Molecular Characterization of *Mycobacterium tuberculosis* complex and Prevalence of Nontuberculous Mycobacteria and other potential pathogenic Bacteria from Tuberculosis suspects in Northeastern, Tanzania

Dissertation for the degree of doctor rerum medicinae (Dr. rer. med)

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Dedication

This thesis is dedicated to my children Hajrah, Harith, and Haris who have always stood by me and dealt with all of my absence from many family occasions with a smile.
‘‘If the number of victims which a disease claims is the measure of its significance, then all diseases, particularly the most dreaded infectious diseases, such as bubonic plague, Asiatic cholera, et cetera, must rank far behind tuberculosis.’’ -ROBERT KOCH, 1882
List of Publications


5. Abubakar S Hoza, Sayoki G. M. Mfinanga, Irmgard Moser, and Brigitte König. Isolation, Biochemical and Molecular identification of Nocardia species among TB suspects in Northeastern, Tanzania. A forgotten or neglected threat? *Submitted to BMC Infectious Diseases*
Bibliographic description

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Abstract

Molecular typing is increasingly essential to tuberculosis (TB) control programmes, providing public health practitioners with a tool to characterize transmission patterns, track the emergence and spread of strains of *M. tuberculosis* complex (MTC) in populations. While molecular typing is already used extensively as a tool for TB control in many developed settings across the globe, its use in resource-poor settings is still limited. Moreover, information on the role, contribution and burden of nontuberculous mycobacteria (NTM) and other pathogens in aetiology of TB-like syndromes is also lacking in such settings.

The broad objective of this dissertation was to determine the genetic diversity of MTC and their drug resistance profiles as well as the prevalence of NTM and other potentially pathogenic bacteria among TB suspects in Northeastern, Tanzania in order to generate insights that may inform the design of a rational TB control programmes.

A total of 18 distinct spoligotypes were identified in this study area, with CAS1-KILI and EAI8 being the most predominant families. Major lineages prediction by conformal Bayesian network (CBN) revealed that 70% of TB infections in this area is due to modern lineages, whereas 30% of TB infections is due to the ancestral lineages mainly of Indo-oceanic lineage. The study also revealed that the overall proportions of any drug resistance and MDR-TB were 12.7% and 6.3% respectively. With the prevalence of any drug resistance and MDR-TB among new cases being 11.4% and 4.3% respectively, among previously, treated cases were 22.2%. The prevalence of NTM was found to be 9.7 %, with HIV being a significant predictor of NTM detection (*P* < 0.001). Four out of 30 patients with NTM diagnosed by culture received 1st line anti-TB treatment suggesting that a proportion of patients diagnosed by smear microscopy (4/65, 6.2%) were mistreated as TB patients. Our findings further showed that 17 (4.6%) out of 372 TB suspects were due to pulmonary nocardiosis.

Overall this dissertation has revealed that TB is still a major problem in Tanga and is characterized by a diverse array of MTB strains. Additionally, modern MTB strains contribute significantly to TB infections in this area. High proportions of anti-TB drug resistance among new treated cases observed suggest that more efforts need to be done to identify individual cases at facility level for improved TB control programmes. Inefficient screening of TB patients and a prevalent increase of NTM may contribute to both unrealistic and mismanagement of TB cases. A diverse array of pathogenic *Nocardia* species among TB suspects further indicates that they are likely cause of human disease in this population. Therefore, need to integrate NTM and pathogens causing TB-like syndromes in diagnosis and management of TB is urgent. Results of these investigations contribute to the understanding of the dynamics of TB transmission in resource poor settings of Tanzania and highlight key factors that should be considered in the development of rational approaches to design effective TB prevention and control programmes in the country.
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<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFB</td>
<td>Acid Fast Bacilli</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired Immune Deficiency Syndrome</td>
</tr>
<tr>
<td>ATS</td>
<td>American Thoracic Society</td>
</tr>
<tr>
<td>BA</td>
<td>Blood agar</td>
</tr>
<tr>
<td>BC</td>
<td>Before Christ</td>
</tr>
<tr>
<td>BCG</td>
<td>Bacillus Calmette-Gue´rin</td>
</tr>
<tr>
<td>CBN</td>
<td>Conformal Bayesian network</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic Obstructive Pulmonary Disease</td>
</tr>
<tr>
<td>CTRL</td>
<td>Central Tuberculosis Reference Laboratory</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DOTS</td>
<td>Direct Observed Treatment Short Course</td>
</tr>
<tr>
<td>DR</td>
<td>Direct Repeat</td>
</tr>
<tr>
<td>DRE</td>
<td>Doble-Repetitive-Element</td>
</tr>
<tr>
<td>DR-TB</td>
<td>Drug Resistant Tuberculosis</td>
</tr>
<tr>
<td>DST</td>
<td>Drug Susceptibility Testing</td>
</tr>
<tr>
<td>DVR</td>
<td>Direct Variable Repeat</td>
</tr>
<tr>
<td>EMB</td>
<td>Ethambutol</td>
</tr>
<tr>
<td>ETRs</td>
<td>Exact tandem repeats</td>
</tr>
<tr>
<td>HBCs</td>
<td>High burdened countries</td>
</tr>
<tr>
<td>HGDI</td>
<td>Hunter-Gaston discriminatory Index</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immune deficiency virus</td>
</tr>
<tr>
<td>Hsp65</td>
<td>Heat shock protein (65)</td>
</tr>
<tr>
<td>IDSA</td>
<td>Infectious Diseases Society of America</td>
</tr>
<tr>
<td>INH</td>
<td>Isoniazid</td>
</tr>
<tr>
<td>IS6110</td>
<td>Insertion Sequence 6110</td>
</tr>
<tr>
<td>ITS</td>
<td>Internal Transcribed Spacer</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>KNTH</td>
<td>Kibong’oto National Tuberculosis Hospital</td>
</tr>
<tr>
<td>LJ</td>
<td>Löwenstein–Jensen</td>
</tr>
<tr>
<td>LTBI</td>
<td>Latent Tuberculosis infection</td>
</tr>
<tr>
<td>MDR-TB</td>
<td>Multidrug resistant tuberculosis</td>
</tr>
<tr>
<td>MGIT</td>
<td>Mycobacteria Growth Indicator Tube</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MIRU-VNTR</td>
<td>Mycobacterial Interspersed Repetitive Unit–Variable Number Tandem Repeats</td>
</tr>
<tr>
<td>MoHSW</td>
<td>Ministry of Health and Socio-welfare</td>
</tr>
<tr>
<td>MTC</td>
<td><em>Mycobacterium tuberculosis</em> Complex</td>
</tr>
<tr>
<td>NIMR</td>
<td>National Institute of Medical Research</td>
</tr>
<tr>
<td>NTLP</td>
<td>National Tuberculosis and Leprosy Programme</td>
</tr>
<tr>
<td>NTM</td>
<td>Non-tuberculous mycobacteria</td>
</tr>
<tr>
<td>NTPs</td>
<td>National Tuberculosis Control Programmes</td>
</tr>
<tr>
<td>PDC</td>
<td>Periphery Diagnostic Centres</td>
</tr>
<tr>
<td>PZA</td>
<td>Pyrazinamide</td>
</tr>
<tr>
<td>QUBs</td>
<td>Queen’s University of Belfast</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction Fragment Length Polymorphism</td>
</tr>
<tr>
<td>RMP</td>
<td>Rifampicin</td>
</tr>
<tr>
<td>Rpoß</td>
<td>RNA polymerase beta-subunit</td>
</tr>
<tr>
<td>RRDR</td>
<td>Rifampicin Resistance Determining Region</td>
</tr>
<tr>
<td>secA1</td>
<td>Essential protein A1</td>
</tr>
<tr>
<td>SM</td>
<td>Streptomycin</td>
</tr>
<tr>
<td>sodA</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>Spoligotyping</td>
<td>Spacer oligonucleotide typing</td>
</tr>
<tr>
<td>TB</td>
<td>Tuberculosis</td>
</tr>
<tr>
<td>TTD</td>
<td>Time to detection</td>
</tr>
<tr>
<td>UPGMA</td>
<td>Unweighted Pair-Group Method using Arithmetic Averages</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>XDR-TB</td>
<td>Extensively Drug Resistant-Tuberculosis</td>
</tr>
<tr>
<td>ZN</td>
<td>Ziehl-Neelsen</td>
</tr>
</tbody>
</table>
Chapter One  Introduction

1.1 A review of *Mycobacterium tuberculosis* Complex and Tuberculosis Disease

1.1.1 *M. tuberculosis* overview

*M. tuberculosis* is known to be the most prominent member of *M. tuberculosis* complex (MTC) causing TB in humans, with an estimated 9.4 million new cases and about 1.8 million deaths worldwide each year [2].

*M. tuberculosis* is an aerobic, slow-growing, acid-fast, rod-shaped bacterium belonging to the genus Mycobacterium, order Actinomycetales that comprises a large number of well-characterized species, several of which are associated with the human and animal disease. Other known human pathogens include *Mycobacterium ulcerans*, the causative agent of Buruli ulcer and *Mycobacterium leprae*, the cause of leprosy. Other classical species of the MTC other than *M. tuberculosis*, include *Mycobacterium africanum* (of various subtypes and variants), *Mycobacterium microti* (voles and other small rodents), and *Mycobacterium bovis* (along with the widely used vaccine strain *M. bovis* bacillus Calmette-Guérin [BCG]).

Newly recognized additions to the MTC include *Mycobacterium caprae* (goats) and *Mycobacterium pinnipedii* (seals and sea lions) [3]. Though not currently an officially described organism, “*Mycobacterium canettii*” is also widely accepted as a member of the MTC [4]. Several studies have shown > 99% DNA homology among subspecies of the MTC [5]. However, they differ in their host range, epidemiology, clinical picture in human, as well as laboratory phenotype.

Like other *Mycobacterium spp*, *M. tuberculosis* is cytochemically Gram-positive. A characteristic feature of these mycobacteria is their extremely resilient cell envelope that contains a rich variety of lipids such as mycolic acids, glycolipids, and polysaccharides. This unique cell wall is also responsible for the acid-fast staining property of mycobacteria. The cell wall lipids bind carbol fuchsin that resists decolorization by the acid-alcohol used in Ziehl-Neelsen (ZN) staining technique, hence bacilli that stain by this method are commonly referred to as acid-fast or ZN-positive [6].
1.1.2 Tuberculosis Disease

Tuberculosis (TB) has afflicted humankind from the time immemorial. It is responsible for millions of death occurring each year worldwide. Evidence of spinal disease has been found in Egyptian mummies of several thousand years BC (Before Christ) and references to TB are found in ancient Babylonian and Chinese writings. With the help of molecular genetic studies *M. tuberculosis*, the most common cause of TB in humans worldwide is believed to have a progenitor dating back to about 3 million years old [7].

1.1.3 TB Transmission and Pathogenesis

The advent of genomic revolution in the last decade has to a large extent enabled the application of a variety of powerful tools, such as proteomics, large-scale transcriptomics, comparative genomics, and structural genomics studies in the field of TB research that have resulted in a great understanding of the biology and pathogenesis of the tubercle bacilli. *M. tuberculosis* has a slow generation time of approximately 24 hours and takes three to four weeks to form colonies *in vitro*.

TB is a communicable disease and patients with pulmonary TB are the most important source of infection. Infection occurs by inhalation of droplet nuclei, which are particles of about 1–5 μm in diameter encompassing *M. tuberculosis*, coughed up by patients with active pulmonary TB. The risk of infection (Figure 1) is a function of several factors such as the infectiousness of the source case, the proximity of contact, the infectious dosage inhaled, and the immune competence of the susceptible host [1,8–10]. The primary route of infection involves the lungs. Inhaled droplet nuclei evade the defences of the bronchi due to their small size and penetrate into the terminal alveoli where are engulfed by phagocytic immune cells (macrophages and dendritic cells). Studies also show that *M. tuberculosis* can infect non-phagocytic cells in the alveolar space like M cells, alveolar endothelial, and type 1 and type 2 epithelial cells (pneumocytes) [11–13].

During the early phase of infection *M. tuberculosis* is internalized by phagocytic immune cells, multiplies intracellularly, and immune cells loaded with the bacteria may cross the alveolar barrier to cause systemic dissemination [1,12,14]. The intracellular replication and simultaneous dissemination of the pathogen to the pulmonary lymph nodes and to various other extrapulmonary sites occur prior to the development of the adaptive immune responses. This elucidates the exceptional competence of *M. tuberculosis* to
establish a protected niche where they can avert elimination by the immune system and persevere indefinitely [15,16].

An effective cell-mediated immune response may develop in the range of 2–8 weeks following infection that ceases further multiplication of the tubercle bacilli (Figure 1). Granulomas that hinder further replication and spread of the tubercle bacilli is finally formed from the activated T lymphocytes, macrophages, and other immune cells. *M. tuberculosis* evolves an effective strategy to evade the immune response leading to the survival and persistence of some bacilli in a non-replicating state within the host called latent TB infection (LTBI), which may take up to 3 years [9,16–18].

During this stage, more slowly developing extrapulmonary lesions, such as those in bones and joints, often characterized by chronic back pain, may occur in some individuals. This assumption is evidenced by the fact that, *M. tuberculosis* has been cultured and the presence of *M. tuberculosis* DNA has been verified from lung tissues of individuals who died from other diseases and who did not manifest with any pathological sign of TB disease [19,20]. When an ensuing defect in cell-mediated immunity occurs, it may elicit reactivation of inert bacilli resulting into an active disease many years after the infection (reactivation TB).

In regards to the HIV/TB co-infection individuals, studies show that one-third of exposed HIV-negative individuals become infected, and 3 to 5% of them develop TB in the first year, an additional 3 to 5% of those infected develop TB later in life. It is speculated that most adult TB in non-HIV-infected patients is caused by reactivation of pre-existing infection [21]. HIV-positive persons infected with *M. tuberculosis* have a 50% chance of developing reactivation (post primary) TB at some time in their lives. Such individuals and those with immunosuppression can also be newly infected with *M. tuberculosis* and may show rapid progression to active disease [22].
Introduction

1.2 Epidemiology and Burden of TB/HIV co-infection

TB remains a major public health problem in many parts of the world regardless of the efforts to combat the disease. Africa alone carries a huge burden of TB estimated at 30% of the global cases in 2009, second after Asia (50%). Approximately 41% of the highest burdened countries (HBCs) with TB worldwide are in Africa [23] (Figure 2). TB burden in Africa is largely fuelled by human immune deficiency virus (HIV) epidemic. In 2009, the region accounted for about 11-13% of the TB deaths associated with HIV-positive [23] (Figure 3).

Figure 1: Progression of natural cause of events and outcome in an immunocompetent individual following exposure to droplet nuclei containing M. tuberculosis expectorated by a source case of sputum smear-positive pulmonary (Open) TB [1].
General weak health care systems, inadequate laboratory services, plus conditions promoting transmission of infection, and the emergence of multidrug-resistant (MDR-TB) strains have compounded the problem [24–26]. Other compounding factors which have aggravated the increased TB trend in the region include poverty, malnutrition, poor living conditions resulting to overcrowding, inadequate access to free and affordable health care services and reliance on traditional healers [27].
1.2.1 World situation of MDR-TB

Multidrug-resistant (MDR) -TB has emerged as a crucial threat to TB control. Drug resistant (DR) -TB is widespread and is found in all countries of the world [23]. MDR-TB caused by tubercle bacilli resistant to at least one of the first-line anti-TB drugs, Rifampicin (RMP) and or Isoniazid (INH) was declared a global burden [29]. Both biological, as well as socioeconomic factors, have been responsible for the emergence of DR-TB, which is a purely manmade phenomenon; a result of sub-optimal chemotherapy [29,30].

Estimates of DR-TB incidence are difficult to obtain largely due to inadequate laboratory facilities for drug susceptibility testing (DST) in most resource-poor endemic countries [31]. For example, only 24,511 cases of the 250,000 expected MDR-TB cases were enrolled for the treatment in 2009 [32]. While the numbers of expected MDR-TB cases rose to 650,000 of which only 46,000 cases were enrolled for treatment in 2010 [28]. These trends indicate the need for continuous improvement in both diagnosis and management of DR-TB, especially in resource-poor countries in order to reduce morbidity, mortality, economic loss, further spreading of infection and emergence of MDR strains [33]. Extensively drug-resistant-TB (XDR-TB) caused by bacterial strains resistant to the two first-line anti-TB (RMP and INH) and to second line drugs (injectable aminoglycosides and fluoroquinolones) is another serious threat to global health [23].

1.2.2 TB and MDR-TB situation in Tanzania

Tanzania is among the 22 HBCs with respect to the number of incident TB cases [34]. Previous reports revealed that up to the mid-eighties, the annual number of cases identified and treated in the country was manageable. However, increased HIV epidemic has reversed the situation [35,36]. For instance, in just 5 years (1980-1985), the number of TB cases notified tripled from 5000 to 15,000. In 2006, over 62,000 TB cases (157 cases per 100,000 population) were reported, while WHO estimated the incidence to be 312 per 100,000 population [34,37]. Although the routine TB surveillance data in the country have been consistent over the years, there are still areas of uncertainty, which makes the routine data not easily translated into an approximation of TB incidence as an indicator for the burden of disease [38].

Information on anti-TB drug resistance levels is a vital management tool for evaluating the performance of National TB control programmes (NTPs) [39]. While resistance in previously treated patients is an essential indicator of current treatment practices in the
community, drug resistance in previously untreated (new) patients mirror the transmission of disease with resistant strains, and indicates the complications that the NTPs will face during administering chemotherapy.

Case detection rate of TB and prevalence of multidrug-resistant (MDR) TB in Tanzania remain low with about 1,300 MDR-TB cases reported in 2007 consequently to improved quality of services and evaluation. Data regarding anti-tuberculosis drug resistance is only available from a handful of selected settings and studies [40,41]. Treatment of MDR cases in Tanzania consists of a standardized MDR-TB regimen based only on drug susceptibility results for the first-line medications, isoniazid, rifampicin, streptomycin and ethambutol [42]. Based on this account, MDR-TB in Tanzania reflects a unique population with little to no exposure to second-line medications and with a relatively low background prevalence of drug resistance.

1.3 Molecular mechanisms of drug resistance in *M. tuberculosis*

Advances in molecular biology techniques as well as the existence of new information generated after deciphering the complete genome sequence of *M. tuberculosis* [43,44], have enabled understanding of the mechanisms of resistance to the principal anti-TB drugs. Specific gene mutations associated with drug resistance have been identified [45]. Drug resistance in *M. tuberculosis* arises from spontaneous chromosomal mutations at a low frequency through the subsequent acquisition of mutations at multiple loci [27]. To date, the most common mutations associated with drug resistance are described and publically accessible on the DR-TB mutation database [46]. Molecular mechanisms of drug resistance have been elucidated for the major first- and second-line drugs [47,48] Table 1.
Table 1 Mechanisms of drug resistance in *M. tuberculosis*

<table>
<thead>
<tr>
<th>Drug (year of discovery)</th>
<th>MIC µg/ml</th>
<th>Genes involved in resistance</th>
<th>Gene function</th>
<th>Role</th>
<th>Mechanism of action</th>
<th>Mutation frequency %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoniazid (1952)</td>
<td>0.02 – 0.2</td>
<td><em>katG</em></td>
<td>Catalase–peroxidase</td>
<td>Pro- drug conversion</td>
<td>Inhibition of mycolic acid biosynthesis and other multiple effects</td>
<td>50 – 95</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>inhA</em></td>
<td>Drug target</td>
<td>Inhibition of RNA synthesis</td>
<td>8 - 43</td>
<td></td>
</tr>
<tr>
<td>Rifampicin (1966)</td>
<td>0.05 - 1</td>
<td><em>rpoB</em></td>
<td>β subunit of RNA polymerase</td>
<td>Drug target</td>
<td>Depletion of membrane energy</td>
<td>95</td>
</tr>
<tr>
<td>Pyrazinamide (1952)</td>
<td>16 – 50 pH 5.5</td>
<td><em>pncA</em></td>
<td>Nicotinamidase/pyrazinamide</td>
<td>Prodrug conversion</td>
<td>Inhibition of RNA synthesis</td>
<td>72 - 97</td>
</tr>
<tr>
<td>Ethambutol (1961)</td>
<td>1 - 5</td>
<td><em>embB</em></td>
<td>Arabinosyltransferase</td>
<td>Drug target</td>
<td>Inhibition of arabinogalactan synthesis</td>
<td>47 - 65</td>
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<tr>
<td>Streptomycin (1944)</td>
<td>2 - 8</td>
<td><em>rpsL</em></td>
<td>S 12 ribosomal protein</td>
<td>Drug target</td>
<td>Inhibition of protein synthesis</td>
<td>52 – 59</td>
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<tr>
<td></td>
<td></td>
<td><em>rrs</em></td>
<td>16S rRNA</td>
<td>Drug target</td>
<td>8 – 21</td>
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<tr>
<td></td>
<td></td>
<td><em>gidB</em></td>
<td>rRNA methyltransferase (G527 in 530 loop)</td>
<td>Drug target</td>
<td>?</td>
<td></td>
</tr>
<tr>
<td>Amikacin/Kanamycin (1957)</td>
<td>2 - 4</td>
<td><em>rrs</em></td>
<td>16S rRNA</td>
<td>Drug target</td>
<td>Inhibition of protein synthesis</td>
<td>76</td>
</tr>
<tr>
<td>Capreomycin (1960)</td>
<td>0.5 – 2.5</td>
<td><em>tlyA</em></td>
<td>2'-O-methyltransferase</td>
<td>Drug target</td>
<td>Inhibition of DNA gyrase</td>
<td>75 - 94</td>
</tr>
<tr>
<td>Quinolones (1963)</td>
<td>0.5 – 2.5</td>
<td><em>gyrA</em></td>
<td>DNA gyrase subunit A</td>
<td>Drug target</td>
<td>Inhibition of DNA gyrase</td>
<td>75 - 94</td>
</tr>
<tr>
<td>Ethionamide (1956)</td>
<td>2.5 - 10</td>
<td><em>gyrB</em></td>
<td>DNA gyrase subunit B</td>
<td>Drug target</td>
<td>Inhibition of mycolic acid synthesis</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>betaA/ethA</em></td>
<td>Flavin monooxygenase</td>
<td>Drug target</td>
<td>Inhibition of mycolic acid synthesis</td>
<td>56</td>
</tr>
<tr>
<td>PAS (1946)</td>
<td>1 - 8</td>
<td><em>inhA</em></td>
<td>Thymidylate synthase</td>
<td>Drug target</td>
<td>Inhibition of folic acid and iron metabolism</td>
<td>36</td>
</tr>
</tbody>
</table>

MIC = Minimum inhibitory concentration; PAS = Para-amino salicylic acid; ? = Mutation frequency not determined. Source:[48]
1.4 Diagnosis of TB

The diagnosis of active TB greatly depends on initial clinical suspicion and radiological examination, followed by subsequent laboratory confirmation using microbiological techniques primarily sputum smear microscopy. TB and MDR-TB are typically common in resource-limited settings, precise diagnosis, and relevant treatment is necessary to prevent the spread of DR-TB within communities. However, these tools do not allow for the prediction of drug resistance.

1.4.1 Radiologic examination

Chest X-ray is the primary radiologic evaluation of suspected or proven pulmonary TB. Radiological presentation of TB may be variable but in many cases is quite characteristic. Radiology also provides essential information for management and follow-up of these patients and is extremely valuable for monitoring complications. Chest X-ray is useful but is not specific for diagnosing pulmonary TB. Moreover, TB can present with symptoms and atypical radiologic findings that are indistinguishable from those of community-acquired pneumonia [49,50]. Therefore, an acid-fast bacilli (AFB) smear and bacteriological culture tests should be performed for patients with symptoms that are compatible with or suggestive of TB.

1.4.2 AFB smear microscopy

Direct sputum microscopy is the primary method for diagnosing pulmonary TB in many resource-poor settings. A major shortfall of conventional microscopy is its relatively low sensitivity compared with culture, especially in patients co-infected with HIV [51,52]. Moreover, smear microscopy may be costly and inconvenient for patients, who must make multiple visits to health facilities and submit multiple sputum specimens over several days. Conventional light microscopy of Ziehl-Neelsen (ZN)–stained smears prepared directly from sputum specimens is the most widely available test for diagnosing TB in resource-limited settings. ZN microscopy is highly specific, but its sensitivity is variable (20%–80%). Conventional fluorescence microscopy is more sensitive (10%) than the Ziehl-Neelsen and takes less time, but it is limited by the high cost of mercury vapor light sources, requires dark room and regular maintenance [53]. Light-emitting diodes (LED) have been developed to offer fluorescence microscopy without the associated costs [54]. The WHO recommends that conventional fluorescence microscopy is replaced by LED microscopy and that LED microscopy be phased in as an alternative for conventional Ziehl-Neelsen light microscopy [54].
1.4.3 Mycobacterial culture

Acid-fast microscopy is easy and quick, but it does not confirm a diagnosis of TB because some acid-fast bacilli are not *M. tuberculosis*. Therefore, a culture is done on all initial samples to confirm the diagnosis. A positive culture for *M. tuberculosis* confirms the diagnosis of TB disease. In an ideal situation, it is recommended that culture examinations be completed on all specimens, regardless of AFB smear results.

Mycobacterial culture is more sensitive, but the growth of TB bacilli on traditional solid medium requires 4–8 weeks, which delays appropriate treatment in the absence of a confirmed diagnosis. Culturing mycobacteria is mainly done on solid media, the Lowenstein-Jensen slope, or in broth media. These methods are slow, with cultures from microscopy-positive specimen ranging from 2–4 weeks and for a microscopy-negative specimen from 4–8 weeks. Liquid media is significantly faster (between 10 and 14 days) and is better for isolation, compared to solid media. For drug susceptibility testing (DST), the delay may be reduced to as little as 10 days compared to 4–6 weeks with conventional solid media. Liquid systems are more sensitive for detecting mycobacteria and may increase the case yield by 10% compared to solid media [55]. With increased sensitivity and reduced delays, liquid systems may contribute significantly to improved patient management. Liquid systems are, however, more prone to contamination by other microorganisms. In experienced laboratories, approximately 5%–10% of specimens fail to yield results because of contamination [56]. Procedures to prevent cross-contamination (due to carryover of bacilli from positive to negative specimens) should also be strictly followed, especially where increased numbers of positive specimens are processed in high-incidence countries [57,58]. Increased bacterial contamination and an increased frequency of nontuberculous mycobacterial (NTM) isolation need to be addressed. A rapid method to differentiate the *M. tuberculosis* complex from other mycobacterial species is essential. Several tools that can automatically detect *M. tuberculosis* growth in the laboratory, such as the Bactec “Mycobacterial Growth Indicator Tube 960” (MGIT 960; Becton-Dickinson, Sparks, MD, USA) and the MB/BacTAlert® 3D (Biomérieux, Durham, NC, USA) have been developed. Unfortunately, these automatized incubators are expensive, and not readily available outside reference centres in resource-poor settings.

1.4.4 Diagnosis of MDR-TB

Early detection of drug resistance TB enables proper treatment that has a great impact in the better control of the disease. Detection of drug-resistant bacilli can be done by culture
Introduction

based or genome-based techniques. Recent advances in molecular biology and a better understanding of the molecular mechanisms of drug resistance TB have provided new tools for its rapid diagnosis and for the rapid detection of drug resistance. Several new genotypic and phenotypic methods for rapid diagnosis and detection of drug resistance have been developed and tested in both *M. tuberculosis* strains and in clinical specimens.

1.4.2.1 Phenotypic drug susceptibility testing

Phenotypic DST or culture-based methods performed on either solid or liquid media as direct or indirect tests are precise and less expensive. However, their turn-around time is long owing to dependence on the growth of *M. tuberculosis* [59].

Commercial automated liquid culture DST methods such as BacT/Alert®3D system (bioMérièux Inc, Durham, North Carolina, USA), Mycobacteria Growth Indicator Tube (MGIT) (Becton Dickinson, Sparks, MD), BACTEC MGIT960 system (Becton Dickinson, Sparks, MD) and Versa TREK system (Trek Diagnostic Systems, West Lake, Ohio, USA) have been developed. The methods have improved the turnaround time because of sensitive automation and the fact that *M. tuberculosis* grows relatively faster in liquid compared to solid media; however, they are expensive both in terms of equipment and expertise for most resource-poor-settings [60].

Alternative ‘in-house’ methods for rapid DST of *M. tuberculosis* using solid culture media have been developed with the aim of shortening the turnaround time. Such methods include Mycobacteriophage-based methods like (*Fast Plaque* assay and *Luciferase* reporter phages), E-test, nitrate reductase assay, microcolony method and the tuberculosis colorimetric culture medium –TK® medium [60–62].

1.4.2.2 Genotypic drug susceptibility testing

Genotypic DST methods target well-characterised resistance-associated mutations to identify drug-resistant *M. tuberculosis*. RMP resistance is notably appropriate for genotypic DST since about 95% of mutations conferring resistance to RMP in RMP-resistant clinical *M. tuberculosis* isolates occur mostly in the 81bp rifampicin resistance determining region (RRDR) of the *rpoB* gene [27,63–66].

Molecular methods, such as Xpert MTB/RIF assay (Cepheid; Sunnyvale, CA, USA), and the GenoType MTBDRplus assay in combination with GenoType MTBDRsl (Hain Life science, GmbH, Nehren, Germany), have shown excellent correlation with phenotypic
DST results with specificities and sensitivities > 90% and have been endorsed by the WHO [34,67].

Molecular detection of resistance to other anti-TB drugs, such as INH and ethambutol (EMB), is, however, more complex and requires detection of mutations in multiple genes for a good correlation with phenotypic results [65]. INH resistance-associated mutations have been found in the katG and inhA genes, however, approximately 15 to 25% of INH-resistant isolates do not contain mutations in these regions, suggesting that other sites may also be involved in resistance [68].

Accurate genotypic DST for first and second-line anti-TB drugs is, still technically demanding. Understanding of the molecular mechanisms of anti-TB drug resistance and improved multiparametric detection technology will make genotypic DST a more powerful technique in future.

1.5 Strain Identification for M. tuberculosis

The distribution of TB in different geographic regions is characterized by the prevalence of different MTB strains with varied virulence and drug resistance. Environmental and host factors are believed to be responsible for the transmission and prevalence of different MTB strains [69]. One of the decisive measures of any TB control program is the capacity to determine where transmission occurs in order to prevent further spread of infection and prevent active disease by identifying newly infected individuals and providing them with timely preventive therapy.

The advent of genetic fingerprinting of MTB has substantially improved the knowledge and ability to determine patterns of transmission in communities, as well as establishing transmission links between individuals [70]. Several molecular techniques have been used for studying the molecular epidemiology of TB.

1.5.1 Genotyping of mycobacteria

Genotyping of isolates from patients is useful in various situations; like to confirm the occurrence of cross-contamination in the laboratory. Approximately 3% of patients from whom MTB is apparently isolated in clinical laboratories do not have TB; the positive cultures are due to cross-contamination [71]. If MTB is isolated from a specimen but the clinical findings do not suggest the presence of disease, genotyping of the isolate as well as other MTB strains handled concurrently in the laboratory can strongly imply the
occurrence of cross contamination and lead to the discontinuation of anti-TB medications [72]. There exist broad variability in the genotypes of MTB isolates from patients with epidemiologically unrelated TB, in which the genotypes of isolates from patients who were infected by a common source are identical [73]. Consequently, clustered cases of TB, defined as isolates with identical or closely related genotypes, are a result of recent transmission. On the contrary, cases in which the isolates have distinctive genotypes generally represent a reactivation of infection acquired in the distant past [74]. Epidemiological studies have exploited DNA polymorphism associated with insertion elements and other repetitive DNA elements to differentiate clinical MTB isolates, based on the assumption that strains with identical DNA fingerprints are epidemiologically related. Strains without identical DNA fingerprints can be classified into clades or genogroups when they partially share polymorphic sites, like IS6110 insertion sites and spacer sequences in the polymorphic direct repeat (DR) region of the MTB genome [75–77].

1.5.2 Genotyping methods

Several repeat sequences have been identified in the genome of the MTC like the transposable elements [75], trinucleotide repeats [78], variable number tandem repeats (VNTR), mycobacterial interspersed repetitive units (MIRU) [79], and the DR region [80]. DR region is one of the most extensively studied loci, it consists of direct repeat sequences (36bp) interspersed with unique spacer sequences (34 to 41bp), referred to as direct variable repeat (DVR) sequences. DR region has evolved through the deletion of DVR sequences by homologous recombination, single nucleotide mutations, and the integration of IS6110 elements [81]. Events believed to be unidirectional and appearing over time, making the DR region an enlightening locus for studying the evolution and epidemiology of the MTC [82,83].

1.5.2.1 Restriction Fragment Length Polymorphism (RFLP)

Restriction-fragment-length polymorphism (RFLP) analysis of the distribution of the insertion sequence IS6110 in different strains is the standard approach used in genotyping MTB isolates. The method is based on differences in the IS6110 copy numbers for each strain, varying from 0 to about 25, and variability in the chromosomal positions of these IS6110 insertion sequences [84]. Isolates from patients infected with epidemiologically
unrelated strains of TB are said to have different RFLP patterns, and patients with epidemiologically related strains generally have identical RFLP patterns [77]. IS6110 as a probe has favourable discriminatory power and is often used as a method of choice to differentiate strains of MTB [77]. However, absence or low copy number of IS6110 element in a significant number of MTB strains limit its usefulness [85].

1.5.2.2 Spoligotyping
The introduction of new PCR-based methods like spoligotyping, MIRU-VNTR and double-repetitive-element PCR (DRE-PCR) typing permit simultaneous detection and epidemiologic typing of MTB [86,87]. Spoligotyping was developed as a tool to provide information on the structure of the DR region in individual MTB strains and in different members of the MTC. Basically, Spoligotyping is a reverse hybridization technique based on polymorphism in the DR locus of the mycobacterial chromosome [86]. The DR locus in MTB consists of 10 to 50 copies of a 36-bp direct repeat, separated from each other by spacers that have different sequences [88]. However, the spacer sequences between any two specific direct repeats are conserved among strains. Considering that strains differ in the presence or absence of specific spacers, the pattern of spacers in a strain can be used for genotyping hence the term spoligotyping (i.e. spacer oligonucleotide typing).

Spoligotyping has two advantages over IS6110. Firstly, a small amount of DNA is required, hence can be performed on clinical samples or on strains of MTB shortly after inoculation into the liquid culture. Secondly, results of spoligotyping can be expressed in a digital format. Furthermore, the method is simple and results can be obtained within a single working day. However, spoligotyping has less power to discriminate among MTB strains than the IS6110-based genotyping [72].

1.5.2.3 MIRU-VNTR
IS6110 fingerprinting, the gold standard method for MTB genotyping is laborious and requires weeks of MTB culturing, hindering the prospective use of typing for more efficient TB control programmes. Moreover, comparison of the fingerprints from large data sets requires highly standardized experimental and computerized procedures [89]. These problems complicate the determination of clustering rates in population-based studies and the exchange of data.
A most promising PCR-based method based on PCR amplification of multiple loci containing MIRU-VNTR repeats offers a potential solution to these drawbacks. The method has proved to be highly reproducible yielding bearable results [77]. It is much faster than IS6110-RFLP typing since it can be directly employed on crude extracts from colonies or early MTB cultures.

Different MIRU-VNTR typing sets have been used, standard MIRU-12 loci set with discriminatory power close to IS6110-RFLP was proposed for molecular epidemiological studies in TB [79,90]. The traditional MIRU-12 set was replaced by the standard MIRU-15 set, which is recommended as the standard for the routine molecular epidemiology of TB, outbreak surveys as well as population-based transmission studies. Presently, standard MIRU-24 loci set has been proposed for optimal discrimination of closely associated strains and for high-resolution phylogenetic studies [91].

1.6 Nontuberculous mycobacteria
Mycobacteria other than MTC referred to as nontuberculous mycobacteria (NTM), have been associated with human diseases for nearly 80 years [92]. NTM are environmental opportunistic pathogens of humans and animals found in a wide variety of habitats that are also occupied by humans; including drinking water distribution systems and household water [93]. Due to the presence of NTM in the environment, various human activities influence their ecology and consequently their epidemiology.

Although regarded as less pathogenic to humans, NTM can cause a wide range of clinical diseases, including chronic, debilitating pulmonary disease among elderly people and immunocompromised individuals, lymphadenitis in children, skin disease, and other extrapulmonary or disseminated infections [94].

Pulmonary NTM disease has been considered in the context of TB for two major reasons. Firstly, lung disease associated with NTM is often characterized by a cough, sputum production, haemoptysis, wasting illness, cavities on lungs and acid-fast organisms on sputum smear microscopy; hence can be mistaken for TB. Secondly, NTM isolates are often resistant to first-line anti-TB drugs, it may result in classifying and treating individuals with these isolates as MDR-TB cases [32].
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1.6.1 Epidemiology of NTM
Although the epidemiology of TB is well documented, the prevalence and epidemiology of NTM disease in most developing countries remain largely unknown. Due to their ubiquitous presence in the environment, exposure to NTM is likely extremely common [95]. NTM can colonize the respiratory tract without causing disease such that finding NTM in respiratory secretions does not necessarily have clinical ramifications in all patients. Likewise in many developing countries, NTM disease is not reportable to public health institutions; consequently, epidemiological and surveillance data are not readily available in such settings.

1.6.2 NTM Pulmonary Disease in Immunocompromised Patients
NTM have been implicated in a large and increasing number of infections in both immunocompetent and immunocompromised hosts, mostly HIV-infected patients throughout the world [95–98]. *Mycobacterium kansasii* (*M. kansasii*), *Mycobacterium xenopi* (*M. xenopi*), *Mycobacterium fortuitum* (*M. fortuitum*) and *Mycobacterium scrofulaceum* (*M. scrofulaceum*) are the common opportunistic mycobacteria associated with HIV infections [99].

Information regarding HIV and pulmonary NTM co-infection from TB-endemic countries heavily stricken by HIV/AIDS are still limited [97]. Reports from Zambia and Tanzania documented pulmonary MAC as well as rarely reported species of *M. sherrisii* and *M. lentiflavum* in HIV-positive patients [100–103].

Findings from a cohort of 1,060 HIV-positive patients in Thailand and Vietnam in southeastern Asian where the liquid culture was used show that, NTM were more frequently isolated than MTB. On the contrary, the report showed that NTM was rare in Cambodia, where only solid media was used [94].

NTM disease has also been reported in individuals with lung cancers, hematologic genitourinary malignancies, and old age [104]. NTM disease is reported to be more predominant among patients receiving anti-Tumour necrosis factor-α (TNF-α) especially in individuals > 50 years old [105].

1.6.3 Diagnosis of NTM
Pulmonary infections due to NTM are diagnosed with increasing frequency, partly due to increased populations at-risk but also because of improved awareness and diagnostic facilities, especially in developed countries. The number of species known to cause NTM infections has increased due to the sprawling use of molecular techniques. However,
diagnosis of NTM in TB/AIDS co-infection is greatly limited by HIV status of a patient. Since disseminated NTM infection is typically detected when the CD4+ T-Lymphocytes declines below 50µl in the majority of cases, the diagnosis of NTM is missed out [97].

According to the American Thoracic Society (ATS) and Infectious Diseases Society of America (IDSA) guidelines, diagnosis of NTM pulmonary disease should be based on a combination of clinical, radiological, histological and bacteriological criteria [106]. Due to the overlapping clinical manifestations of the diseases caused by *M. tuberculosis* and NTM, the specific diagnosis of NTM is complicated. Chances that NTM species are missed during diagnosis is largely attributed to:

(i) poor diagnostic capabilities for culture and identification of NTM
(ii) overburden by other diseases like Malaria, TB and HIV such that more focus from the health sector system and governments are paid to such other diseases
(iii) lack of awareness among the public health personnel’s, and
(iv) lack of standardized or accepted criteria to properly define and report NTM diseases.

Conventional identification of mycobacteria is achieved by standard culture and biochemical methods, which is time-consuming. Increased NTM prevalence demands faster methods for identification and selection of appropriate therapy [107].

Molecular techniques complement conventional methods for appropriate species identification and may be considered as a new gold standard for NTM diagnosis. Several gene targets are known to be suitable for species differentiation. Partial genes sequencing for 65 kDa heat shock protein (*hsp65*), RNA polymerase beta-subunit (*rpoB*), essential protein secA1, superoxide dismutase (*sodA*), 16S rRNA and the 16S–23S ribosomal RNA internal transcribed spacer (ITS) have been evaluated for different NTM species [108–112].

### 1.6.4 Management of NTM disease

Despite the availability of advanced diagnostic tools and revised diagnostic criteria for NTM lung infection, the decision whether to treat a patient remains a subject of careful individual assessment taking into account the NTM species, the severity of disease, general condition, and underlying disorders.

Clinical management approaches of patients diagnosed with MTC or NTM infections are different. Therefore, proper and timely identification of mycobacterial species causing
infections is a key to appropriate antimicrobial therapy [113]. As the understanding of the transmission of NTM between patients and their environment increases, the need for interventional trials designed to interrupt transmission in those susceptible to disease is increasingly evident.

Since NTM are ubiquitous in nature and many questions pertaining NTM acquisition and pathophysiology remain unravelled, it has been difficult to devise practical and effective control strategies for diseases due to NTM. Increasing understanding of different NTM diseases has, however, given room for realistic discussion of NTM diseases prevention. Better understanding and correct identification of the reservoir(s) responsible for causing most NTM diseases (especially lung disease), factors associated with the disease transmission, and host susceptibility risks demand further research.

1.7 Pulmonary Nocardiosis

*Nocardia* species are increasingly isolated as infectious agents in immunocompromised patients, and at times, even among healthy individuals [114], causing infections ranging from pulmonary, cutaneous and subcutaneous human diseases [115]. Members of genus *Nocardia* are characteristically gram-positive, weakly acid-fast, strictly aerobic, filamentous branching bacilli that fragment into rod or coccoid shaped forms. *Nocardia* species are ubiquitous environmental bacteria capable of causing opportunistic infections in both human and animals [116,117]

The most commonly isolated species in human include *N. asteroides*, *N. farcinica*, *N. cyriacigeorgica*, *N. nova*, *N. brasiliensis* [118–120] and *N. ignorata* [121]. Pulmonary nocardiosis has been reported in patients with debilitating conditions, such as those with organ transplants, diabetes mellitus, leukaemia, and other malignancies [122].

The incidence of nocardiosis varies geographically according to a number of factors, like the prevalence of HIV infections, transplants, cancer, climate as well as socio-economic status, and laboratory capacity for *Nocardia* species detection and identification.

Increased incidence of human nocardiosis may be attributed to the wide use of immunosuppressive drugs, improved diagnostic tests, and increased awareness among microbiologists and health professionals. Nonetheless, in many developing countries where other chronic lung diseases, particularly TB, are prevalent, *Nocardia* species are either missed or misidentified during diagnosis [123,124].
1.8 Problem Statement and Justification

1.8.1 TB situation in Tanzania

The last two decades in Tanzania like in other developing countries are marked by huge socioeconomic differences. These differences are reflected in the increased gap between the poor and the rich, lack of access to primary health care services, rapid population growth, as well as rural-urban migration. Consequently, this has negatively impacted the endemic diseases status like TB and HIV/AIDS pandemic.

Early detection of TB has great potential to improve individual outcomes and reduce transmission within communities. Although improved diagnostics has potential to increase TB detection in patients with symptoms and signs of active TB, many patients, however, have few symptoms that fulfill definitions of suspected TB, or cannot reach the relevant health services because of barriers to access.

Prevalence surveys of areas endemic for TB show that 50–60% of people with culture-confirmed TB do not report a chronic cough, and 15–25% report no symptoms [125]. Characteristic TB symptoms are less frequently observed in people with HIV [126], paediatric cases, and may be concealed during pregnancy. This implies that if only passive case finding is used to identify new TB cases, then a large proportion of patients with active TB will continue to be undiagnosed. Tanzania continues to rely on passive case finding using clinical and radiological (where available) algorithm with the assistance of less sensitive smear microscopy for detection and identification of TB patients. In most cases, this has provided the unrealistic status of the actual prevalence of MTB among TB suspects [38]. Moreover, information on the different spoligotype families of MTC in Tanzania is limited, where available is restricted to small geographical settings. The absence of current data on the genetic structure of MTC limits identification of genotypes as well as understanding the transmission dynamics among patients who remain undetected by conventional methods.

1.8.2 NTM situation in Tanzania

Although notification of NTM disease is not required in many resource-poor countries, reports indicate that the prevalence of NTM diseases is increasing [127–129]. Since the habitats occupied by NTM (e.g. drinking water) are also shared by humans. Furthermore, the number of individuals with aggravated susceptibility to mycobacterial disease is also increasing (e.g. through immunosuppression) especially where the prevalence of HIV infection is high [93,130]. NTMs play a great role in TB-like disease than was previously
thought [131]. Despite evidence of isolation of NTM especially among HIV-infected individuals in Tanzania, no efforts have been made to establish the actual prevalence and clinical significance of NTM infections in the country [102,132]. Data on clinical infections due to NTM in Tanzania and in sub-Saharan Africa, in general, is still limited. NTM infections can confuse the diagnosis of TB as the tools for culture and identification of mycobacteria are often not available in these settings. Incomplete identification of mycobacterial species causing infections may have serious consequences, such as longer hospitalization time, the risk of co-infections as well as a selection of MDR strains. Clinical management approaches of patients diagnosed with MTC or NTM infections are different, therefore, proper and timely identification of mycobacterial species causing infections is likely to impact antimicrobial therapy [113]. The prevalence of diseases caused by NTM in the country will continue to increase as long as there is no guidelines, which are set in place to diagnose report and manage NTM infections. This trend, combined with antibiotic resistance, will continue to create havoc and difficulties in the management of mycobacterial infections.

1.8.3 Pulmonary nocardiosis in Tanzania
Pulmonary nocardiosis mimic pulmonary tuberculosis in most clinical and radiological manifestations. In Tanzania, where tuberculosis is one of the major public health threat clinical impact of nocardiosis as the cause of the human disease remains unknown, suggesting that infections due to this genus may be underdiagnosed and/or neglected as a cause of human diseases.

1.8.4 Justification
It is, therefore, apparent that correct and rapid identification of MTB, NTM and other bacterial pathogens causing TB-like syndromes such as Nocardia species is urgent, as they all constitute a clinical emergency that cannot be underrated. Need to carry out continuous surveys to provide correct information on the current genetic diversity of MTC, anti-TB drug resistance and the prevalence of NTM and other bacterial pathogens causing TB-like syndromes among TB suspects in Tanzania is particularly urgent in strengthening the fight against mycobacterial diseases and MDR-TB.
Chapter Two  Objective of the Study

This chapter describes the main objective and the specific objectives of this study.

2.1 Main Objective

The main objective of this study was to determine the current genetic biodiversity of *M. tuberculosis* complex and their drug resistance profiles as well as the prevalence of Nontuberculous Mycobacteria (NTM) and other potentially pathogenic bacteria among TB suspects in Northeastern, Tanzania.

2.1.1 Specific Objectives

i. To determine the genetic diversity of the *M. tuberculosis* complex strains in Tanzania using microarray-based spoligotyping and the mycobacterial interspersed repetitive units-variable number of tandem repeats (MIRU-VNTR).

ii. To assess the magnitude of anti-TB drug resistance and associated risks among newly, and previously treated pulmonary TB patients at a facility-base level in Northeastern, Tanzania.

iii. To determine the frequency and diversity of nontuberculous mycobacterial species among TB suspects in Northeastern, Tanzania.

iv. To determining the role of nontuberculous mycobacteria in diagnosis, management and quantifying risks for TB transmission in Tanga, Tanzania.

v. To determine the frequency of other potentially pathogenic bacteria among smear negative TB suspects in Northeastern, Tanzania.
Chapter Three    Materials and Methods

This chapter describes the summary of the general methods used in the study.

3.1 Study area and Patients

3.1.1 Tanga city and Muheza District

Tanga Region is located on the northeastern side of Tanzania mainland. The region lies between Latitudes 4° and 6° south of the Equator, and between longitudes 37° and 39° east of Greenwich. The Region is bordered by the Republic of Kenya in the north, Kilimanjaro Region in the northwest, Manyara Region in the west, Morogoro, and Coast Regions in the south and the Indian Ocean in the east (Figure 4). Muheza District and Tanga city have respectively a residential catchment population of 204,461 people with the average household size of 4.3 persons per household and 273,332 people with the average household size of 4.4 persons per household [133].

Figure 4. Map of Tanga indicating the study sites, the two hospitals, and two health centres are shown by octagon shapes and marked by letters A: Muheza Designated District Hospital (MDDH); B: Bombo Referral Hospital; C: Makorora Health Centre and D: Ngamiani Health Centre.
3.2 Inclusion and exclusion criteria

All suspects aged 7 years and above, residing in Tanga city and Muheza District during the study period and informed consent from the patients were the inclusion criteria into the study; whereas temporary residents like visitors, unwillingness to consent and patients who had been on TB treatment for more than one week were excluded from the study.

3.3 Study design

A cross-sectional study was carried out between November 2012 and January 2013; suspects presenting with any of the following symptoms were recruited: the presence of symptoms suggestive of TB like a chronic cough for a period of ≥ 2 weeks, night sweats, fatigue, unexpected loss of weight, and fever.

3.4 Sample collection

Two sputum samples were collected from the consenting patients at the respective clinics, one spot sputum during the first visit and patients were provided with another sterile container to collect an early morning specimen. Direct smear microscopy using Ziehl Nelsen (ZN) - stain (Ngamiani and Makorora health centres) and fluorescence stain (Muheza Designated District hospital and Bombo referral hospital) were performed. Smear results were recorded in accordance with the WHO/IUTLD and National Tuberculosis and Leprosy Programme (NTLP) guidelines [134]. All early morning sputum samples were stored at -20°C and later transported to the mycobacteriology laboratory, University hospital Leipzig, Germany for culture and molecular analyses. Seven hundred and forty-four sputum specimens were collected from 372 TB suspects. Patients were also requested for HIV testing after pre-test counselling, with only 159 patients consented for HIV testing.

3.5 Sputum culture and identification of mycobacterial isolates

Sputum specimens were decontaminated using N-acetyl-l-cysteine-sodium hydroxide method [135] and concentrated by centrifugation. All specimens were re-examined for the presence of acid-fast bacilli by fluorescence stain in Leipzig. Subsequently, all isolates were cultured in BacT/Alert 3D liquid culture system (BioMe´rieux, Marcy-l’Etoile, France) and on Löwenstein- Jensen and Gottsacker slants (Artelt-ENCLIT GmbH, Wyhra, Germany). Gottsacker slopes contain sodium pyruvate for isolation of M. bovis. Cultures were incubated at 37°C for up to 8 weeks. GenoType®MTC (Hain Lifescience, Nehren, Germany) was used for confirmation of M. tuberculosis complex, whereas GenoType®CM/AS (Hain Lifescience, Nehren, Germany)
for confirmation of either common or accessory NTM. Additionally, NTM isolates not detected to the species level by Hain kits were subjected to the 16S rRNA and hsp65 gene sequencing.

3.6 Drug susceptibility testing of M. tuberculosis complex isolates

3.6.1 Phenotypic drug susceptibility testing
Isolates were tested for resistance to rifampicin (RMP), isoniazid (INH), streptomycin (SM), ethambutol (EMB) and pyrazinamide (PZA) using BacT/ALERT 3D system [136,137] in concentrations of 1μg/ml for RMP, INH, and SM; 2μg/ml for EMB and 200μg/ml for PZA. Bottles were incubated at 37°C and growth were monitored daily. An isolate was considered resistant to a drug under test when drug-containing bottle had a time to detection (TTD) that was less or equal to the TTD of 1% control bottle.

3.6.2 Genotypic drug susceptibility testing
Genotypic DST for RMP and INH was performed by using Genotype® MTBDRplus assay (Hain Life Science GmbH, Nehren, Germany). The assay uses a set of probes to affirm the test procedures (amplification and hybridization), a probe to identify MTC, eight rpoB wild-type (WT) probes encompassing the RRDR with no mutations, and four mutant probes expressly targeting the most common mutations conferring RMP resistance (D516V, H526Y, H526D, S531L). Additionally, it includes one WT and two mutant probes (S315T1 and S315T2) for the 315 region of the katG gene conferring high INH resistance, plus two other WT probes and four mutant probes (C15T, A16G, T8C and T8A) for the promoter region of the inhA gene, conferring low INH resistance. Genotypic detection of resistance to ethambutol (EMB) was done by using GenotypeMTBDRsl assay (Hain Life Science GmbH, Nehren, Germany).

3.7 Genetic Analyses
Eighty MTB isolates were eligible for molecular typing by using microarray-based spoligotyping and the conventional MIRU-VNTR typing.

3.7.1 Microarray-based spoligotyping
Spoligotyping of the MTB isolates was performed by using microarray-based spoligotyping format of the Array Strip platform (Alere Technologies GmbH, Jena, Germany) [138]. Briefly, genomic DNA of MTB isolates were amplified using polymerase chain reaction (PCR) with the primers DRa (5’-biotin labelled) and DRb. The
Materials and Methods

PCR products were hybridized on Array Strips using hybridization kit (Alere) at 60°C for 1 hr and wash steps at 55°C, otherwise following manufacturer’s instructions. Recording of stained microarrays was done by using an Array Mate transmission reader (Alere). The binary code data were automatically compared with SpolDB4.0 and MIRU-VNTRplus (http://www.miru_vntrplus.org/MIRU/index.faces) database to identify concordant species and lineages.

3.7.2 MIRU-VNTR typing

MIRU-VNTR typing was performed by PCR amplification of a panel of 21 MTB MIRU-VNTR loci using primers as described in the MIRU-VNTR standard protocol [91,139,140]. PCR mixture was prepared using the HotStar Taq DNA polymerase kit (Qiagen, Hilden, Germany). PCR products were analysed on 1.5% agarose gel against 100bp DNA ladder (PEQ lab Biotechnologie GmbH, Erlangen, Germany) in TAE buffer electrophoresis for two hours at 100 constant voltage. The allele calling table provided in the MIRU-VNTR standard protocol [91] was used to assign the number of alleles that correspond to the amplicon’s size. Results were entered into Ms. Excel sheet in a digital format. MIRU-VNTRplus database (http://www.miru-vntrplus.org/) was used to identify the MTB strains by similarity search and by a phylogenetic tree, using a categorical coefficient of one and a distance cut-off of zero. The UPGMA dendrogram was constructed from the strains genotypes using the online MIRU-VNTRplus database [141].

3.8 Norcadia isolates identification

Nocardia isolates were presumptively identified based on their cultural and microscopic characteristics on Löwestein-Jensen media, blood agar and chocolate agar plates. Definitive identification was performed using biomérieux ID 32C yeast identification system (Biomérieux, Marcy-l’Etoile, France) following manufactures instructions and confirmed by 16S rRNA gene sequencing.

3.9 Ethical considerations

The protocol for this study was reviewed and approved by National Institute for Medical Research (NIMR), Tanzania (Reference number NIMR/HQ/R.8a/Vol.IX/1401). Informed consent was obtained from all participants of this study, the consent allowed for use of the collected sputum samples for the performance of other unspecified, TB, NTM and other bacterial pathogens diagnostic tests for research purposes. This consent was
obtained as written consent or, for illiterate participants, witnessed verbal consent. For illiterate participants, there was an independent witness present during the consenting process, who then signed the relevant witness section of the consent form. Ethical approval was obtained for the secondary use of the study data from the same ethical committee, which approved the consent form, including the section on the use of witnessed verbal consent for illiterate participants.
Materials and Methods

A: Patient recruitment at PDCs

372 TB suspects

312 New cases 60 Repeat cases

Direct sputum smear microscopy

Decontamination & Concentration method + Fluorescent microscopy

SOLID CULTURE
LJ & Gottsacker

372 samples

LIQUID CULTURE
BacT/Alert 3D system

Genotype MTBC

Phenotypic DST

MTB

80 MTB isolates, 26 from smear–ve

Spoligotyping & MIRU-VNTR

Genotypic DST

36 NTM isolates

16S rRNA Sequencing
(8 isolates)

Screening for Other Potential Bacterial Pathogens

20 Nocardia isolates

Staphylococcus aureus & Mycoplasmas

B: Smear results at PDCs

Each patient gave two sputum samples (One spot + Early morning)

C: Leipzig

Figure 5. A flow chart summarizing Approach of the study

Key: PDC= Periphery diagnostic centre; DST= drug susceptibility testing; MTBC = Mycobacterium tuberculosis complex; CM/AS = common or accessory nontuberculous mycobacteria; MIRU-VNTR = mycobacterial interspersed repetitive units-variable number of tandem repeats
Chapter Four  Results and Discussion

4.1 Publication I

Molecular characterization of *Mycobacterium tuberculosis* isolates from Tanga, Tanzania: First insight of MIRU-VNTR and microarray-based spoligotyping in a high burden country

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**ARTICLE INFO**

**SUMMARY**

Molecular typing of *Mycobacterium tuberculosis*(MTB) has greatly enhanced the understanding of the population structure of MTB isolates and epidemiology of tuberculosis (TB). To characterize prevalent genotypes of MTB, microarray-based spoligotyping and mycobacterial interspersed repetitive unit-variable tandem repeat (MIRU-VNTR) were applied on 80 isolates collected from primary health care facilities in Tanga, North-eastern Tanzania. A total of 18 distinct spoligotypes were identified. The lineages by order of their predominance were EAI and CAS families (26.25%, 21 isolates each), LAM family and T super-family (19%, 8 isolates each), MAAO family (5.75%, 3 isolates), Beijing family (2.5%, 2 isolates) and 5 family (1.25%, 1 isolate). Overall, sixteen (20%) strains could not be allocated to any lineage according to the STIIVT_web database. The allelic diversity (h) for specific MIRU-VNTR loci showed a considerable variation ranging from 0.825 of VNTR locus 3102 to 0.641 of VNTR locus 3605. The allelic diversity for 11 loci (VNTR 2302, 2396, 2165, 560, 4052, 424, 4152, 2531, 6644, 802 and 3605) exceeded 0.6, indicating highly discriminatory power. Seven loci (VNTR 2302, 2396, 2165, 560, 4052, 424, 4152) showed moderate discrimination (0.5 ≤ h ≤ 0.6), and three loci (VNTR3007, 154 and 2805) were less polymorphic. The present study suggests that the TB cases in Tanga might be caused by a diverse array of MTB strain families that may be indicative of a cosmopolitan population with frequent migration and travel. Microarray-based spoligotyping and MIRU-VNTR could be reliable tools in detecting different MTB genotypes in high burden settings.

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1. Introduction

Tuberculosis (TB) caused by bacteria belonging to the *Mycobacterium tuberculosis* complex (MTC) in humans primarily by *M. tuberculosis* (MTB), remains a major public health threat globally and a leading cause of morbidity and mortality in many sub-Saharan African countries. Co-infection with HIV/AIDS and the emergence of multi-drug resistant (MDR) MTB strains have worsened the situation [1]. Tanzania is among 22 countries with the highest burden of disease [2]. In 2013, the prevalence of bacteriologically confirmed TB in Tanzania was 295 per 100,000 adult persons [3]. Notification of TB cases, has increased from about 618,380 cases in 2011 to 63,892 in 2012 which is about 3.1% increase [4]. This increase may be attributable to either increased TB/HIV co-infection [5] or reactivation of latent MTB infection or due to better diagnostic tools.

Molecular genotyping of MTC isolates has proven to be a valuable tool with great potential to significantly impact both individual clinical management and public health [6]. Techniques such as IG610 restriction fragment length polymorphism (RFLP) typing [7], spoligotyping and mycobacterial interspersed repetitive unit - variable tandem repeats (MIRU-VNTR) analysis [8,9], and more recently whole genome sequencing [10] have become powerful tools in understanding and predicting disease transmission dynamics. IG610-RFLP is the gold standard among the MTB genotyping tools; however, the method is laborious and requires
weeks for culturing the isolates since it demands large amounts of DNA and has poor inter-laboratory reproducibility hence rendering its use limited [11]. In addition, it is also less discriminatory among the MTB isolates having a low copy number (≤5) of IS6110 [12].

PCR-based methods such as spoligotyping [13] and MRU-VNTR typing [19] have enabled to overcome the drawbacks shown by the IS6110 fingerprinting. The methods are faster to perform and interpret, demand small quantities of genomic DNA and can easily be presented in digitalized format allowing for comparison between different laboratories [14]. Furthermore, availability of freely accessible web-based database for analysing data generated from these methods makes strains identification rather easier and quicker [13,15].

Spoligotyping is based on polymorphism among 43 spacers in the direct repeat (DR) locus among species of the MTC [13], whereas MRU-VNTR is based on the number of repetitive units present in multiple defined loci [10]. The discriminatory power of this method is close to that of IS6110-based fingerprinting [12,17] and produces better resolution between strains with low IS6110 copy number [18]. Availability of an online tool for classification and analysis of strains of MTC http://tbinsight.csru.cf.ac.uk/mu_1t_1/lineage.html [15] plus an international database at http://www.pasteur-guadeloupe.fr/biopspdb4.html maintained at Pasteur Guadeloupe, allow comparison of spoligotypes of MTB [19].

Microarray-based spoligotyping offers a powerful high-throughput molecular typing alternative that is suitable for studying strain diversity in high burden populations and geographical areas to reveal epidemiological trends [20].

Only a limited number of studies have been conducted in Tanzania using modern molecular DNA fingerprinting techniques that are capable of directly tracing TB transmission routes. Information on important aspects of TB epidemiology, such as role of recent transmission in reactivation of latent cases is still lacking. Available data on the different spoligotype families of MTB strains in Tanzania are limited, and where available restricted to small geographical areas [21].

The present study aimed at characterizing the causative agent from TB patients in Tanga, Tanzania, by using microarray-based spoligotyping together with conventional (i.e. agarose gel-based) MIRU-VNTR typing. This is the first study in which a recently developed microarray-based spoligotyping and MIRU-VNTR are employed in a high burden country. Findings of this study provide informative epidemiological data urgently needed for improved TB control programmes and for prediction of future epidemiological trends.

2. Materials and methods

2.1. Study population

Eighty MTB isolates collected from 372 new and recurrent TB patients during a cross-sectional study conducted in Tanga, Tanzania from November 2012 through January 2013 were eligible for this study. All patients with clinical signs and symptoms suggestive of TB self-referred to the primary health care facility were recruited in the study. The facilities included: Regional Referral Hospital, Makororo and Ngamiangani. A map of Tanga indicating the number of isolates from each site is provided as Supplementary File S1. Demographic information and data were collected only after provision of informed consent.

2.2. Specimen and data collection

Structured questionnaires were administered to the patients attending four TB clinics who provided informed consent to the study. Two sputum samples (one during the initial visit to the clinic and one early morning) were collected into small autoclavable wide mouth glass bottles. The specimens were examined by direct smear microscopy at the respective clinics using either Ziehl Neelsen ZN -stain (at Makorora and Ngamiangani health centres) or fluorescent stain (at Bombo referral and MDHH). All morning sputum samples were then shipped to the mycobacteriology laboratory at the University Hospital Leipzig, Germany, for culture and molecular analysis.

2.3. Specimen processing and culture

Sputum specimens were digested and decontaminated using N-acetyl-l-cysteine-sodium hydroxide method [22] and were re-examined for the presence of acid-fast bacillus (AFB) by fluorescent stain in Leipzig. The isolates were cultured in Bact/Alert 3D liquid culture system (bioMérieux, Marcy-l’Etoile, France) and on Löwenstein-Jensen and Gotsacker slants (Arcel ENICHT GmbH, Würzbur, Germany). Gotsacker slants contain sodium pyruvate for improved isolation yield of Mycobacterium bovis. Cultures were incubated at 37°C for up to 8 weeks and confirmed as MTB by using line probe assay Genotyper® MTBRC (Hain Lifescience, Nehren, Germany). Isolates included those from smear-positive patients (n = 54), as well as those from smear-negative but culture positive patients (n = 26).

2.4. Extraction of genomic DNA from MTB isolates

Mycobacterial cells from positive Bact/Alert bottles and from LJ and Gotsacker slants were used for DNA extraction. Bacterial DNA was extracted from heat-inactivated AFB isolates. Briefly, a loopful of colony material was placed into a labelled screw-cap eppendorf tube containing 500 μl sterile distilled water or by taking 500 μl from a positive Bact/Alert bottle. Each specimen was incubated on a heat block at 95°C for 20 min to inactivate the bacteria. Heat-killed cells were stored at 4°C until analysis. DNA extraction was done using a High Pure PCR Template Preparation kit (Roche Diagnostics GmbH, Mannheim, Germany) according to manufacturer’s instruction. Genomic DNA of the H37Rv strain and sterile distilled water were used as positive and negative controls, respectively, for all genotyping procedures.

2.5. Microarray-based spoligotyping

Spoligotyping of the MTB isolates was performed by using the newly developed microarray-based spoligotyping format of the Array Strip platform (Arete Technologies GmbH, Jena, Germany) [20]. Briefly, genomic DNA of MTB isolates was amplified using polymerase chain reaction (PCR) with the primers DR1 (5’-biotin labelled) and DR8. The PCR products were then hybridized on Array Strips using hybridization kit (Arete) at 60°C for 1 h and washed steps at 55°C, otherwise following manufacturer’s instructions. Recording of stained microarrays was done by using an Array Mate transmission reader (Arete). Signal intensities equal and higher than 0.3 (on a scale from 0 to 1.0) were considered positive for the respective probe [20]. The binary code data were automatically compared with SpolDB4.0 [19] and the updated version MITBWEB [23] databases to identify concordant species and lineages.

2.6. MIRU-VNTR typing

To identify a suitable MIRU-VNTR focus panel for genotyping MTB isolates in this geographical area, 12 and 15 loci were chosen for analysing the 80 MTB isolates. MIRU-VNTR typing was
performed by PCR amplification of the selected MIRU loci using primers as described in the MIRU-VNTR standard protocol [16,24,25]. PCR mixture was prepared using the HotStar Taq DNA polymerase kit (Qiagen, Hilden, Germany). PCR products were analysed on 1% agarose gel against 100bp DNA ladder (FEQ Lab Biotechnologie GmbH, Erlangen, Germany) in TAE buffer electrophoresis for 2 h at 100 constant voltage. The allele calling table provided in the MIRU-VNTR standard protocol [16] was applied to assign the number of alleles that correspond to the amplicon’s size.

Results were entered into Ms Excel sheet in a digital format. MIRU-VNTRplus database (http://www.mini-vntrplus.org) was used to identify the MTB strains by similarity search and phylogenetic tree, using a categorical coefficient of one and a distance cut-off of zero. The UPGMA dendrogram was constructed from the strain genotypes using the online MIRU-VNTRplus database [1]. The MIRU-VNTR allelic diversity (h) at a particular locus was calculated as follows: \[ h = 1 - \sum x_i^2 / n(n-1) \], where \( x_i \) is the frequency of the \( i \)th allele at the locus and \( n \) is the number of isolates [26]. To determine the discriminatory power of the MIRU-VNTR for this patient population, the Hunter–Gaston discriminatory index (HCDI) was used [27,28]. The HCDI was calculated using the following formula:

\[ HCDI = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} n_j(n_j - 1) \]

where \( D \) is the numerical index of discrimination, \( N \) the total number of strains in the typing scheme, \( s \) is the total number of different strain types, and \( n_j \) is the number of strains belonging to the \( j \)th type.

2.7. Major lineages prediction

An online tool for classification and analysis of strains of MTC (http://hbsite.cl/mbblineage.html) was used to determine the major lineages based on Conformal Bayesian Network (CIN) [15,29]. To determine the major lineages in our patient population, we used a combination of spoligotype and 12-loci of MIRU as a feature in the CBN method, since the complete data set for the 24 loci of MIRU was not available.

2.8. Ethical consideration

The study was reviewed and approved by the national ethical review committee with secretariat at the National Institute for Medical Research (NIMR), Dar es Salaam, Tanzania and provided with a certificate number NIMR/HQ/R.8a/Vol.7/1401. All subjects

![Figure 1](image-url)
Results and Discussion

Table 2
Most prevalent spoligotype families in Tanzania according to the SITVIT_WEB database and their corresponding major lineages predicted by CBN method.

<table>
<thead>
<tr>
<th>SIT</th>
<th>SITVIT_WEB</th>
<th>CBN</th>
<th>NE</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>EAI</td>
<td>Info-Denic</td>
<td>18 (22.5)</td>
</tr>
<tr>
<td>21</td>
<td>CAS1, KIU</td>
<td>East-African Indian</td>
<td>16 (20.0)</td>
</tr>
<tr>
<td>46</td>
<td>NE</td>
<td>Euro-American</td>
<td>6 (7.5)</td>
</tr>
<tr>
<td>Orphan</td>
<td>NE</td>
<td>Euro-American</td>
<td>6 (7.5)</td>
</tr>
<tr>
<td>53</td>
<td>T1</td>
<td>Euro-American</td>
<td>5 (6.25)</td>
</tr>
<tr>
<td>4</td>
<td>NE</td>
<td>Euro-American</td>
<td>4 (5.0)</td>
</tr>
</tbody>
</table>

ND, not defined.
1 SIT, spoligotype international-type from SITVIT_WEB database.
2 Spoligotype families as assigned in the SITVIT_WEB database.
3 Major lineage predicted by CBN.
4 Number of isolates with common SIT.

The test results of the respective strains were plotted on a graph using signal intensity (i.e., presence or absence of a particular space) each spacer for each particular spoligotype with cut-off point value of 0.3. The experimental output of the microarray-based spoligotyping of MTB for the representative predominant spoligotype lineage (CAS1, KIU) in the study population as well as the positive and negative controls are shown exemplarily in Figure 2. We also compared the results from genotyping studies conducted previously in Tanzania using conventional membrane-based spoligotyping (Table 4).

3.1. Description of the isolates
A total of 80 MTB isolates from 56 male and 24 female TB patients with a range 10–90 years (median age of 33) from four TB clinics were included in the study. Seventy-two of the patients were new cases, eight were recurrent cases; fifty-four of the subjects were smear-positive cases by direct smear microscopy, while 26 were smear-negative (Table 1). All 80 isolates were genotyped by microarray-based spoligotyping and MIRU-VNTR typing.

3.2. Interpretation of the array results

Among the 80 isolates, the majority of strains belonged to the EAI and CAS1 lineages (26.25%, 21 isolates) each, LAM and T1 lineages comprised eight (10% each) strains, three (3.75%) were assigned to MANU lineage; two (2.5%) strains belonged to Beijing genotype and one (1.25%) strain was assigned to S-lineage. Sixteen (20%) isolates were not assigned to any lineage using the SITVIT_WEB database and were named as “not defined” in this study. The predominant spoligotype families in Tanzania based on SITVIT_WEB database, their respective major lineages as predicted by CBN method are shown in Table 2, and information of all the 80 isolates is shown in Supplementary Table S2.

3.3. Microarray-based spoligotyping results

Figure 1. We also compared the results from genotyping studies conducted previously in Tanzania using conventional membrane-based spoligotyping (Table 4).

3.4. Major lineages prediction by CBN method

Among the 80 isolates predicted by the CBN method, majority of the strains belonged to the modern lineages, with Euro-American (33 (41.25%)) being the most prevalent TB lineage, followed by East-African Indian (21 (26.25%)) and East-Asian (Beijing) 2 (2.5%)
Results and Discussion

Table 3

Proportions of first-line anti-TB drug resistance among different genotypes in Tonga.

<table>
<thead>
<tr>
<th>Site</th>
<th>SIT</th>
<th>% Resistance to:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>RH</td>
</tr>
<tr>
<td>46</td>
<td>ND</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>ND</td>
<td>4</td>
</tr>
<tr>
<td>42</td>
<td>LAM</td>
<td>3</td>
</tr>
<tr>
<td>156</td>
<td>CAI</td>
<td>2</td>
</tr>
</tbody>
</table>

INH, isoniazid; RMP, rifampicin; SM, streptomycin; EMB, ethambutol; PZA, pyrazinamide.
ND, not defined.
* SIT from STITIT_WEB database.
† Representing families annotated in STITIT_WEB database.
1 Number of isolates.
2 MDR, multidrug resistant, represent isolates resistant to at least isoniazid and rifampicin.

being the least prevalent. On the other hand 24 (30%) belonged to the ancestral lineages of the Indo-Oceanic lineage. Results of major lineages prediction and distribution by site are shown in Figure 2.

3.5. Drug resistance profile of the spoligotype families

All isolates were tested for their resistance to rifampicin (RMP), isoniazid (INH), streptomycin (SM), ethambutol (EMB) and pyrazinamide (PZA) by a proportion method using Bact/ALERT 3D system [Biomérieux, Marcy-l’Étoile, France]. Critical concentrations of 1 μg/ml for RMP, INH, and SM; 2 μg/ml for EMB and 350 μg/ml for PZA were respectively used as described before [30]. Results showed that the majority of the genotype families found in this population were susceptible to first-line anti-TB drugs. One of the three isolates assigned to LAM9 genotype was found to be a MDR case, and in addition was resistant to SM and PZA. Another resistant isolate was found in one of the two isolates designated to CAS genotype, which was both MDR case and EMB resistant. Other resistant strains were found among the isolates "not defined" in the STITIT_WEB, with four isolates being MDR cases. The proportions of first-line anti-TB drugs among different genotypes are shown in Table 3.

Figure 3. This dendrogram was generated by using unweighted pair group method with arithmetic average (UPGMA) algorithm in the MIRU-VNTRplus database [http://www.mirem-spc.org]. The tree was built on 80 M. tuberculosis clinical isolates from Tonga based on 15 loci MIRU-VNTR genotypes and Microsatellite-based spoligotyping. The order of MIRU loci is as follows, left to right: 424, 577, 580, 802, 960, 1644, 1955, 2353, 2615, 2401, 2996, 3192, 3690, 4052 and 4156.
3.6 MIRU-VNTR typing

In order to evaluate and determine the most suitable loci for genotyping the M. tuberculosis isolates in Tanga, we analysed 12 and 15 loci panels of MIRU-VNTR loci. The allelic diversity (h) calculated for 80 MTB isolates for specific MIRU-VNTR loci showed a considerable variation ranging from 0.826 of VNTR locus 3192 to 0.141 of VNTR locus 2059. The allelic diversity for 11 loci (VNTR 3192, 2996, 2165, 968, 4062, 424, 4156, 2531, 1644, 800 and 3690) exceeded 0.6, indicating that they are highly discriminating. Seven loci (VNTR 2163b, 2401, 1955, 577, 4148, 2687 and 5801) showed moderate discrimination (0.3 < h < 0.6), three loci (VNTR 3007, 154 and 2059) were even less polymorphic (Table 5).

Due to low discriminatory power of the 12-loci MIRU-VNTR set seen for this patient population, the 15 loci MIRU-VNTR set with the overall HDGI of 0.8889 and 21.3% clustering rate was deemed suitable for genotyping MTB isolates in this patient population.

VNTR locus 3192 was the most discriminatory locus with seven alleles; one to seven repeats of this loci were observed with allele number three being the most common found in 19 isolates. VNTR locus 2996 exhibited in eight alleles was the second most discriminatory loci with allele number one being the most common found in 21 isolates followed by locus 2165 exhibited in eight alleles, with allele two being the most common found in 24 isolates. VNTR locus 968 exhibited in nine alleles, with allele four being most common observed in 28 isolates. The least variable locus was VNTR 5801 exhibited in two to five alleles, with allele two being the most common found in 60 isolates. Other loci with their respective discriminatory indices are shown in Table 5.

Of the 80 M. tuberculosis strains genotyped, a total of 58 different VNTR genotypes were identified. Of these, 46 (79.3%) were unique (i.e. for a single strain) and 34 strains were clustered into 12 (20.7%) clusters of two to six representatives. The largest cluster observed comprised of six isolates all from the same location whereas other clusters comprised between two to four strains. The UPGMA dendrogram was constructed using 15 loci MIRU-VNTR (Figure 3).

4. Discussion

In the present study, we report an insight into the MTB strains isolated in Tanga, Tanzania by combining microarray-based spoligotyping and MIRU-VNTR typing method. To the best of our knowledge, this is the first study on the genetic diversity of MTB in a high burden setting where the two approaches have been employed.

The results obtained reveal that the population structure of MTB isolates in Tanga appears to be heterogeneous, as 18 known spoligotype families based on the SITVIT-Web database were obtained from 80 MTB isolates as depicted in Supplementary Table S2. Similar families have been reported in variable proportions in other countries bordering Tanzania [31–35], indicating a geographical widespread of this group of spoligotypes in the region. These findings are also in agreement with those of other studies conducted in Tanzania which reported the predominance of these families [21,36,37].

The predominance of the CAS1, KIL1 in this area and other parts of the country as previously reported [36] may be enhanced by virulence, transmissibility and/or specific adaptation to a host population. Increased reporting of CAS1, KIL1 strain in Tanzania is, however, not clear, whether it is a result of chance of this strain to spread following introduction or is due to its phylogeographical specificity for Tanzania. Six (37.5%) of the “not defined” isolates in the SITVIT-Web had unique patterns and were predominant in Muhesa District and was the most clustered family suggesting a recent transmission.

Strain families previously reported in Tanzania but not reported in this study include the Haarlem genotype [21,37] and the X genotypes [21,36–38]. We detected a high proportion of T family isolates as found in previous studies [21,37]. Furthermore, we report sub-lineage EAI1 BGD1 which was reported in a recent study [21] and sub-lineage EAI1 SOM [21,37]. We also report a relative high proportion of MANU family as compared to those reported previously [36,37] (Table 4).

The presence of MANU family in this geographical area suggests the predominance of this family in northern part of the country, as Tanzania lies in the north-eastern corner of Tanzania and the previous MANU families were detected in Kilimanjaro and Serengeti ecosystem in northern Tanzania as well [36,37]. The diverse distribution of strains observed in this and other studies in Tanzania so far underscore the need to map the distribution of MTC genotypes in the country.

Generally, the results of major lineages prediction by CBN method suggest that 70% of TB infections in this geographical area are due to the modern lineages, whereas 30% of TB infections is due to the ancestral lineages mainly of Indo-oceanic lineage. This suggests that modern M. tuberculosis strains contribute significantly to TB infections in Tanga. Modern M. tuberculosis strains represent epidemic strains that were introduced into the same geographical regions more recently as a consequence of the world-wide spread of the tuberculosis epidemic [19]. Over the past decades, Tanzania has experienced a tremendous improvement in infrastructure development and expanding local and international trade, which

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**Table 4**

Comparison of prevalence of MTB families based on microarray-based spoligotyping (this study) and in other studies in Tanzania.

<table>
<thead>
<tr>
<th>Family</th>
<th>(Dithain et al., 2008)</th>
<th>(Kibiki et al., 2007)</th>
<th>(Mitanga et al., 2014)</th>
<th>(Mnge et al., 2014)</th>
<th>Present study</th>
</tr>
</thead>
<tbody>
<tr>
<td>EAI</td>
<td>25 (17.0)</td>
<td>13 (10.0)</td>
<td>49 (19.1)</td>
<td>25 (11.7)</td>
<td>21 (26.3)</td>
</tr>
<tr>
<td>CAS</td>
<td>52 (35.4)</td>
<td>40 (37.7)</td>
<td>195 (49.0)</td>
<td>55 (25.7)</td>
<td>21 (26.3)</td>
</tr>
<tr>
<td>LAM</td>
<td>33 (22.4)</td>
<td>30 (29.2)</td>
<td>84 (17.5)</td>
<td>38 (17.8)</td>
<td>8 (10.0)</td>
</tr>
<tr>
<td>T</td>
<td>21 (14.3)</td>
<td>16 (12.3)</td>
<td>58 (13.9)</td>
<td>52 (24.3)</td>
<td>8 (10.0)</td>
</tr>
<tr>
<td>Beijing</td>
<td>7 (4.8)</td>
<td>7 (5.4)</td>
<td>33 (8.4)</td>
<td>8 (13.7)</td>
<td>2 (2.5)</td>
</tr>
<tr>
<td>S</td>
<td>3 (2.0)</td>
<td>0</td>
<td>4 (0.8)</td>
<td>1 (0.5)</td>
<td>1 (1.3)</td>
</tr>
<tr>
<td>MANU</td>
<td>0</td>
<td>3 (2.3)</td>
<td>0</td>
<td>1 (0.5)</td>
<td>3 (3.7)</td>
</tr>
<tr>
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* Membrane-based spoligotyping.
* Membrane-based spoligotyping and 24 loci typing MIRU-VNTR.
* Microarray-based spoligotyping and 15 loci MIRU-VNTR typing (this study).
Results and Discussion

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HGD1: Hunter-Gaston discriminatory index

permit movement of people from within and outside the country. Consequently, this might have played an important role in the introduction of new M. tuberculosis strains in this region.

Spoligotyping, being microarray-based or membrane-based, is a rapid diagnostic method most suitable for studying TB disease in high burden countries. However, an important component for technology transfer, especially for resource-poor settings like Tanzania, will be the cost/output ratio required to introduce a new technique (microarray-based high-throughput) compared to the established one (membrane-based low throughput). Microarray-based method has a potential of: (i) producing results within a single working day (high turnaround time), (ii) ease of operation and use, since no need of handling membrane in a dot blot manifold and developing a chemi-luminescence film in a darkroom, (iii) data are automatically processed using an online database, and (iv) relatively low cost (~5–10 euros per assay). Given its upper hand advantages, microarray-based method could be used as a relatively inexpensive “first screen” genotyping of MTC, and still remain an informative assay that could be suited to resource-poor countries. Moreover, digital-numerical data output makes it less prone to interpretation errors, in addition allows easy interpretation of data (Figure 1).

MIRU-VNTR typing has been used in epidemiology studies, and has shown adequate stability in tracking recent transmission and distinguishing relapses and reinfections [9]. Different VNTR typing sets have been used in the present study; we evaluated a 15 loci panel for its ability to discriminate the different MTB genotypes in Tanzania. Many loci had high discriminatory index with VNTR locus 3192 being the most discriminatory, while only VNTR locus 380 showed low discriminatory index (H = 0.412) (Table 5).

The high genetic diversity (i.e. HGD1) of 0.989 in this study is relatively close to those observed in Kwaempe, Uganda (0.996) [33] and in Niola, Zambia (0.998) [32], which are similar settings in endemic countries bordering Tanzania, in the north and south, respectively. The diversity of MTB isolates in Tanzania population may be related to reactivation of latent MTB infection, and/or may be due to increased human population movement. Consequently, this may predispose the population to possible introduction of potentially new different strains [39].
Results and Discussion

The standard MIRU 15 loci set has been recommended as the standard for routine molecular epidemiology of TB, including outbreak investigations and population-based transmission studies [40]. As shown in this study, the 15 loci panel offers a better discriminatory power in this patient population. Therefore, we recommend that this 15 loci panel should be used as a first-line panel for genotyping MTB isolates in this setting, especially for routine epidemiological investigation, as it may be more cost effective than the full set of 24 loci. It should also be noted that not all 24 loci are required for MTB genotyping in any given situation [16,41], as the number of loci required depends on the lineage known to be prevalent in the investigated area. Furthermore, based on the allelic diversity of individual MIRU-VNTR locus, different combinations of MIRU-VNTR loci offering high resolution and cost effective analyses for the different MTB lineages need to be sought [41].

However, it is important to note that the accuracy of the phylotyping group by MIRU-VNTR is more exact than that of spoligotyping but depends on the number of loci included in the analysis and classification errors are reduced when analysing 24 loci [42]. Strains with ambiguous spoligotype signatures like the unknown family isolates in our study belonging to the Euro-African lineages may require a more discriminatory marker such as the 24 loci.

The fact that 26/80 (32.5%) patients were smear-negative suggests that a substantial number of patients may be missed out if we continue relying on insensitive smear microscopy for TB screening (Table 1). Establishing culture facilities at least at regional levels may help detecting such patients. This underscores the need to consider smear-negative patients when conducting future surveillance programmes in the country.

The findings of this study suggest that TB cases in Tanga might be caused by a diverse array of MTB strains as an indication of a cosmopolitan population with frequent migration and travel. Furthermore, the dominant genotypes such as CAS1, KIU and EA15 may have been present in this population for an extended period or there is an ongoing transmission of closely related strains. Although these strains are not representative of all strains in the population, they provide preliminary insights into the strain diversity of MTB genotypes in Tanga. The emergence of new subgroup strains familiarized may well confirm the dynamic nature of this population.

In conclusion, this study provides a preliminary insight into the MTB genotypes circulating in Tanga and that microarray-based spoligotyping and MIRU-VNTR 15 loci panel could be reliable tools in detecting different genotypes in settings like Tanga. However, we propose a large sample size with a long recruitment period for the whole country, in order to provide a detailed population structure of MTB circulating in Tanzania, and an informative epidemiological data needed for improved TB control programmes. We also call for strengthening efforts on early case finding through enhanced public health education campaigns and promotion of accessible diagnostic services with enhanced treatment compliance in the country.

Acknowledgement

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Ethical approval: Not required.

Competing interests: The authors declare that there is no conflict of interests regarding the publication of this paper.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.tube.2016.02.002.

References

Results and Discussion
Results and Discussion

4.2 Publication II

**Anti-TB drug resistance in Tanga, Tanzania: A cross sectional facility-base prevalence among pulmonary TB patients**

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3National Institute for Medical Research (NIMR), Dar es Salaam, Tanzania

**ABSTRACT**

**Objective:** To determine the prevalence and risk factors associated with drug resistance tuberculosis (TB) at facility-base level in Tanga, Tanzania.

**Methods:** A total of 79 Mycobacterium tuberculosis (MTB) isolates included in the study were collected from among 772 (312 new and 460 previously treated) TB suspects self-referred to four TB clinics during a prospective study conducted from November 2012 to January 2013. Culture and drug susceptibility test of the isolates was performed at the institute of medical microbiology and epidemiology of infectious diseases, University hospital, Leipzig, Germany. Data on the patient's characteristics were obtained from structured questionnaire administered to the patients who gave informed verbal consent. Unadjusted bivariate logistic regression analysis was performed to assess the risk factors for drug resistant-TB. The significance level was determined at P < 0.05.

**Results:** The overall proportions of any drug resistance and MDR-TB were 12.7% and 6.3% respectively. The prevalence of any drug resistance and MDR-TB among new cases were 11.4% and 4.3% respectively, whereas among previously treated cases was 22.2% respectively. Previously treated patients were more likely to develop anti-TB drug resistance. There was no association between anti-TB drug resistances (including MDR-TB) with the risk factors analysed.

**Conclusions:** High proportions of anti-TB drug resistance among new and previously treated cases observed in this study suggest that, additional efforts still need to be done in identifying individual cases at facility-base level for improved TB control programmes and drug resistance survey should continuously be monitored in the country.

1. Introduction

Tuberculosis (TB) continues to be a major health challenge globally despite the efforts to combat the disease. Increased drug resistant strains in many parts of the world has worsened the situation [1]. Escalating human immunodeficiency virus (HIV) infection, increased prevalence of nontuberculous mycobacteria (NTM), poverty, and inadequacy public health infrastructure have also contributed greatly in worsening the situation [1].

During the last two decades, the World Health Organization and the International Union Against Tuberculosis and Lung Disease set up a global project to monitor the development of drug-resistant tuberculosis (DR-TB). Since that time approximately 60% of all countries in the world have implemented surveillance activities [3].

HIV epidemic and the emergence of drug resistant TB threaten the efforts to reduce the global burden of TB by 2015 that aims at ensuring that all TB patients benefit from universal access to high-quality diagnosis and patient-centred treatment [4]. Treatment of multi-drug resistant-TB (MDR-TB) is
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undoubtedly costly and requires longer treatment with more toxic drugs compared to drug susceptible TB [59]. Incorrect drug regimes, non-adherence to treatment, transmission in congested settings, substandard drug quality, as well as erratic drug supply are key risk factors for drug resistance development [74]. Several studies have reported unacceptably high mortality rates among HIV-infected patients with MDR-TB [60,61].

Principally, drug resistance data are obtained through continuous surveillance by routine testing of all TB patients. However, in resource-poor settings like Tanzania, periodic drug resistance surveys (DRS) based on random, representative drug susceptibility testing (DST) among previously untreated smear-positive cases are seldom performed. Laboratories performing culture and DST are scarce and very often overcrowded with divergent tasks for the TB control program. Consequently, only phenotypic DST has been customarily performed; frequently faced with notable logistics and operational challenges [13], and therefore impeding regular DRS and precise surveillance. Generally, DST of Mycobacterium tuberculosis (M. tuberculosis) (MTB) demands the presence of bio-safety laboratories, which are rarely found outside reference centres in many resource-poor settings. Moreover, rapid transport of sputum from remote areas of the country to the reference laboratory is required to minimize losses due to contamination or growth failure. Poor infrastructure and unsustainable logistics in these settings hamper this.

Tanzania is among the 22 high TB burden countries, with an estimated incidence (all forms) in 2007 and 2012 of 297 and 295 per 100,000 population respectively [12,13]. Available data on the prevalence of DR-TB and MDR-TB in Tanzania are still low owing to improved case management. Although the levels of anti-TB drug resistance in the country are still low, the need for continuous monitoring has always been emphasized [11,62]. The most effective strategies for limiting further spread of drug-resistant TB include rapid detection of drug resistance followed by prompt and effective therapy of each case. Routine surveillance linked to patient care, represents the best approach to monitor drug resistance [23].

Limited anti-TB drug resistance surveys have been conducted in Tanzania, the last one being in 2007 as part of national representative sample of TB patients [11]. Since that time, no survey has been conducted outside the national survey. The present study aimed at assessing the magnitude of anti-TB drug resistance and associated risks among newly, and previously treated pulmonary TB patients at facility-base level in Tanganyika, Tanzania.

2. Materials and methods

2.1. Study design, area and study population

A total of 79 M. tuberculosis isolates collected from 372 new and previously treated TB patients during a prospective study conducted in Tanzania from November 2012 to January 2013 were eligible for this study. All patients with clinical signs and symptoms suggestive of TB self-referred to four TB clinics were eligible for the study. The clinics included Makorora and Ngami health centres, Bombo Regional referral hospital and Muhiza designated District hospital. Demographic information and data were collected only after provision of informed consent. A careful cross examination of patient history for previous anti-TB treatment using a structured questionnaire was used to classify patients as ‘new’ or ‘repeat’ TB cases. A patient was considered as ‘new case’ if he had not received anti-TB treatment for a period >1 month and was considered ‘repeat’ if he had received anti-TB drugs in a period less or equal to 1 month. No restrictions on inclusion criteria regarding clinical symptoms and age of the patients.

2.2. Sputum and data collection

Demographic data was obtained by using structured questionnaire administered to the patients attending four TB clinics who provided informed verbal consent to the study. Two sputum samples (one spot during the initial visit to the clinic and one early morning) were collected into small autoclavable wide mouth glass bottles. The specimens were examined by direct smear microscopy at the respective clinics using either Ziehl-Neelsen stain (Makorora and Ngami health centres) or fluorescence stain (Bombo referral hospital and MDHH). Diagnosis of smear-positive TB was performed based on the national tuberculosis and leprosy programme guidelines [15]. All morning sputum samples were kept at −20 °C at the respective clinics. No preservative was added until shipped to the Institute of Medical Microbiology and Epidemiology of Infectious Diseases, University hospital, Leipzig, Germany for culture and molecular analysis. HIV status of the patients was determined by rapid HIV screening method at the treatment and care centres of the respective clinics.

2.3. Sputum culture and identification of mycobacterial isolates

Sputum specimens were digested and decontaminated using N-acetyl-l-cysteine-sodium hydroxide method [10] and were reexamined for the presence of acid-fast bacilli by fluorescence stain in Leipzig. The isolates were cultured in Bact/Alert® 3D liquid culture system (bioMérieux) and on Löwenstein-Jensen and Ogontz-Agar (Arthel-ENCLIT GmbH, Wyhra, Germany). Gotsacker slopes contains sodium pyruvate for isolation of Mycobacterium bovis. Cultures were incubated at 37 °C for up to 8 weeks. Confirmation of MTD was done based on presumptive phenotypic appearance of colonies and by line probe assay (GenoType® MTBC; Hain Life science, Nehren, Germany).

2.4. Phenotypic drug susceptibility testing by Bact/Alert® 3D system

M. tuberculosis isolates were tested for their resistance to rifampicin (RMP), isoniazid (INH), streptomycin (SM), ethambutol (EMB) and pyrazamide (PZA) by a proportion method using Bact/Alert® 3D system. Critical concentrations of 1 μg/mL for RMP, INH, and SM; 2 μg/mL for EMB and 200 μg/mL for PZA were respectively used [17]. The PZA bottles were incubated in Bact/Alert® 3D automated system at 37 °C, as mycobacteria designed blood culture bottles to inactivate the delta-algorithm of the system for growth detection in order to avoid false drug-resistant results. Growth was monitored daily, and an isolate was considered resistant to a drug under test when the drug-containing bottle had a time to detection (TTD) that was less or equal to the TTD of the 1% control bottle.

Definitions: Any resistance was defined as resistance to one or more first-line anti-TB drugs. Mono-resistance was defined as
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2.5. *M. tuberculosis* genomic DNA extraction

Mycobacterial cells from positive BacT/Alert® bottles and from LJ slopes showing visible positive growth were used for DNA extraction. Briefly, bacterial DNA was extracted from heat-inactivated AFB isolates. A loopful of colony material was placed into a labelled screw caped eppendorf tube containing 500 µL sterile distilled water or by taking 500 µL from a positive BacT/Alert® bottles. Each specimen was incubated on a heat block at 95 °C for 20 min to inactivate the bacteria. Then centrifuged at 14,000 g for 15 min, the supernatant was discarded using pasteur pipette, followed by addition of 200 µL distilled water to resuspend the pellets. This was followed by maximum vortexing for 10 s to homogenize the sediments. The tubes were incubated in an ultrasonic water bath at 95 °C for 15 min in order to rupture the inactivated mycobacterial cells to release the genomic DNA. The tubes were finally centrifuged at 14,000 g for 15 min. The supernatant was immediately used for PCR or transferred to a new sterile eppendorf for longer storage at −20 °C until used.

2.6. Genotypic drug susceptibility testing

Genotypic drug susceptibility testing for RMP and INH was performed using Genotype®MTBDRplus assay (Hain Life Science GmbH, Nehren, Germany). Genotypic detection of resistance to EMB was done by using Genotype®MTBDRsl assay (Hain Life Science GmbH, Nehren, Germany). PCR amplification, step hybridization and interpretation of profiles were done by following manufacturer's instructions.

2.7. Data management and analysis

Data were first entered and cleaned by using Ms Excel, then analysed using SPSS version 20 (SPSS Inc, Chicago, IL, USA). Unadjusted bivariate logistic regression analysis was performed to determine the risk factors for drug-resistant TB and the strength of the association was determined by odds ratio (OR) with 95% confidence interval (95% CI) and P-value of <0.05 was considered statistically significant.

The study was approved by the Ethical Review Committee of the National Institute for Medical Research, Dar es Salaam, Tanzania. All patients gave verbal informed consent to participate in the study.

3. Results

3.1. *M. tuberculosis* culture results

From among 372 TB suspect patients enrolled in the study 312 (83.9%) were new and 60 (16.1%) were previously treated cases. Of the 312 new cases enrolled, 182 (58.3%) were culture negative, 33 (10.6%) contaminated and 97 (31.1%) had culture positive results. Of the 97 cases with culture positive results, 27 (27.8%) were excluded from the study because they were NTMs; only 70 (72.2%) cases from this category were eligible for DST. Of the 60 previously treated cases, 42 (70%) were culture negative, 5 (8.3%) were contaminated and 13 (21.7%) were culture positive. Four (30.8%) cases were excluded from the test because they were NTMs and nine (69.2%) were eligible for the DST. Overall, 79 (21.2%) out of 372 patients with positive *M. tuberculosis* isolates were eligible for phenotypic and genotypic DST as illustrated in Figure 1.

3.2. Demographic characteristics of the patients

The demographic characteristics of 79 (21.2%) patients who had positive MTB isolates were analysed for DST of the first line anti-TB drugs in this study. 70 (88.6%) were new and 9 (11.6%) were previously treated (repeat) cases. These included 55 (69.6%) males and 24 (30.4%) females with the mean age of (35.8 ± 12.6) years. Other demographic characteristics of the patients are shown in Table 1.

3.3. Drug resistance prevalence by phenotypic DST

Of the 79 patients with DST data, the overall proportion of any drug resistance was 12.7% (n = 10/79) and that of MDR-TB was 6.3% (n = 5/79). The proportion of any drug resistant anti-TB drug among new cases was 11.4% (n = 8/70) and the proportion of MDR-TB in this category was 4.3% (n = 3/70); whereas resistance to any drug and that of MDR-TB among previously treated cases were respectively 22.2% (n = 2/9). The proportions of any drug resistance and MDR-TB among HIV sero-positive patients were 30.8% (n = 4/13) and 23.1% (n = 3/13) respectively, while among the sero-negative group
Results and Discussion

Table 1


<table>
<thead>
<tr>
<th>Characteristic</th>
<th>New (n = 76) (%)</th>
<th>Repeat (n = 91) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>47 (62.1)</td>
<td>8 (8.8)</td>
</tr>
<tr>
<td>Female</td>
<td>29 (37.9)</td>
<td>1 (1.1)</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;35 years</td>
<td>37 (51.3)</td>
<td>4 (4.4)</td>
</tr>
<tr>
<td>≥35 years</td>
<td>39 (51.3)</td>
<td>5 (5.6)</td>
</tr>
<tr>
<td>Residence</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rural</td>
<td>36 (51.4)</td>
<td>5 (5.5)</td>
</tr>
<tr>
<td>Urban</td>
<td>40 (52.6)</td>
<td>6 (6.6)</td>
</tr>
<tr>
<td>Site</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Makomoro HC</td>
<td>3 (4.0)</td>
<td>0</td>
</tr>
<tr>
<td>Ngamia HC</td>
<td>20 (26.3)</td>
<td>6 (6.6)</td>
</tr>
<tr>
<td>Bombo RH</td>
<td>14 (18.4)</td>
<td>0</td>
</tr>
<tr>
<td>MDHH</td>
<td>31 (41.0)</td>
<td>3 (3.3)</td>
</tr>
<tr>
<td>Disease type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smear+ Culture+</td>
<td>48 (63.2)</td>
<td>6 (6.6)</td>
</tr>
<tr>
<td>Smear+ Culture−</td>
<td>22 (29.4)</td>
<td>3 (3.3)</td>
</tr>
<tr>
<td>HIV status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>11 (15.7)</td>
<td>2 (2.2)</td>
</tr>
<tr>
<td>Negative</td>
<td>59 (79.3)</td>
<td>7 (7.7)</td>
</tr>
</tbody>
</table>

were 9.1% (n = 6/66) and 3.0% (n = 2/66) respectively (Table 2).

3.4. Analysis of risk factors associated with any drug resistance and MDR-TB

Using univariate logistic regression model, risk factors associated with any drug resistance and MDR-TB were determined. The patients were grouped into seven groups with the following characteristics in each group. Sex: males 55 (69.9%) cases, females 20 (26.3%) cases; age <35 years: 41 (51.9%) cases; age ≥35 years: 38 (48.1%) cases. With respect to residence, 40 (51.3%) were from urban and 36 (47.4%) from rural. Seventy (88.6%) were new and 9 (11.4%) were previously treated cases. With respect to HIV status 13 (16.9%) were HIV-sero-positive, and 66 (87.5%) were sero-negative. With respect to disease type 55 (69.9%) were both smear and culture positive, while 24 (30.4%) were smear-negative but culture positive. Regarding disease type, we interestingly detected 8.0% (n = 2/25) cases with any drug resistance and 4.0% (n = 1/25) case of MDR-TB among smear negative but culture positive patients. There was no significant difference among all variables analyzed with development of any drug resistance or with MDR-TB in this population as shown in Table 2.

3.5. Resistance patterns of M. tuberculosis isolates

Table 3 shows different resistance patterns among new and previously treated (repeat) cases. Overall 87.3% of all cases were susceptible to all first-line anti-TB drugs. Among the newly registered patients, any resistance to INH and RMP was each found in four (5.7%) isolates, resistance to SM in three (4.3%), any resistance to EMB and PZA was each found in two (2.9%) isolates and 4 (5.7%) isolates showed MDR-TB. Among the previously treated cases, any resistance to INH, SM and EMB were each found in two (22.2%) cases, any resistance to RMP and PZA was each found in one (11.1%) case and MDR-TB was found in one (11.1%) case, which was resistant to all first line anti-TB drugs. The overall prevalence of any resistance and MDR-TB when new and previously treated cases were combined was 12.7% and 6.3% respectively. Mono-resistance was only observed for SM and PZA in 1.4% each, all being from newly registered cases as shown in Table 3.

3.6. Comparison between phenotypic and genotypic drug susceptibility testing

Congruent results between phenotypic DST performed by BacT/Alert® 3D system and Genotype® MTBDRplus assay was obtained in 72 (91.1%) of the 79 subjects. Of 79 isolates identified as being susceptible to both INH and RMP by BacT/Alert® 3D, 66 (87.5%) cases were congruent by Genotype® MTBDRplus; 3 (4.2%) isolates were detected as MDR-TB by both methods and all with mutations in the rpoB gene and katG gene as shown by Genotype® MTBDRplus. Two (4.0%) isolates detected by BacT/Alert® 3D as MDR-TB, were detected by the

Table 2

Unadjusted bivariate logistic regression analysis of risk factors associated with any drug resistance and MDR-TB.

<table>
<thead>
<tr>
<th>Variable</th>
<th>n</th>
<th>Susceptible n (%)</th>
<th>OR, 95% CI</th>
<th>P value</th>
<th>OR, 95% CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>55</td>
<td>48 (87.3)</td>
<td>6.44 (0.87-22.72)</td>
<td>0.32</td>
<td>0.37 (0.1-2.7)</td>
<td>0.33</td>
</tr>
<tr>
<td>Female</td>
<td>21</td>
<td>3 (14.3)</td>
<td>Ref</td>
<td></td>
<td>Ref</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;35 years</td>
<td>37</td>
<td>29 (78.4)</td>
<td>6.06 (0.21-18.47)</td>
<td>0.97</td>
<td>1.86 (0.2-24.9)</td>
<td>0.61</td>
</tr>
<tr>
<td>≥35 years</td>
<td>38</td>
<td>14 (36.8)</td>
<td>Ref</td>
<td></td>
<td>Ref</td>
<td></td>
</tr>
<tr>
<td>Residence</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rural</td>
<td>39</td>
<td>14 (35.9)</td>
<td>6.29 (0.01-43.76)</td>
<td>0.46</td>
<td>0.34 (0.004-28.73)</td>
<td>0.64</td>
</tr>
<tr>
<td>Urban</td>
<td>40</td>
<td>35 (87.5)</td>
<td>Ref</td>
<td></td>
<td>Ref</td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
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<td></td>
</tr>
<tr>
<td>New case</td>
<td>70</td>
<td>62 (88.6)</td>
<td>6.03 (0.35-29.47)</td>
<td>0.36</td>
<td>0.44 (0.03-74.2)</td>
<td>0.57</td>
</tr>
<tr>
<td>Previously treated</td>
<td>9</td>
<td>7 (77.8)</td>
<td>Ref</td>
<td></td>
<td>Ref</td>
<td></td>
</tr>
<tr>
<td>Site</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Makomoro HC</td>
<td>5</td>
<td>3 (60)</td>
<td>0</td>
<td></td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Ngamia HC</td>
<td>28</td>
<td>24 (85.7)</td>
<td>Ref</td>
<td></td>
<td>Ref</td>
<td></td>
</tr>
<tr>
<td>Bombo RH</td>
<td>14</td>
<td>13 (92.9)</td>
<td>1.01 (0.003-2.45)</td>
<td>0.35</td>
<td>0.30 (0.001-28.11)</td>
<td>0.52</td>
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<tr>
<td>MDHH</td>
<td>34</td>
<td>29 (85.3)</td>
<td>5.14 (0.11-24.7)</td>
<td>0.32</td>
<td>4.1 (0.01-64.1)</td>
<td>0.09</td>
</tr>
<tr>
<td>Disease type</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Smear+ Culture+</td>
<td>54</td>
<td>48 (87.3)</td>
<td>0.18 (0.03-23.5)</td>
<td>0.33</td>
<td>3 (0.3)</td>
<td>0.21 (0.03-1.43)</td>
</tr>
<tr>
<td>Smear− Culture+</td>
<td>25</td>
<td>3 (12.0)</td>
<td>Ref</td>
<td></td>
<td>Ref</td>
<td></td>
</tr>
<tr>
<td>Smear− Culture−</td>
<td>41</td>
<td>38 (92.7)</td>
<td>Ref</td>
<td></td>
<td>Ref</td>
<td></td>
</tr>
<tr>
<td>HIV status</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seropositive</td>
<td>13</td>
<td>9 (69.2)</td>
<td>4.02 (0.88-16.6)</td>
<td>0.19</td>
<td>0.21 (0.03-1.43)</td>
<td>0.11</td>
</tr>
<tr>
<td>Sernegative</td>
<td>66</td>
<td>60 (90.9)</td>
<td>Ref</td>
<td></td>
<td>Ref</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>79</td>
<td>10 (12.7)</td>
<td>5.63</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

DST, drug susceptibility testing; DR, drug resistance; MDR-TB, multidrug-resistant tuberculosis; OR, odds ratio.
Results and Discussion

Table 3

<table>
<thead>
<tr>
<th>Resistance pattern</th>
<th>New n (%)</th>
<th>Repeat n (%)</th>
<th>Total n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total patients</td>
<td>n = 70</td>
<td>n = 9</td>
<td>n = 79</td>
</tr>
<tr>
<td>Susceptible to all drugs</td>
<td>62 (88.6)</td>
<td>7 (77.8)</td>
<td>69 (87.3)</td>
</tr>
<tr>
<td>Any resistance</td>
<td>8 (11.4)</td>
<td>2 (22.2)</td>
<td>10 (12.7)</td>
</tr>
<tr>
<td>Any resistance to:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>INH</td>
<td>4 (5.7)</td>
<td>2 (22.2)</td>
<td>6 (7.6)</td>
</tr>
<tr>
<td>RMP</td>
<td>4 (5.7)</td>
<td>1 (11.1)</td>
<td>5 (6.3)</td>
</tr>
<tr>
<td>SM</td>
<td>3 (4.3)</td>
<td>2 (22.2)</td>
<td>5 (6.3)</td>
</tr>
<tr>
<td>EMB</td>
<td>2 (2.9)</td>
<td>2 (22.2)</td>
<td>4 (5.1)</td>
</tr>
<tr>
<td>PZA</td>
<td>2 (2.9)</td>
<td>1 (11.1)</td>
<td>3 (3.8)</td>
</tr>
<tr>
<td>All INH + RMP</td>
<td>4 (5.7)</td>
<td>1 (11.1)</td>
<td>5 (6.3)</td>
</tr>
<tr>
<td>resistant (MDR-TB)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>INH + RMP (only)</td>
<td>1 (1.4)</td>
<td>0</td>
<td>1 (1.3)</td>
</tr>
<tr>
<td>INH + RMP + SM</td>
<td>2 (2.8)</td>
<td>0</td>
<td>2 (2.5)</td>
</tr>
<tr>
<td>INH + RMP + EMB</td>
<td>1 (1.4)</td>
<td>0</td>
<td>1 (1.3)</td>
</tr>
<tr>
<td>INH + RMP + PZA</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>INH + RMP + SM +</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>EMB + PZA</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>INH + Other resistance</td>
<td>0</td>
<td>2 (22.2)</td>
<td>2 (2.5)</td>
</tr>
<tr>
<td>INH + SM</td>
<td>0</td>
<td>1 (11.1)</td>
<td>1 (1.3)</td>
</tr>
<tr>
<td>INH + EMB</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>INH + PZA</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>INH + SM + EMB</td>
<td>0</td>
<td>1 (11.1)</td>
<td>1 (1.3)</td>
</tr>
<tr>
<td>INH + SM + EMB + PZA</td>
<td>0</td>
<td>1 (11.1)</td>
<td>1 (1.3)</td>
</tr>
<tr>
<td>RMP + Other resistance</td>
<td>0</td>
<td>1 (11.1)</td>
<td>1 (1.3)</td>
</tr>
<tr>
<td>RMP + SM</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>RMP + EMB</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>RMP + PZA</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>RMP + SM + EMB + PZA</td>
<td>0</td>
<td>1 (11.1)</td>
<td>1 (1.3)</td>
</tr>
<tr>
<td><em>Mono-resistance to</em></td>
<td>2 (2.9)</td>
<td>0</td>
<td>2 (2.5)</td>
</tr>
<tr>
<td>INH</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>RMP</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SM</td>
<td>1 (1.4)</td>
<td>0</td>
<td>1 (1.3)</td>
</tr>
<tr>
<td>EMB</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PZA</td>
<td>1 (1.4)</td>
<td>0</td>
<td>1 (1.3)</td>
</tr>
</tbody>
</table>

INH, isoniazid; RMP, rifampicin; SM, streptomycin; EMB, ethambutol; PZA, pyrazinamide. *Any resistance: Resistance to any of the first-line anti-TB either in combination or as single; *Mono-resistance: Resistance to only one anti-TB drug.

Genotyping assay as being RMP mono-resistant. Overall, Genotyping detected 3 (3.8%) isolates as RMP mono-resistant, whereas no isolate could be detected as RMP mono-resistant by the BacT/Alert MD 3D. On the other hand, 2 (2.5%) isolates were detected by either methods: as INH mono-resistant; with one (1.4%) having mutation in katG gene and one missed by the genotypic method as depicted in Table 4.

4. Discussion

Our key findings show that the prevalence of resistance to any anti-TB drug among new patients was 11.4% and that of MDR-TB was 4.3%, whereas among previously treated patients the resistance to any anti-TB drug and MDR-TB were respectively 22.2%. The overall resistance to any anti-TB drugs and MDR-TB when newly and previously treated cases were combined was 12.7% and 6.3% respectively. The overall prevalence to any resistance of 7.6% for INH; 6.3% for RMP and SM; 5.1% for EMB and 3.8% for PZA were high. The levels reported in our study are higher than those reported during the last national DST survey [11]. These proportions are alarming, hence calling for immediate intervention to reverse the trend, as it raises concern on the increased transmission of drug-resistant MTB in the settings.

High proportion of resistance to any drug (22.2%) and MDR-TB among previously treated patients, also raises concerns on continual reliance on home-based supervision of TB treatment as previously advocated [9], as patient adherence to treatment may be difficult to monitor, since the supervision of patients at home is made by non-medical professionals. Moreover, the existence of other resistance patterns with INH and or RMP among previously treated patients further reiterates the concern over the home-based supervision of TB patients. Although no association to any anti-TB drug and or MDR-TB was found between newly and previously treated cases, our findings show that 22.2% (n = 29) of the previously treated cases were more likely to develop resistance to first-line anti-TB drugs. Lack of association between history of treatment with DR-TB and MDR-TB observed could be due to low number of previously treated patients enrolled during the study.

Observed mono-resistance to SM and PZA in 2.9% among new patients is plausibly an indication of growing use of these antibiotics in the treatment of other bacterial infections in the community. Generally, our findings are well in agreement with those of the nation-wide survey, which showed the prevalence of any resistance among new and previously treated patients in the range of 8.3% and 20% respectively [10]. The observed resistance to PZA in this study is, to the best of our understanding reported for the first time in Tanzania. The standard practice for drug resistance surveys has been to test for four first-line drugs (INH, RMP, EMB and SM); and for the purposes of surveys NTPs are required to perform, at minimum DST for INH and RMP on all cases included in the survey.

From the findings of this study, it may be worth including DST for PZA especially among HIV positive patients.

Table 4

<table>
<thead>
<tr>
<th>Genotypic resistance pattern</th>
<th>INH/RMP n (%)</th>
<th>INH/RMP n (%)</th>
<th>INH/RMP n (%)</th>
<th>MDR-TB n (%)</th>
<th>Total n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>INH/INH</td>
<td>65 (91.7)</td>
<td>1 (50.0)</td>
<td>0</td>
<td>2 (40.0)</td>
<td>68 (86.1)</td>
</tr>
<tr>
<td>INH/INH</td>
<td>3 (4.2)</td>
<td>0</td>
<td>0</td>
<td>3 (60.0)</td>
<td>6 (76.6)</td>
</tr>
<tr>
<td>INH/RMP</td>
<td>1 (1.4)</td>
<td>1 (50.0)</td>
<td>0</td>
<td>0</td>
<td>2 (2.5)</td>
</tr>
<tr>
<td>INH/RMP</td>
<td>3 (4.2)</td>
<td>0</td>
<td>0</td>
<td>3 (60.0)</td>
<td>6 (76.6)</td>
</tr>
<tr>
<td>Total</td>
<td>72 (91.1)</td>
<td>2 (2.5)</td>
<td>0</td>
<td>5 (6.3)</td>
<td>79 (100)</td>
</tr>
</tbody>
</table>

INH, isoniazid sensitive; INH, isoniazid resistance; RMP, rifampicin sensitive; RMP, rifampicin resistance; INH, isoniazid wild type gene without mutation; INH, isoniazid with mutation band detected; RMP, rifampicin wild type gene without mutation; RMP, rifampicin with mutation band detected; MDR-TB, multidrug-resistant tuberculosis.
The findings of any drug resistance of 8.0% and of MDR-TB 4.0% among smear-negative but culture-positive patients in this study need to be addressed with special concern, as this suggests the inadequacy of peripheral microscopy performance. Several studies have shown that peripheral smear microscopy in Tanzania has frequently more problems in false-negative slides than in false-positive [22].

Although several reports from other countries, have documented that HIV positivity is an important risk factor associated with primary MDR-TB [23–30], and that HIV infection has been associated with MDR-TB outbreaks in institutional settings, such as hospitals and prisons [27,28]. Our findings showed lack of association between anti-TB drug resistance in patients with or without HIV and these results are in agreement with studies conducted in Mwanza, Tanzania [34]. This lack of association could be explained by the fact that majority of HIV infected TB patients are likely to be smear negative and tend to have lower rate of sputum smear positivity [29].

Of the 5 (6.3%) MDR isolates detected in our study, 2/5 isolates showed discordant results by BacT/Alert® 3D system and Genotypen® MTBDRplus. While BacT/Alert® 3D detected all isolates as being INH resistant, the later detected one of the isolate as INH mono-resistant and the other as INH susceptible. Possible explanation of this disparity could be due to presence of mutations outside the 81-bp “hot-spot” of the rpoB gene, though this does occur less frequently [30,31]. Such mutations may occur for example at codon 490 CAG to TAG [32], codon 534 (GGG to GAG), codon 535 (CCC to CAC) [33] and at codon 572 (ATC to TTC) [34]. Another possible explnation could be due to changes occurring in genes whose products participate in antibiotic permeation or metabolism [35]. Several studies have also indicated presence of high discordance in RMP susceptibility testing [36–37]. Since the Genotypen® MTBDRplus asy we used is not very robust; such methods like DNA sequencing may be considered for isolates with discordant results.

Limitations: The small sample size may have limited the generalization of the observed results in our study. High rates of culture negative and contaminated specimens may be attributed to such factors as: (i) inclusion of specimens with very low bacilli load resulting to loss of viability during culture, (ii) understaffed and overburdened of the clinics with other non-TB patients might have led to delayed sample processing, (iii) frequent power interruption may have resulted into inappropriate cold chain maintenance at the clinic level, (iv) some patients failing to deliver early morning samples within the specified period, due to among other factors long distance and costs of travel to and from the clinics. Despite the limitations of the study, it has provided important information regarding the resistance profiles at the facility-base level where majority of the patients may not have opportunity to be covered during the national drug resistance surveys. High proportions of anti-TB drug resistance among new and previously treated cases, gives an insight of DR-TB situation at facility levels in the country. Therefore, it provides for a better planning of the drug resistance surveys including improving smear microscopy performance for case detection and improvement of TB control programmes. Frequent drug resistance survey need to be done in order to monitor the situation of DR-TB in the country.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

We are grateful to the management and technical personnel of the TB clinics and TB coordinators in Muhura and Tanga. We thank Ms. Elizabeth Kraftschek and Dr. Joerg Beer of the Department of Medical Microbiology and Epidemiology of Infectious Diseases, University Hospital Leipzig for their technical support throughout the study. We acknowledge the financial support from the German Academic Exchange Service (DAAD).

References

Results and Discussion
4.3 Publication III

Increased isolation of nontuberculous mycobacteria among TB suspects in Northeastern, Tanzania: public health and diagnostic implications for control programmes

Abubakar S. Hoza, Sayoki G. M. Mfinanga, Arne C. Rodloff, Irmgard Moser and Brigitte König

Abstract

Background: Non-tuberculous mycobacteria (NTM) are increasingly reported worldwide associated with human disease. Defining the significance of NTM in settings with endemic tuberculosis (TB) requires the discrimination of NTM from TB in suspect patients. Correct and timely identification of NTM will impact both therapy and epidemiology of TB and TB-like diseases. The present study aimed at determining the frequency and diversity of NTM among TB suspects in northeastern Tanzania.

Methods: A cross-sectional study was conducted between November 2012 through January 2013. Seven hundred and forty-four sputum samples were collected from 372 TB suspects. Detection was done by using phenotypic GenoType® Mycobacterium CM/AS kits, 16S rRNA and hsp65 gene sequencing for identification of isolates not identified by main kits. Binary regression model was used to analyse the predictors of NTM detection.

Results: The prevalence of NTM was 9.7 % of the mycobacterial isolates. Out of 36 patients with confirmed NTM infection, 12 were HIV infected with HIV being a significant predictor of NTM detection (P < 0.001). Co-infection with Mycobacterium tuberculosis (M. tb) was found in five patients. Twenty-eight NTM isolates were identified using GenoType® Mycobacterium CM/AS and eight isolates could not be identified. Identified species included M. gordonae and M. interjectum 6 (16.7%), M. intracellulare 4 (11.1%), M. avium spp. and M. fortuitum 2 (5.5%), M. kansasii, M. lentiflavum, M. simiae, M. celatum, M. marinum 1 (2.8%) each. Of isolates not identified to subspecies level, we identified M. kumamotoense (2), M. intracellulare/kansasii, M. intermedium/triplelex, M. aviumcomplex/avium, M. stomatopneumoniae, M. colombiensis and M. tereae complex (1 each) using 16S rRNA sequencing. Additionally, hsp65 gene sequencing identified M. kumamotoense, M. scrofulaceum/M. avium, M. avium, M. flavescentes/novocastrense, M. kumamotoense/hibernae, M. lentiflavum, M. colombiensis/M. avium and M. kumamotoense/teneae/hibernae (1 each). Results of the 16S rRNA and hsp65 gene sequencing were concordant in three and discordant in five isolates not identified by GenoType® Mycobacterium CM/AS.

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Results and Discussion

Conclusion: NTM infections may play a vital role in causing lung disease and impact management of TB in endemic settings. GenoType® Mycobacterium CM/AS represents a useful tool to identify clinical NTM infections. However, 16S rRNA gene sequencing should be thought for confirmatory diagnosis of the clinical isolates. Due to the complexity and inconsistency of NTM identification, we recommend diagnosis of NTM infections be centralized by strengthening and setting up quality national and regional infrastructure.

Keywords: Nontuberculous mycobacteria, Peripheral diagnostic centres, Human immunodeficiency virus (HIV), Tanzania

Background

Over the past decades, the prevalence of pulmonary nontuberculous mycobacteria (NTM) is increasingly reported worldwide. However, epidemiological and surveillance data of NTM infections are still limited [1].

Defining the epidemiology of NTM diseases in most resource poor settings like Tanzania is more challenging than its well documented relative Mycobacterium tuberculosis complex (MTC) [2].

Due to their ubiquitous presence in the environment, exposure to NTM is likely common, as they can colonize the respiratory tract without causing disease such that finding NTM in respiratory secretions does not necessarily have clinical implications in all patients [3–5]. Differentiating true NTM lung infection from contamination and/or colonization is difficult; hence, the presence of acid-fast bacilli (AFB) positive by microscopy of a respiratory sample or culture poses major diagnostic challenge. Increasingly, NTM are becoming recognized as true pathogens and important causes of human infections [6, 7].

Information on the role, contribution and burden of NTM in aetiology of TB–like syndromes is limited in many sub-Saharan African countries endemic to TB and HIV [8–10]. Lack of rapid and accurate methods to diagnose AFB positive pulmonary infections due to NTM results in misdiagnosis and mismanagement of pulmonary TB in such settings. Correct and timely identification of NTM is particularly urgent for both therapy and epidemiology, since infections with different mycobacterial species demand different management approaches [11, 12].

Tanzania is among the 22 high burden countries (HBCs) in the world with high prevalence of TB. On average, 61,500 new TB patients are notified annually [13]. Patients with AFB positive sputum or with chest radiographic findings presumptive of active TB, who do not respond to general antituberculotics, are generally assumed positive for TB. As a general guideline, patients are empirically treated with first line anti-TB drugs for 6 months.

Since several TB-like syndromes could also be due to NTM, inconclusive diagnosis of pulmonary TB would result in over-diagnosis of TB and hence miss-management of both TB and NTM infections.

Invention of DNA strip technology (line probe assays) based on reverse hybridization of PCR products to their complementary oligonucleotide probes has revolutionized the diagnosis of NTM. Commercial DNA strip assay kits GenoType® Mycobacterium CM/AS (Hain Life science GnH, Nehren, Germany); GenoType® CM/AS for detection of common and additional NTM species have been widely and successfully used as rapid molecular tools in the diagnosis of NTM both in resource poor and developed countries. However, cross-reactivity of DNA probes between mycobacterial species has been reported, leading to incorrect diagnosis and treatment of patients [14, 15]. Use of molecular techniques targeting the 16S rRNA and hsp65 genes have been useful in diagnosis and speciation of NTM, including those which are dead or uncultivable [16].

The objective of the present study was therefore to determine the frequency and diversity of NTM among TB suspects in Northeastern Tanzania using conventional phenotypic methods, GenoType® Mycobacterium CM/AS kits and 16S rRNA as well as hsp65 gene sequencing.

Our findings suggest that NTM infections may play a vital role in causing lung disease and hence impacting management of TB in endemic settings like Tanzania. Therefore, the need to consider NTM in TB control programmes in such settings is urgent.

Methods

Study settings

The study was conducted in two peripheral diagnostic centres (PDCs) of Ngamiani and Makorora. The centres serve as primary catchment for TB diagnosis in Tanga municipal and two hospitals of Muheza Designated District Hospital (MDDH) and Bombo referral hospital. Tanga is among the top 10 regions with high TB noticification in Tanzania with annual TB noticification cases for the entire region being about 3852 cases, and the TB prevalence of 217/100,000 and 244/100,000 in urban and rural settings respectively as per national prevalence survey, 2012 [17].
Study design
A cross-sectional study was carried out between November 2012 through January 2013. A total of 744 spot and morning sputum samples were collected from 372 TB suspected patients self-presenting at the PDCs and hospitals. All patients presenting to the diagnostic centre with any of the following symptoms were included: presence of symptoms suggestive of TB for a period of ≥2 weeks, night sweats, fatigue, unexpected loss of weight, and fever. Three sputum samples were collected from patients (one on the spot during the first visit, one early morning and another spot the following morning). Early morning spuTa were stored at −20 °C and later transported to the Mycobacteriology laboratory at the University hospital Leipzig, Germany for culture and molecular identification.

Smear microscopy and culture isolation
Direct smear microscopy using Ziehl Neelsen (ZN) or fluorescent stains were performed at the respective study sites by experienced laboratory technicians and the results recorded in accordance to the WHO/IUATLD and National Tuberculosis and Leprosy Programme (NTLP) guidelines [18, 19].

In Leipzig, all the sputum specimens were processed by the standard N-acetyl-L-cysteine (NALC)-NaOH method [20]. Briefly, 10 ml of 0.5 % N-acetyl-cysteine (NALC) solution was added to each sample, specimens were then incubated at room temperature on a shaker for 20 min, followed by addition of 30 ml of phosphate buffered saline (PBS) pH 6.8 for neutralization and subsequently centrifuged at 3000 × g for 20 min. The sediment were resuspended in 1 ml PBS after discarding the supernatant. All the decontamination procedure followed the Deutsches Institut für Normung (DIN) recommendations for the detection of mycobacteria [21].

About 2–3 drops of resuspended specimens were inoculated on Lowenstein-Jensen (LJ), Gottacker, and Coletto slants (Artec ENCLIT GmbH, Germany) supplemented with antibiotics (Polymixin B 200,000 IU/liter), Amphotericin B (10 mg/liter), Carbencillin (90 mg/liter), and Trimethoprim (10 mg/liter) (PACT). Cultures were incubated for 8 weeks at 37 °C and read weekly.

Examination and reporting of smear microscopy was performed by fluorescent microscopy. Subsequently, 0.5 ml of each specimen was inoculated into BacT/Alert bottles supplemented with antibiotics PACT and incubated in an automated BacT/Alert 3D System (Biomerieux, Marcy l’Etoule, France) liquid culture system for 8 weeks. Culture not showing any growth after 8 weeks of incubation was considered negative. Positive BacT/Alert bottles were checked for purity by plating a drop from each positive bottle onto blood agar (BA) plate to rule out false positive results due to bacterial contamination and simultaneously ZN stain was performed to confirm presence of AFB positive results.

Phenotypic identification
Slants containing pure cultures of AFB were assessed for growth rate and pigment accumulation on LJ, Gottacker or Coletto slants (at 30 °C, 37 °C and 45 °C). NTM isolates were grouped based on Runyon classification and results compared with those of molecular methods.

DNA extraction
Mycobacterial DNA was extracted from heat–inactivated AFB isolates. Briefly, bacteria suspended in 500 µl sterile water or 1 ml directly from positive BacT/Alert bottles were inactivated at 80 °C for 20 min, then ultra-sonicated at 35 kH and heated at 100 °C for 10 min each treatment and centrifuged at 16,100 × g two times for 5 min. The supernatant was taken as template DNA. Genomic DNA the H37Rv strain and sterile distilled water were used as positive and negative controls respectively for all molecular procedures.

Identification of NTM by Genotype® Mycobacterium CM/AS assay
All isolates identified as NTM based on their cultural characteristics and confirmed by ZN staining were subjected to further definitive identification using two commercial kits, the Genotype® Mycobacterium CM for detection of common NTM. Isolates not identified by Genotype® CM assay were further tested with the Genotype® AS assay for additional NTM. All the procedures followed manufacturer’s instructions.

DNA sequence analysis
All isolates not identified to the species level by the CM/AS assay were analyzed by 16S rRNA and hsp65 gene sequencing. For sequence analysis of the 16S rRNA gene was done according to [22] using the primers 265 and 264 for generating the PCR product (1037 bp) and primer 271 for sequencing. For hsp65 gene sequencing, PCR was done according to [23] using the primers 21M13TB11/M13TB12 generating a 441 bp fragment. For sequencing, the primer M13 pUC forward was used. The primers were purchased from Jena Bioscience, Jena, Germany. The PCR products were purified for sequencing using QIAquick PCR Purification Kit or alternatively QIAquick Gel Extraction Kit according to the manufacturer’s instructions. Sequencing was done by GATC Company (Konstanz, Germany). The raw data were analyzed at the EBI using the National Centre for Biotechnology Information (NCBI) BLAST software optimized for highly
similar sequences (http://blast.ncbi.nlm.nih.gov). Strain identification was based on the BLAST hit with the highest score, combined with the greatest sequence coverage and identity. Reference strains with the highest similarity score as present in the NCBI database were used as references for the similarity assessment.

Data analysis
Demographic and clinical data were cleared and analyzed by SPSS version 11.5 for Windows (SPSS Inc., Chicago, IL) software package. Binary regression model was used to analyse the predictors of NTM isolation. One-way analysis of variance (ANOVA) was used to determine trend analysis across ordered groups. P value <0.05 was considered statistically significant. GenoType® Mycobacterium CM/AS assay results were interpreted based on manufacturers’ instruction.

Ethical clearance
The protocol for this study was reviewed and approved by the Ethical Committee of the National Institute for Medical Research (NIMR), Dar es Salaam, Tanzania. Written informed consent was obtained from the patients or relatives of the patients, where the patients could not read and write.

Results
A total of 744 sputum samples were collected from 372 TB suspects who self-presented at two PDCs and two hospitals in Tanga, northeastern, Tanzania. The proportion of males 196 (52.7%) was higher than that of females 176 (47.3%). The median age of the patients was 40 years (range 7–88 years).

Demographic characteristics of patients and risk factors associated with NTM
Demographic data and risk factors associated with NTM infections are shown in (Tables 1, 2). The overall frequency of the patients with NTM in the study was 9.7%. HIV positivity was found to be associated with NTM infection among the factors analysed, with a statistical significance of (OR 3.86, 95% CI [1.79–8.3], P < 0.001). There was no association between NTM and other factors analysed.

Culture Results
Of the 372 patients, positive mycobacterial cultures were obtained in 121 (32.5%) patients and among these 36 (9.7%) patients harboured NTM, which are subject of this paper. From each patient three sputum samples were analysed for NTM growth. In total, 101 sputum samples were positive for NTM by culture. In this regard, 29 patients (80.6%) were positive for NTM growth in all three samples and seven patients (19.4%) were positive for NTM only in two out of the three samples. NTM without any other co-infections were detected in 21 (58.3%) patients. NTM coinfection with HIV was found in 11 (30.6%) patients, with MTBC in three (8.3%) patients and NTM with both HIV and MTBC coinfections in one (2.8%) patient. One patient was simultaneously positive for NTM plus Nocardia spp. (data not shown) (Table 3). None of the patients had mixed NTM infections.

Of the 121 patients, 81 (66.9%) patients had positive smear microscopy for AFB and 40 (33.1%) patients were smear negative at the laboratory in Leipzig. On the other hand, of these 121 patients with positive culture results, 64 (52.9%) patients had positive smear microscopy for AFB and 56 (46.3%) patients had negative smear microscopy for AFB at local PDCs/hospitals, whereas only one (0.8%) patient had smear positive AFB at local PDCs/hospitals but negative smear microscopy and culture in Leipzig.

Phenotypic identification of the NTM
Based on their growth characteristics and pigment production, the isolates were grouped into different Runyon groups [24]. Eighteen isolates were classified as scotochromogenic (Runyon II), 13 as nonphotochromogenic (III), 4 as photochromogenic (Runyon I) and 1 as rapid growing mycobacteria (Runyon IV). The results were comparable to the molecular detection by Hain kit results (Table 4).

Distribution of mycobacterial isolates by site
Results of genotyping using GenoType® MTBC for the MTC and GenoType® Mycobacterium CM/AS for NTM isolates at each site showed that the prevalence of M. tuberculosis alone were 10.4, 22.7, 42.4 and 26.9% at Makorora HC, Ngamian HC, Bombo RH and MDDH respectively. The prevalence of NTM alone were 10.4, 11.0, 1.6 and 8.2% at Makorora HC, Ngamian HC, Bombo RH and MDDH respectively (Table 1).

Identification of NTM isolates GenoType® Mycobacterium CM/AS
Identification of NTM isolates to species level by GenoType® Mycobacterium CM/AS kits was achieved in 28 (77.8%) isolates with GenoType® Mycobacterium CM identifying 23 (63.9%) of the isolates and GenoType® Mycobacterium AS identifying 5 (13.9%) isolates. On the other hand, eight (22.2%) out of 36 isolates could not be identified by either kit. M. gordonae and M. interjectum were the most frequently identified with 6 (16.7%) isolates each, followed by M. intracellulare 4 (11.1%), M. scrofulaceum 3 (8.3%), M. avium spp. 2 (5.5%), M.
Results and Discussion

Table 1: Demographic characteristics and laboratory findings of the patients with NTM infections stratified by site

<table>
<thead>
<tr>
<th>Demographic and risk factors</th>
<th>Makorora HC n = 48 (%)</th>
<th>Ngamian HC n = 128 (%)</th>
<th>Bombo RH n = 62 (%)</th>
<th>Muheza DDH n = 134 (%)</th>
<th>Total n = 372</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>20 (41.7)</td>
<td>56 (44.7)</td>
<td>40 (64.5)</td>
<td>60 (44.8)</td>
<td>176</td>
</tr>
<tr>
<td>Male</td>
<td>28 (58.3)</td>
<td>72 (55.3)</td>
<td>22 (35.5)</td>
<td>74 (55.2)</td>
<td>196</td>
</tr>
<tr>
<td>Age, Mean (SD)</td>
<td>45.1 (18.9)</td>
<td>39.4 (17.6)</td>
<td>38.3 (15.6)</td>
<td>42.9 (16.9)</td>
<td></td>
</tr>
<tr>
<td>Age groups (years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;20</td>
<td>1 (2.1)</td>
<td>8 (6.3)</td>
<td>6 (9.7)</td>
<td>6 (4.5)</td>
<td>21</td>
</tr>
<tr>
<td>20-40</td>
<td>13 (27.1)</td>
<td>69 (53.9)</td>
<td>23 (37.1)</td>
<td>51 (38.1)</td>
<td>156</td>
</tr>
<tr>
<td>40-60</td>
<td>22 (45.8)</td>
<td>29 (22.6)</td>
<td>27 (43.5)</td>
<td>57 (42.5)</td>
<td>135</td>
</tr>
<tr>
<td>&gt;60</td>
<td>12 (25.0)</td>
<td>22 (17.2)</td>
<td>6 (9.7)</td>
<td>20 (14.9)</td>
<td>60</td>
</tr>
<tr>
<td>Residence</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urban</td>
<td>39 (81.3)</td>
<td>97 (75.8)</td>
<td>43 (69.4)</td>
<td>28 (20.8)</td>
<td>207</td>
</tr>
<tr>
<td>Rural</td>
<td>9 (18.7)</td>
<td>31 (24.2)</td>
<td>19 (30.6)</td>
<td>106 (79.1)</td>
<td>165</td>
</tr>
<tr>
<td>Occupation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peasants</td>
<td>24 (50.0)</td>
<td>31 (25.8)</td>
<td>31 (50.0)</td>
<td>68 (50.0)</td>
<td>156</td>
</tr>
<tr>
<td>Housewife</td>
<td>11 (22.9)</td>
<td>16 (12.5)</td>
<td>18 (29.0)</td>
<td>24 (17.9)</td>
<td>69</td>
</tr>
<tr>
<td>Business</td>
<td>5 (10.4)</td>
<td>24 (18.8)</td>
<td>5 (8.1)</td>
<td>11 (8.2)</td>
<td>45</td>
</tr>
<tr>
<td>Others</td>
<td>8 (16.7)</td>
<td>55 (42.9)</td>
<td>8 (12.9)</td>
<td>31 (23.1)</td>
<td>102</td>
</tr>
<tr>
<td>Prevalence of MTBC alone</td>
<td>5 (10.4)</td>
<td>29 (22.7)</td>
<td>15 (24.2)</td>
<td>36 (26.9)</td>
<td>85</td>
</tr>
<tr>
<td>Prevalence of NTM alone</td>
<td>5 (10.4)</td>
<td>14 (11.0)</td>
<td>1 (1.6)</td>
<td>11 (8.2)</td>
<td>31</td>
</tr>
<tr>
<td>Prevalence of MTB and NTM</td>
<td>0</td>
<td>3 (2.4)</td>
<td>1 (1.6)</td>
<td>1 (0.7)</td>
<td>5</td>
</tr>
<tr>
<td>Concurrent conditions</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>HIV+ ve</td>
<td>12 (25.0)</td>
<td>14 (10.9)</td>
<td>13 (30.0)</td>
<td>14 (10.4)</td>
<td>53</td>
</tr>
<tr>
<td>HIV- ve</td>
<td>7 (14.6)</td>
<td>87 (68.0)</td>
<td>1 (1.6)</td>
<td>19 (14.2)</td>
<td>114</td>
</tr>
<tr>
<td>Unknown</td>
<td>29 (60.4)</td>
<td>27 (21.1)</td>
<td>48 (77.4)</td>
<td>101 (75.4)</td>
<td>205</td>
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<tr>
<td>Previous TB</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>9 (18.7)</td>
<td>33 (26.5)</td>
<td>1 (1.6)</td>
<td>16 (11.9)</td>
<td>59</td>
</tr>
<tr>
<td>No</td>
<td>39 (81.3)</td>
<td>95 (73.5)</td>
<td>51 (98.4)</td>
<td>118 (88.1)</td>
<td>313</td>
</tr>
</tbody>
</table>

H: Health Centre, DDH: Muheza Designated District Hospital, MTBC: Mycobacterium tuberculosis complex, NTM: Nontuberculous Mycobacterium

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Table 2: Risk factors associated with NTM infection among suspected TB patients attending FDCs/hospitals in north-eastern Tanzania from November 2012 to January 2013

<table>
<thead>
<tr>
<th></th>
<th>OR</th>
<th>95% CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>0.63</td>
<td>0.42–1.66</td>
<td>0.6</td>
</tr>
<tr>
<td>Age</td>
<td>1.02</td>
<td>0.52–2.04</td>
<td>0.95</td>
</tr>
<tr>
<td>Location</td>
<td>0.96</td>
<td>0.40–1.99</td>
<td>0.9</td>
</tr>
<tr>
<td>Occupation</td>
<td>0.95</td>
<td>0.43–2.11</td>
<td>0.8</td>
</tr>
<tr>
<td>HIV positive</td>
<td>3.86</td>
<td>1.70–8.33</td>
<td>0.001*</td>
</tr>
<tr>
<td>Previous TB</td>
<td>0.64</td>
<td>0.31–2.26</td>
<td>0.7</td>
</tr>
</tbody>
</table>

OR: odd ratio, CI: confidence interval
* P value is statistically significant at P < 0.05

---

Table 3: Comorbidity of NTM with either M. tb or and HIV among individuals with NTMs

<table>
<thead>
<tr>
<th>Type of Infection</th>
<th>No. of Individuals (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTM alone</td>
<td>21 (58.3)</td>
</tr>
<tr>
<td>HIV + NTM</td>
<td>11 (30.6)</td>
</tr>
<tr>
<td>NTM + M. Tb</td>
<td>3 (8.3)</td>
</tr>
<tr>
<td>HIV + NTM + M. Tb</td>
<td>1 (2.8)</td>
</tr>
<tr>
<td>Total</td>
<td>36</td>
</tr>
</tbody>
</table>

*M. fortuitum 2 (5.5 %), M. kansasii 1 (2.8 %), M. lentiflavum 1 (2.8 %), M. simiae 1 (2.8 %), M. celatum 1 (2.8 %) and M. marinum 1 (2.8 %) (Table 4).
Discussions

While the epidemiology of TB is well documented, the prevalence and epidemiology of NTM in Tanzania is largely unexplored [25]. NTM pulmonary infections are increasingly reported because of increased populations at risk due to HIV infection, old age, other immunosuppressive conditions, increased awareness and improved diagnostic facilities especially in developed countries [26, 27].

In Tanzania, the existing supposition is that most individuals presenting with pulmonary symptoms reflecting mycobacterial diseases are infected with MTC. Changes that NTM are missed during diagnosis is to a great extent attributed to poor diagnostic capabilities for culture and identification of NTM, endemic nature of MTC, overburden by diseases like malaria and HIV. Furthermore, lack of awareness among public health personnel and lack of standardized or accepted criteria to properly define and report NTM have all resulted in less attention on the NTM infections.

In this study, the overall frequency of patients with NTM detected by Hain kits among pulmonary TB suspects population was 9.7%. We identified M. gordonae and M. intermedium with 6 (16.7%) isolates each accounting for about one-third of all NTM isolates, M. intracellulare 4 (11.1%), M. avium spp. and M. fortuitum 2 (5.5%), M. kansasi, M. lentiflavum, M. simiae, M. celatum, M. marinum 1 (2.8%) isolate each. Additionally, eight isolates, which gave no signal with GenoType® Mycobacterium CM/AS kits were identified by 16S rRNA gene sequencing. These isolates were assigned to the species M. kumamotoensis (2), M. intracellulare/kansasi (1), M. intermedium/M. triplex (1), M. acapulcensis/M. faveolaciens.

### Table 4 Species distribution of the NTM isolated from PDCs/hospitals in northeastern Tanzania based on Runyon grouping and GenoType® Mycobacterium CM/AS

<table>
<thead>
<tr>
<th>Strain ID</th>
<th>16S rRNA gene sequencing</th>
<th>Identity/sequence length (bp)</th>
<th>GenoType CM/AS</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. gordonae</td>
<td>IL</td>
<td>6 (16.7%)</td>
<td>GenoType CM</td>
</tr>
<tr>
<td>M. intermedium</td>
<td>IL</td>
<td>6 (16.7%)</td>
<td>GenoType CM</td>
</tr>
<tr>
<td>M. intracellulare</td>
<td>I</td>
<td>4 (11.1%)</td>
<td>GenoType CM</td>
</tr>
<tr>
<td>M. scrofulaceum</td>
<td>IL</td>
<td>3 (8.3%)</td>
<td>GenoType CM</td>
</tr>
<tr>
<td>M. avium spp.</td>
<td>I</td>
<td>2 (5.5%)</td>
<td>GenoType CM</td>
</tr>
<tr>
<td>M. fortuitum</td>
<td>I</td>
<td>2 (5.5%)</td>
<td>GenoType CM</td>
</tr>
<tr>
<td>M. kansasi</td>
<td>IL</td>
<td>1 (2.8%)</td>
<td>GenoType AS</td>
</tr>
<tr>
<td>M. lentiflavum</td>
<td>IL</td>
<td>1 (2.8%)</td>
<td>GenoType AS</td>
</tr>
<tr>
<td>M. simiae</td>
<td>IL</td>
<td>1 (2.8%)</td>
<td>GenoType AS</td>
</tr>
<tr>
<td>M. celatum</td>
<td>IL</td>
<td>1 (2.8%)</td>
<td>GenoType AS</td>
</tr>
<tr>
<td>M. marinum</td>
<td>I</td>
<td>1 (2.8%)</td>
<td>GenoType AS</td>
</tr>
<tr>
<td>NTM not identified</td>
<td>I</td>
<td>8 (22.2%)</td>
<td>GenoType AS</td>
</tr>
</tbody>
</table>

### Table 5 NTM species identified based on 16S rRNA gene and hsp65 gene sequencing

<table>
<thead>
<tr>
<th>Strain ID</th>
<th>16S rRNA gene sequencing</th>
<th>Identity/sequence length (bp)</th>
<th>GenoType CM/AS</th>
</tr>
</thead>
<tbody>
<tr>
<td>TZA09</td>
<td>M. kumamotoensis</td>
<td>592/559</td>
<td>317/357</td>
</tr>
<tr>
<td>TZA14</td>
<td>M. intracellulare</td>
<td>912/928</td>
<td>345/359</td>
</tr>
<tr>
<td>TZA16</td>
<td>M. kansasi</td>
<td>924/928</td>
<td>345/359</td>
</tr>
<tr>
<td>TZA145</td>
<td>M. intracellulare/medium/triplex</td>
<td>982/916</td>
<td>300/315</td>
</tr>
<tr>
<td>TZA147</td>
<td>M. acapulcensis</td>
<td>918/918</td>
<td>360/372</td>
</tr>
<tr>
<td>TZA19</td>
<td>M. faveolaciens</td>
<td>912/919</td>
<td>360/372</td>
</tr>
<tr>
<td>TZA190</td>
<td>M. kumamotoensis</td>
<td>928/928</td>
<td>360/372</td>
</tr>
<tr>
<td>TZA217</td>
<td>M. stotaei</td>
<td>570/570</td>
<td>386/386</td>
</tr>
<tr>
<td>TZA224</td>
<td>M. coloebiensis</td>
<td>928/928</td>
<td>386/386</td>
</tr>
<tr>
<td>TZA294</td>
<td>M. terrae complex</td>
<td>923/931</td>
<td>386/386</td>
</tr>
</tbody>
</table>
Results and Discussion

(1), M. stomatopiae (1), M. colombiensis (1) and M. terrae (1). Additionally, hyp65 gene sequencing was conducted if the 16S RNA sequences were not discriminatory in order to confirm especially the ambiguous species assignments. However, only three of eight isolates both genes indicated the same species, for five isolates discordant results were achieved by sequencing both genes (Table 5). Sequencing of different genes used for bacterial species identification may yield inconsistent results. The problem is even more complicated with mycobacteria which are characterized by interspecies similarity clearly higher than in other bacteria [28, 29]. These results emphasise the difficulties of species identification for NTM. Therefore, it is of great importance to standardize the methods in order to generate comparable results between different laboratories, countries and continents.

M. kumamotonense, M. acapulcanis M. novocastrense, M. stomatopiae, and M. kiberialis determined by either 16S RNA or hyp65 in this study (Table 5) have been reported elsewhere in the world. Some of which have been associated with human diseases and others isolated from the environment [30–33]. To the best of our understanding, these species have not previously been reported in Tanzania.

Our findings are in agreement with findings from other studies in Tanzania and Africa which indicated increasing prevalence of NTM [9, 34, 35]. Whether there exist geographic variations in the diversity of NTM in Tanzania is not clear since this study focused only on patients residing in a more or less similar geographic location along the northeastern coast of Tanzania.

Understanding the distribution and clinical impact of NTM is of public health significance, as it addresses concerns of over diagnosis of tuberculosis and potential under treatment of NTM infections. As reported previously [35], there exist quite diverse species of NTM among humans, livestock and wildlife [36]; this therefore stresses need to investigate the distribution and clinical impact of different NTM species in Tanzania.

Analysis of the predictors of NTM infection in this study shows lack of association among gender, age, area of residence and occupation in patients diagnosed with NTM. However, need to study NTM infections in TB endemic settings like Tanzania through a larger cohort and evaluation of their impact on TB disease is particularly urgent. Though in the present study we did not perform drug susceptibility testing for the isolated NTM, such patients may erroneously regarded as having MDR isolates.

Immunocompromised individuals due to HIV/AIDS are at a high risk of NTM infections, with M. intracellulare, and M. avium complex (MAC) being frequently reported [37–39]. Findings from this study show that individuals with HIV positive status had a varying range of NTM infections and HIV was an important predictor of NTM detection (OR 3.86, 95% CI: [1.79–8.3], p < 0.001). The identified NTM included M. scrofulaceum (2), M. avium spp. (2), M. gordonae (2), M. intracellulare (1), M. lentiflavum (1), M. celatum (1) and M. interjectum (1). Two of the isolates from HIV positive cases, not identified by the Hain kits, were found to belong to M. kumamotonense and M. intracellulare by 16S RNA sequencing.

MAC and rarely identified species of M. lentiflavum and M. sherrisi were reported among HIV positive patients with pulmonary disease in Zambia and Tanzania [9, 34, 40], M. sherrisi which was found to be commonly associated with HIV individuals in northern Tanzania [34], was not detected in this patient population. M. celatum commonly isolated from human respiratory tract specimens is also known to be pathogenic to HIV patients and sometimes non-immunosuppressed patients [41], whereas, M. gordonae a common contaminant of water supply, soil, casual resident in human sputum and gastric luminal specimens is rarely (e.g. AIDS) if ever implicated in disease processes [41].

NTM co-infections with M. tuberculosis disease are rarely diagnosed owing to overlapping clinical manifestations [10]. Our results show that five (13.3%) individuals had both M. tuberculosis and NTM which included M. scrofulaceum (2), M. interjectum (1), M. gordonae (1) and one isolate not identified by Hain kits was identified as M. kumamotonense by 16S RNA sequencing. Although, it may be assumed that patients with such co-infections in many cases manifest symptoms mainly due to M. tuberculosis, the role of such co-infections underscore the need for further research to determine their contribution in the disease pathogenesis, severity and progression.

NTMs pose a major challenge for TB treatment programmes since such patients are managed mainly on the basis of smear microscopy which is not suitable to differentiate between MTC and NTM, but also major drawbacks lie on limited sensitivity and specificity of symptoms and radiology [8]. Culture on the other hand, is the gold standard but is time consuming, demands use of different types of media and longer incubation for optimization of growth. Since such elaborate culture algorithms are scarce outside the reference centres, NTMs warrant a special emphasis as possible cause of disease.

Although we cannot certainly conclude whether the isolated NTM merit to be classified as cause of infection/ disease in each particular case, a mere presence of NTM in a particular case could make the decision on the diagnosis more complex. Need for public hygiene education is particularly high, since NTM are mostly found in the environment (water, soil). If people exert better hygiene
management in their homes and food preparation, animal keeping, water boiling before drinking and so on, the risk of being infected would decrease, especially for persons at risk such as children, elderly, and immunocompromised individuals.

Limitations
A number of factors could have limited our findings. Firstly, lack of preliminary laboratory data on the chronic patients presented limited us from determining whether they had been infected only by an NTM or they primarily had coinfection with M. tuberculosis. Secondly, lack of follow up limits the capacity to establish patients’ outcomes especially with NTM disease in many resource poor settings. Moreover, the true prevalence of NTM can only be assessed through a wider epidemiological study. These findings were communicated to the Medical Research Coordinating Committee where National Tuberculosis Control Program is represented. However, we are not sure if NTLP guided starting of appropriate treatment for NTM since there are no clear national guidelines for management of NTMs. This makes these findings very important as they may serve as baseline data for the NTLP to develop guidelines for management of NTMs.

In conclusion, our findings suggest that there is a diverse range of NTM infections, which may play a vital role in causing lung disease and impact the management of TB in TB-endemic settings leading to misdiagnosis and inappropriate treatment of MDR cases particularly the clinically “chronic cases”. This highlights the need to consider NTM when treating patients with putative TB treatment failures. Moreover, fundamental information that meets ATS/IDSA diagnostic criteria for diagnosis of pulmonary NTM is needed to improve the understanding of NTM disease. There is an urgent need of formulating standardized criteria for defining and reporting NTM infections. We recommend that laboratory diagnosis of NTM infections be centralized by strengthening and setting up quality national and regional infrastructure.

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Competing interests
The authors declare that they have no competing interest.

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Results and Discussion

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The role of nontuberculous mycobacteria in the diagnosis, management and quantifying risks of tuberculosis in Tanga, Tanzania

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Abstract

Background: The role of nontuberculous mycobacteria (NTM) in tuberculosis (TB) diagnosis is well documented in many developing settings. However, this has not been the case in many resource poor settings like Tanzania. This study aimed at understanding the role of NTM in the diagnosis and management of TB in resource poor settings of Tanzania.

Methods: A cross-sectional study was conducted in Tanga, Tanzania. Patients with symptoms suggestive of TB self-referred to health care facilities were recruited. Two sputum samples were collected for standard direct smear microscopy. Culture was performed using BacT/Alert 3D system, Löwenstein-Jensen and Gottleber slopes. Identification of Mycobacterium tuberculosis and NTM was done by using GenoType MTBC and GenoType CM/AS, respectively.

Results: A total of 372 patients were involved in the study. Eighty-one (21.8%) patients were diagnosed as having M. tuberculosis by the isolation of the organism from cultures of sputum. Further analysis of culture showed that 8.1% (30/372) were NTM with 7/372 (1.9%) cases of NTM classified as pulmonary tuberculosis (PTB) patients. Ziehl Neelsen stain had a sensitivity of 58.8% and produced 10 false negative results. On the other hand, Fluorescence stain had a sensitivity of 85.7% and gave seven false negative samples when compared with culture results. Weight loss (p = 0.0001), fatigue (p = 0.003), fever (p = 0.038) and night sweats (p = 0.004), young population (18-40 years) (p = 0.035), males (p = 0.003) were important risk factors for TB. Four out of 30 NTM diagnosed by culture received first line anti-TB treatment suggesting that a good proportion of patients (4/65, 6.2%) were mistreated as TB patients.

Conclusion: Inefficient screening of TB patients in resource poor settings and prevalent increase of NTM may contribute to over diagnosis of TB cases. The need to integrate NTM diagnosis in the routine management of TB is urgently needed for designing effective tuberculosis prevention and control strategies in the country.

Keywords: tuberculosis, nontuberculous mycobacteria, management, Tanzania

Introduction

Tuberculosis (TB) caused by Mycobacterium tuberculosis (MTB) complex remains a major public health challenge worldwide and a leading cause of morbidity and mortality throughout sub-Saharan Africa (WHO, 2009). The emergency of multidrug-resistant (MDR) TB and co-infection with HIV have continued to threaten global TB control, especially in resource poor settings of sub-Saharan Africa (Cobelens et al., 2008; Migliori et al., 2008a,b; WHO, 2010a). The problem in many resource poor settings like Tanzania is further compounded by weak health care systems, and inadequate laboratory infrastructure.

The incidence of all forms of TB in Tanzania is estimated at 295 cases per 100,000 persons per year (NTLP, 2011). The country has achieved commendable landmarks in fighting...
against TB, and listed among 13 countries from the high-burden countries (HBCs) that achieved treatment success rate target of 85% set by the World Health Organization for new sputum smear-positive cases of pulmonary TB (WHO, 2010a). Case detection rate for new smear positive TB cases remains low at 51%, far below the WHO's target of 70%. The WHO report further shows an alarming trend of increased active TB cases in Tanzania due to HIV/AIDS epidemic, which is associated with 60 percent increase in active TB (WHO, 2010a).

In spite of these achievements, a number of challenges face the TB control programme. The current surveillance system still relies on passive case finding among individuals who self-referred to health facilities, followed by diagnosis based on the presumptive clinical TB symptoms and laboratory diagnosis using insensitive direct sputum smear microscopy (Mfinanga et al., 2007; Matee et al., 2009). Typically, this method has been shown to be poor in performance, with low detection rate of 56 to 58% resulting in missing of half of the cases (WHO, 2009). The sensitivity is reported to be further reduced to 43-51% among TB/HIV co-infected patients (Sonnenberg et al., 2001).

Several studies have demonstrated that serious infections with non-tuberculosis mycobacteria (NTM) remain a major public health concern in many TB-endemic countries (Crump et al., 2009; Buitjerts et al., 2010; Maiga et al., 2012). NTM can present with clinical and radiologic manifestations comparable to M. tuberculosis. Most often, it is not feasible to differentiate between NTM and MTB microscopically in situations where sputum culture and other diagnostic tests are not done. Increased prevalence of nontuberculosis mycobacteria (NTM) infections among TB patients which are difficult to differentiate from MTBC further jeopardises the control efforts to contain the disease (Migliori et al., 2008).

It is apparently therefore, that the need for a quick and accurate diagnosis of TB is critically important. Accurate diagnosis of MTB will not only ensure accurate and timely management of TB patients, but also will reduce morbidity, mortality, economic loss, further spreading of infection and emergence of MDR strains (Kox et al., 1994; Moulding et al., 2004). This study therefore aimed at determining the role of nontuberculosis mycobacteria (NTM) in diagnosis, treatment and quantifying risks for TB transmission in Tanga, Tanzania.

Materials and Methods

Study area, design and population
This cross-sectional study was conducted in Tanga, Tanzania from November 2012 to January 2013. The study involved four health facilities, namely Makorora and Ngamian health centres and Bombo Regional Referral hospital in Tanga City and the Designated District Hospital in the nearby Muheza district. All patients with clinical signs and symptoms suggestive of TB self-referred to four primary health care facilities were eligible for the study. Demographic information and data were collected only after provision of informed consent. A careful cross examination of patient history for previous anti-TB treatment using a structured questionnaire was used to classify patients as 'new' or 'previous treated' TB cases. A patient was considered as 'new case' if he had no prior anti-TB treatment for more than 1 month and was considered 'previous treated' if had received anti-TB treatment lasting at least 1 month (Abdel-Aziz, 2003). There were no restrictions on inclusion criteria regarding clinical symptoms and age of the patients.

Sputum and data collection
Demographic data were obtained using structured questionnaire administered to the patients attending the health care facilities who provided informed consent to the study. Two sputum samples (one spot during the initial visit to the clinic and one early morning) were collected into small autoclavable wide mouth glass bottles. The specimens were examined using the standard direct smear microscopy at the respective facilities using either Ziehl Neelsen (ZN) stain (Makorora and Ngamian health centres) or fluorescence stain (Bombo and Muheza). Diagnosis of smear-
positive TB was performed based on the National Tuberculosis and Leprosy Programme Guidelines (NTLP, 2006). All morning sputum samples kept at -20°C at the respective clinics. No preservatives were added until shipped to the mycobacteriology laboratory at the Institute of Medical Microbiology and Epidemiology of Infectious Diseases, University Hospital, Leipzig, Germany for culture and molecular analysis. HIV status of the patients was determined by rapid HIV screening method at the treatment and care centres (CTC) of the respective clinics.

**Sputum culture and identification of mycobacterial isolates**

Sputum specimens were digested and decontaminated using N-acetyl-L-cysteine-sodium hydroxide method (Kent & Kubic, 1985) and were re-examined for the presence of acid-fast bacilli by fluorescence stain in Leipzig. The isolates were cultured in Bact/Alert 3D liquid culture system (bioMe’rieux, Mercy-I’ Etoile, France) and on Löwenstein-Jensen and Gottsacker slants (Artelt-ENCLIT GmbH, Wyhra, Germany). Gottsacker slopes contains sodium pyruvate for isolation of M. bovis. Cultures were incubated at 37°C for up to 8 weeks. Confirmation of M. tuberculosis and NTM was done based on presumptive phenotypic appearance of colonies and by line probe assay GenoType®MTBC for identification of M. tuberculosis and GenoType®CM/AS for the identification of common and additional NTMs. The assay kits were obtained from Hain Lifescience, Nehren, Germany.

**Data analysis**

Descriptive statistics such as frequencies, means and proportions for different study attributes were computed by using Open Epi software version 3. A 2x2 contingency table for comparison of direct smear microscopy and the gold standard cultural method was built and prevalence, sensitivity, specificity and kappa statistic of agreement were calculated by using SAS software version 9. The Chi-square test of association between TB status of study participants and clinical signs were computed at significant level of 5%. The risk factors associated with positive TB status were quantified by logistic regression using SAS software version 9. The model building process followed a backward stepwise strategy whereby univariable analysis of all variables preceded the multivariable analysis. The variables included age, gender, occupation, previous TB history, smoking, diabetes and HIV/AIDS status. A variable qualified for multivariable analysis when it had a p-value of less than 0.25. A multivariable model was run and an iterative process of removing a variable with highest p-value was done until all variables in the model had p-value of less than 0.05. A variable was regarded as a confounder and retained in the final model when its removal from the multivariable model caused a more than 25% or between -0.4 and 0.4 changes in coefficient of any other variable. Model fitness test was assessed by Hosmer-Lemeshow test.

**Ethical considerations**

The protocol for this study was approved by the Medical Research Coordinating Committee of National Institute for Medical Research, Tanzania (Certificate No. NIMR/HQ/R.8a/Vol. IX/1401). Written informed consents were obtained from all participants of this study. For illiterate participants, there was an independent witness present during the consenting process, who then signed the relevant witness section of the consent form.

**Results**

**Comparison of results of staining techniques and culture**

A total of 372 TB suspects including 312 new and 60 previously treated cases were recruited. Eighty-one of the 372 (21.8%) patients were diagnosed as having M. tuberculosis by the isolation of the organism from cultures of sputum. A total of 176 samples were analysed by ZN staining while 196 samples were analysed by fluorescence staining. ZN stain was able to detect 22 out of 32 positive samples by culture technique. This means ZN method had a sensitivity of (68.8%) and produced 10
false negative results. On the other hand, Fluorescence staining was able to detect 42 out of 49 positive samples by culture technique (sensitivity = 85.7%) and seven (7) samples were false negative (Table 1). Moreover, culture results showed that 8.1% (30/372) of the sputum was positive for NTM, with 7/372 (1.9%) of culture confirmed NTM recorded as TB positive case by the health facilities. The remaining 68.3% (254/372) were culture negative for M. tuberculosis at 8 weeks.

<table>
<thead>
<tr>
<th>Stain</th>
<th>Culture</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZN</td>
<td>Positive</td>
<td>22</td>
<td>0</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>10</td>
<td>144</td>
<td>154</td>
</tr>
<tr>
<td>Fluorescence</td>
<td>Positive</td>
<td>42</td>
<td>0</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>07</td>
<td>147</td>
<td>154</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>81</td>
<td>291</td>
<td>372</td>
</tr>
</tbody>
</table>

**Assessment of criteria for suspecting TB cases in Tanga**

Currently, one of the defining criteria for suspecting TB is cough for over 2 weeks. In attempt to establish how correctly the clinics were able to assess the patients presented with TB cardinal symptoms and the actual TB cases, we analysed the association of TB status with the clinical manifestations presented during patients visit to the TB facilities. Results show that weight loss (p = 0.0001), fatigue (p=0.001), fever (p=0.038) and night sweats (p=0.004) were statistically significantly associated with TB positive status of patient in Tanga (Table 2).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Category</th>
<th>n</th>
<th>TB+</th>
<th>TB-</th>
<th>Df</th>
<th>X^2 value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cough ≥ 2 weeks</td>
<td>Yes</td>
<td>327</td>
<td>75</td>
<td>252</td>
<td>1</td>
<td>2.14</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>45</td>
<td>6</td>
<td>39</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bloody sputum</td>
<td>Yes</td>
<td>44</td>
<td>11</td>
<td>33</td>
<td>1</td>
<td>0.3</td>
<td>0.58</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>328</td>
<td>70</td>
<td>258</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Night sweats</td>
<td>Yes</td>
<td>249</td>
<td>65</td>
<td>184</td>
<td>1</td>
<td>8.29</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>123</td>
<td>16</td>
<td>107</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fatigue</td>
<td>Yes</td>
<td>208</td>
<td>57</td>
<td>151</td>
<td>1</td>
<td>8.78</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>164</td>
<td>24</td>
<td>140</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fever</td>
<td>Yes</td>
<td>254</td>
<td>63</td>
<td>191</td>
<td>1</td>
<td>4.31</td>
<td>0.038</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>118</td>
<td>18</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dyspnoea</td>
<td>Yes</td>
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<td>46</td>
<td>130</td>
<td>1</td>
<td>4.04</td>
<td>0.045</td>
</tr>
<tr>
<td></td>
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<td>34</td>
<td>160</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight loss</td>
<td>Yes</td>
<td>138</td>
<td>49</td>
<td>89</td>
<td>1</td>
<td>24.16</td>
<td>0.0001</td>
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<td>31</td>
<td>197</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

**Quantification of risk factors for occurrence Tuberculosis in patients in Tanga**

Univariable analysis of TB status (dependent variable) against age, gender, occupation, TB history, smoking, diabetes, HIV/AIDS (independent variables) was run. Results of this analysis revealed that age, sex and occupation were significantly associated with positive TB status in patients in study area (Table 3).
Results and Discussion

Table 3. Single and modelled multiple risk factors for occurrence of TB

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>Response</th>
<th>n (%)</th>
<th>TB positive</th>
<th>OR</th>
<th>95% CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-17</td>
<td>16</td>
<td>4 (4.9)</td>
<td>0.211</td>
<td>0.046 - 0.962</td>
<td>0.3537</td>
<td></td>
</tr>
<tr>
<td>18-40</td>
<td>184</td>
<td>49 (60.5)</td>
<td>0.193</td>
<td>0.067 - 0.561</td>
<td>0.0352</td>
<td></td>
</tr>
<tr>
<td>41-60</td>
<td>111</td>
<td>24 (21.6)</td>
<td>0.254</td>
<td>0.084 - 0.772</td>
<td>0.3896</td>
<td></td>
</tr>
<tr>
<td>&gt; 60</td>
<td>61</td>
<td>4 (6.9 )</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>192 (51.6)</td>
<td>54 (66.7)</td>
<td>2.217</td>
<td>1.313 - 3.715</td>
<td>0.0025</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>180 (48.4)</td>
<td>27 (33.3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Occupation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High contact</td>
<td>147 (39.5)</td>
<td>46 (68.8)</td>
<td>2.472</td>
<td>1.497 - 4.083</td>
<td>0.0004</td>
<td></td>
</tr>
<tr>
<td>Low contact</td>
<td>215 (60.5)</td>
<td>35 (43.2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Previous TB history</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>61</td>
<td>11 (19.6)</td>
<td>0.757</td>
<td>0.374 - 1.533</td>
<td>0.3999</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>311</td>
<td>70 (23.4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>79</td>
<td>22 (28.5)</td>
<td>1.558</td>
<td>0.881 - 2.753</td>
<td>0.1173</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>293</td>
<td>58 (20.1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>2</td>
<td>1 (50.0)</td>
<td>3.613</td>
<td>0.233 - 58.396</td>
<td>0.3657</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>370</td>
<td>80 (21.7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIV/AIDS status</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>57</td>
<td>7 (12.3)</td>
<td>2.335</td>
<td>0.954 - 5.335</td>
<td>0.0682</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>110</td>
<td>23 (21.0)</td>
<td>1.047</td>
<td>0.597 - 1.857</td>
<td>0.2691</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>215</td>
<td>51 (23.7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Final Model Multiple risk†</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>192 (51.6)</td>
<td>54 (28.1)</td>
<td>1.099</td>
<td>1.124 - 3.244</td>
<td>0.0167</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>180 (48.4)</td>
<td>27 (15.0)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Occupation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High contact</td>
<td>147 (39.5)</td>
<td>46 (31.3)</td>
<td>2.188</td>
<td>1.31 - 3.654</td>
<td>0.0028</td>
<td></td>
</tr>
<tr>
<td>Low contact</td>
<td>215 (60.5)</td>
<td>35 (15.6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

† The multivariable analysis resulted into a final model with two variables, namely gender and occupation. Male patients had 1.9 times higher risk of suffering TB while people involved in high contact activities had 2.188 times odds of getting TB. This model well fitted the variables as shown by Hosmer-Lemeshow test with χ² = 0.0092, df = 2 and p-value of 0.99.

Comparison of diagnostic results and treatment

We evaluated the treatment of different subjects based on smear microscopy results and culture results. Among the 65 subjects receiving first line anti-TB drugs, 25 (38.5%) were diagnosed by ZN staining and 40 (61.5%) by Fluorescence staining. Moreover, 64 subjects whose samples were processed by ZN staining and 47 subjects by fluorescence staining were empirically treated with broad spectrum antibiotics. Out of 196 subjects who did not receive any medication, 87 and 109 subjects had their samples processed by ZN and fluorescence staining, respectively (Table 4).

Table 4. Distribution of treatment type by staining technique (n=372)

<table>
<thead>
<tr>
<th>Smear technique</th>
<th>ZN</th>
<th>Fluorescence</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st line anti-TB drugs</td>
<td>15</td>
<td>40</td>
<td>65</td>
</tr>
<tr>
<td>Broad spectrum antibiotics</td>
<td>64</td>
<td>47</td>
<td>111</td>
</tr>
<tr>
<td>No treatment</td>
<td>87</td>
<td>109</td>
<td>196</td>
</tr>
<tr>
<td>Total</td>
<td>176</td>
<td>196</td>
<td>372</td>
</tr>
</tbody>
</table>

Of the 81 patients diagnosed as TB positive by culture, 54 were already on first line anti-TB drugs, while 11 were empirically treated with broad spectrum antibiotics and 16 were not given any medication following smear microscopy results. Out of 291 TB negative subjects, 11 were given first line anti-TB drugs, 100 were given broad spectrum antibiotics and 180 received no therapy (Table 5).
### Results and Discussion

#### Table 5. Comparison of diagnostic results and treatment for the study subjects (n=372)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Culture results</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td>Total</td>
</tr>
<tr>
<td>First line anti-TB</td>
<td>54</td>
<td>11</td>
<td>65</td>
</tr>
<tr>
<td>Broad spectrum antibiotics</td>
<td>11</td>
<td>100</td>
<td>111</td>
</tr>
<tr>
<td>No treatment</td>
<td>16</td>
<td>180</td>
<td>196</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>81</td>
<td>291</td>
<td>372</td>
</tr>
</tbody>
</table>

Further analysis indicated that subjects diagnosed as NTM positive by culture had already received different medication following smear microscopy results (Table 6). Four subjects received first line anti-TB drugs, 12 received broad spectrum antibiotics and 14 received no medication.

#### Table 6. Association of NTM diagnosis and treatment for the study participants (n=372)

<table>
<thead>
<tr>
<th>NTM diagnosis</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st line anti-TB</td>
<td>4</td>
<td>61</td>
</tr>
<tr>
<td>Broad spectrum antibiotics</td>
<td>12</td>
<td>99</td>
</tr>
<tr>
<td>No medication</td>
<td>14</td>
<td>196</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>30</td>
<td>342</td>
</tr>
</tbody>
</table>

### Discussion

Sputum smear microscopy is the mainstay for pulmonary tuberculosis screening in high-burden countries (WHO, 2011). The method has proven to be rapid and easily affordable means of diagnosing PTB in many resource poor settings, where TB is still a major public health burden. Findings from this study showed that the sensitivity of ZN method was 68.8% and that of Fluorescence staining was 85.7% when compared to culture results. This finding is not surprising since ZN stain is known to have a low level of sensitivity compared to the Fluorescence method (Ulukenigil et al., 2000). Finding that about one-fifth of the patients were diagnosed with M. tuberculosis by the isolation of the organism from cultures of sputum suggest that a significant proportion of the patients had sputum culture positive for M. tuberculosis. These findings reaffirms the consolidate the findings that the performance characteristics of smear microscopy is poor in the diagnosis of PTB (WHO, 2009). In patients co-infected with HIV the performance has shown to be further lower (Sonnenberg et al., 2001).

Generally, smear-negative PTB accounts for 30% of PTB cases per annum in developing countries (Scherer et al., 2007). The findings suggest that, while from the public health point of view smear-positive cases are considered more significant in diagnosing PTB, smear-negative patients on the other hand may be actual cases of TB as proved at some cases by culture (Tadesse et al., 2011). Since smear microscopy does not only play a pivotal role in the initial diagnosis of PTB but also in monitoring of anti-TB treatment, false negatives can continue to be infection source while false positives may be subjected to unnecessary anti-TB medication. Our results show that over half of the patients diagnosed by Fluorescence method received anti-TB treatment compared to about a third when ZN method was used. In general, passive case finding strategy combined with sub-optimal diagnostic procedures at diagnostic clinics may pose a serious problem in underestimating the burden of the disease.

The diagnostic value of smear microscopy in general is reportedly being eroded not only by increased cases of HIV-related smear negative PTB, but also due to the influence of NTM (Lalit, 2001; Valadas et al., 2003). The finding that a few of the patients had culture confirmed NTM elucidates an important public health and diagnostic challenge of continuing relying on smear microscopy for diagnosis of PTB in settings where dual infections are on the increase. Furthermore, classification of a few of culture confirmed NTM as PTB patients based on the smear microscopy results means that, such patients were managed as individuals with PTB.
NTM can present with clinical and radiologic manifestations comparable to M. tuberculosis. Since it is not feasible to differentiate between NTM and MTB microscopically in situations where sputum culture and other identification tests are not done, it could imply therefore, that NTM cases may have been treated as PTB for long periods of time. Essentially, NTM treatment differ from PTB treatment. Thus, such patients may show unfavourable response to anti-TB therapy. Additionally, since NTM isolates are often resistant to first-line anti-TB drugs, it may result into classifying and treating these individuals as MDR-TB cases (Tabarsi et al., 2009), that may require the use of second-line drugs which are more toxic and expensive, consequently adding up to waste of resources due to mismanagement of such patients. Several studies have demonstrated that serious infections with NTM remain a major public health concern in many TB-endemic countries (Crump et al., 2009; Buitjels et al., 2010; Maiga et al., 2012).

The presumptive diagnosis of PTB has in practice been based on an algorithm of clinical and radiological (where available) criteria coupled with smear microscopy. Based on this fact, we assessed the defining criteria in predicting PTB positivity at the primary care facilities. Our results showed that, weight loss, fatigue, fever and night sweats were the most likely criteria for TB positivity among patients in Tanga. Since one of the defining criteria for suspecting TB is cough for ≥ 2 weeks, it can be argued that, this generalization might be contributing to missing a reasonable number of TB cases. This is because patients reporting at the primary diagnostic facilities with cough of less than two weeks are likely to have not been screened for TB under routine programme (Ngadaya et al., 2009). Conversely, experience from the study shows that, a tendency of generalizing all complaints of cough for ≥ 2 weeks as a gold standard for PTB suspect leads not only to unnecessary increase of workload to already overwhelmed clinics, but also to unrealistic case detection. Inclusion of all patients with cough of more than two weeks may include patients with simple chest infections that are sometimes complicated with cough (Ngadaya et al., 2009; Swai et al., 2011). Furthermore, it may also contribute to increased negative results, delayed reporting and ultimately eroding patient’s faith in the services of the laboratory and of the general health system (Wandwalo & Morkve, 2000).

Results of risk factors quantification indicated that age, sex and occupation were the important risk factors associated with TB positivity. Young population at the age between 18 to 40 years and males were at most risk of TB infection. Similar trend was also reported during the 2012 prevalence survey (NTLP, 2011). These findings suggest that the patient population in this setting is marked with active transmission; hence, TB is largely seen in younger population. Furthermore, “high contact” subjects like students, community gatherings and other crowd related human activities (including public commute transports) remain at high risk of contracting TB.

In conclusion, unrealistic TB case detection may create a cluster of infectious cases in the community that maintain active TB transmission. Furthermore, classification of 1.9 % of culture confirmed NTM as TB patients at the primary health care facilities suggest that, a substantial number of patients may have been wrongly managed as PTB cases. An inefficient screening plus sub-optimal diagnostic procedures at the primary health care facilities may lead to unrealistic detection of TB cases. There is an urgent need to integrate NTM diagnosis in management of TB for designing effective tuberculosis prevention and control strategies in the country. Promoting of favourable health seeking behaviours and education especially among males, younger population and those at high risk such as crowded human activities need to be strengthened.

Acknowledgements

We are grateful to the management and technical personnel, of the TB clinics as well as the TB Coordinators in Muheza and Tanga. We thank Ms. Elizabeth Kraftschek from the Department of Medical Microbiology and Epidemiology of Infectious Diseases, University of Leipzig for her technical support.
Results and Discussion

Competing interests

All authors declare that they have no competing interests.

References


Results and Discussion

4.5 Publication V

(Submitted to *BMC Infectious Diseases*)

Isolation, Biochemical and Molecular identification of *Nocardia* species among TB suspects in Northeastern, Tanzania. A forgotten or neglected threat?
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Abstract

**Background:** Pulmonary nocardiosis mimic pulmonary tuberculosis in most clinical and radiological manifestations. In Tanzania, where tuberculosis is one of the major public health threat clinical impact of nocardiosis as cause of human disease remains unknown. Objective of the present study was to isolate and identify *Nocardia* isolates recovered from TB suspects in Northeastern, Tanzania by using biochemical and molecular methods.

**Methods:** The study involved 744 sputum samples collected from 372 TB suspects from four periphery diagnostic centres. Twenty patients were diagnosed as having presumptively *Nocardia* infections based on microscopic and cultural characteristics and confirmed using biomèriques ID 32C Yeast Identification system and 16S rRNA gene specific primers for *Nocardia* species and sequencing.

**Results:** Biochemically, the majority of the isolates were *N. asteroides* (n = 8, 40%), *N. brasiliensis* (n = 4, 20%), *N. farcinica* (n = 3, 15%), *N. nova* (n = 1, 5%). Other aerobic actinomycetales included *Streptomyces cyanescens* (n = 2, 10%), *Streptomyces griseus*, *Actinomadura madurae* each (n = 1, 5%). 16S rRNA sequencing identified five members of the species (29.4%) *N. cyriacigeorgica*, four (23.5%) *N. farcinica*, two (11.7%) *N. flavorosea* and *N. testacea* each, *N. asteroides*, *N. nova*, *N. carnea* and *N. brevicatena* one (5.9%) each.

**Conclusions:** Our findings suggest that *Nocardia* species may be an important cause of pulmonary nocardiosis that is underdiagnosed or ignored. This underscore needs to consider
pulmonary nocardiosis as differential diagnosis when there is failure of anti-TB therapy and as possible cause of human infections.

Key words: Nocardia. Tuberculosis. biochemical. molecular. northeastern. Tanzania

Introduction

Members of genus Nocardia are characteristically gram-positive, weakly acid-fast, strictly aerobic, filamentous branching bacilli that fragment into rod or coccoid shaped forms. Nocardia species are ubiquitous environmental bacteria capable of causing opportunistic infections in both human and animals [1,2]. Nocardia species are increasingly isolated as infectious agents in immunocompromised patients, and at times, even among healthy individuals [3], causing infections ranging from pulmonary, cutaneous and subcutaneous human diseases [4].

The most commonly isolated species in human include N. asteroides, N. farcinica, N. cyriacigeorgica, N. nova, N. brasiliensis [5–7] and N. ignorata [8]. Pulmonary nocardiosis has been reported in patients with debilitating conditions, such as those with organ transplants, diabetes mellitus, leukaemia, and other malignancies [9].

The incidence of nocardiosis varies geographically according to a number of factors, like the prevalence of HIV infections, transplants, cancer, climate as well as socio-economic status, and laboratory capacity for Nocardia species detection and identification.

Increased incidence of human nocardiosis may be attributed to wide use of immunosuppressive drugs, improved diagnostic tests, and increased awareness among microbiologists and health professionals. Nonetheless, in many developing countries where other chronic lung diseases, particularly TB, are prevalent, Nocardia species are either missed or misidentified during diagnosis [10,11].

Accurate detection and identification of Nocardia species have become increasingly important for prediction of antimicrobial susceptibility since different species have emerged in terms of their virulence and epidemiology. Furthermore, prompt and timely species identification can significantly influence the choice of therapy.

Apart from Gram and modified acid-fast staining, identification of Nocardia species depends largely on biochemical tests and cellular fatty acid analysis, which have proved to be laborious with long turnaround time, and less definitive.

Several molecular identification methods have been successfully employed to identify and characterize Nocardia species. Multilocus sequence analysis (MLSA) of 16S rRNA, the 65-kDa heat shock protein (hsp65), gyrase B of the β subunit of DNA topoisomerase (gyrB), subunit A of Sec A preprotein translocase (secA1) and RNA polymerase (rpoB) have previously been used
to identify *Nocardia* species [8,12,13]. However, 16S rRNA gene sequencing, which is the most often used system of bacterial identification, cannot discriminate many species [14], therefore identification based on the basis of the DNA sequence of a single housekeeping gene is hindered by stochastic genetic variation as well as horizontal gene transfer and recombination [15]. Pulmonary nocardiosis mimics pulmonary tuberculosis in both clinical manifestations, being chronic in nature and radiological characteristics makes it difficult to differentiate from *M. tuberculosis* and may as well be often wrongly treated with anti-TB drugs [16,17]. Patients might also be confused with other chronic lung infections such as invasive fungal infection [18,19].

In Tanzania, where tuberculosis is one of the major public health threats and a third leading cause of adult morbidity and mortality after Malaria and HIV/AIDS; clinical reports of *Nocardia* are rare if found at all. Moreover, the knowledge as well as the clinical impact of nocardiosis is unknown, suggesting that infections due to this genus may be underdiagnosed and/or neglected as a cause of human diseases.

Due to the lack of information on pulmonary nocardiosis in Tanzania, the objective of the present study was to isolate and identify *Nocardia* isolates recovered from TB suspects in Northeastern, Tanzania by using biochemical and molecular methods.

**Materials and Methods**

**Collection and decontamination of clinical specimens**

Three hundred and seventy two (n = 372) self-presented TB suspects were included in this study after presumptively diagnosed as pulmonary tuberculosis cases by clinical symptoms and microscopically by acid-fast Ziehl-Neelsen (ZN) and or fluorescence staining method from four peripheral diagnostic centres (PDCs) in north eastern Tanzania. From each patient two sputum specimens were collected one spot during the first visit at the respective TB facilities and one early morning specimen. The specimens were decontaminated by N-acetyl L-cystiene (NALC) sodium hydroxide (NaOH) method as described previously [20]. Decontaminated specimens were concentrated by centrifugation at 3000xg for 20 min after discarding the supernatant the sediments were re-suspended by adding 1.5ml of phosphate buffered saline (PBS). All procedures were carried out in a certified level II biosafety cabinet.

**Culture and identification of the isolates**

Two drops of the centrifuged sediments were inoculated on Löwestein-Jensen (L-J) and incubated at 37°C for 8 weeks. Subsequently 500µl of each specimen was inoculated in a
BacT/Alert bottle, incubated in the BacT/Alert® 3D system for 8 weeks. Sample was considered negative when no bacterial growth or positive signal from the BacT/Alert instrument was observed after 8 weeks incubation. Positive cultures were stained by ZN for the confirmation of the acid-fast bacilli (AFB). Confirmation of *M. tuberculosis* was performed by using GenoType® MTBC (Hain Life science GmbH, Nehren, Germany) and GenoType® Mycobacterium CM/AS assay (Hain Life science GmbH, Nehren, Germany) for the detection of common and accessory nontuberculous mycobacteria (NTM).

Negative cultures for AFB were cultured on blood agar (BA) and chocolate agar plates and incubated at 37°C for 2 to 4 days. Isolates were diagnosed as *Nocardia* based on the presence of non-acid-fast branching filamentous bacilli using Gram and modified Kinyoun staining, as well as their colony morphology.

**Biochemical identification**

The BioMérieux ID 32C yeast identification system was used to identify the presumptively 20 *Nocardia* isolates diagnosed in this study. All procedures followed manufacturer’s recommendations. Briefly, test isolates were grown on brain heart infusion agar or chocolate agar plates at 35°C. A Mc Farland no.4 was prepared in a standard physiological saline. One ml of the suspension was inoculated into ID 32C medium, and 3 drops were dispensed into each well of the strip using an automated dispenser ATB Vitek® 1574 (biomérieux). The strips were incubated at 35°C for 7 days in a sealed container to avoid evaporation. The strips were read to give an eight-digit profile, as per manufacturer’s instruction.

**DNA extraction**

DNA extraction was performed by using Ultracean® Microbial DNA isolation kit (MOBIO Laboratories, Inc.) following manufacturer’s instructions, after an initial heat inactivation of a loopful colony suspended in 500μl-distilled water in a 2ml Eppendorf tube then incubation at 95°C for 20 min followed by centrifugation at 15,000 x g for 5 min at room temperature (r.m.t). 50μl of collected DNA was then stored at -20°C until use. *M. tuberculosis* reference strain H37Rv genomic DNA and distilled water were used as positive and negative controls respectively.

**PCR for 16S rRNA**

A 606-bp fragment of the 16S rRNA gene specific for *Nocardia* species was amplified with primers Noc1 (5’-GCTTAAACCATGCAAGTCG-3’) (positions 46 to 46, *Escherichia coli* numbering system) and Noc2 (5’-GAATTCCAGTCTCCTCCCTG-3’) (positions 663 to 680, *E.*
coli numbering system) [1]. PCR reaction was performed in a 25μl volume with master mix contained final concentration of 5U Taq DNA polymerase (Applied Biosystems), 10x buffer, 10mM dNTPs mix, 100pmol/μl of each primer, 25mM MgCl₂, and 100ng template DNA. PCR amplification was carried out with an initial denaturation of 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, extension at 72°C. Then followed by a final extension at 72°C for 5 min. PCR products were purified with Qiagen PCR purification kit (Qiagen, CA, USA) following manufacturer’s recommendations and were submitted for sequencing at the Department of Biochemistry, University of Leipzig, Germany.

Sequencing of 16S rRNA PCR product fragments

PCR products were purified and sequenced on both strands. The resulting 16S rRNA sequences of the 20 isolates were analysed using CodonCode Aligner software Version 5.1.5 (http://www.codoncode.com/aligner/) and compared with the corresponding sequences of the Nocardia species deposited in the GenBank database (www.ncbi.nlm.nih.gov) using the Basic Local Alignment Sequencing Tool for nucleotide sequence queries (BLASTN). The GenBank query type strain or culture collection strain with the highest score was downloaded and compared to the subject sequences and the percentage similarity was then determined for each strain. Strains with greater than 99% homology to a single type strain were considered definitive for identification to species level.

Ethical clearance

The protocol for this study was reviewed and approved by the Ethical Committee of the National Institute for Medical Research (NIMR) (Ref. No. NIMR/HQ/R.8a/Vol.IX/1401), Dar es Salaam, Tanzania. Written informed consent was obtained from the patients or relative of the patients, where the patients could not read and write. Ethical approval was obtained for the secondary use of the study data from the same ethical committee.

Results

Detection of Mycobacteria and Nocardia species

Of 372 decontaminated samples one hundred eleven (n = 65) were AFB positive by ZN staining, whereas one-hundred thirty-seven (n = 137) including the AFB positive were culture positive by either L-J or BacT/Alert 3D system. Two hundred and two (n = 202) samples were both AFB and culture negative and 33 samples were contaminated. Eighty-one of the AFB isolates were confirmed to be M. tuberculosis using GenoType® MTBC (Hain Life science GmbH, Nehren, Germany) and 36 isolates were confirmed as NTM by GenoType® Mycobacterium
CM/AS (Hain Life science GmbH, Nehren, Germany). Twenty (5.4%) out of 372 TB suspects which are subject of this manuscript were presumptively diagnosed as having *Nocardia* infection. Diagnosis of *Nocardia* species based on the presence of non-acid-fast branching filamentous bacilli using Gram and modified ZN staining, as well as their colony morphology on LJ, BA and Chocolate agar (Fig 1). Eleven isolates grew well on LJ media, and all isolates grew on both BA and Chocolate agar.

Profiles of isolates obtained by using the biomérieux ID 32C Yeast Identification system

Twenty isolates presumptively diagnosed as *Nocardia* species in this study were tested by the Biomérieux ID 32C Yeast Identification system and results obtained showed that majority of the isolates were *N. asteroides* (n = 8, 40%), *N. brasiliensis* (n = 4, 20%), *N. farcinica* (n = 3, 15%), *N. nova* (n = 1, 5%). Other aerobic actinomycetes identified included *Streptomyces cyanescens* (n = 2, 10%), *Streptomyces griseus*, and *Actinomadura madurae* each (n = 1, 5%). The profiles of the twenty isolates obtained using the biomérieux ID 32C yeast identification system in this study are shown in Table 1.

Identification of *Nocardia* species by 16S rRNA sequencing

Since *Streptomyces* species are difficult to identify by the biomérieux ID 32C Yeast Identification system because they give a less distinct profile pattern than *Nocardia* isolates [21], we decided to submit all 20 isolates tested by this method for the 16S rRNA gene sequence analysis using the GenBank database (www.ncbi.nlm.nih.gov). Sequencing results revealed that of the 17 confirmed *Nocardia* isolates sixteen (n = 16) isolates had closest species similarity identity score of >99% with the type or culture collection strain in the database, whereas, one *Nocardia* isolates had species with similarity identity score of < 99% with type or culture collection strain in the database. On the other hand, two *Streptomyces* isolates had the same similarity identity for this gene. (Table 2). Of the 17 *Nocardia* isolates, 16S rRNA gene sequencing identified five (n = 5, 29.4%) *N. cyriacigeorgica*, four (n = 4, 23.5%) *N. farcinica*, *N. flavorosea* and *N. testacea* were two (n = 2, 11.7%) each, whereas *N. asteroides*, *N. nova*, *N. carnea* and *N. brevicaten*a each was identified in one isolate (n = 1, 5.9%).

Demographic and clinical data of patients diagnosed with *Nocardia* infection

Based on the 16S rRNA sequencing results the confirmed 17 cases of *Nocardia* belonged to *N. cyriacigeorgica* (n = 5), *N. farcinica* (n = 4), *N. testacea* and *N. flavorosea* each (n = 2), *N. nova*, *N. asteroides*, *N. carnea* and *N. brevicaten*a one each. We revised the demographic and clinical data obtained during the study period of all the patients as shown in (Table 3). The clinical symptoms
and concurrent conditions show that the proportion of Nocardia infection was higher (58.8%) in individuals with ≥ 35 years of age than among those ≤ 35 years of age (41.2%), and both sexes are equally affected. The data further showed that nearly all patients presented with clinical syndromes mimicking those of pulmonary tuberculosis. The proportion of individuals with Nocardia infection alone was 70.6% (n = 12), three (n = 3, 17.6%) cases had co-infection with HIV and two (n = 2, 11.7%) cases were co-infected with TB and they had commenced anti-TB treatment. Ten (n = 10, 58.8%) other cases were initiated with broad-spectrum antibiotic treatment whereas, for five (n = 5, 29.6%) cases records showed that they had no treatment whatsoever.

Discussion

Nocardia infections causing both human and animal diseases are increasingly reported owing to improved diagnostics especially in developed settings which has emphasized the need for rapid characterization of clinically isolated Nocardia [4,9,11,22,23]. However, data regarding nocardiosis from resource poor settings like Tanzania heavily stricken by HIV and TB pandemic are rare if available at all. Such diseases are either underdiagnosed or neglected due to similarity of clinical and radiological features between pulmonary nocardiosis and pulmonary tuberculosis on one hand and on the other due to poor diagnostic capabilities. In Tanzania where TB is still a major public health threat, anti-TB treatment is initiated based on clinical symptoms, direct smear microscopy and radiological diagnosis where available.

In this study, all 372 TB suspects self-presented at four health facilities were diagnosed based on clinical symptoms and direct smear microscopy by acid-fast staining method at the respective clinics. Eighty one (n = 81, 21.8%) patients were positive for M. tuberculosis by culture and confirmed by GenoType® MTBC and 36 (9.7%) patients had NTM infections confirmed by GenoType® Mycobacterium CM/AS. 202/372 patients sputum samples were negative by culture and 33 other samples were contaminated.

Twenty (5.4%) isolates out of 372 TB suspects were presumptively diagnosed as Nocardia species based on their colony morphology on different culture media and by microscopic appearance using modified ZN for weak acid-fast bacilli.

Isolates of Nocardia species in this study showed good growth on LJ, BA and chocolate agar (Fig 1). Decontamination of sputum samples with NALC-NaOH in this study did not seem to affect growth of Nocardia species as observed in a previous study [24].

Samples from 17 suspects were presumptively diagnosed as having Nocardia infection and three patients an infection with other actinomycetales. Findings that majority of Nocardia
species grow well on LJ in this study correspond with findings of other authors [23,25]. In this scenario, such growth may be confused with that of mycobacteria species, making the diagnosis more complicated. Furthermore, careful microscopic analysis should be thoroughly performed, since these two genera may present difficulties for an inexperienced microscopist [22]. As observed in the previous study [23], where *Nocardia* species were detected by the modified ZN-method for weak acid fast bacteria, we also detected *Nocardia* species by the ZN-method employed for mycobacteria in this study.

All the *Nocardia* species identified in this study have been reported to be associated with pulmonary nocardiosis in many parts of the world [22,24–27].

The finding that the biomèrioux ID 32C yeast identification system identified correctly five *Nocardia* isolates to species level and two isolates to least genus level as *Streptomyces* (Table 1) when compared to 16S rRNA sequencing results is in agreement with those in a study conducted in Brazil [13]. In the Brazilian study, seven isolates were identified by 31 different phenotypic tests using six identification systems with only two isolates correctly identified by phenotypic method, compared to multilocus sequence analysis (MLSA) results. However, the biomèrioux ID 32C yeast identification system used in this study appears to be useful in identifying *Nocardia* species and other aerobic actinomyceetales as previously determined [21].

The discrepancies between the ID 32C yeast identification system and sequencing results suggest that a careful interpretation of results indicating different *Nocardia* species and other actinomyceetales is needed, since such methods are not accurate. Molecular identification is necessary for definitive identification of *Nocardia* species [11,28–30].

Important to note is that the following species: *N. cyriacigeorgica, N. farcinica, N. brevicatena* and *N. nova* identified by 16S rRNA sequencing in this study were separated from the originally referred *Nocardia asteroides* and later found to be a group of bacteria with a heterozygous pattern of antimicrobial drug susceptibilities [31]. *N asteroides* complex was further separated and reorganized into different species on the basis of drug susceptibility patterns: *Nocardia abscessus, Nocardia brevicatena-pauviorans*complex, *Nocardia nova* complex (includes *N nova, Nocardia veteranana, Nocardia africana, Nocardia kruizakiae*), *Nocardia transvalensis* complex, *Nocardia farcinica*, and *N asteroides* [5]. Moreover, *Nocardia cyriacigeorgica*, which comprise majority of *Nocardia* species in this study (n = 5, 29.4%) was also differentiated from *N asteroides* and is becoming a more frequently identified clinically significant pathogen [32].
This separation may explain the observed discrepancy between the two methods. While phenotypic identification leads to misidentification, molecular identification can improve the diagnostic accuracy since some molecular targets can present high sequence similarity [2].

Looking into the demographic data of all 17 *Nocardia* cases in this study, it was observed that nine (52.9%) were males and eight (47.1%) were females. Seven (41.1%) were ≤ 35 years and ten (58.8%) were ≥ 35 years. This incidence suggests that nocardiosis can occur in both sexes and in different age groups with more or less the same frequency. However, of importance is that nearly all cases of nocardiosis identified in this study reported similar clinical symptoms as those of TB suspects with chronic coughing featuring in all cases (Table 3). This is not surprising since pulmonary nocardiosis is the most common clinical presentation acquired primarily by inhalation. The onset of symptoms maybe subacute to more chronic and can include productive or non-productive cough, shortness of breath, chest pain, haemoptysis, fever, night sweats, weight loss, and progressive fatigue [4].

In this study, 12 patients had *Nocardia* alone; two had co-infection with TB and were AFB positive, whereas three had co-infection with HIV. Similar findings have been reported in other studies [9,33]. It is important that irrespective of a patient’s immunologic status, the isolation of *Nocardia* from the respiratory tract or other body source should not be regarded as a contaminant or commensal organism [4].

**Conclusion**

In conclusion, our study reveals that *Nocardia* species are important causes of pulmonary disease and that may have been underdiagnosed and/or ignored all together. It is also clear that mycobacterial infections and nocardiosis treatment differ. Correct identification of the causative agent is therefore critical to avoid treatment failure. *Nocardia* species identified in this study, underscore the need to consider pulmonary nocardiosis as differential diagnosis especially when there is treatment failure with the standard anti-TB therapy, and as possible cause of human infections.

**Acknowledgement**

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Declarations

Ethics and consent to participate

The protocol for this study was reviewed and approved by the Ethical Committee of the National Institute for Medical Research (NIMR) (Ref. No. NIMR/HQ/R.8a/Vol.IX/1401), Dar es Salaam, Tanzania. Written informed consent to participate in the study was obtained from the patients or relative of the patients, where the patients could not read and write. Ethical approval was obtained for the secondary use of the study data from the same ethical committee.

Consent to publish

All authors have agreed to the submission and publication of this manuscript.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

ASH designed the study, conducted fieldwork, carried laboratory experiments, analysed the results and drafted the manuscript; SGSM designed the study and reviewed the manuscript; IM analysed the results and critically reviewed the manuscript; BK designed the study, supervised laboratory work, analysed the results and reviewed the manuscript.

Availability of data and materials

All data supporting the conclusions drawn from of this study can be found as supplementary file S1.

Abbreviations

AFB: acid-fast bacilli; BA: blood agar; GenoType® Mycobacterium CM: (for common mycobacteria) and AS (for addition species of mycobacteria); gyrB: gyrase B of the β subunit of DNA topoisomerase; hsp65: 65-kDa heat shock protein; L-J: Löwestein-Jensen; MLSA: Multilocus sequence analysis; MTBC: Mycobacterium tuberculosis complex; NALC: N-acely L-cystiene; NaOH: sodium hydroxide; NTM: nontuberculous mycobacteria; PBS: phosphate buffered saline; PDCs: peripheral diagnostic centres; rpoB: RNA polymerase r.m.t: room temperature; secA1: subunit A of Sec A prepotin translocase; Z-N: Ziehl-Neelsen

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Results and Discussion

Figure 1. Growth of *Nocardia* spp isolated from TB suspects on three different media used in this study (A) LJ medium (see arrows), (B) Blood agar plate and (C) Chocolate agar plate.

Table 1. Profiles of different *Nocardia* species and other aerobic *Actinomycetes* identified using the biomèrieux ID 32 C Yeast Identification system in this study

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Profile</th>
<th>Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>37735522</td>
<td><em>Streptomyces cyanescens</em></td>
</tr>
<tr>
<td>2</td>
<td>37735522</td>
<td><em>Streptomyces cyanescens</em></td>
</tr>
<tr>
<td>3</td>
<td>37516506</td>
<td><em>S. griseus</em></td>
</tr>
<tr>
<td>4</td>
<td>75112246</td>
<td><em>A. madurae</em></td>
</tr>
<tr>
<td>5</td>
<td>71414140</td>
<td><em>N. brasiliensis</em></td>
</tr>
<tr>
<td>6</td>
<td>20004004</td>
<td><em>N. nova</em></td>
</tr>
<tr>
<td>7</td>
<td>26420142</td>
<td><em>N. asteroides</em></td>
</tr>
<tr>
<td>8</td>
<td>10000304</td>
<td><em>N. farcinica</em></td>
</tr>
<tr>
<td>9</td>
<td>61400304</td>
<td><em>N. farcinica</em></td>
</tr>
<tr>
<td>10</td>
<td>00000106</td>
<td><em>N. asteroides</em></td>
</tr>
<tr>
<td>11</td>
<td>00040100</td>
<td><em>N. asteroides</em></td>
</tr>
<tr>
<td>12</td>
<td>20000100</td>
<td><em>N. asteroides</em></td>
</tr>
<tr>
<td>13</td>
<td>00000104</td>
<td><em>N. asteroides</em></td>
</tr>
<tr>
<td>14</td>
<td>31014100</td>
<td><em>N. brasiliensis</em></td>
</tr>
<tr>
<td>15</td>
<td>31414100</td>
<td><em>N. brasiliensis</em></td>
</tr>
<tr>
<td>16</td>
<td>20000401</td>
<td><em>N. asteroides</em></td>
</tr>
<tr>
<td>17</td>
<td>20000100</td>
<td><em>N. asteroides</em></td>
</tr>
<tr>
<td>18</td>
<td>10000204</td>
<td><em>N. farcinica</em></td>
</tr>
<tr>
<td>19</td>
<td>71414140</td>
<td><em>N. brasiliensis</em></td>
</tr>
<tr>
<td>20</td>
<td>00040100</td>
<td><em>N. asteroides</em></td>
</tr>
</tbody>
</table>
Table 2. List of the species with the highest similarity score species obtained by using Codon Code Aligner software applied to the 16S rRNA GenBank database for 20 clinical presumptive Nocardia isolates from TB suspects in Northern, Tanzania

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Species</th>
<th>% similarity identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>IsoN.04</td>
<td>Streptomyces cacaoi subsp cacaoi</td>
<td>99</td>
</tr>
<tr>
<td>IsoN.014</td>
<td>Nocardia farcinica</td>
<td>99</td>
</tr>
<tr>
<td>IsoN.064</td>
<td>S. cacaoi subsp cacaoi</td>
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<td>IsoN.177</td>
<td>N. cyriacigeorgica</td>
<td>99</td>
</tr>
<tr>
<td>IsoN.200</td>
<td>N. asteroides</td>
<td>99</td>
</tr>
<tr>
<td>IsoN.205</td>
<td>N. farcinica</td>
<td>96</td>
</tr>
<tr>
<td>IsoN.208</td>
<td>N. flavoforsea</td>
<td>99</td>
</tr>
<tr>
<td>IsoN.233</td>
<td>N. farcinica</td>
<td>99</td>
</tr>
<tr>
<td>IsoN.239</td>
<td>N. cyriacigeorgica</td>
<td>99</td>
</tr>
<tr>
<td>IsoN.240</td>
<td>N. nova</td>
<td>99</td>
</tr>
<tr>
<td>IsoN.260</td>
<td>N. carnea</td>
<td>99</td>
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<tr>
<td>IsoN.281</td>
<td>N. farcinica</td>
<td>99</td>
</tr>
<tr>
<td>IsoN.290</td>
<td>N. cyriacigeorgica</td>
<td>99</td>
</tr>
<tr>
<td>IsoN.300</td>
<td>N. cyriacigeorgica</td>
<td>100</td>
</tr>
<tr>
<td>IsoN.324</td>
<td>N. testacea</td>
<td>99</td>
</tr>
<tr>
<td>IsoN.329</td>
<td>N. testacea</td>
<td>99</td>
</tr>
<tr>
<td>IsoN.334</td>
<td>N. brevicatena</td>
<td>99</td>
</tr>
<tr>
<td>IsoN.342</td>
<td>N. flavorosa</td>
<td>99</td>
</tr>
<tr>
<td>IsoN.350</td>
<td>N. cyriacigeorgica</td>
<td>100</td>
</tr>
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</table>

IsoN; Isolate number

Table 3. Clinical data of the 17 patients with Nocardia infections as confirmed by the 16S rRNA gene sequencing

<table>
<thead>
<tr>
<th></th>
<th>N. cyriacigeorgica (n = 5)</th>
<th>N. farcinica (n = 4)</th>
<th>N. testacea (n ≥ 2)</th>
<th>N. flavoforsea (n = 2)</th>
<th>N. nova (n = 1)</th>
<th>N. asteroides (n = 1)</th>
<th>N. carnea (n =1)</th>
<th>N. brevicatena (n = 1)</th>
<th>Total</th>
<th>N (%)</th>
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<tbody>
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<td>Age group:</td>
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<td></td>
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<td></td>
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</tr>
<tr>
<td>≤ 35</td>
<td>2</td>
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<td>2</td>
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<td>1</td>
<td>7</td>
<td>(41.2)</td>
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<tr>
<td>&gt; 35</td>
<td>3</td>
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<td>0</td>
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<td>1</td>
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<td>10</td>
<td>(58.8)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>0</td>
<td>8</td>
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<td>0</td>
<td>1</td>
<td>1</td>
<td>9</td>
<td>(52.9)</td>
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<td>Clinical symptoms:</td>
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<td></td>
<td></td>
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<tr>
<td>Chronic cough ≥ 2 weeks</td>
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<td>4</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>17</td>
<td>(100)</td>
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<td>1</td>
<td>10</td>
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<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>11</td>
<td>(64.7)</td>
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<td>1</td>
<td>1</td>
<td>14</td>
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<td>2</td>
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<td>1</td>
<td>1</td>
<td>11</td>
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<td>Concurrent conditions</td>
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<td>0</td>
<td>5</td>
<td>(29.4)</td>
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<tr>
<td>TB-Infeciton</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>(11.7)</td>
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<td>0</td>
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<td>0</td>
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<tr>
<td>Nocardia alone</td>
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<td>2</td>
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<td>1</td>
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<td>1</td>
<td>12</td>
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<tr>
<td>Smear Microscopy</td>
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<td>AFB positive</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>(11.7)</td>
<td></td>
</tr>
<tr>
<td>AFB negative</td>
<td>5</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>15</td>
<td>(88.2)</td>
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<td>Treatment initiated</td>
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<td></td>
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<tr>
<td>Anti-TB drugs</td>
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<td>0</td>
<td>0</td>
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<td>1</td>
<td>0</td>
<td>1</td>
<td>(5.9)</td>
<td></td>
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<td>Broad-spectrum antibiotics</td>
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<td>1</td>
<td>1</td>
<td>0</td>
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Results and Discussion

References
Summary

Tanzania is one of the countries with the highest burden of tuberculosis (TB) in sub-Saharan Africa, ranked 14th among the 22 high-burden countries. The last two decades in developing countries like Tanzania are marked by enormous socio-economic differences. These differences are reflected in the increased gap between the poor and the rich, lack of access to primary health care services, rapid population growth, as well as rural-urban migration. Consequently, this has negatively influenced the endemic diseases like TB and HIV/AIDS pandemic.

Early detection of TB has great potential to improve individual outcomes and reduce transmission within communities. Although improved diagnostics has potential to increase TB detection in patients with symptoms and signs of active TB, many patients, however, have few symptoms that fulfil definitions of suspected TB, or cannot reach the relevant health services because of barriers to access. Passive case finding is the mainstay of case finding in most developing countries. This relies largely on sputum examination by smear microscopy, with culture only performed at reference centres for patients failing therapy of anti-TB drugs. Tanzania continues to rely on passive case finding using clinical and radiological (where available) algorithm with the assistance of less sensitive smear microscopy for detection and identification of TB patients. In most cases, this has provided the unrealistic status of the actual prevalence of MTB among suspected TB patients.

Moreover, different species, strain families, and lineages of MTC are known to have differences in virulence, clinical presentation as well as transmission potential. However, information on the different spoligotype families of MTC in Tanzania is limited, where available is restricted to small geographical settings. The absence of current data on the genetic structure of MTC limits identification of genotypes as well as understanding the transmission dynamics among patients who remain undetected by conventional methods. Although notification of NTM disease is not required in many resource-poor countries, reports indicate that the prevalence of NTM diseases is increasing. NTMs play a great role in TB-like disease than was previously thought since the habitats occupied by NTM (e.g. drinking water) are also shared by humans. Furthermore, the number of individuals with aggravated susceptibility to mycobacterial disease is also increasing (e.g. through immunosuppression) especially where the prevalence of HIV infection is high. Despite
evidence of isolation of NTM especially among HIV-infected individuals in Tanzania, efforts have not been made to establish the actual prevalence and clinical significance of NTM infections. Data on clinical infections due to NTM in Tanzania is limited.

NTM infections can confuse the diagnosis of TB, as the tools for culture and identification of mycobacteria are often not available in these settings. Incomplete identification of mycobacterial species causing infections may have serious consequences, such as longer hospitalization time, the risk of co-infections as well as a selection of MDR strains. Clinical management approaches of patients diagnosed with MTC or NTM infections are different, therefore, proper and timely identification of mycobacterial species causing infections is likely to affect antimicrobial therapy. This trend, combined with antibiotic resistance will continue to create havoc and difficulties in the management of mycobacterial infections. The prevalence of diseases caused by NTM will continue to increase in many resource-poor settings like Tanzania unless guidelines are set in place to diagnose, report and manage NTM infections.

The contribution of other potential pathogens known to cause TB-like symptoms such as pulmonary nocardiosis in many resource-poor settings like Tanzania remains unknown, largely due to insufficient diagnostic capabilities and overburdened with other diseases like malaria, TB, and HIV such that more focus from the health sector system, governments are paid to such other diseases. Several studies have shown that pulmonary nocardiosis which mimics pulmonary TB to be a common cause of human diseases in both developed and developing settings. Increased incidence of human nocardiosis may be attributed to the wide use of immunosuppressive drugs, improved diagnostic tests, and increased awareness among microbiologists and health professionals. Nonetheless, in many developing countries where other chronic lung diseases, particularly TB, are prevalent, Nocardia species are either missed or misidentified during diagnosis. Need for correct and rapid identification of MTB, anti-TB drug resistance and the prevalence of NTM and other pathogens causing TB-like symptoms among TB patients in Tanzania is urgent, as they, all constitute a clinical emergency.

Therefore, this research was conducted to bridge the information gaps urgently required in designing effective prevention and control strategies against tuberculosis and NTMs among TB suspects in Tanzania. Furthermore, it demonstrates the need to investigate the involvement of other potentially pathogenic bacteria characterized by TB-like symptoms that are seldom diagnosed in health facilities and hospitals.
Publication I: Molecular typing of *Mycobacterium tuberculosis* (MTB) has greatly enhanced the understanding of the population structure of MTB isolates and epidemiology of tuberculosis (TB). To characterize prevalent genotypes of MTB, microarrays-based spoligotyping and mycobacterial interspersed repetitive unit-variable number of tandem repeats (MIRU-VNTR) were applied on 80 isolates collected from primary health care facilities in Tanga, Northeastern Tanzania. A total of 18 distinct spoligotypes were identified. The lineages by order of their predominance were EAI and CAS families (26.25%, 21 isolates each), LAM family and T superfamily (10%, 8 isolates each), MANU family (3.75%, 3 isolates), Beijing family (2.5%, 2 isolates) and S family (1.25%, 1 isolate). Overall, sixteen (20%) strains could not be allocated to any lineage according to the SITVIT_WEB database. Results of major lineages prediction by CBN method suggest that 70% of TB infections in Tanga are due to the modern lineages, whereas 30% of TB infections is due to the ancestral lineages mainly of Indo-oceanic lineage. This suggests that modern MTB strains contribute significantly to TB infections in Tanga. The allelic diversity ($h$) for specific MIRU-VNTR loci showed a considerable variation ranging from 0.826 of VNTR locus 3192 to 0.141 of VNTR locus 2059. The allelic diversity for 11 loci (VNTR 3192, 2996, 2165, 960, 4052, 424, 4156, 2531, 1644, 802 and 3690) exceeded 0.6, indicating highly discriminatory power. Seven loci (VNTR 2163b, 2401, 1955, 577, 4348, 2687 and 580) showed moderate discrimination ($0.3 \leq h \leq 0.6$), and three loci (VNTR3007, 154 and 2059) were less polymorphic. Findings of this study suggest that the TB cases in Tanga might be caused by a diverse array of MTB strain families that may be indicative of a cosmopolitan population with frequent migration and travel. Microarray-based spoligotyping and MIRU-VNTR could be reliable tools in detecting different MTB genotypes in high burden settings.

Publication II: To determine the prevalence and risk factors associated with drug resistance tuberculosis (TB) at a facility-base level in Tanga, Tanzania. A total of 79 *Mycobacterium tuberculosis* (MTB) isolates collected from among 372 (312 new and 60 previously treated) TB suspects self-referred at four TB clinics during a cross-sectional study conducted from November 2012 to January 2013 were included. Drug susceptibility testing of the isolates to first-line anti-TB drugs was performed using BacT/Alert 3D system. Data on the patient's characteristics were obtained from a structured questionnaire administered to the patients who gave informed verbal consent. Unadjusted bivariate logistic regression analysis was performed to assess the risk factors.
for drug resistant-TB. The significance level was determined at \( P < 0.05 \). The overall proportions of any drug resistance and MDR-TB were 12.7\% and 6.3\% respectively. The prevalence of any drug resistance and MDR-TB among new cases were 11.4\% and 4.3\% respectively, whereas among previously treated cases were 22.2\% respectively. Previously treated patients were more likely to develop anti-TB drug resistance. There was no association between anti-TB drug resistances (including MDRTB) with the risk factors analysed. High proportions of anti-TB drug resistance among new and previously treated cases observed in this study suggest that additional efforts still need to be done in identifying individual cases at a facility-base level for improved TB control programmes and drug resistance survey should continuously be monitored in the country.

**Publication III:** To determine the frequency and diversity of nontuberculous mycobacteria (NTM) among TB suspects in Northeastern, Tanzania. A cross-sectional study was conducted from November 2012 through January 2013. Seven hundred and forty-four sputum samples collected from 372 TB suspects were included in the study. Detection of NTM was done by using phenotypic, and Geno-Type® Mycobacterium CM/AS kits, additionally 16S rRNA and hsp65 gene sequencing was performed for isolates not identified by Hain kits. A binary regression model was used to analyse the predictors of NTM detection. The prevalence of NTM was 9.7\% of the mycobacterial isolates. Out of 36 patients with confirmed NTM infection, 12 were HIV infected with HIV being a significant predictor of NTM detection (\( P < 0.001 \)). Co-infection with *Mycobacterium tuberculosis* (*M. tb*) was found in five patients. Twenty-eight NTM isolates were identified using Geno-Type® Mycobacterium CM/AS and eight isolates could not be identified. Identified species included *M. gordonae* and *M. interjectum* 6 (16.7\%), *M. intracelullare* 4 (11.1\%), *M. avium* spp. and *M. fortuitum* 2 (5.5\%), *M. kansasii*, *M. lentiflavum*, *M. simiae*, *M. celatum*, *M. marinum* 1 (2.8\%) each. Of isolates not identified to subspecies level, we identified *M. kumamotonense* (2), *M. intracellulare/kansasii*, *M. intermedium/triplex*, *M. acapulcensis/flavescens*, *M. stomatopaeiae*, *M. colombiense* and *M. terrae* complex (1) each using 16S rRNA sequencing. Additionally, hsp65 gene sequencing identified *M. kumamotonense*, *M. scrofulaceum/M. avium*, *M. avium*, *M. flavescens/novocastrense*, *M. kumamotonense/hiberniae*, *M. lentiflavum*, *M. colombiense/M. avium* and *M. kumamotonense/terrace/hiberniae* (1) each. Results of the 16S rRNA and hsp65 gene sequencing were concordant in three and discordant in five isolates not identified by
GenoType® Mycobacterium CM/AS. NTM infections may play a vital role in causing lung disease and impact management of TB in endemic settings. GenoType® Mycobacterium CM/AS represents a useful tool to identify clinical NTM infections. However, 16S rRNA gene sequencing should be thought for confirmatory diagnosis of the clinical isolates. Due to the complexity and inconsistency of NTM identification, we recommend a diagnosis of NTM infections be centralized by strengthening and setting up quality national and regional infrastructure.

**Publication V:** To understanding the role of NTM in diagnosis and management of TB in resource-poor settings a cross-sectional study was conducted at four peripheral diagnostic centres in Tanga, Tanzania. Two sputa (spot and one early morning) samples were collected for standard direct smear microscopy from 372 TB suspects. The culture was performed using BacT/Alert 3D system, Löwenstein-Jensen, and Gottsacker slopes. Identification of *M. tuberculosis* and NTM was done by using GenoType® MTBC and GenoType® CM/AS respectively. Eighty-one of the 372 (21.8%) patients were diagnosed as having *Mycobacterium tuberculosis* by the isolation of the organism from cultures of sputum. Further analysis of culture showed that 30/372 (8.1%) were NTM with 7/372 (1.9%) cases of NTM classified as PTB patients. ZN stain had a sensitivity of 68.8% and produced 10 false negative results. On the other hand, Fluorescence stain had a sensitivity of 85.7% and gave seven false negative samples when compared with culture results. Weight loss (p = 0.0001), fatigue (p = 0.003), fever (p = 0.038) and night sweats (p = 0.004), young population at the age of 18-40 years (p = 0.0352), males (p = 0.0025) were important risk factors for TB. Four out of 30 NTM diagnosed by culture received 1st line anti-TB treatment suggesting that a good proportion of patients (4/65, 6.2%) were mistreated as TB patients in Tanga. Inefficient screening of TB patients in resource-poor settings and a prevalent increase of NTM may contribute unrealistic TB cases. Need to integrate NTM diagnosis in the management of TB is urgently needed for designing effective tuberculosis prevention and control strategies in the country.

**Publication V:** In this study, we report for the first time the isolation and identification of *Nocardia* isolates recovered from TB suspects in Northeastern, Tanzania. Twenty patients diagnosed as having presumptively *Nocardia* infections based on microscopic and cultural characteristics were confirmed using biomèrieux ID 32C Yeast Identification
system and 16S rRNA gene specific primers for *Nocardiia* species and sequencing. Biochemically, the majority of the isolates were *N. asteroides* (n = 8, 40%), *N. brasiliensis* (n = 4, 20%), *N. farcinica* (n = 3, 15%), *N. nova* (n = 1, 5%). Other aerobic actinomycetales included *Streptomyces cyanescens* (n = 2, 10%), *Streptomyces griseus, Actinomadura madurae* each (n = 1, 5%). 16S rRNA sequencing identified five members of the species (29.4%) *N. cyriacigeorgica*, four (23.5%) *N. farcinica*, two (11.7%) *N. flavorosea* and *N. testacea* each, *N. asteroides, N. nova, N. carnea* and *N. brevicatena* one (5.9%) each. Our findings suggest that *Nocardia* species may be an important cause of pulmonary nocardiosis that is underdiagnosed or ignored. This underscore needs to consider pulmonary nocardiosis as a differential diagnosis when there is a failure of anti-TB therapy and as a possible cause of human infections.

In general, this dissertation has provided important information gaps that hinder the efforts to prevent and control TB disease in Tanzania. The study further shows that MTB is the predominant species of the MTC in Tanga and the CAS1_KILI spoligotype the predominant strain type. Moreover, the study has demonstrated that majority of TB infections in this area are due to the modern TB lineages. The study has also highlighted the need to conduct drug susceptibility testing of patients diagnosed at the health facility levels since a good proportion of self-presented TB suspects may have already been infected with resistant TB strains. The prevalent increase of NTM and pulmonary nocardiosis showed in this study has demonstrated that NTMs and *Nocardia* species may be potential causes of human disease in this population, therefore calling for the national control programmes to design an integrated preventive and control strategy urgently required to combat the disease in the country. Further research in determining the prevalence of other pathogens causing TB-like symptoms and the role of NTM and or *Nocardia* co-infections in the progression of TB disease is also required in strengthening national’s efforts to prevent and control the disease.
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Appendices

Annex I: Declaration of Authorship

I hereby declare that the work presented in this dissertation has been designed and performed independently, without help from others and without other materials than stated in the text. To the best of my knowledge and belief, thoughts and ideas from other people and colleagues that have been adopted directly or indirectly in this dissertation were specifically indicated and acknowledged in any case. I confirm that others did not either directly or indirectly receive any payment in kind for any work related to the content of this dissertation. This dissertation has never been submitted before for the award of any other degree or during any kind of examination procedure in any other institution.


Date

........................................

Signature
Annex II: Specimen request and reporting form for TB suspects in Muheza and Tanga, Tanzania study 2012/13

Patient Identification No: _________

Patient Name __________________________  Age (yr): ________ Sex: ___

Ethnicity: ___________________________ Occupation: ______________

Village/Ward: ________________________

Clinical signs and symptoms (Tick where appropriate)

1) Chronic cough (> 2 weeks): Yes:_______ No:______

2) Blood streaked sputum: Yes:____  No:________

3) Night sweat: Yes:____  No:______

4) Fatigue: Yes:_____ No:______

5) Fever: Yes:______ No:_______

6) Dyspnoea (Difficult breathing): Yes:_____ No:______

7) Unexpected weight loss: Yes:_______ No:____

Concurrent conditions

1) Tobacco smoking (previous or current): Yes:____  No:_____

2) Diabetes: Yes: _______ No: _____

3) *HIV-status: Positive/ Negative / Unknown (Tick where appropriate)

NB:

*Information that can be disclosed optionally.

Type of TB disease and history

New TB register No: ________________ Previous TB register No: ________________

MDR case No: _____________________

☐ Pulmonary TB: ________________  ☐ New case (never treated for ≥ 1 month)

☐ Relapse case

Treatment history:

☐ First-line drugs: _____________  ☐ Second-line (Mention if any) ____________

☐ Other treatment: _____________  ☐ Failure of treatment  ☐ MDR

Origin of request: Research (PhD study)

Laboratory performing investigation: __________________________

92
Type of specimen: [ ] Sputum
[ ] Sputum in preservative (Type of preservative if any):

_____________________

Date specimen was collected: _____/_____/___________
Specimen ID number: ___________

Local laboratory microscopy results:

[ ] Negative
[ ] Doubtful
[ ] 1+
[ ] 2+
[ ] 3+

Microscopy technique used (Tick where appropriate)

[ ] Hot Ziehl- Nelsen
[ ] Kinyoun (Cold staining)
[ ] Fluorescence
[ ] Direct smear
[ ] Concentrated smear

Person requesting examination:

Name: _____________________________
Signature: _________________________
Position: __________________________
**PERSONAL PARTICULARS**

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<td>April 2014 to present:</td>
<td>Ph.D. student, Faculty of Medicine, University of Leipzig, Germany</td>
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<td>2006 to 2008:</td>
<td>MSc in Molecular Biology, Katholieke Universitat Leuven, Belgium</td>
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<td>1998 to 2003:</td>
<td>Bachelor of Veterinary Medicine, Sokoine University of Agriculture, Morogoro, Tanzania</td>
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**SHORT TRAININGS**

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<td>October 2011 to March 2012:</td>
<td>German Language course, InterDaF inteDaf e.V am Herder-Institut der Universitat Leipzig, Germany</td>
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<tr>
<td>May 2006 to June 2006:</td>
<td>Diploma in Global Health, Tampere University, Finland</td>
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**EMPLOYMENTS AND WORK EXPERIENCE**

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<td>April to July 2016:</td>
<td>Scientific Assistant</td>
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<td>July 2011 to date:</td>
<td>Lecturer Veterinary Microbiology, Molecular Biology &amp; Biotechnology</td>
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<tr>
<td>July 2007 to June 2011:</td>
<td>Assistant Lecturer Veterinary Microbiology &amp; Molecular Biology</td>
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Appendices

December 2003 to June 2011: Tutorial Assistant Veterinary Microbiology | Department of Microbiology & Parasitology, Faculty of Veterinary Medicine, Sokoine University of Agriculture.

July - November 2003: Research Assistant Tick and Tick-borne Disease Project (TARP II)

SCHOLARSHIPS AND AWARDS

April 2012 to March 2016: German Academic Exchange Service (DAAD) scholarship to pursue Ph.D. degree at the University of Leipzig, Germany.

October 2011 to March 2012: German Academic Exchange Service (DAAD) Scholarship to pursue Germany Language Course at the InterDaF inteDaF e.V am Herder-Institut der Universität Leipzig

2006 to 2008: Flemish Interuniversity Council (VLIR) scholarship to pursue an Interuniversity Programme in Molecular Biology (IPMB) in Belgium

May- June 2006: The Finnish Medical Society Duodecim and University of Tampere Medical school Scholarship, to pursue a Diploma Course in Global Heath, Tampere, Finland

1998 to 2003: United Republic of Tanzania Government Scholarship to pursue a Bachelor degree in Veterinary Medicine, Sokoine University of Agriculture.

PUBLICATIONS AND SCIENTIFIC WRITINGS


Appendices


Other Published Works


SEMINARS | WORKSHOPS | CONFERENCES ATTENDED

12 March 2016: 17. Bad Lippspring Tuberculosetag Teilbereich Plenarvorträge | Bad Lippsringe

11 March 2016: Seminar Presentation on “Chronic cough in Low-Income countries” Do you think only on TB? Prof. Dr. Brigitte König/ Abubakar Hoza. Cepheid Symposium, Tuberculosis day | 33175 Bad Lippsringe

11 March 2016: Tuberculosis in Focus workshop | Bad Lippsringe

25 -26 June 2015: The 2nd Global Health Conference Faculty of Medicine, University of Barcelona | Barcelona, Spain.

17 June 2015: Good Scientific Practices workshop, Faculty of Medicine, University of Leipzig.

2 June 2015: Seminar Presentation on “Molecular characterization of Mycobacterium tuberculosis isolates from North-eastern, Tanzania” | FLI, Jena, Germany

25-26 September 2013: Citavi Reference Manager Workshop organized by University of Leipzig Main Library

12-13 April 2013: Academia Career Path International Workshop University of Leipzig

15 March 2013: Academic writing workshop organized by the Kompetenzschule, Research Academy Leipzig.

21 - 25 February 2011: Laboratory Biosafety course for Laboratory Technicians and Livestock field officers | Surveillance for diseases in Wild Birds populations workshop | ICE, SUA, Morogoro, Tanzania. Sponsored by Minnesota Centre of Excellence in Influenza Research Surveillance
2 to 27 August 2010: Organized “The 2nd Global Health Course 2010 in Tanzania” an International course held at Sokoine University of Agriculture that involved health specialists from Finland and Tanzania.


28th April 2016
Date

[Signature]
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Annex IV: Acknowledgements

A journey is smoother when you sail together. This dissertation is the result of a four years work whereby I had countless support and accompany of several people. It is a gracious attitude to have now the opportunity to express my gratitude to all of them.

First, it is with immense gratitude that I acknowledge the support and help of my supervisor Professor Dr. Brigitte König for guiding and supporting me over the years. Thank you for your patience, support, and guidance. Your constant enthusiasm and encouragement have always inspired me to work hard.

To my co-supervisor Dr. Sayoki G. Mfinanga, it gives me great pleasure to have worked with you. I cannot find words to express how grateful I am for the support you offered me. You always availed yourself for me when I was in need of your enormous experience and expertise in the field of TB in Tanzania. Your inputs and advice have not gone unnoticed.

My gratitude also goes to Dr. Vet. Irmgard Moser for accepting me to her lab at the Friedrich-Loeffler Institute in Jena, where I had an enormous exposure into Microarray-based spoligotyping and MIRU-VNTR typing. You entrenched an example of excellence as a supervisor, instructor, researcher, mentor, and above all a role model.

I am indebted to Ms. Elizabeth Kraftschek of the Department of Medical Microbiology and Epidemiology of Infectious Diseases, University of Leipzig for her technical support and cooperation during the whole period of my lab work. Let me assure you, Elisabeth, I have learnt a lot from you and I will always remain grateful for the opportunity I had to work with you in S3 lab.

To Dr. Joerg Beer, I consider it an honour to have worked with you even for the very short period you managed to impact a lot on me, even after your retirement you did not hesitate to come to the lab when I mostly needed your help especially with drug susceptibility testing of *M. tuberculosis*.

I am indebted to the technical support I got from Gesine Kauth and Uta Brommer of the Friedrich Loeffler Institute (FLI, Jena). Your tireless efforts to help me even amid your busy schedules will always be cherished.

I also want to acknowledge the staff at the Department of Medical Microbiology & Epidemiology of Infectious Disease, University of Leipzig and FLI in Jena for their cooperation, without their support life in Germany would not have been easy.
To my friends, Belay Tessema, Fantahun, Theodwros, Efunshile, Sabrina and Kerstin who have been there every step of the way, during my lab work in Leipzig and Jena. Thank you.

To my friends Gamal Wareth and Sobhy Kholaiif thank you for making my three months stay in Jena comfortable and enjoyable. Also to a friend that is very dear to my heart, Consolata R. Sulley, your support and encouragement have been truly priceless; thank you for everything.

In my opinion, doing a Ph.D. is a sacred task and this was definitely one of the best judgements of my career. Supplementary motivation and endurance for this research were provided externally through my involvement in several social activities. The different Germans and International groups whom we formed football teams have played a substantial role and provided me the opportunity to meet many good friends. I thank them all for having shared many experiences and thoughts with me throughout my four years in Leipzig.

I sincerely thank the German Academic Exchange Service (DAAD) for sponsoring my Ph.D. studies. My employer Sokoine University of Agriculture for granting me the opportunity to pursue my studies in Germany.

I would especially like to thank my amazing family for the affection, backing, and continual inspiration they have given me over the years. You are the salt of the earth, and I undoubtedly could not have done this without you. To my lovely children Hajrah, Harith and Haris for all the lonely times endured while I was away to school.

The chain of my gratitude would be definitely incomplete if I would forget to thank the first cause of this succession, hiring Aristotle's words, “The Prime Mover”. My sincere gratitude for inspiring and guiding this humble being.

Thank you, Allah the Almighty!