=94.4%, accuracy=94%) suggests that studies of target volatiles of *Mtb* for TB detection should include different growth stages of *Mtb* and control microorganisms.

3.4 Odour compounds of mycobacteria and other microorganisms, and detection by rats

3.4.1 Volatile compound profiles, frequencies and distribution

Microorganisms cultured in different media produced 26 different volatile compounds. Different compounds were also produced by microorganisms in different growth phases. Half of the volatile compounds were specific to *Mtb* only (n=13), whereas another half (n=13) were found in *Mtb*, NTM and other microorganisms (Table 15, Fig. 14).

Table 15 Frequencies of volatile compounds detected in *M. tuberculosis* strains and in the cluster of nontuberculous mycobacteria, *Nocardia, Rhodococcus, Streptomyces, Staphylococcus* and *Candida* species, named "NTM and non-mycobacteria" including isolates from smear-and *Mtb* culture-negative sputa detected by rats.

No.	Compound		*Mtb* (n=35)			NTM and non-mycobacteria (n=56)			
	Mtb-specific volatiles		Present (+)	Absent (-)	Occurrence frequency (%)	Present	Absent	Occurrence frequency (%)	Tested by rats (TR)
1	Methyl nicotinate	^	18	17	51.4	0	56	0	TR
2	Methyl 4-anisate		17	18	48.6	0	56	0	TR
3	2-Phenylanisol		16	19	45.7	0	56	0	TR
4	4-Methylanisol		10	25	28.6	0	56	0	TR
5	Ethyl 4-anisate		9	26	25.7	0	56	0	TR
6	2-Methyl-1,6-dioxaspiro-4-nonane		8	27	22.9	0	56	0	TR
7	Methyl 2-amino benzoate		4	31	11.4	0	56	0	n/a
8	Trimethyloxazole		3	32	8.6	0	56	0	n/a
9	Benzothiazole		3	32	8.6	0	56	0	TR
10	4-Hydroxy-4-methylpentan-2-one		2	33	5.7	0	56	0	n/a
11	Dimethyldihydro-furanone		2	33	5.7	0	56	0	n/a
12	Dimethyltetra-hydropyranone		2	33	5.7	0	56	0	n/a
13	Cyclic proline-glycine *		2	1	66.7	0	3	0	TR*
	Overlapping/shared odour compounds produced by *Mtb*, NTM and other respiratory tract microorganisms								
14	2-Phenylethanol	^	27	8	77.1	16	40	28.6	TR
15	Methyl benzoate		23	12	65.7	3	53	5.4	TR
16	4-Pentanolide		21	14	60	19	37	33.9	TR
17	Methyl phenyl acetate		16	19	45.7	4	52	7.1	TR
18	Methyl 2-furoate		8	27	22.9	3	53	5.4	TR
19	Dimethyl-pentanolide		6	29	17.1	2	54	3.6	TR
20	Methyl salicylate		5	30	14.3	6	50	10.7	TR
21	Camphor		4	31	11.4	1	55	1.8	TR
22	Methylbutenoilide		2	33	5.7	5	51	8.9	n/a
23	Methyl dimethyl benzoate		2	33	5.7	2	54	3.6	n/a
24	Benzyl alcohol		2	33	5.7	6	50	10.7	n/a
25	Ethyl benzoate		1	34	2.8	2	54	3.6	n/a
26	Aciphyllene **		0	18	0	7	1	87.5	n/a

^ Nawrath et al., (*in prep*.)

* Cyclic proline-glycine was obtained in 2 out of 3 *Mtb* cultures in PBSGG minimal nutrient medium

** Aciphyllene was obtained in 7 out of 8 *Nocardia* cultures in Sauton medium

TR* tested by rats as combination of proline and glycine

3.4.2 Clustering of microorganisms by volatile compounds produced

There was a good clustering of microorganisms based on volatile compounds produced. Random forest cluster analysis of these volatile data shows a significant clustering of *Mtb* strains only; a cluster of *Mtb* and NTM only, and a cluster of *Mtb*, NTM, *Nocardia* spp., *Rhodococcus* sp., *Staphylococcus* sp. and *Candida* sp. from

sputum samples detected by rats. This is indicated by higher AU (approximately unbiased) *p*-value greater than 95% (Suzuki and Shimodaira 2006). There are 7 clusters (Fig. 13) with highly significant probability values (100%) indicating that these clusters are strongly supported by data. Indeed some volatile compounds were only found (specific) in certain microorganisms such as *Mtb*, and other volatile compounds had a wider occurrence in different microorganisms, hence causing the observed linkage between microorganisms' clusters.

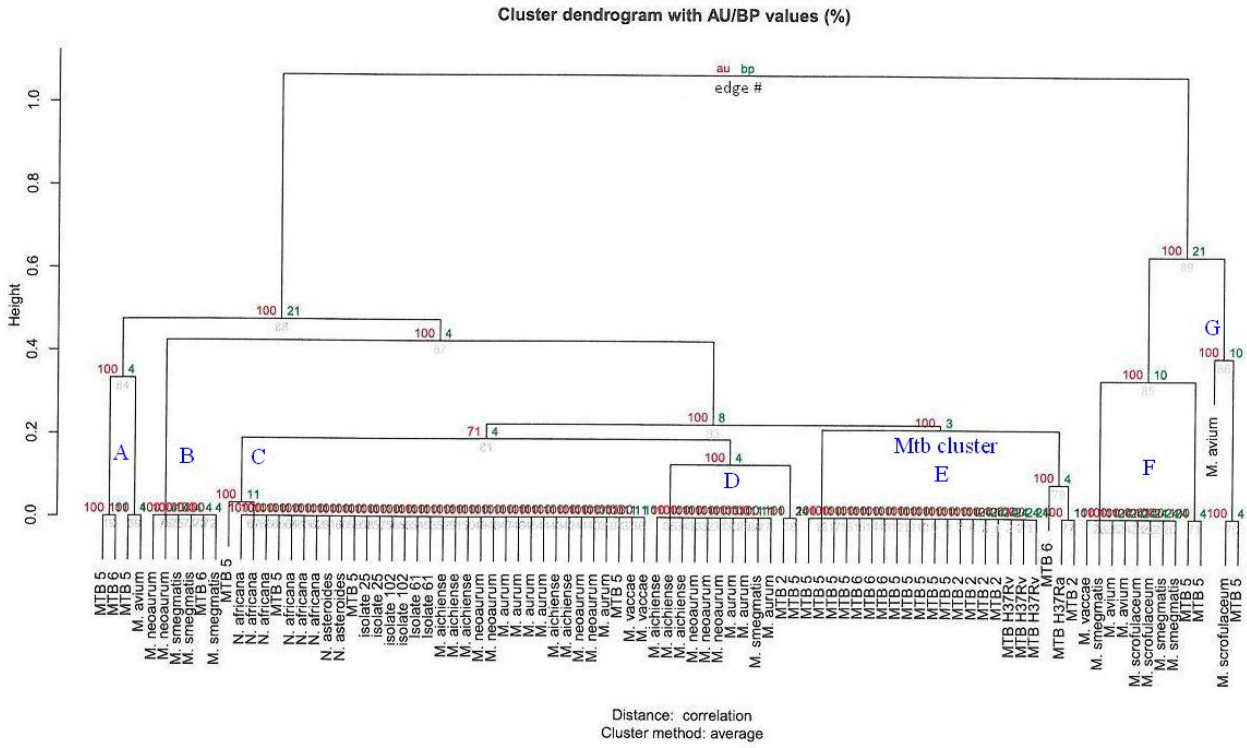

Figure 13 Clusters of *M. tuberculosis*, nontuberculous mycobacteria and other microorganisms based on volatile odour compounds produced by these microorganisms. The volatile compounds data were subjected to cluster analysis performed at 0.05 significance level or *alpha* (α). There are 7 clusters with AU *p*-values > 95%. Only one of the 7 clusters is specific to *Mtb* strains (cluster E). Other clusters consist of *Mtb* and *M. avium* (A); *Mtb* and 2 NTM (B); *Mtb*, 4 NTM, *Nocardia* spp., *Candida* sp. (isolate 25), *Staphylococcus* sp. (isolate 102), *Rhodococcus* sp. (isolate 61) (C); *Mtb* and 4 NTM (D and F); *Mtb* and 2 NTM (G). AU (approximately unbiased) *p*-values, BP (bootstrap probability) values.

The frequencies of occurrence of these volatiles in *Mtb* and other microorganisms is shown in Fig. 14, which also depicts the proportion (percent contribution) of the overlapping/shared volatiles found in *Mtb* and in other microorganisms including clinical isolates from sputum samples detected by rats.

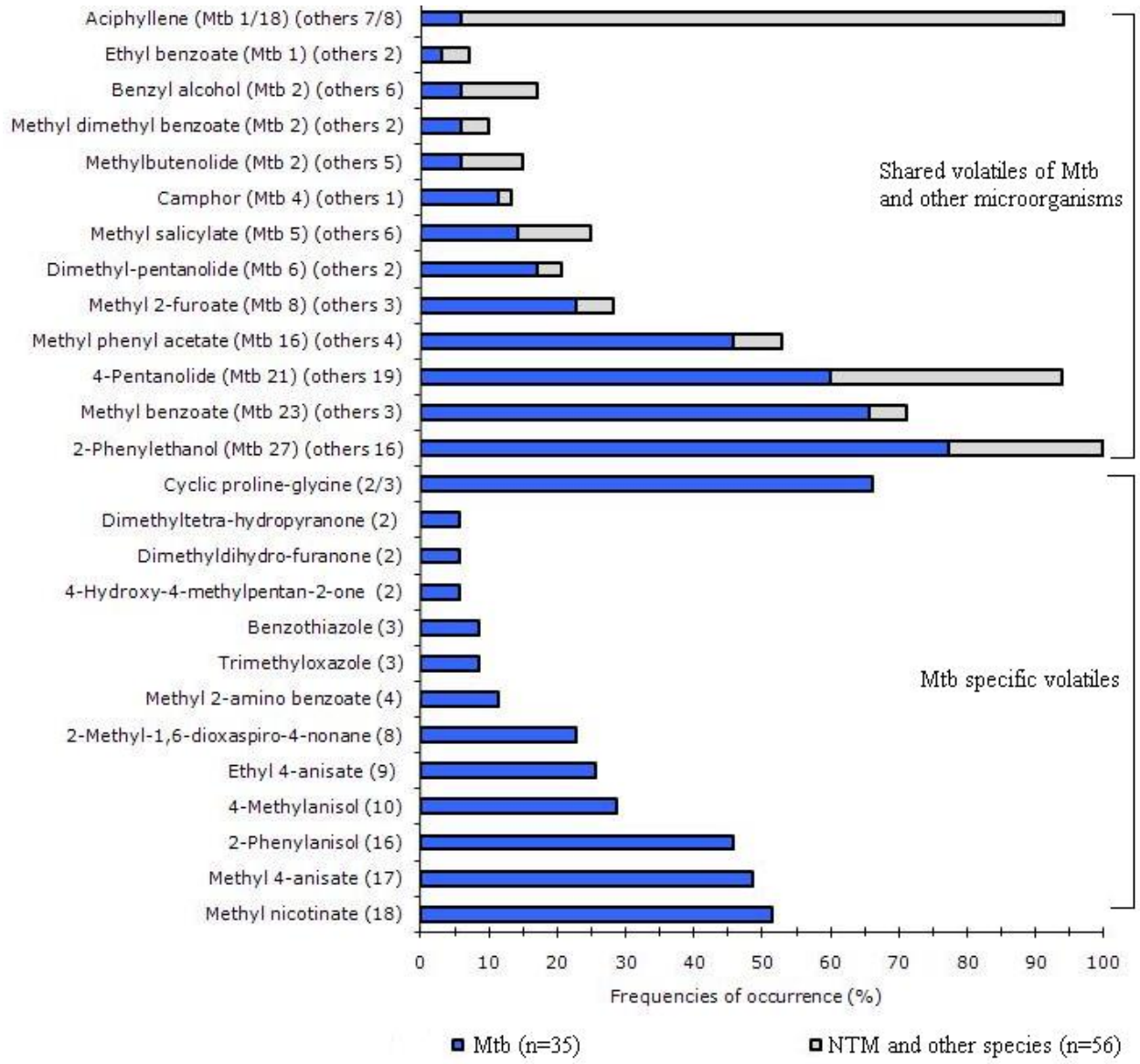

Figure 14 Occurrence and proportion (percent) of specific and shared volatile compound in a cluster of *Mtb* strains and NTM and other microorganisms including *Nocardia* spp., *Rhodococcus* sp., *Streptomyces* spp, *Staphylococcus* sp. and *Candida* sp. The numbers in brackets (after compounds) indicate total samples with the compounds.

3.4.3 Olfactory detection of candidate odour compounds by rats

A total of 7 specific volatiles of *Mtb* and 7 shared volatiles were presented individually to five trained TB detection rats in different concentrations in TB-negative sputum. Coded compounds (see Table 16) were first presented individually to the 5 rats and subsequently in combinations (blends). Rats' detected 2 *Mtb*-specific volatile compounds repeatedly (2-phenylanisol and benzothiazole) but detection was not statistically significant (P =0.155). Only one shared compound (Camphor) was also detected by rats in 1 out of 5 tests (days) (50 presentations for this compound) and 92 tests (920 presentations for all overlapping compounds). Table 16 shows the cumulative sum of presentations of individual and combinations of compounds to the rats in different concentrations. Each individual compound or combination of compounds was minimally tested three times, with exception of paired overlapping compounds found in most microorganisms which were presented once to the 5 rats which performed two sessions on each sample. Hence these samples were technically tested ten times in that single presentation (5 rats x 2 sessions).

Table 16 Presentation of volatile compounds to TB detection rats to determine TB odour compounds detected by rats in sputum. 10 tests per sample per presentation.

Code	Compound name	Species specificity	Presentation	Cumulative ^	Rats test	+ Conc. $10^{-1} - 10^{-5}$
1	Methyl nicotinate (99%)	*Mtb*-specific	Presented individually in different concentration	16	–	
2	Methyl 4-anisate (99+ %)			16	–	
3	2-Phenylanisol (2-Methoxybiphenyl,98%)			16	±	10^{-2} NS
4	4-Methylanisol			16	–	
5	Ethyl 4-anisate (97%)			16	–	
6	Chalcogran			16	–	
7	Benzothiazole (96%)			21	±	10^{-2} NS
8	2-Phenylethanol	Overlapping compounds /found in *Mtb*, NTM and other microorganisms	Presented individually in different concentration	10	–	
9	Methyl benzoate (99%)			10	–	
10	4-Pentanolide (γ-valerolactone) (99%)			10	–	
11	Methylphenylacetate (99+ %)			10	–	
12	Methyl 2-furoate (98%)			10	–	
13	Methyl salicylate (99%)			10	–	
14	Camphor (96%)			10	±	10^{-2} NS
15	Proline + Glycine	*Mtb*-specific	Presented in pair	10	–	
16	Methyl nicotinate + Methyl 4-anisate			6	–	
17	Methyl nicotinate + 2-Phenylanisol (2-methoxybiphenyl)			6	–	
18	Methyl nicotinate + 4-Methylanisol			6	–	
19	Methyl nicotinate + Ethyl 4-anisate			6	–	
20	Methyl nicotinate + Chalcogran			6	–	
21	Methyl nicotinate + Benzothiazole			6	–	
22	Methyl nicotinate Methyl 4-anisate 2-Phenylanisol (2-Methoxybiphenyl) 4-Methylanisol Ethyl 4-anisate Chalcogran Benzothiazole	*Mtb*-specific	Presented in combination (blend) of 7 compounds	23	+ *	10^{-3} (P = 0.001)
23	2-Phenylethanol + Methyl benzoate	Overlapping compounds /found in *Mtb*, NTM and other microorganisms	Presented in pair	1	–	
24	2-Phenylethanol + Pentanolide (γ-Valerolactone)			1	–	
25	2-Phenylethanol + Methylphenylacetate			1	–	
26	2-Phenylethanol + Methyl 2-furoate			1	–	
27	2-Phenylethanol + Methyl salicylate			1	–	
28	2-Phenylethanol + Camphor			1	–	
29	2-Phenylethanol Methyl benzoate 4-Pentanolide (γ-Valerolactone) Methylphenylacetate Methyl 2-furoate Methyl salicylate Camphor	Overlapping compounds/found in *Mtb*, NTM and other microorganisms	Presented in combination (blend) of 7 compounds	6	–	
30	Methyl nicotinate Methyl 4-anisate 2-Phenylanisol (2-Methoxybiphenyl)	*Mtb*-specific	Combination of 3 most abundant compounds	10	±	10^{-3} NS
31	Methyl nicotinate Methyl 4-anisate 2-Phenylanisol (2-Methoxybiphenyl) 4-Methylanisol Ethyl 4-anisate Chalcogran Benzothiazole 2-Phenylethanol Methyl benzoate 4-Pentanolide (γ-Valerolactone) Methylphenylacetate Methyl 2-furoate Methyl salicylate Camphor	*Mtb*-specific and Overlapping compounds /found in *Mtb*, NTM and other microorganisms	Presented in combination (blend) of 7 *Mtb*-specific compounds and 7 overlapping compounds	3	–	

+ * Detection with statistical significant difference; P = 0.001). ± Slightly detection not statistically significant (NS).

(–) All concentrations were not detected by rats. + Conc. = concentrations of volatile compounds detected by rats.

^ The cumulative presentation includes different concentrations of the same compound or combinations.

The combination of volatile compounds in pairs consisting of the most abundant methyl nicotinate and each of the 6 remaining *Mtb*-specific volatiles was not detected by rats. Proline-glycine volatile compound of *Mtb*, prepared by mixing the two constituent compounds was also not detected by rats (Table 16). The combination of all 7 *Mtb*-specific volatiles was repeatedly detected better than individual compounds (2-phenylanisol and benzothiazole) and the blend of 3 *Mtb*-specific volatile compounds (P = 0.001, Fisher's exact test). The rats detected better the mild concentration (10^{-3}) of this blend than the higher (10^{-1} and 10^{-2}) and lower concentrations (10^{-4} and 10^{-5}) (P = 0.001, Fisher's exact test). This blend was detected in 4 out of 7 test days. In one occasion, the rats detected a slightly higher concentration (10^{-2}) of this blend but the detection was not significant compared to mild concentration (10^{-3}) of this blend (Fig. 16).

Among individual volatile compounds, benzothiazole was detected twice by rats with 6 out of 10 scores (60%) on the first day and 7 out of 10 (70%) on the second day, but was not detected in the subsequent 5 days. The blend of 3 most abundant volatiles (methyl nicotinate, methyl 4-anisate and 2-phenylanisol) was also detected in 1 out of 4 days which was also not statistically significant. Significant detection was obtained only when the 7 selected *Mtb*-specific compounds were combined together and presented to rats as blend (Fig. 15).

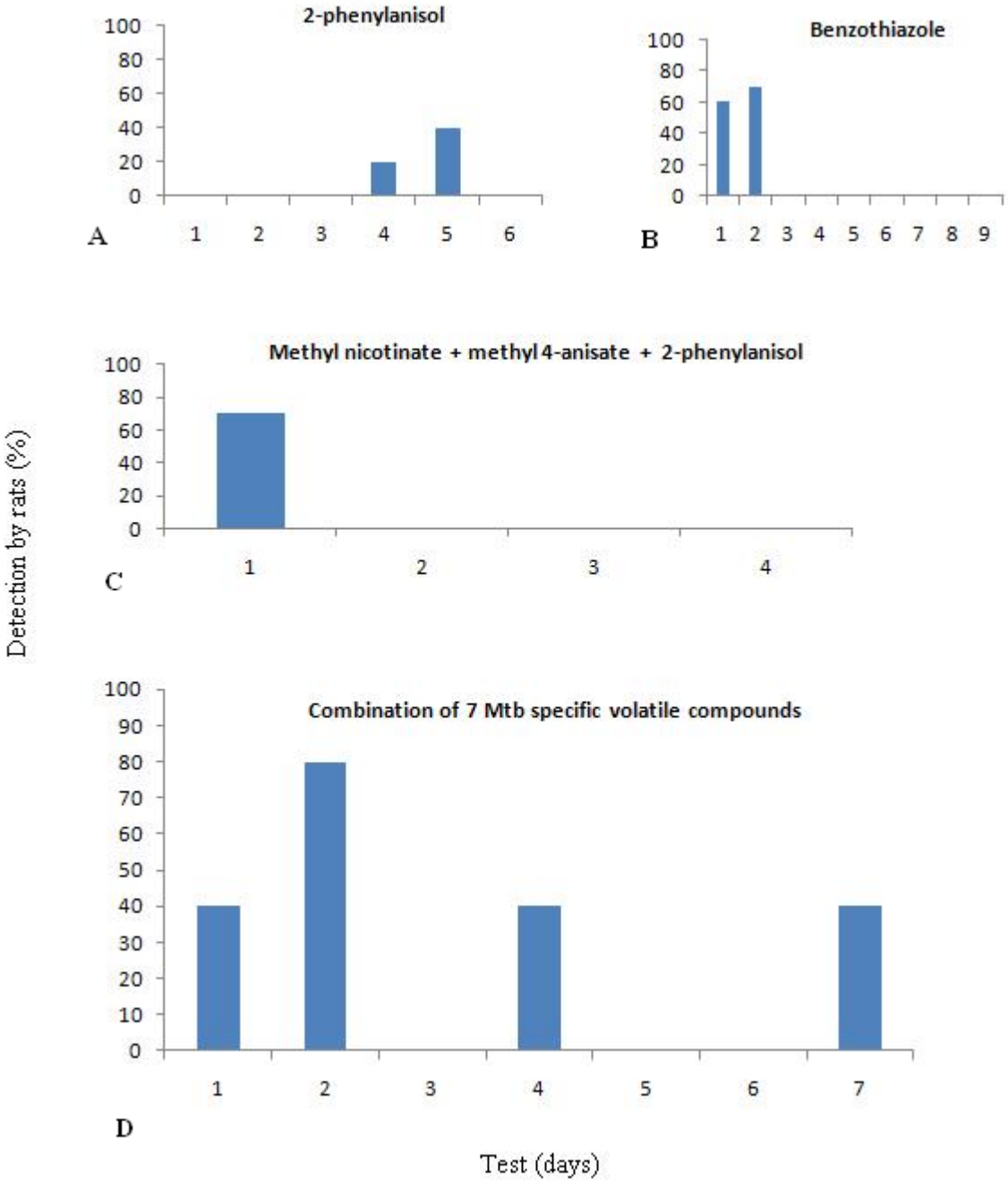

Figure 15 Presentation of *Mtb*-specific volatile compounds individually shows that two compounds are slightly detected (A) and (B). None of these compounds was detected when presented in pairs. Combination of three most abundant *Mtb*-specific volatiles induced slight detection (C), whereas the detection increased significantly when the 7 *Mtb*-specific compounds were combined (D) (code No. 22, Table 16). The overlapping volatile compounds were not detected in 92 tests involving individual compounds (excluding Camphor which was detected in one out of five tests) and combination of the overlapping compounds. The blend consisting 7 *Mtb*-specific volatiles and 7 overlapping compounds was also not detected by rats.

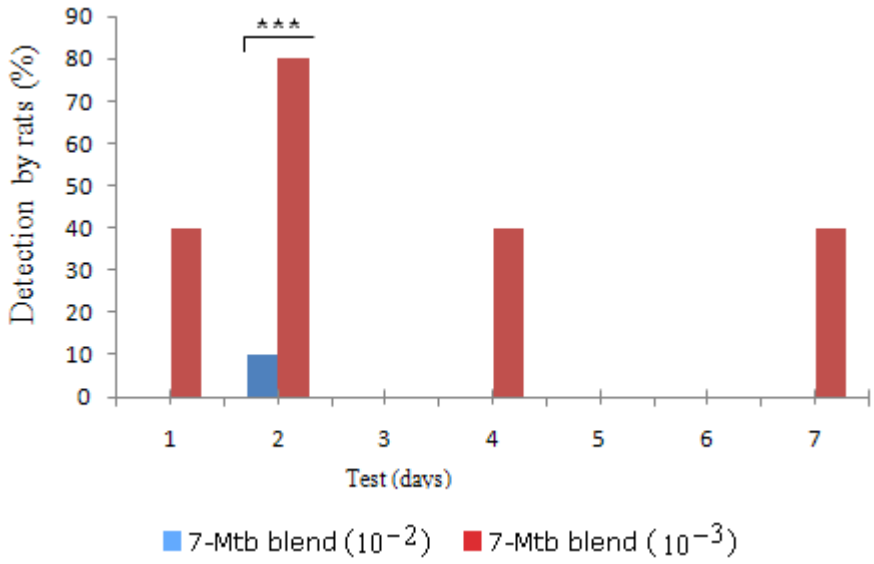

Figure 16 Detection of blend of 7 *Mtb*-specific volatile compounds by rats. Mild concentration (10^{-3}, red bars) was repeatedly detected better than higher concentration (10^{-2}, blue bar) and other concentrations of this blend (*P* = 0.001, Fisher's exact test). The mild concentration (10^{-3}; red bars) of the 7-*Mtb* blend was detected in 4 out of 7 tests. Other blends and individual compounds of this blend were not detected (except 2-phenylanisol and benzothiazole) when presented alone to the rats . A cut-off point of two rats was used to determine the positive detection.

Of the 7 overlapping/shared volatile compounds tested, only one compound (camphor) was detected once out of 5 tests (50 presentations). However, this detection was not statistically significant. Rats did not detect other shared compounds also presented in various concentrations in 5 test days (50 presentations). The blends of shared volatile compounds were also not detected by rats in 92 repeated tests. The combination of 7 *Mtb*-specific volatile compounds and 7 overlapping volatile compounds was also not detected by these rats in three presentations (30 tests). This shows that overlapping volatile compounds are not components of the TB odour detected by rats. This corroborates results of another

experiment of this study in which rats significantly discriminated cultures of *Mtb* from NTM and other related species. These results also corroborate findings of another experiment which shows that majority of the detected smear-negative sputum contained *Mtb* as revealed by culture. The detection of sputum with other microorganisms was also associated with *Mtb* (microscopy and culture) as indicated by lower prevalence of non-mycobacterial species which cannot account for the total number of false positive sputa detected by rats. Further investigations including recovery of dormant *Mtb* in sputum may give insights on actual cause of detection of negative sputa currently judged by microscopy which has low sensitivity and conventional *Mtb* culture in which dormant *Mtb* bacilli in sputum cannot grow in absence of resuscitation promoting factors (*rpfs*) (Mukamolova et al. 2010).

3.4.4 Sensitivity, specificity and accuracy of rats to detect TB in clinical control samples

Rats detected 118 of the 119 TB positive control sputum samples which were included in this experiment. The rats detected all 7 TB-positive sputa (100%) in 16 out of 17 presentations (days), and 6 out of 7 TB-positive sputa (85.7%) once. Rats also detected 61 out of 785 negative control sputum samples (false positive) in 17 presentations (days) with average false positive rate of 7.8% (negative control sputum samples detected by rats) throughout the study. These findings include evaluations performed by all 5 rats which tested each sample twice per test. The sensitivity of rats' detection of typical TB-positive sputa was 99.2%, specificity was 92.2% and the accuracy was 93.1%. These results show the profound potential of rats

in TB detection. The results also show that the ability of rats to detect TB was not affected by compounds spiked in negative sputum because the observed sensitivity and specificity is not lower than sensitivity and specificity of 81.6 and 88%, respectively, of these rats before the experiment on volatile compounds (Table 9).

4 DISCUSSION

In the following part I will discuss the major findings of this thesis including: analysis of the diversity of *Mycobacterium* species and *Mtb* genotypes and their detection by trained *Cricetomys gambianus* rats for TB detection; occurrence and diversity of respiratory tract microorganisms from smear-negative sputum samples detected by rats and assessment of whether opportunistic pulmonary pathogens are detected by rats; analyses of the ability of the rats to discriminate cultures of *Mtb*, NTM spp., *Nocardia* spp., *Rhodococcus* spp., *Streptomyces* spp., and other respiratory tract pathogens which are frequently found in sputum; identification of TB odour compounds in mycobacteria and other microorganisms; comparison of the profiles of the compounds from *Mtb* and other microorganisms; and testing of the candidate volatile compounds by trained rats for identification of the target TB odour markers detected by rats in sputum.

4.1 Mycobacterial species diversity and detection by rats

Diverse *Mycobacterium* species which exist in Dar es Salaam enabled evaluation of performance of the TB detection *Cricetomys* rats on sputa with a variety of tubercle bacilli. Ten genotypes of *Mtb* and 2 nontuberculous mycobacterial species (*M. avium* subspecies *hominissuis* and *M. intracellulare*) were identified in this study. This data is considered authentic because all negative controls included in molecular analysis of mycobacterial DNA were negative. The genotyping of *Mtb* isolates was done using the multispacer sequence typing (MST) method which enabled determination of the genotypes (Djelouadji et al. 2008). The MST method led to discovery of three

new MST profiles not previously described with this method. There was a significant correlation between MST 67 and one DOTS centre (Mwananyamala, Fig. 4), strongly suggesting circulation of a single *Mtb* clone around this DOTS centre or potential cross-contamination (Djelouadji et al. 2009). The putative cross-contamination among the MST 67 genotype is mainly suggested due to genotypic relatedness of the 9 isolates from the same DOTS centre. These findings suggest the need for reinforcing preventive measures in DOTS/TB laboratories to avoid dissemination of TB. Proper implementation of standard mycobacteriological operating procedures can control potential cross-contamination in TB laboratories (Ruddy et al. 2002, de Boer et al. 2002) and avoid false diagnosis. This finding further suggests that this *Mtb* clone is not widely circulating in Dar es Salaam, in agreement with the large diversity of MST genotypes found, which may also reflect population diversity in this cosmopolitan city. Three new MST genotypes, which have not previously been described using this method, were found during this study providing evidence that data did not result from cross-contamination during genotyping. These data likely reflect circulating mycobacteria in Dar es Salaam. The inclusion and exclusion criteria of study participants (quality and quantity of sputum samples) may have no effects on the distribution of mycobacteria reported in this study because these criteria were applied to all samples from the four DOTS centres before isolation and genotyping and there was no prior knowledge of presence or absence of mycobacteria in the specimens during selection.

This study reveals that 5.8% of mycobacterial isolates from patients suspected of pulmonary TB were not MTC organisms, which is not statistically different from

0.3% rate of NTM previously reported in Dar es Salaam (Matee et al. 2008). Although information on clinical manifestation/history of patients is not reported here, the identification of MAC-infected patients suggests the need for further studies and consideration of these species during TB diagnosis. The increasing occurrence of NTM in Dar es Salaam corroborates recent reports on the occurrence of NTM species in invasive diseases in northern Tanzania (Crump et al. 2009, 2011).

Assessment of performance of TB detection rats on sputum samples with different *Mycobacterium* species and genotypes of *Mtb* shows that rats detect sputum with different genotypes of *Mtb*. Rats detected 80% of the MST genotypes of *Mtb* (8/10). Two MST genotypes (MST 59 and MST 3) represented by 2 and 1 isolate, respectively, were the only genotypes not detected by rats (Fig. 5). However, MST 59 and MST 3 genotypes belong to clusters of *Mtb* genotypes from rat-positive sputa (Fig. 5B) suggesting that the two genotypes can also be detected by rats. Further studies are needed to determine the rat's ability to detect sputa with MST 59 and MST 3 genotypes using more isolates to provide robust assessment. The use of mycobacterial isolation procedures that also target recovery of dormant *Mtb* in sputa (Mukamolova et al. 2010) could enhance recovery of sufficient isolates for such studies. The potential effects of cross-contamination on TB detection rats could not be sufficiently evaluated in this study because 6/7 (85.7%) of the detected sputum samples with MST 67 genotype were smear-positive at the DOTS centre, which suggests that patients were indeed TB-positive and samples were not cross-contaminated. The rat-positive, MST 67 genotype (n=7) specimens were collected in three different periods [(March (n=2), April (n=3) and June (1)] from one clinic and

only one MST 67 isolate (June 2009) from a different clinic. This time frame further indicates that MST 67 genotype is either a circulating *Mtb* clone in areas surrounding this DOTS centre or cross-contamination is persistent in this DOTS centre. Further investigation is needed to determine the extent of cross-contamination and the origin of this MST 67 genotype. Three smear-negative sputa with MST 67 were not detected by rats. This may suggest that cross-contamination could lower the sensitivity of rats assessed using cross-contaminated samples which may not be detected by rats due to low load of *Mtb* insufficient to produce detectable odours but yields positive culture. Two smear-negative sputa (2/8) (25%) with two different genotypes (MST 4 and MST 53) were detected by rats indicating that typical TB smear-negative sputa with different MST genotypes can be detected by these rats. This corroborates other findings of this thesis which show that rats detect better the naturally infected TB-positive sputa than TB-negative sputa spiked with pure cultures of *Mtb* (100–1000 µl). This also indicates that minimum levels of cross-contamination of TB-negative sputum may not be detected by rats and the sensitivity of the rats will be lower if assessed using culture positive cross-contaminated samples. The reported ability of rats to discriminate typical TB sputa ranges from 82% to 90% sensitivity and 91% to 95% specificity (Weetjens et al. 2009, Poling et al. 2010).

4.2 Respiratory tract microorganisms in sputum detected by rats

Responses of trained *Cricetomys* rats to sputum samples with different respiratory tract microorganisms reveal that trained rats specifically target *Mtb* in sputum samples and not other microorganisms also found in sputa. Sputa with *Mtb* isolates

were more frequently detected compared to those with other microorganisms (Fig. 6). Most of the rat-positive sputum samples containing opportunistic pulmonary pathogens also contained *Mtb* as confirmed by either smear microscopy or culture (Table 12, Fig. 10). The detection by rats of smear and culture-negative sputum samples containing other pulmonary pathogens, such as *M. catarrhalis*, *Nocardia* spp., *Rhodococcus* spp., *Enterococcus* spp. and pathogenic *S. pneumoniae*, at least in part appears to be due to *Mtb*, which could be below the detection threshold of microscopy and culture (Martin et al. 1975, Mukamolova et al. 2010). Sputum samples with these species alone, excluding those with *Rhodococcus* spp., *Nocardia* spp., which have low prevalence (Table 12) and *Streptomyces* spp. and *Candida* spp. were less frequently detected (Table 12, Fig. 10). This association was more evident with the most abundant *S. pneumoniae* of which 23 isolates (33.3%) were from rat-positive sputa and 46 isolates (66.7%) were from rat-negative sputa. Thirteen of 23 rat-positive sputa with streptococcal isolates (56.5%) also contained tubercle bacilli. Sputa containing *Enterococcus* spp. and *Staphylococcus* spp. were also detected in the presence of *Mtb*. These species were isolated in the majority of the sputa from TB negative individuals not detected by rats. The distribution of rat-positive and rat-negative sputa with non-mycobacterial species shows that the detection of *M. catarrhalis*, *S. pneumoniae*, *Candida* spp., *Enterococcus* spp., *S. succinus* and other *Staphylococcus* spp. is significantly different from the detection of sputa with *Mtb* ($P < 0.05$) (Table 12, Fig. 6). This indicates that these non-mycobacterial species do not cause false detection of sputa by rats.

The prevalence of the emerging pulmonary pathogens was lower (Table 12): *Nocardia* spp. (1%) and *Rhodococcus* spp. (1%) in Dar es Salaam, Tanzania. This prevalence is lower than in other sub-Saharan African countries (4–5%) (Jones et al. 2000). However, the present study used randomly chosen sputum samples based on sample volume rather than patients' symptoms. Isolation of *Nocardia* spp. increases in samples from patients with bronchopneumonia (Osoagbaka and Njoku-Obi 1985). While the prevalence of *Nocardia* sp. in this study could be an underestimate, the low prevalence of *Rhodococcus* sp. cannot be adequately discussed since there are no previous data from Tanzania regarding this pathogen in humans. The prevalence of *Streptomyces* spp. and *Candida* spp. was higher (7.3% and 7.8%, respectively) than that of *Nocardia* spp. and *Rhodococcus* spp. but cannot account for the high proportion of smear-negative, rat-positive sputum samples (i.e. 28.6% of smear-negative *Mtb* culture-positive TB cases detected by rats). The smear-negative, culture-positive *Mycobacterium* sp. contributed to 44.7% of the total mycobacterial isolates. The detection of four sputum samples with NTM species may suggest co-existence of these species with *Mtb* bacilli which were not recovered in culture due to competition with the fast-growing NTM and/or presence of dormant *Mtb* which requires resuscitation promoting factors (*rfps*) to grow (Mukamolova et al. 2010). Existence of slowly replicating or nonreplicating *Mtb* bacilli in sputum samples has also been confirmed by transcriptomic studies (Garton et al. 2008). *Mtb* culture-negativity can also be caused by low *Mtb* abundance in sputum, which is common in immunocompromised populations (Elliott et al. 1993, Johnson et al. 1999, Colebunders and Bastian 2000). Loss (death) of the few available *Mtb* during sample processing for culture (decontamination and neutralization) may also lead to culture-

negativity but rat-positive. An overlap in volatile compounds exists between *Mtb*, NTM, *Rhodococcus* sp., *Candida* sp., *Staphylococcus* sp., *Nocardia* spp. and *Streptomyces* spp. which were also isolated from smear and *Mtb* culture-negative sputa detected by rats. Yet the detection of sputa with these microorganisms cannot be due to the overlapping odour compounds because rats did not detect these overlapping/shared compounds and cultures of these microorganisms compared to significant detection of volatile compounds from *Mtb* (Fig. 16) and pure cultures of *Mtb* by these rats (Fig. 11). This rules out the potential of these microorganisms to cause false detection of sputum compared to *Mtb*. Co-existence of *Mtb* and NTM is most likely the cause of detection of these sputum samples considering the increasing prevalence of NTM species in clinical cases (Buijtels et al. 2009, Crump et al. 2009). Moreover, these NTM are often present in smear-negative TB patients especially in TB/HIV co-infections associated with low *Mtb* load in sputum. Use of a nucleic acid test such as Xpert MTB/RIF for *Mtb* (Boehme et al. 2010) and *rpfs* in culture medium (Mukamolova et al. 2010) could assist in determining the presence of *Mtb* in smear-negative, culture-negative, rat-positive sputum samples and consequently provide additional information on the actual false-positive rate of the rats.

The sensitivity and specificity of TB diagnosis by rats in this part of the study was high (80.4% and 72.4%, respectively) but lower than the previously reported 86.6% and 93.8%, respectively (Weetjens et al. 2009). This lower level could be attributed to modifications in sample treatment before conducting the first part of this study from April to June 2009. Indeed, the sensitivity during this period was lower (72.2%) compared to 95% sensitivity found during the second part of the study in July 2010.

However, specificity was higher (80.6%) during the first part of this study than the second part (62.5%). Lower specificity can be influenced by cross-contamination of negative sputa with smear-positive sputa, particularly with nonreplicating *Mtb* which are prevalent in sputum samples of TB patients (Garton et al 2008, Mukamolova et al. 2010). Thus, use of a culture method that can recover dormant *Mtb* sputa is crucially needed to determine presence of *Mtb* in sputa for evaluation of TB detection rats. TB case definition may also affect sensitivity and specificity. In this study I categorized patients using smear-microscopy and culture method which have several limitations as outlined in this thesis. Thirty-nine of 104 TB negative cases based on microscopy and cultures were falsely detected by rats in the second part of this study (low specificity), whereas 19 out of 20 confirmed TB cases were correctly detected in this part (highest sensitivity). It is possible that some of the false positive sputa had *Mtb* below detection limits of microscopy and conventional culture. The sensitivity and specificity of these rats is consistently higher (>80%) when assessed using control smear-positive and TB negative sputa (smear, culture and rat-negative) used in other experiments of this study such as spiking experiments. Positive predictive values (PPV) and negative predictive values (NPV) are largely affected by the prevalence of a given disease in population and longitudinal studies of a particular population over a long time period to determine the PPV and NPV are needed. Yet, this study indicates that harnessing rats for early TB diagnosis could have a significant impact on TB control. This is supported by the higher NPV (93.8%), which indicates that individuals with rat-negative sputum have a 93.8% likelihood of not having active TB disease. The shorter time needed for the rats to detect TB (70 sputa analysed twice by one rat at an average rate of 16 min) could

enable screening of a larger population and reduce new TB transmissions that occur from undetected TB cases.

4.3 Detection of cultures of *Mtb* and related microorganisms by rats

Trained *Cricetomys gambianus* rats demonstrate that they detect TB causing agent (*Mtb*) and not other microorganisms. The rats discriminated pure cultures of *Mtb* from NTM and related *Nocardia* and *Rhodococcus* species. Detection of pure cultures by rats was influenced by the growth stage of this bacterium, suggesting that the specific odour of *Mtb* detected by rats is below the detection threshold or is absent from other growth stages of this species. Hence not all cultures of *Mtb* can be used to determine the diagnostic performance of these rats. The trained rats repeatedly detected pure cultures of *Mtb* at exponential and early stationary phase more frequently than early- and late-phase (older) cultures ($P < 0.001$) (Table 14, Figs. 11 and 12). The age-based detection of *Mtb* cultures was revealed when an attempt was made to establish the causes for variation in rats' detection of cultures of the same strains, which were cultured and harvested at different time-points (days). These findings corroborate data that variations in odour profiles of *Mtb* is related to growth phase and type of media used. A difference in volatile profiles of bacteria related to growth phases was also recently reported on species of bacteria from human skin which produce mosquito-attractant odour (Verhulst et al. 2010). Another study has also shown that endogenous respiration of *Mtb* bacilli in cultures and infected lung varies in different time-points (Segal and Bloch 1956). This study attempted to mimic conditions under which rats are trained to detect TB in sputum samples by spiking TB-negative sputa, derived from different individuals, with test

microorganisms. Rats were able to consistently detect suitable growth phase of *Mtb* organisms spiked in negative sputa. However, average positive scores on mimicked samples were slightly lower compared to those obtained from typical TB-positive sputa. Thus, rats are conditioned to detect *Mtb* odour in naturally infected TB-positive sputa compared to mimicked sputa spiked with pure culture of *Mtb*. The false-positive rate (detection of TB-negative sputum) in the present study was lower (5.6%), which also indicates the higher specificity of these rats to TB detection (94.4%). These results corroborate those of the clinical respiratory tract microorganisms which show that most of detected smear-negative sputum had *Mtb* isolated in sputum cultures. When other microorganisms were isolated in such samples, detection was linked with *Mtb* either through microscopy or culture results, meaning that rats targeted *Mtb* volatiles. The slightly lower detection rate of the spiked samples suggests differences in background odour of the typical TB-positive sputum and the simulated one. This is well possible since *Mtb* inhabiting the host body can present different odour profiles from that of the *Mtb* grown in synthetic culture medium. The interaction of *Mtb* with host cells and that of *Mtb* in culture medium is also different, as are the substrates that determine the type of volatiles produced in the two milieus. This phenomenon has also been reported for other microbial species such as *Trichoderma* fungi (Wheatley et al. 1997, Bruce et al. 2000). Previous comparative studies show that *Mtb* bacilli grown in artificial medium lack several characteristic chemical compounds found in *Mtb* bacilli from host tissue (Anderson et al. 1943). The lipid content of *Mtb* bacilli grown *in vitro* also differs from bacilli in pathological lesions (Sheehan and Whitwell 1949). Similarly, there is a remarkable difference in biochemical activity of *Mtb* grown *in*

vitro to that found in lungs (Segal and Bloch 1956). Despite these causes of differences in *Mtb* from different conditions, rats were able to discriminate *Mtb* from other microorganisms which show higher olfactory ability of these rats considering that detection of TB by these rats is learned behaviour which differs from inert behaviour in which the olfactory system responds to specific odour stimuli by default. This underlines further the potential capacity of trained rats in TB diagnosis.

The detection of some NTM, for example, *M. smegmatis*, *M. aurum* and *M. neoaurum*, which was not statistically significant [OR = 1.42 (95% CI: 0.72–2.61), *P*= 0.23)] was by chance not due to odour from these species. This is also supported by findings that volatile compounds produced by other mycobacterial species including these ones do not induce rat's detection of spiked negative sputa compared to volatiles from *Mtb* (Fig. 16). Failure of the shared volatile compounds to induce rat's detection of spiked negative sputa like the *Mtb*-specific volatiles which were detected in spiked negative sputa (Table 16, Figs. 15 and 16) also indicate that NTM are not detected by trained rats. The ability of these rats to distinguish cultures of *Mtb* from NTM and related *Nocardia* and *Rhodococcus* species, which are emerging pulmonary pathogens, shows the potential of these rats in TB diagnosis. NTM as well as *Nocardia* spp. and *Rhodococcus* spp. can be ruled out in sputum samples detected by trained rats but the present isolation of these pathogens in Dar es Salaam indicates the need for considering these pathogens in patients with pulmonary disease who are ruled out from TB infection.

The sensitivity, specificity and accuracy of the rats in detecting TB assessed with *Mtb* and NTM which were grown in parallel was high. For example, five out of six

exponential- and early stationary-phase (21–30 days) of *Mtb* cultures were detected with a sensitivity of 83.3%, specificity of 94.4% and accuracy of 94%. These are in accordance with the recently reported sensitivity value of 82% and specificity of 90% obtained from testing of clinical sputum samples from over 10 000 individuals using these rats (Poling et al. 2010). The growth-phase dependent detection of *Mtb* cultures indicates the need for inclusion of cultures in different growth phases when investigating volatile compounds of *Mtb* detected by rats and for other marker-based tests. This approach was employed in the analyses of odour compounds of *Mtb* and other microorganisms in this study.

4.4 Odour compounds of mycobacteria, other microorganisms and detection by rats

4.4.1 Specificity of volatile compounds

The analyses of volatile compounds of *Mtb*, NTM, and respiratory tract microorganisms including *Nocardia* spp., *Rhodococcus* sp., *Staphylococcus* sp., *Streptomyces* spp. and *Candida* sp. also found in sputa detected by rats (Tables 6 and 12) revealed at least 26 compounds produced in different media and growth phases. Thirteen compounds are specific to *Mtb* and other 13 compounds overlap or are also found in different microorganisms (Tables 13 and 15). This suggests that the target TB odour comprises at least 13 specific volatile compounds and is distinct from odours of other microorganisms including NTM species found in sputa. This is supported by the evidence that *Cricetomys* rats also detect cultures of *Mtb* but not cultures of other species (Fig. 11), and that they discriminate sputum samples spiked

with *Mtb*-specific volatile compounds (Fig. 16) from those spiked with shared compounds of NTM and other microorganisms (Table 16). The microorganisms were cultured in different types of medium including nutrient-rich and less-nutritious culture medium which enabled wider-assessment of volatile compounds produced in different substrates which determines the type of volatiles produced (Wheatley et al. 1997, Bruce et al. 2000). The 26 volatile compounds (Table 15) were repeatedly produced by the selected microorganisms (Table 6) and were not identical to volatile compounds found in the blank media. Multiple volatiles were found in enriched medium (7H11 and 7H9) which supported well the growth of *Mycobacterium* spp., *Nocardia* spp., and *Streptomyces* spp. as indicated by higher optical densities and colony forming units for liquid cultures and growth of the mycobacteria on solid medium. The headspace samples of microorganisms in less-nutritious medium that included Sauton (without glycerol) and phosphate buffered saline glycerol glucose (PBSGG) medium produced few types of volatile compounds with some rare compounds for bacteria such as aciphyllene which was mostly produced by *Nocardia* spp. in Sauton medium, and cyclic proline-glycine, cyclic proline-leucine and uric acid produced by *Mtb* in PBSGG. These compounds are probably associated with *Mtb* adaption to survive in extreme harsh/starvation condition. For example, L-proline is an osmoprotectant while glycine induces L-forms in bacteria (Want and May 1975). Conversion to L-form enables mycobacteria to survive in host tissues (Beran et al. 2006, Markova et al. 2008).

The microorganisms from smear-negative sputa detected by rats do not produce the *Mtb*-specific compounds and their isolation was associated with isolation of *Mtb*

and/or smear-positive TB indicating that such microorganisms do not cause rat detection of sputum.

4.4.2 Olfactory detection of volatile compounds by rats

Trained *Cricetomys* rats can distinguish *Mtb*-specific volatile compounds from the overlapping/shared volatile compounds found in *Mtb* and other mycobacteria and related species (*Nocardia* spp., *Rhodococcus* sp. and *Streptomyces* spp.) from clinical sputa. Presentation of shared volatile compounds which were also found in microorganisms from clinical smear-negative, mycobacterial culture-negative sputa detected by rats did not induce positive signals in rats compared to *Mtb*-specific volatiles ($P = 0.001$). This suggests that the detection of the smear-negative and *Mtb* culture-negative sputa could be due to *Mtb* odour produced by few *Mtb* in sputa which are probably below the detection limit of microscopy (Elliott et al. 1993, Johnson et al. 1999, Colebunders and Bastian 2000) and culture (Martin et al. 1975). Two of the 7 *Mtb*-specific volatiles (2-phenylanisol and benzothiazole) presented to the rats individually were detected but not significantly. Benzothiazole compound was collected in the growth phase of *Mtb* cultures (early exponential and stationary phase), which is the most detectable growth phase of cultures of *Mtb* by rats (Fig. 11). This suggests that benzothiazole could be among the constituent compounds of the detected growth phase of *Mtb*. This compound was detected in the first 2 of the 7 tests with 60 and 70% detection scores but not in subsequent 5 tests (Fig. 15 B), thus the lack of statistical significance. The rats also detected slightly (not significantly) the blend of 3 most abundant *Mtb*-specific compounds (methyl nicotinate, methyl 4-anisate and 2-phenylanisol) (Table 2), suggesting that blends of volatile compounds

at least induce rats' detection. Indeed, the blend of 7 *Mtb*-specific volatiles was detected more frequently and significantly than the blend of 3 most frequently occurring *Mtb*-specific volatiles and the two individual volatile compounds which were detected by these rats (2-phenylanisol and benzothiazole) (P = 0.001). This suggests that the *Mtb* odour detected by trained rats is likely a combination of several volatile compounds, not a single compound. The blend of 7 *Mtb*-specific compounds was detected in 4 out of 7 test days with 40 to 80% of the expected detection (Fig. 16). The disrupted detection trend (Fig. 16) suggests that the blend of 7 *Mtb*-specific volatiles is either semi-identical to that of typical TB-positive sputum which the rats are trained to detect, or the ratios of odour components in this blend were closely related but not identical to that of typical TB-positive sputum. Hence the rats did not consistently detect this blend. The ratios and constituents of the odour produced by *Mtb in vivo* in the host could differ to those of *Mtb* grown *in vitro* due to differences in growth substrates which can determine the types of volatile compounds produced by microorganisms (Wheatley et al. 1997, Bruce et al. 2000). Anderson and co-workers (1943) reported that *Mtb* grown *in vitro* do not produce the typical chemical compounds such as phthioic acid, phthiocol, tuberculostearic acids and polysaccharides found in *Mtb* bacilli in human tissue. Indeed, none of these compounds were found in the present study whereby *Mtb* were grown in four different types of artificial medium. The gene expression of *Mtb* in lung also differs from that of *Mtb* in culture medium (Rachman et al. 2006) suggesting also for possible differences in volatile compounds produced by *Mtb* in host tissue and artificial medium environment. Such difference in gene expression profiles is further observed even in *Mtb* bacilli from different parts of the lung (Rachman et al. 2006).

Hence, the detection of the blend of *Mtb*-specific compounds by rats strongly indicates ability of the rats to distinguish *Mtb* from other microorganisms based on these odour markers.

The influence of food reward may also affect the detection quality since the rats were denied food reward on detection of the test volatiles to avoid training them on unknown compounds which could be TB odour markers or not. Hence rewarding could impair their pre-conditioned recognition and responses to typical TB-odour. Food reward was only provided when the rats detected the TB-positive control sputum samples. However, the repeated detection of this blend with 80% detection rate and failure of the shared/overlapping compounds to induce such detection in 192 repeated tests (overall for individual and combinations of shared compounds) indicate that the blend of *Mtb*-specific compounds is the potential odour marker of *Mtb* detected by these rats in human sputum. Further studies are needed to determine optimal ratios of *Mtb*-specific odour components that can produce consistently high detection rates similar to that of typical TB-positive sputum which was in most cases 100% and rarely 85.7%. Inclusion of other *Mtb*-specific volatiles is also imperative. Only 8 of the 13 *Mtb*-specific volatile compounds were tested by rats. Other 5 *Mtb*-specific volatile compounds mainly with low frequency of occurrence were not tested. The contribution of the untested compounds to the target odour detected by rats in sputum cannot be ruled out because this study has revealed that high abundance of individual compounds alone, is not sufficient to induce detection by rats as indicated by failure of methyl nicotinate with occurrence frequency of 51.4%, methyl 4-anisol (48.6%) and 2-phenylanisol (45.7%) to induce rats' detection. There

was a slight detection when these 3 most abundant *Mtb*-specific volatile compounds were mixed together and statistically significant detection was obtained when 7 *Mtb*-specific volatiles were combined. These findings corroborate recent reports which show that blends of volatile compounds are capable of eliciting positive signals not induced by individual compounds that make up the blend (Webster et al. 2010, Johnson et al. 2011). Webster and co-workers (2008) also found that presentation of individual compounds at the same concentration as in the natural sample did not induce comparable response to that of natural sample in aphid's olfactory recognition of host plants. This indicates the potential differences in odour of natural TB-positive sputa and spiked negative sputa such that the detection of the blend of 7 *Mtb*-specific volatiles strongly show relatedness of this blend (odour) to that of *Mtb* detected by rats in sputum. This finding differs from previous reports which suggest that single volatile compounds are potential odour markers for TB diagnosis (Syhre and Chambers 2008, Syhre et al. 2009). My study also shows that the concentration of volatile compounds is an important factor in inducing TB-odour detection. This was shown by failure of higher and lower concentrations of the detected individual and blend of *Mtb*-specific compounds to induce detection. Rats repeatedly detected mild (10^{-3}) concentrations of the candidate volatiles but not higher and lower concentrations ($P = 0.001$). These findings corroborate a previous study which shows that different concentrations of pheromone induce distinct behavioural responses in mice (He et al. 2010).

The spiking of TB-negative sputum with different volatile compounds did not affect the ability of rats to detect typical TB positive sputa which was 81.6% (sensitivity) and 88% (specificity) (Table 9) before starting the experiment of presenting to the

rats volatile compounds spiked into negative sputa. Rats detected 118 of the 119 positive control sputa and 61 out of 785 TB-negative sputum samples (sensitivity = 99.2%, specificity = 92.2% and accuracy = 93.1%). Thus further studies on target volatile compounds can be accomplished by spiking into negative sputa. This also mimics the odour background encountered by rats during the diagnosis of TB in sputum samples.

The present study demonstrates that *Mtb* produce specific volatile compounds which are detected by rats in sputa of TB patients and that rats use this specific odour to distinguish sputa from TB patients and those from healthy individuals (TB-negative). Higher sensitivity (99.2%), specificity (92.2%) and accuracy (93.1%) of the rats discriminating TB-positive sputum (positive control) of varying bacilli counts and the negative sputum controls support this interpretation. Further studies are needed to determine the optimal ratios of the candidate volatiles that produce similarly higher sensitivity and specificity as the ones achieved in clinical TB detection by these rats. The present findings form the basis for comparative studies of volatile profiles from clinical TB-positive sputum samples. Such studies could deepen our knowledge of TB odour markers and further evaluation of the potential of trained *Cricetomys gambianus* rats for rapid diagnosis of TB in resource-limited settings. This is urgently needed for controlling this poverty-related disease and achieving the millennium development goal (MDG 6).

4.5 Conclusions

- From the findings of this study it is concluded that *Mtb* shows a large diversity of genotypes not widely circulating in Dar es Salaam. The population in Dar es Salaam city provides diverse *Mtb* genotypes (n = 10) and other mycobacterial species suitable for evaluation of the diagnostic potential of TB detection rats.

- Trained rats detect sputum with different genotypes of *Mtb* but not with nontuberculous mycobacteria (NTM). The *Nocardia* spp. and *Rhodococcus* spp. which are closely related to *Mtb* are not detected by these rats.

- The discrimination of *Mtb* from NTM and other microorganisms from sputum suggest that smear-negative sputum detected by trained rats could be due to low concentration of *Mtb* bacilli below detection limit of microscopy or dormant *Mtb* not culturable in conventional medium.

- *Nocardia* and *Rhodococcus* sp. should be considered in diagnosis when TB is ruled out in patients with pulmonary disease symptoms.

- *Mtb* and other species produce different volatiles under different conditions and growth phase. Some volatile compounds are specific markers of *Mtb* and are targeted by *Cricetomys gambianus* to distinguish sputum of TB-positive patients from that of healthy individuals.

- The blend of *Mtb*-specific volatile compounds is responsible for TB detection in human sputum by *Cricetomys* rats. Further studies are needed to determine the optimal ratios of the candidate volatiles that could produce similar higher

sensitivity (99.2%), specificity (92.2%) achieved in clinical TB detection by these rats (see 3.4.4).

- The present findings make feasible comparative studies of volatile profiles from clinical TB-positive sputum samples. Such studies could deepen our knowledge of TB odour markers and further evaluation of the potential of *Cricetomys gambianus* for rapid diagnosis of tuberculosis in resource-limited settings that is urgently needed for controlling this poverty-related disease.

- The recorded lifespan of approximate 8 years of trained *Cricetomys* rats and the short period of time required for these rats to diagnose TB (32 min) show that these rats once trained can help diagnosis of TB in a cost efficient way.

- The adaptability of *Cricetomys* rats to wide range of climate allows application of this novel diagnostic technology in many areas with high TB burden. The use of the TB detection rats in resource-limited settings such as Sub Saharan Africa can contribute to achieving the goal of increasing TB detection and reducing TB incidences.

4.6 Contribution of this thesis

This thesis has contributed the following knowledge:

- This thesis has provided the first wider search and comparison of volatile chemical compound profiles of *Mtb*, NTM spp., *Nocardia* and *Rhodococcus* sp., and other respiratory tract microorganisms, which demonstrates specificities of some compounds with potential application as biomarkers for rapid diagnosis of TB.

- The thesis has provided the first insights into the ability of trained rats to discriminate cultures of *Mtb* from cultures of NTM and other related microorganisms and that this detection is based on the growth phase of cultures. This knowledge can enable selection of suitable age of cultures for determining specific volatile compounds.

- This thesis has provided evidence that trained rats specifically detect clinical sputum samples with *Mtb* and not other microorganisms which can be found in smear-negative samples detected by rats. This study has shown that these microorganisms do not produce the specific volatile compounds of *Mtb* which are discriminated by these rats from volatile compounds of other mycobacteria and respiratory tract microorganisms.

- The thesis has provided knowledge that trained rats detect clinical sputum samples with different genotypes of *Mtb* and not sputa with *M. avium* complex (*M. avium* subsp. *hominissuis* and *M. intracellulare*) which indicates that rats can be reliably used to diagnose TB.

- This thesis provides the knowledge that odour of *Mtb* detected by rats in sputum consists of a combination (blend) of several volatile compounds rather than single compounds. When the constituents of the detectable odour blend were tested individually rats could not detect these compounds.

- The concentration of the compounds is important in inducing the detection. Higher and lower concentrations are evasive compared to mild (10^{-3}) concentrations which allow detection.

- This thesis shows that different *Mtb* strains of Beijing family which are the most abundant and widely distributed causative agents of TB worldwide produce similar specific volatile compounds. Thus TB detection rats which detect these compounds can detect TB caused by different strains.

4.7 Future studies/outlooks

Further studies are needed on the target volatile compounds of TB. Future studies aiming at the following areas will provide in-depth knowledge needed for assessing the detection of TB by rats:

- Determination of optimal ratios of *Mtb*-specific volatiles to match detection rate of typical TB samples.

- Determination of the presence of the *Mtb*-specific volatiles in clinical sputum samples.

- Investigation of the nature of the new multispacer sequence typing (MST) genotypes found in Dar es Salaam (MST 67), and the detection by rats of two genotypes **(**MST 59 and MST 3**)** which were represented by few isolates in this study.

- Further determination of the extent/potential cross-contamination in sputum samples in TB clinic and consequences for diagnosis of TB by rapid tests.

- Investigation of the HIV status of the patients with smear-negative sputum samples detected by rats and potential presence of dormant *Mtb*.

- Expansion (scaling-up) of the evaluation of the TB detection rats technology in selected population/area with high TB burden.

- Investigation into prevalence and management of *Nocardia* and *Rhodococcus* pathogens in pulmonary infections.

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