Conventional and molecular characterization of *Mycobacterium* spp. with special reference to nontuberculous mycobacteria isolated from animal, human and environmental samples

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By

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DECLARATION

I declare the thesis entitled, **"Conventional and molecular characterization of *Mycobacterium* spp. with special reference to nontuberculous mycobacteria isolated from animal, human and environmental samples, "** has been prepared by me under the supervision of Dr. Akshay Garg, Assistant Professor, School of Bioengineering and Biosciences, Lovely Professional University, Punjab, and co-supervision of Dr. Samir Das, Scientist, Division of Animal Health, Indian Council of Agricultural Research (ICAR) Research Complex for Northeastern Hill (NEH) Region, Meghalaya. The thesis submitted presents an original contribution of research.

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Certified that the thesis entitled **"Conventional and molecular characterization of *Mycobacterium* spp. with special reference to nontuberculous mycobacteria isolated from animal, human and environmental samples"** submitted by **Miss Esther Vise, Reg. No. 11211370**, was carried out under the supervision of Dr. Akshay Garg, Assistant Professor, School of Bioengineering and Biosciences, Lovely Professional University, Punjab, and co-supervision of Dr. Samir Das, Scientist, Division of Animal Health, Indian Council of Agricultural Research (ICAR) RC for Northeastern Hill (NEH) Region, Meghalaya. No part of this thesis has formed the basis for the award of any degree or fellowship previously.

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To,

Those afflicted by the disease

ABSTRACT

The work falls under the broad field of tuberculosis (TB) research where several members of the *Mycobacterium* genus have mainly been explored. Tuberculosis, caused by *Mycobacterium tuberculosis* still claims 14 lakh lives annually and remains the major cause of death among HIV patients (WHO, 2017). Major hurdles in controlling the disease is attributed to poverty, substance abuse like smoking, co-infection with Human immunodeficiency Virus (HIV) and emergence of multi drug-resistant TB (MDR-TB). Tuberculosis can also be caused by *M. bovis* in humans which cannot be distinguished clinically or radiologically from tuberculosis caused by *M. tuberculosis*. *M. bovis* is zoonotic in nature and causes tuberculosis in cattle which is known as bovine tuberculosis. *M. tuberculosis and M. bovis* belong to the *Mycobacterium tuberculosis* complex (MTBC) along with five other tuberculosis-causing strains. Other members of the genus apart from *M. leprae* are the Nontuberculous mycobacteria (NTM), which were previously believed to be harmless ubiquitous environmental bacteria but are now emerging as opportunistic pathogens. They cause infections in immunocompromised individuals and presents clinical manifestations similar to TB. Our study was aimed to explore the distribution of this entity from two locations of the Northeastern Region (NER), India. The two locations comprised of Khasi Hills of Meghalaya and Dimapur Region of Nagaland. The NER of India is rather isolated from the main land and data availability on scholarly articles of NTM is also rare. A ground level study on the presence and distribution of NTM mainly was required as rise in infections with NTM along with the existing burden of *M. tuberculosis* needs to be addressed. Designing of the study was done to obtain all cultivable mycobacteria from sources which could depict the nature of NTM distribution in the study sites.

In our study a total of 555 samples were collected from geographically dispersed sites in and around Khasi Hills of Meghalaya and the Dimapur district of Nagaland. Samples were collected throughout the year irrespective of the season from three different sources—human, animal and environment. Clinical source comprised of 121 samples from suspected tuberculosis patients, collected from Revised National Tuberculosis Control Programme (RNTCP) centre of Dimapur, Nagaland. Approval was obtained from the physician in charge, and a few samples (12) from Meghalaya state were voluntarily submitted to our laboratory from whom consent forms were also taken. From the animal sources, 220 samples were collected of which 207 raw milk samples were from small dairy herds. Thirty two (32) samples of individual cow and pooled sample were collected from Dimapur district of Nagaland, and 175 samples from the Khasi Hills of Meghalaya. Suspected tuberculous tissue and swabs of animals consisted of 13 samples, largely swine and two of dog. The sampling locations were selected on the basis of significant dairy farms present in the area and accessibility to the institute where the samples could be transferred in cold chain within 24 hours. Environmental sources comprised of a total 214 samples. Soil samples (136) were collected in pre-sterilised sampling vials. Water samples (78) were collected from sources which were representative of the water source utilized in the area for drinking, household chores, farming or for livestock. The samples were grown on egg-based Lowenstein Jensen (LJ)-glycerol, LJ-pyruvate media, agar-based Middlebrook media and Middlebrook 7H9 broth according to the requirement. Primary identification of the isolates was done by acid fast staining, followed by a series of identification PCR amplification tests with targeting genes like *hsp65, IS6110, rpoB, RD9, RD4, 500bp,*

16sRNA, and *rrl* gene was used for checking acquisition of base pair mutation for clarithromycin resistance. Biochemical and susceptibility test was also done for selected clinically relevant and unique isolates. Sequencing of specific genes ultimately identified all the species isolated in the study. The genes for PCR identification included the genus-specific *hsp65* and MTBC-specific IS6110, while differentiation of NTM and MTBC involved *hsp65*-PRA and largely through sequencing and differential PCR. Out of the total 69 (12.43%) isolates obtained in culture, 62 (11.17%) isolates were confirmed to belong to the genus *Mycobacterium* through molecular identification methods. A series of identification and differentiating PCR were employed for initial identification but sequencing and BLAST similarity search ultimately confirmed 38 (6.85%) isolates to be NTM, 24 (4.32%) as MTBC and 7 (1.26%) to be non-mycobacterial bacteria. Out of the 38 NTM, 28 were of animal origin, 02 from human and 08 from environmental source. Of the 24 MTBC, 23 were from human and 01 from bovine milk. The 24 MTBC isolates were differentiated as *M. tuberculosis* and *M. bovis* by differentiation PCR like RD9, RD4 and 500bp fragment PCR. All these three targeted regions identified the 24 MTBC as *M. tuberculosis*. The 38 NTM were speciated mostly by *hsp65* sequencing and less frequently by *hsp65*-PRA. Some of the NTM required additional *rpoB* sequencing for identification. Out of these 38 NTM, the 08 NTM belonged to environmental species which comprised a diverse range of species such as *M. immunogenum* (01), *M. pyrenivorans* (01), *M. saopaulense* (01), *M. chelonae* (04) and *M. fortuitum* (01). Out of the 02 NTM species from human, one was identified as the rare *M. novocastrense* and another could not be speciated which was presumed as novel. The 26 NTM from milk were speciated as *M. chelonae* (21), *M. lentiflavum* (03), *M. goodii* (01) and *M. peregrinum* (01). 02 NTM isolates of canine origin could not be speciated by these approaches and were also presumed as novel NTM strains. All these sequenced *hsp65* and *rpoB* genes were subjected to phylogenetic analysis in the freely available MEGA7 software and a total of 119 gene sequences from the study has been deposited to GeneBank NCBI. Our study obtained three isolates which have been presumed to be novel/unique species through multiple gene study involving three genes-*hsp65, rpoB* and *16sRNA*. Concatenated phylogenetic analysis was performed where all these isolates formed unique phyletic lines distinct from the reported species of mycobacteria thereby assigning to be unique.

A significant number of NTM have also been isolated from milk of which *M. chelonae* was the dominant species with 03 *M. lentiflavum* and 01 each of *M. peregrinum*, *M. goodii* and a *M. tuberculosis* isolation. These finding clearly depicts *M. chelonae* to be one of the dominant NTM in nature and correlates with the large number of human infections reported from the country. Mycobacterial isolation from milk indicates importance of hygienic standards in dairy farms from food safety point of view. Isolation of 03 very unique isolates which did not identify itself to any of the reported mycobacterial isolates shows the need for better equipped laboratories. To designate such isolates there is a need to perform intensive conventional, cultural, biochemical, molecular and proteomics profile of the isolate which cannot be attained if technical resources are limited. The isolation of rare and novel NTM from human cases in this region points to a possible underlying problem of NTM which could be larger than comprehended. NTM infections globally and in India are increasing and observations from a relatively small scale study like this shows the occurrence of both common and rare NTM species in the population of this region calling for careful observation during treatment of mycobacterial infections.

PREFACE

For the fulfilment of my doctoral degree in biotechnology, the thesis titled, "Conventional and molecular characterization of *Mycobacterium* spp. with special reference to nontuberculous mycobacteria isolated from animal, human and environmental samples", has been carried out from August 2012 to July 2017. This work is based on exploring the diversity of mycobacterial species, keenly the nontuberculous mycobacteria (NTM), known to be ubiquitous in the environment causing infections in animal and human alike. My research on mycobacteria began during my M.Phil dissertation, under the guidance of Dr. Akshay Garg who continued to mentor me through my doctoral programme. The dissertation was mainly based on NTM isolation from water sources. It was during this endeavour I realized their emerging potential as clinical and zoonotic entities, the gaps on clear understanding of their pathogenicity, and the lack of data from the Northeastern Region, where I belong. Both my supervisors are trained veterinary specialists and acquires fine background on mycobacterial research. Through their constant support, this study was carefully planned and formulated keeping in mind the geographical location and the type of studies that would hold significant to the region and the country at large.

I would always remain thankful to my supervisor Dr. Akshay Garg for extending all possible help and providing the opportunity to conduct my research in the best suited laboratory. The knowledge gained from all the scientists and specially my seniors Dr. Anuj Ahuja, Dr. Uttaran Bhattacharjee, Dr. Amarjit Karam, Dr. Jayanti Dutta Roy, Dr. Rajkumar Pegu, Dr. Khetbadem, Dr. Valreleiyn Siao and Dr. Aparajita Dawra from Division of Animal Health would always be appreciated and valued. I am thankful to Dr. Arnab Sen, HOD, Division of Animal Health, ICAR, Meghalaya, Dr. Neeta Raj Sharma, HOS, School of Biosciences and Bioengineering, LPU, Punjab, Dr. Dhami from Department of Academic affairs, LPU, Punjab, and Dr. Rekha from School of Research degree programme, LPU, Punjab, for sensibly managing my academic and administrative needs. I am obliged to my colleague Shivika, Parvinder and my dear friend Pooja who went out of their way to ensure my scholarship forms and official documents were always submitted on time. My sincere gratitude to Dr. Khaleto Sema and Dr. Panger Longchar from RNTCP unit, Dimapur, Dr. George Thira from District Hospital, Dimapur and Dr Michael Mawlong from Nazareth Hospital, Meghalaya, for providing access to clinical samples intended for the research. I will always cherish my closest companions George, Priyanka, Pooja, Tulsi and Amit who went through with me in the finest and unfavourable times making my journey meaningful. I am in debt and extend my earnest acknowledgement towards Dr. Samir Das, my co-supervisor and mentor for wisely overseeing all requirements through these years. A very kind-hearted researcher with infinite patience, coolest temperament, liked by all. My source of emotional support and pillars of strength, my family— Abba, Oja, Oya, Naro, Nungsang and Apotsa, without whom I would not have achieved my aspirations. Thank you all for the earnest Prayers and constant encouragement that brought out the best in me. With wishes of blessings, my sincere apologies also goes out to all who have not being credited here but extended their support directly or indirectly. My utmost gratefulness to The God Almighty for the divine intervention all through my academic endeavour, sending wonderful people along the journey…

Dated: 30th November 2017 Esther Vise

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CHAPTER 1

INTRODUCTION

Tuberculosis (TB), is an bacterial infectious disease caused by the bacillus *Mycobacterium tuberculosis* belonging to the *Mycobacterium tuberculosis* complex (MTBC) consisting of *M. africanum, M. bovis, M. canetti, M. tuberculosis, M. mungi, M. microti, M. pinnipedii, M. orygis* and *M. caprae* which are all known to show vast genetic similarities. *M. tuberculosis* primarily involves the lungs and is capable of affecting many other sites which is then called as extrapulmonary tuberculosis. The disease is mainly airborne where majority of the infections is caused by the bacteria expelled in the air by coughing. One third of the population is exposed to the bacilli but only a small portion (5-10%) of them develops the disease (Comstock et al., 1974) which is assigned as latent tuberculosis. TB is one of the major global health problem despite noteworthy socio economic development and advances in medical sciences. Tuberculosis stands as one of the top ten causes of death worldwide in 2015 (WHO report, 2016). Every year it causes ill health among lakhs of people impacting the economic and social growth. Globally between 2000 to 2015, around 490 lakh individuals were saved from succumbing to TB, but still in the year 2015, 104 lakh new TB cases were notified with 14 lakh deaths (WHO report, 2017).The report also estimates higher TB cases (59 lakh) and deaths (10.62 lakh) among men than women and children globally. In women, the disease is also high with male:female ratio as 1.6:1. There were around 35 lakh cases and 4.96 lakh TB deaths among women, as well as an estimated 10 lakh cases and 2.10 lakh deaths among children. Of the new 104 lakh TB cases, over 12 lakh were among Human immunodeficiency Virus (HIV) positive people and 4 lakhs mortality occurred due to TB among this group. Globally in 2015, an estimated 4.80 lakh developed multi drug resistant tuberculosis (MDR-TB) and 20% of those previously treated patients had MDR-TB (WHO report, 2017).

Globally India accounts for more TB cases (27%) annually than any other country. Out of the estimated global annual incidence of 104 lakh TB cases, 28 lakhs were estimated to be in India with a mortality of 4.8 lakh in 2015. The Revised National Tuberculosis Control Programme (RNTCP) noted wide variation from state to state in terms of proportionate reporting of TB patients from public and private sector. In India there was a 34% surge of TB cases in 2015 when 'missing cases' from private sector were recorded. In 2015, among the 28 lakh TB cases, 1.1 lakh were estimated to be co-infected with HIV and 1.3 lakh patients were notified to be suffering from MDR-TB (RNTCP report, 2017). MDR-TB is 100 times more pathogenic than TB and requires at least 18-24 months of chemotherapy, which is more expensive and often shows a high failure rate.

Tuberculosis can also be caused by *M. bovis* in humans which cannot be distinguished clinically or radiologically from tuberculosis caused by *M. tuberculosis*. *M. bovis* is a member of MTBC and basically causes tuberculosis in cattle which is known as bovine tuberculosis. The World Organization for Animal Health (OIE) considers it to be an important zoonotic disease with a socioeconomic and public health impact that affects the international trade of livestock and animal products (OIE, 2017). Gallagher and Jenkins (1998) reported that among the MTBC complex, 95% percent of the cases in human are caused by *M. tuberculosis* and, around 5% of all deaths from this disease were reported to be due to *M. bovis*. However globally, incidence of tuberculosis caused by *M. bovis* varies regionally and many counties lack the data. The true incidence of *M. bovis* infections are very likely to be underestimated as in most countries the cases are not reported or missed. Several developed countries have eradicated bovine TB with strong mandatory regulations.

The incidence of bovine tuberculosis comprises around 1% of all tuberculosis cases in developed countries and reactivation of latent infections or transmission of zoonotic tuberculosis from these infected individuals is also known to occur sporadically (de la Rua-Domenech, 2006).

M. bovis has a very wide host range and can infect almost all mammals with the highest incidence seen in the bovine population which acts as a principle reservoir (Olea-Popelka et al., 2017). Other species susceptible to *M. bovis* include cats, dogs, buffalo, bison, goats, pigs, deer, wild boars, badgers, possums etc. and infection caused in these organisms, including man are spill over hosts (Palmer et al 2012; Broughan et al., 2013; Brunton et al., 2017). Cattle-to-cattle transmission is thought to occur through inhalation of infected nuclei (Menzies and Neill, 2000). *M. bovis* infection is known to be a public health problem as transmission to humans can occur through ingestion of milk from infected cows. The pasteurization process of milk has controlled the spread of infection through this mode to a certain extend where raw milk intake is not practiced. Infection by *M. bovis* is also mostly observed in those living under close proximity with infected livestock (Ashford et al., 2001; Fritsche et al., 2004). Individuals working in slaughter houses and handling infected abattoirs are also at higher risk.

Eradication programs for bovine tuberculosis has been implemented in several countries which involves farm visits, inspection in abattoirs, controlled movement of herds, testing and culling of infected herds. However in countries like India, slaughtering of cows are prohibited due to religious sentiments, due to which the sick herds are isolated in separate sheds. Although vaccination is practiced in humans, treatment of these infected cattle are rarely attempted as it is expensive, requires long duration and care. India has the largest cattle population globally and ranks first in total milk production. With raw milk intake still practiced in many communities, milk borne mycobacterial species mainly *M. bovis* use it as a ploy for spread of disease.

The genus *Mycobacterium* has around 184 species as per the standing nomenclature list (www.bacterio.net/*Mycobacterium*) and the number increasing constantly. It is not only the members of MTBC that causes diseases in human. Mycobacterial infections other than those caused by MTBC are attributed to the larger group of the genus comprising the Nontuberculous Mycobacteria (NTM). These organisms grow naturally in the environment and are also occasionally responsible for infection in man, animal, birds, fishes and other animals as opportunistic pathogen. However, for a long time due to limited knowledge on identification and differentiation techniques, disease conditions caused by any NTM or any 'atypical' *Mycobacterium* species such as *M. avium* were named as tuberculosis if they were associated with granulomatous lesions and presence of acid-fast organisms was noted. *M. lepare* which causes leprosy is a non-cultivable bacteria of this remaining large group.

NTM are recovered from both natural and human-influenced environment for which they are also called as environmental mycobacteria. Important distinguishing features of NTM and *M. tuberculosis* includes lower virulence of NTM; human-to-human transmission of *M. tuberculosis* caused by inhaling bacilli-containing expectorated aerosol, while NTM infections are mainly acquired directly from the environment. Nevertheless, given these features and described asopportunistic in nature, these entities cannot be overlooked as large number of infections have been known to occur in previously healthy individuals who

do not present immunologic deficiencies usually associated with opportunistic infections (Kartalija et al., 2013) and recent literatures present cases both from immuno- and nonimmuno- compromised individuals (Narang and Narang, 2017).Environmental opportunistic mycobacteria are distinguished as slow growing mycobacteria (SGM) and rapidly growing mycobacteria (RGM);those which takes more than 7 days to form distinct colonies and those taking less than 7 days, respectively.

NTM are known to be ubiquitous in the environment with isolation from numerous sources such as water, soil, cigarettes, vegetables, animals, amoeba, bronchoscopes etc. (Falkinham, 2002). Exploring their wide adaptations to various habitats is crucial to understand and identify factors which influence the geographical distribution of these organisms. While several NTM are known to be intrinsically resistant to several drugs (Nash et al., 2006; Nessar et al., 2012) and disinfectants, they are also known to be relatively resistant to heavy metals (Taylor et al., 2000) with members of the *Mycobacterium avium intracellulare* complex (MAIC) like *M. intracellulare* and *M. avium* being highly resistant to mercury and cadmium. These members are also known to be hydrophobic which probably increases their persistence in water delivery systems (Hall-Stoodley and Lappin-Scott, 1998).

NTM causes clinical disease in humans after exposure which include pulmonary, cervical lymphadenitis, joint infections, cutaneous granulomas, or other disseminated infections (Brown-Elliott and Wallace, 2002). Although NTM infections are also observed in normal healthy individuals, most patients include those having predisposing lung conditions or are occupationally acquired. The era of acquired immunodeficiency syndrome (AIDS) epidemic saw the rise in NTM infections too with increased immunocompromised individuals. A number of human NTM infections and outbreaks have being pounding from India as well since the beginning of the century (Raman et al., 2000; Kalita et al., 2005; Lavania et al., 2008; Narang, 2008; Mendiratta et al., 2009; Shah et al., 2010; Gopinath et al., 2010; Narang et al., 2010; Shenai et al., 2010; Simons et al., 2011; Lai et al., 2014; Kannaiyan et al., 2015; Achra et al., 2016; Bhandari et al., 2016; Garg et al., 2016; Umrao et al., 2016; Kotwal et al., 2017). Most of the isolation of NTM from human being in India goes around *M. chelonae*, *M. fortuitum* and *M. absecessus*, *M. avium* complex (MAC) (Saravanakumar et al., 2000; Jesudason and Gladstone, 2005; Srinivasan et al., 2005; Chauhan et al., 2007; Verghese et al., 2014).

However, very limited reports of nontuberculous mycobacterial infections have been documented from North Eastern region of India (Kalita et al., 2005), which is considered remote in the country. While isolated case reports are available, the country lacks a concrete data on NTM infections involving large scale population studies and data from the animal sector owing to its zoonotic potential is also very scarce. In addition, nontuberculous infections are often undermined and many a times misdiagnosed as tuberculosis. In most resource-limited settings, TB diagnosis is done by a simple smear microscopic detection of the bacilli, however, a positive acid fast bacilli (AFB) smear or a slant showing culture growth does not always indicate infection by *M. tuberculosis*. Further investigation is absolutely essential to identify if the infection is caused by *M. tuberculosis* or other mycobacteria. Identification of mycobacteria upto species level is of diagnostic importance and several Polymerase Chain Reaction (PCR) assays targeting specific genes have been

reported for species identification of mycobacteria.The identification methods for mycobacteria in diagnostic centers and clinical laboratories have advanced over the recent past. Conventional identification approaches such as biochemical tests have been replaced by rapid and reliable molecular methods. However, while WHO approves rapid diagnosis of TB by molecular approach like Xpert® MTB/RIF assay (Cepheid, Sunnyvale USA) to be used for TB diagnosis and has laid down guidelines for TB treatment, such recommendations are not available to distinguish its closest entities like NTM and zoonotic TB which may manifests TB caused by *M. tuberculosis*.

Although *M. tuberculosis* and *M. leprae* are the best known pathogens from the genus, an increasing number of NTM are being constantly designated as human and animal pathogens making it crucial for identification and discrimination of the pathogenic ones from the usual contaminants or the saprophytes. Despite their clinical, zoonotic and public health significance, the identification of mycobacteria has always been challenging. This work has been planned to explore the overall presence of every cultivable mycobacteria from human, animal and their interacting environment so as to understand their distribution, pathogenic behaviour and diversity in different hosts and niches. Likewise, an objective was dedicated for mycobacterial isolation from three sources— human, animal and environment, with special interest given to isolating NTM. An approach for profiling the mycobacterial species from animals with special focus on raw cow milk was done to comprehend the presence of pathogenic MTBC and NTM in milk from food safety point of view. NTM infections are well established in industrialized countries and gradually increasing in developing or under-developed nation where *M. tuberculosis* is still the main pathogen. Increased number of reports of NTM infections including hospital outbreaks is being reported from India which could be due to higher awareness among the clinicians and researches, and improved diagnostic tools. For capturing the NTM cases from humans for assessing the ground reality of mycobacterial infection, we have targeted suspected TB and chronic cough patients to aid in understanding the overall presence of mycobacterial infections in this region. Conventional culture on solid media and staining method followed by series of molecular confirmation was the identification scheme employed in the study. In addition to attempts made for understanding the drug-resistance pattern of clinically important mycobacterial isolates, we have also tried to establish the simplest diagnostic approaches and tried combination of molecular techniques which could be adopted by majority of the resource-constrained laboratories. Simultaneously, keeping in mind the accuracy, rapidity and cost effectiveness, we employed the trending biotechnology tools to understand these emerging pathogenic forms.To explore any transmission which might occur at the human-animal-environment interface, the isolates from the three sources were correlated through speciation and phylogenetic analysis. In brief, the study has been aimed to identify the species circulating in the selected geographical sites and understand the possible threats to public and animal health posed by these agents.

CHAPTER 2

REVIEW OF LITERATURE

2.1. Mycobacteria

Mycobacteria are a group of non-motile organisms presenting straight or mildly curved rods which are at times branched depending on the growth stage. They are opportunistic pathogens or intracellular parasites with size ranging from 0.2 to 0.6 x 1.0 to 10 µm. Falling under the suborder of *Corynebacterineae* their cell walls possess a distinctive feature of containing very high lipid content made up of waxes with 60 to 90 carbon mycolic acids that makes up to 60% the cell wall (Minnikin, 1982). They respond abnormally to gram stain and are classified as 'acid-fast' due to their acid-alcohol resistance for which characteristic Ziehl-Neelsen staining is used for detection of the bacilli. The genus *Mycobacterium* comprises over 184 different species according to cataloguing of 'The List of Prokaryotic Names with a Standing in Nomenclature (LPSN)' (Parte, 2013). On the basis of clinical importance, these organisms are well classified into three main groups— strict pathogens, opportunistic pathogens and rare pathogens including saprophytes (Rastogi et al., 2001). The genus is best known by strict pathogens like *Mycobacterium tuberculosis* which causes tuberculosis (TB), and *M. leprae* including the currently included *M. lepromatosis* (Han et al., 2008), which are the pathogenic agent of leprosy (Hansen's Disease). Both these diseases are linked to severe impact on mankind with high loss of lives and morbidity. TB is endemic to mainly the developing countries leading to highest number of deaths due to an infectious disease (WHO, 2017). *M. leprae* is known to infect the Schwann cells in peripheral nerves causing nerve damage and disabilities. In 2016, 2.1 lakh new cases of leprosy was reported from 106 countries and considered as a country with "high burden for leprosy", India accounted for 60% of these new cases (WHO, 2016).

M. tuberculosis, including *M. bovis*, and other genetically related tuberculosis-causing species belongs to the *Mycobacterium tuberculosis* complex (MTBC) and the rest of the species, apart from *M. leprae*, comprises of opportunistic and saprophytic agents called the Nontuberculous Mycobacteria (NTM). Known to be ubiquitous in the environment they are also responsible for infection in human, animals, birds as opportunistic pathogens (Falkinham, 1996; Prevots et al., 2015). Mycobacteria are known to encompass a wide range of hosts. In animals, *M. bovis*, *M. avium* and *M. avium* subsp. *paratuberculosis* are the principle mycobacterial pathogens. *M. bovis* causes bovine tuberculosis and is known to be zoonotic in nature. *M. avium* and *M. paratuberculosis* are classified under opportunistic pathogens and *M. avium* is often associated with diseases in poultry and pigs, while *M. paratuberculosis* leads to Johne's disease in cattle. Considered to be third most common mycobacterial disease following leprosy and TB is the vector-borne Buruli Ulcer (BU) suggested to be linked between *M. ulcerans* and insect bite. NTM infections are commonly being reported from industrialized country and is increasingly reported from developing or under-developed nation where *M. tuberculosis* is the main pathogen. Infections by NTM appear to be higher in the developed countries than underdeveloped or developing country where the burden of tuberculosis by *M. tuberculosis* is large (Cassidy et al., 2009; Kendall et al., 2011). NTM causes clinical disease in humans after exposure which include pulmonary, cervical lymphadenitis, joint infections, cutaneous granulomas, or other disseminated infections (Wolinsky, 1979; Weber et al., 1989; Zaugg, M. et al., 1993; Saritsiri et al., 2006; Barry et al., 2011; Lai and Hsueh 2014; Halstrom et al., 2015).

2.2 *Mycobacterium tuberculosis* bacillus complex (MTBC)

Given the genetic relatedness of the tuberculosis causing species, nine species currently are classified as members of the MTBC. The human-adapted ecotypes include *M. tuberculosis, M. canetti* and *M. africanum* while animal-adapted ecotypes comprises *M. bovis, M. microti, M. caprae, M. orygis, M. pinnipedi,* and *M. mungi* (Yruela et al., 2016). *M. canetti* is considered to be an ancestral lineage from which the members of MTBC emerged. *M. tuberculosis* causes the highest number of tuberculosis in humans with *M. canetti* and *M. africanum* restricted to populations of the East- and West-African countries (de Jong et al., 2010; Supply et al., 2013). *M. microti* is known to be a rodent pathogen, *M. caprae* of goats and *M. bovis* an established pathogen in cattle with zoonotic risk to humans (de la Fuente et al., 2015). Given the economic burden and public health importance of human, zoonotic and bovine TB, they have been briefly discussed in the following sections.

2.2.1 Tuberculosis

Tuberculosis, a universal disease of man and animals is caused by an aerobic, acid fast, rod shaped '*Mycobacterium*' (Lehmann and Neumann, 1896). Only 5-10% exposed to *M. tuberculosis* in their lifetime have the risk of developing active tuberculosis in the following one to two years after suffering from primary tuberculosis infection (Flynn et al., 2001). The burden of latent tuberculosis is predicted to about 23% with approximately amounting to 1.7 billion individuals (Houben and Dodd, 2016). The usual presentation of tuberculosis by *M. tuberculosis* can also be observed in other sites of the body as in case of extrapulmonary tuberculosis (ETB) cases. Among notified tuberculosis cases in 2016, ETB amounted to 15% of these cases (WHO, 2017). There are also instances where an individual can suffer only from bone, stomach, genital TB with no involvement of the lung. After an individual inhales the TB bacilli from air, several possibilities may follow. A healthy person with strong immunity may destroy the bacteria and escape infection and when not immediately killed will get localised in the lymphnode. These calcifications are visible in radiographic images which assists clinicians to diagnosed the illness. Most often the infection gets localized but few develop active TB or latent TB due to reactivity (van Crevel et al., 2002). Of those exposed to the bacilli, approximately 90% of the individuals evades active infection and never suffers from the disease (Frieden et al., 2003). Manifestations of tuberculosis ranges from mild infiltration to severe destructive disease which depends upon status of the hosts immune health. Cellular immunity is important in tuberculosis and acquired immunodeficiencies mainly through human immunodeficiency virus (HIV) infection predisposes individuals dramatically. The advent of tuberculosis begins with ingestion of the bacilli and several events follows thereafter (Fig 1). If the alveolar macrophages do not destroy the bacilli a complex comprising a draining lymphnode and infiltrate develops. These becomes visible as tiny calcifications in X-ray imaging and purified protein derivative (PPD) skin test also shows positive. A latent infection may develop in later years if the infection is not contained at this stage under failing conditions of the immune system (van Crevel et al., 2002). There are several determined risk factors of tuberculosis which includes undernourishment (due to poverty), HIV infection, diabetes, smoking, harmful use of alcohol (Lönnroth et al., 2010). In 2016, out of the 104 lakh TB incidence globally, 19 lakh were attributed to undernourishment, 10 lakh to HIV infection, 8 lakhs each to smoking and diabetes. Evidences suggests a close

link of the disease to poverty. This deprivation of basic necessities is known to incite risky behaviors directly associated to major social, medical and habits (WHO, 2017). Due to better diagnostics, efforts put in for early detection, better follow ups, improvement in social conditions and hygienic standard, tuberculosis mortality in countries like Europe and USA has been controlled. However eliminating the disease in TB endemic countries like India is a herculean challenge. This year the government's plan to eliminate tuberculosis by 2025 in the country was presented during the "Union budget for 2017-2018" which comes as a bold objective (The Hindu, 2017).

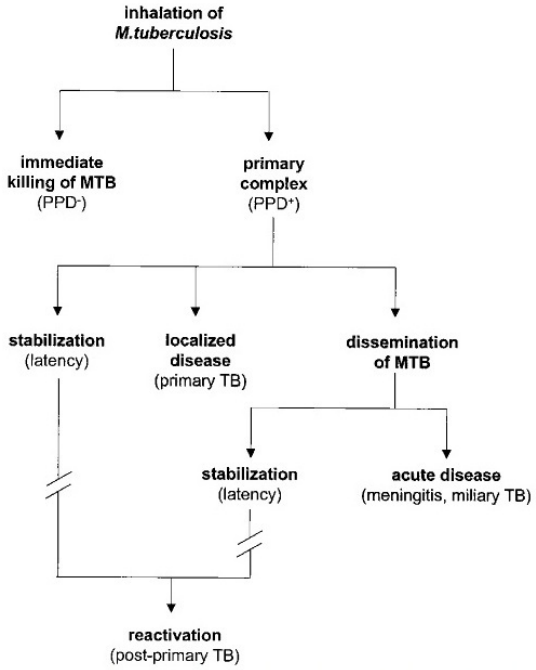


Fig 1. Sequential events following inhalation of *M. tuberculosis*. Adapted from van Crevel et al. (2002).

The Revised National Tuberculosis Control Programme (RNTCP) initiated by presenting a 'New National Strategic Plan (NSP) for tuberculosis elimination 2017-2025'(RNTCP, 2017) which if implemented is estimated to cost 2.5 billion USD (Coarasa and Pai, 2017). India treats over 1.5 patients in the public sector and the plan is expected to support millions from the private sector as well. It is estimated that in 2017 the total amount required for mainly diagnosis and treatment of drug-sensitive TB is 9.2 billion USD, while for multidrug resistant tuberculosis (MDR-TB) it is 2.0 billion USD. The average reduction in decline of TB per year in 2000-2016 was 1.4% per year and by 2020, management of the disease and also for TB research and development the amount is expected to rise (WHO, 2017).

2.2.2 Zoonotic tuberculosis

Zoonotic tuberculosis is chiefly caused by *M. bovis* leading to tuberculosis mainly in bovine animals which is the principle host and also affects other wild or domesticated animals. Human can be infected with "classic bovine strains" (*M. bovis*) where cattle act as a principal reservoir, which can transmit the infection to any of the mammalian species.

Development of tubercles in any body part is a characteristic feature of zoonotic TB caused by *M. bovis*. The clinical, radiological and pathological presentations of tuberculosis caused by *M. bovis* and *M. tuberculosis* cannot be distinguished (Grange et al., 1994). Further laboratory tests like biochemical and molecular identification is therefore required to distinguish these two entities. *M. bovis* is underreported from many countries as the methods to identify these pathogen is not easily accessible to resource constrained laboratories (Gallagher and Jenkins, 1998). In 2016, zoonotic TB was known to account for 1.49 lakh new zoonotic TB cases and 12500 deaths due to the disease (WHO, 2017). Region-wise distribution of the estimated incidence and mortality caused by tuberculosis due to zoonotic tuberculosis by *M. bovis* is available in the WHO, global TB report, 2017. *M. bovis* primarily infects cattle, which can transmit the agent to humans largely through the consumption of unpasteurized, contaminated dairy products (Cosivi et al., 1998). Majority of the milk borne mycobacterial infections are attributed to classical bovine strains of *M. bovis* which is being secreted into milk by infected cows, while *M. tuberculosis* is also known to be isolated from milk which usually is entered through human contamination (Srivastava et al., 2008; Ameni et al., 2013). Zoonotic tuberculosis is known to be associated with those in close proximity to livestock, such as farmers, veterinary surgeons and abattoir workers. Transmission between individuals have also been observed and documented (Robinson et al., 1988; Smith et al., 2004; Etchechoury et al., 2010; Evans, 2011; Ameni et al., 2013). However zoonotic tuberculosis caused by *M. bovis* is being underestimated in human population and several countries lacks accountable data (De Kantor et al., 2010; Perez-Lago et al., 2014; Olea-Popelka et al., 2017). It has been observed through comprehensive meta-analysis and systematic reviews of global zoonotic TB that we still face the same challenges and concerns that existed fifteen years earlier (Muller et al., 2013). However, Zoonotic potential of *M. tuberculosis* is well documented from various zoo, sentinel, non-human primates and wild animals (Furley, 1997; Oh et al., 2002). Even though human are the main reservoir of TB, animals can also be infected if they are exposed to *M. tuberculosis*, a phenomenon which is known as reverse zoonosis. Progressive form of TB by *M. tuberculosis* is not usually observed in animals but leads to sensitization. Other than cattle, pigs and other household livestock which are fed with wastes of human house dwellings can lead to tuberculosis infection in the food tract and closely located lymph nodes. Pet dogs, birds, non-human primates, can be infected with TB from their infected caretaker (Thoen et al., 2009). Zoo animals are prone to infection by TB as they are constantly interacting with large numbers of visitors and are exposed to stressful surrounding through continuous activities under enclosed structures. They also can be infected with the foods fed which is contaminated with the bacilli (Liu et al., 1980; Turinelli et al., 2004). Co-transmission is very common and likely when animals and humans live in closely situated or overlapping habitats. The bovine tuberculosis is seen more commonly in bovine population i.e, cattle and buffalo, although infection are seen in wild species such as llama, alpacas, wild boar and badgers are common, and other domestic animals like pigs and goat as well. Culture confirmation of feline tuberculosis caused by *M. bovis* is also documented (Roberts et al., 2014)

2.3 The Nontuberculous Mycobacteria (NTM)

Apart from causing major opportunistic infection largely in immunocompromised hosts, the NTM implies several other significance. NTM received its terminology from the

International Working Group on Mycobacterial Taxonomy. NTM comprises of those mycobacteria which are not members of the MTBC. They are also sometimes referred as "mycobacteria other than tuberculosis" (MOTT) (Wolinsky, 1979), atypical or potentially pathogenic environmental mycobacteria (PPEM). NTM were reported and have been recognized since its first description in 1868 which was later on described as *M. avium*. The first case of NTM in human was described in 1943 in a man suffering from pulmonary infection of the *M. avium* complex with underlying silicosis. Following this report by 1950s, several other reports became available on its infective nature. In 1979 presentation of such several cases in international conference in Denver and other hospital documentations provided as the proof for the basis of NTM research as a pathogen (Griffith et al., 2007; Kazda et al., 2009). With advancement in molecular based identification techniques like polymerase chain reaction, restriction digestion, sequencing, proteomic studies, over 184 species have been identified till date. NTM comprises both slow growers and rapid growers of which around 90 are pathogenic and clinically significant. Species like *M. intracellulare, M. ulcerans, M. marinum, M. ulcerans,* and complexes like the MAIC are important slow growers. While *M. abscessus, M. chelonae, M. fortuitum* are few of the important rapid growers.

2.3.1 NTM from Environment

The source of NTM infections appear to be environmental exposure either from the water or soil, but no definitive investigation have been reported on NTM transmission between human or from the environment. NTM have been isolated from various water sources including bottled water, drinking water pipelines, municipal water to households, hospitals, laboratories, shower heads, fresh, stored water, sea water, including ice and hot tubs (Falkinham, 2015; Halstrom et al., 2015). Due to their lipid-rich cell wall, these organisms form biofilms making them sturdy and are also known to be intrinsically capable of withstanding commonly used disinfectants such as chlorine (Lumb et al., 2004; Schreiber et al., 2016). Reduction in the load of other microorganisms in such treated water could aid in multiplication of NTM and possess infection risk to the users when the inoculum is high. Capable of surviving even when engulfed by protozoa and amoeba (Strahl et al., 2001; Drancourt, 2014), their hydrophobic nature helps them thrive in high water temperatures. Aerosolization of water bodies like running streams, or man-made structures like shower faucets, spa setting are also known to promote their existence (Briancesco et al., 2014; Soto-Giron et al., 2016). However NTM are seldom detected in groundwater. A wide variety of mycobacterial species are being isolated from environment, of which *M. avium* complex (MAC) and *M. fortuitum*, are commonly isolated in both developing and developed countries from varied water distribution systems (Gopinath et al. 2010; Thomson et al., 2013; Velayati et al., 2015; Donohue et al., 2015). *M. fortuitum* which is classified as a rapidly growing mycobacteria (RGM) shows visible colonies within a week. Apart from being observed largely in environmental samples from geographically dispersed sites, this species is also isolated from clinical settings causing infections in human, animals and fishes (Decostere et al., 2004; El Helou et al., 2013; Zhang et al., 2015). The persistence of the mycobacteria in bronchoscopes is undoubtedly caused by their resistance to disinfectants. Boreal coniferous forest soils are shown to have very high numbers of environmental opportunistic mycobacteria. The anaerobic river sediments including dusts have been found to be thriving with *M. avium* and *M. intracellulare* (Iivanainen et al.,1999).

Other mycobacteria were also recovered from water-damaged, moldy buildings and even in instruments like bronchoscopes and catheter. From the natural environment they are also known to enter the plumbing systems of household and also commonly thrives in shower heads (Falkinham et al., 2001, 2008). Although no prove exist in human-to-human transmission of this disease, due to their wide distributions, they can commonly infect almost all living beings.

Tuberculosis caused by *M. tuberculosis* complex is generally treated with multiple drugs for six months but most NTM infections are firstly, overlooked or misdiagnosed (Maiga et al., 2012; Shahraki et al., 2015) and secondly require different drug regimen and longer duration (18 months) than *M. tuberculosis* complex infection (Griffith et al., 2007). Global reports show *M. fortuitum* and *M. avium* complex is observed to be common isolates from the drinking water or household distribution systems and swimming pools in both developing and developed countries (Halstrom et al., 2015). Falkinham (2001) interestingly observed that the households with water heater temperatures ≤50°C were significantly more likely to harbour NTM compared to temperatures ≥55°C, and households whose water came from a public or private water system but hardly in those whose water source was underground. It is hypothesized that NTM infections are acquired through environmental water sources and household water outlets by inhaling aerosols containing mycobacteria (Marras et al., 2005). Several comparisons on various culturing methods of NTM has been reported and tested and hence one can easily follow any of the standardized protocol according to the sample types used and similarly with the culture media selection. This selection is important for isolation as the susceptibility to decontamination would vary with different geographical location. A gentle decontamination procedure documented by Schulze Robbecke et al. (1995) is a commonly employed protocol which involves cetylpyridinium chloride (CPC) and yields fairly high number and variety of mycobacteria from water.

In India, tuberculosis is endemic with few reporting of NTM from environment (Kamala et al., 1994; Bannalikar et al., 2003; Lavania et al., 2008; Narang et al., 2009). Parashar et al. (2004) through conventional and several molecular approaches isolated 119 environmental mycobacterial (water and soil samples) comprising nonpathogenic species, like *M. smegmatis, M. kansasii* and *M. terrae* and several other potentially pathogenic species such as *M. manitobense, M. chelonae, M. fortuitum, M. avium* and *M. marinum*. *M. gilvum* has been isolated from leprosy patients living in the leprosy endemic region from the stagnant drain water flowing through bathing area (Lavania et al., 2008). Kamala et al. (1994) studied environmental samples (soil, water and dust) from south India and could isolate 577 NTM isolates (58 different NTM species) from 717 samples. They also found *M. fortuitum* complex to be of highest isolation followed by *M. avium intracellure* complex. This study still remains an important documentation which was carried out from environmental as well as clinical samples from BCG trial area in south India. Both soil and water samples yielded a greater proportion of pathogenic mycobacteria than the non-pathogenic agents. The results from this study observed mycobacterial isolation profile for water resembling the profile for sputum samples in certain aspect. Isolation of NTM from both soil and water from the sites of people suffering from AIDS have been reported in India by Narang et al. (2009). A study in JALMA, India, in 2004 demonstrated isolation of NTM from environmental samples. Isolated samples included the common *M. fortuitum*

and *M. chelonae* from water and *M. kansasii, M. avium*, *M. terrae* from soil. In 2007, Narang et al., conducted a study in Sewagram, Wardha to correlate isolation of NTM isolated from clinical samples and from the environment samples of these patients. In this study, five isolates were recovered from water while 20 were isolated from soil samples, of which only one isolate of *M. avium* complex was the same isolate recovered from both the patient and the patient's environment. However typing of the isolate did not indicate the same strain.

2.3.2 NTM in animals

Apart from the pathogenic species of MTBC, some NTM are also important pathogens of man and animals. In animals, *M. avium* subsps. *paratuberculosis* (MAP) is the main infectious agent among the NTM causing chronic granulomatous enterocolitis in ruminants leading to chronic diahhroea and progressive weight loss and is usually fatal. MAP is sometimes known to be associated with human form of Crohn's diseases but debated to be zoonotic (Biet and Boschiroli, 2014). *M. kansasii* infections has been documented in calves (Houlihan, 2010) and *M. fortuitum* is also noted to be an infectious cause of mastitis, including *M. gordonae* and *M. vaccae* which have been observed in nodular tuberculous thelitis (inflammation of teats) (Biet and Boschiroli., 2014). Other infrequently NTM observed in animals are *M. lentiflavum, M. simiae, M. neoaurum, M. nonchromogenicum, M. terrae* etc. (Katale et al., 2014). *M. avium* is commonly isolated from pigs with lymphadenitis. This presents an economical burden, as such meats are considered inappropriate for consumption. Findings of the close genetic relatedness of *M. avium*, which was isolated from animal and human for comparative study indicates the risk of transmission either by shedding or by intake of poorly cooked meat (Johansen et al., 2007; Cvetnic et al., 2009). Detection of large number of *M. avium* from pig lymphnodes is also noteworthy as many production or slaughter houses have poor inspection for hygienic and quality meat (Muwonge et al., 2012). Occurrence of other pathogenic NTM like *M. chelonae* and *M. kansasii* from the surrounding areas of pig slaughter houses and even from the processed meat (Shitaye et al., 2009) indicates high risk to the consumers and a public health concern. The animal reservoir for MAIC being very large, mycobacterial infections including tuberculosis in companion animals poses a serious health risk to the in-contact animals and humans concerned. In India there have been a few reports of MAIC from human origin (Latawa et al., 2014) and ruminants (Singh et al., 2013), and infection by *M. avium* in dogs are also available in world literature (O'Toole et al., 2005; Haist et al., 2008). Infection by *M. tuberculosis* is rarely observed in cats as they are considered resistant to TB. However reports of *M. bovis* infection and *M. microti* have been observed (Gunn-Moore et al., 2014). While members of the *M. tuberculosis* complex are transmitted by direct host contact, MAIC species are acquired predominantly from environmental sources such as soil, water, dust, and even feed. Birds commonly suffer infections by *M. genavense*, mainly in pet birds, or *M. avium* subsp. *avium* in domestic birds (Evans, 2011). Other NTM such as *M. fortuitum*, *M. gordonae*, *M. nonchromogenicum* and *M. avium* subsp. *hominissuis* are also isolated from necropsied pet birds (Shitaye et al. 2009). Other mycobacterial species, particularly *M. marinum* found in the aquarium environments, may cause chronic diseases in fish and cutaneous infections in humans known as the 'fish tank granuloma'. *M. marinum* is commonly present in fish tanks and aquariums and is responsible for disseminated infections in fishes and its handlers (Slany et al., 2013). Apart

from this species, twenty other species have been reported comprising of main pathogens like *M. fortuitum* and *M. chelonae* (Phung et al., 2013). Reptiles can also get tuberculosis like other animals but generally is very uncommon (Hassl et al., 2004).

There have been isolations of many of these NTM from milk of which *M. avium* subsp *paratuberculosis* is the most common with other reported isolation of *M. chelonae, M. fortuitum, M. peregrinum, M. smegmatis, M. scrofulaceum, M. lentiflavum, M. mucogenicum, M. novocastrense, M. vaccae* etc. (Leite et al., 2003; Junior et al., 2009; Okura et al., 2012; Franco et al., 2013; Sgarioni et al., 2014; Bolaños et al., 2017). Mycobacteria can get access to milk through an infected animal mainly those suffering a tuberculosis mastitic udder or may get exposed from the animal handler (filthy hands, sputum of an infected individual) or from contaminated environment (unclean milk utensils, contaminated water, dust, manure etc.) (Alexander et al., 2002; Pavlik et al., 2005). It is necessary for detailed understanding of the flora of different mycobacterial species in raw milk and to identify the dominant species that might have potential role in transmission of mycobacterial infection to the consumer. Though pasteurization kills *M. tuberculosis* bacilli, there are known reports of other NTM such as *Mycobacterium avium* subsp. *paratuberculosis* surviving the pasteurization temperature (Shankar et al., 2010; Carvalho et al., 2012) and also species like *M. xenopi, M. phlei, M. chelonae* being more heat tolerant in milk (Schulze-Robbecke and Buchholtz, 1992) thereby exhibiting potential threat in transmission to humans. Many fermented milk products are directly made from raw milk and some of the mycobacterial species can survive this process also (Klanicova et al., 2012). Even though mycobacteria do not multiply in milk, if the initial load of these heat tolerant NTM are high, than there would be adequate chance for their survival upon pasteurization as well.

2.3.3 NTM in Humans

Nontuberculous infections is common in the developed world and increasing number of such NTM reports are being observed from the developing world where *M. tuberculosis* is the main pathogen. It has been observed that in US and South Korea, where the incidence of tuberculosis is low, NTM infection in human is exceeding *M. tuberculosis* complex infections (Cassidy et al., 2009; Kendall et al., 2011; Won-Jung Koh et al., 2013). As the incidence of *M.tuberculosis* fell, infection by these NTM became more increased and recognized. Through intensive literature search it has been observed repeatedly and reported that NTM infections have shown a drastic rise during later part of 1980s with AIDS epidemic coming up (Gopinath and Singh, 2010). NTM infections been increasingly reported since then and are now recognized as emerging opportunistic pathogen and is usually associated with person of weak immune system, chronic lung diseases and elderly infirm people. NTM causes clinical disease in humans after exposure which include pulmonary, cervical lymphadenitis, joint infections, cutaneous granulomas, or other disseminated infections. Given their ubiquitous nature in every possible environment they are known to infect almost every part of the body from skin to bones. The infection can be seen in the lymphatic system as bursa, lymphadenitis, lungs, skin, soft tissue, joints, tendon sheath, bones etc. (Falkinham 1996; Barry et al., 2011; Guglielmetti et al., 2015). *M. avium* complex (MAC) is reported to be the most common isolation from human (Chong et al., 2015; Sheu et al., 2015), following which the pathogenic *M. abscessus, M. intracellulare,*

M. chelonae and lesser pathogenic ones like *M. peregrinum, M. lentiflavum, M. kansasii and M. gordonae* are common isolations from human (Sawahata et al., 2010; Thomas et al., 2014; Chong et al., 2015; Sheu et al., 2015; Duan et al., 2016). Separated from the *M. chelonae* group is *M. abscessus* which is known to be one of the highly drug resistant entities among NTM (Brown-Elliott and Wallace, 2002). They are responsible for a wide range of diseases including disseminated infection or soft skin tissue infections mainly among immunocompromised individuals and compromised lung conditions (Griffith et al., 2007). Usually infections of NTM are non-transmissible but some zoonotic cases of NTM infection being transmitted from animal to man had been seen (Iyengar et al., 2013). An observation also arises that disseminated infection by NTM occurs when $CD4^+$ T lymphocyte count goes below 50/µl, due to which in countries where TB and HIV are endemic, AIDS patients succumb to the infection before their CD4 count falls low enough for NTM to cause infection. Several factors accounts for under- or non-reporting of NTM from mostly TB-endemic countries. While NTM infections are not reportable in any country, lack of awareness among physicians and clinicians added to weak laboratory conditions are most common reasons. Additionally, NTM often remain neglected due to the high burden of other mycobacterial infections like tuberculosis and several other communicable or non-communicable diseases diverts and draws larger attention and funds (Gopinath and Singh, 2010). Apart from immunocompromised individuals, NTM infections are also commonly observed in HIV negative and healthy individuals. Accidents or occupational activities have also been attributed to NTM infections. In Thailand skin and soft tissue infections were observed in HIV-negative farmers where exposure to contaminated environment was observed to be from open lesions of any injury sites during farming (Chetchotisakd et al., 2007). Veterinarians are more often infected when exposed to infected carcasses and individuals suffering injury during hobby exploration and recreational activities also acquire such infections. *M. marinum* is commonly present in fish tanks and aquariums and is responsible for disseminated infections in fishes and its handlers known as the 'fish tank granuloma' (Slany et al., 2013). NTM infections were also more commonly observed in patients with predisposing lung conditions or long exposure to dust or occupations related to asbestos or silica related work or with chronic lung diseases (Shelton, et al., 1999). Ethnicity is also considered by some researchers as one of the proposed reasons for NTM prevalence (Howell et al., 1997). In another study, low body mass index (BMI) has also been associated with pulmonary NTM infections with thinner, older women appearing more susceptible (Chan et al. 2010; Dirac et al., 2012). Pulmonary NTM infections have been noted in patients with lower sub-cutaneous fats as compared to the controls (Lee et al. 2014) and several other host traits and immune phenotypes have been assumed to make patients susceptible to NTM patients (Kartalija et al., 2013; Lake et al., 2016) (Fig. 2). However reports indicate NTM exposure to provide cross-protection against leprosy and tuberculosis as an adaptive immunity (Fine et al., 2001).

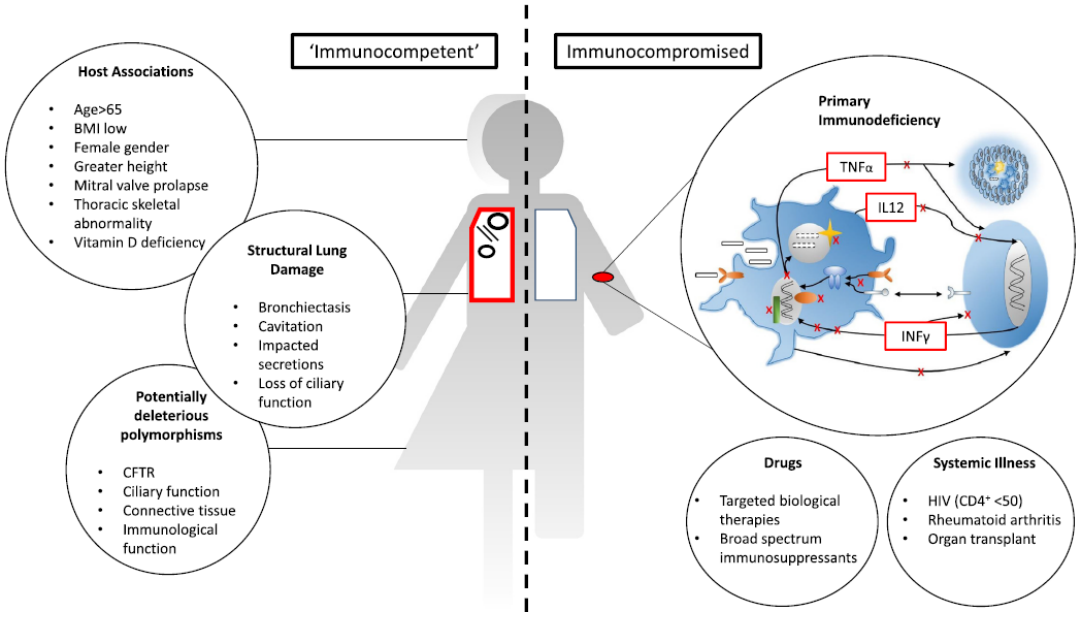


Fig 2. A representation of factors contributing to NTM infection in both immunocompromised and immunocompetent individuals. Adapted from Lake et al. (2016).

Global distribution

The global distribution of NTM species is diverse and true prevalence data of NTM infections is limited to few countries mainly associated with infections in healthcare settings and also surgical tourism. While NTM infection in AIDS patients are caused commonly by *M. avium*, infectious entity depend widely according to the geographical locations (Hoefsloot et al., 2013). *M. kansasii* is known to be a common pathogen in England and Wales but in Scotland, *M. malmoense* is known to be the dominating species.

In Asia isolation of varied NTM species has been observed which is different from the common isolations of Europe and North America. Wide scale studies from tertiary health care study from Japan, Taiwan and South Korea indicates an upsurge in pulmonary NTM infections. The most common isolations of NTM include *M. chelonae* and *M. fortuitum* in India and China, *M. fortuitum* and *M. kansasii* in Iran, *M. abscessus* in Saudi Arabia and MAC being the most common in South Korea, Taiwan, Hong Kong and Singapore (Lai and Hsueh, 2014). Outbreaks in hospital settings and communities have also been observed in the region. Cutaneous infection by *M. abscessus* following acupuncture and *M. chelonae* after tattoo inking has been noted in a wide community in Japan including USA and Europe (Song et al., 2006; Kennedy et al., 2012). Nosocomial infections involving contamination through water distribution system and through contamination of invasive instruments like bronchoscopes and catheters have also been reported (Raman et al., 2000; Simons et al., 2011). A wide scale literature survey of NTM epidemiology in Asia presents a data differing from the developed countries with a wide distribution of species within the region and also variation in antibiotic resistance pattern (Lai and Hsueh, 2014). Data from Iran on NTM prevalence have been reported through a large scale data review between 1992 to 2014. An upsurge of NTM prevalence in clinical settings from 8% in 1995 to 18% in 2014

was noted (Velayati et al., 2015). Between 1995 to 2005, the NTM infections have been known to rise in the European region covering Northern Ireland, England and Wales with a steep rise to 2.9% per 1 lakh population has been noted (Moore et al., 2010). Prevalence of NTM infections in Australia and North America is higher than the reported prevalence in Europe falling under 3.2 to 9.8 per 1 lakh population. The prevalence of pulmonary infections due to NTM is also known to be increasing from Australia, Europe and North America based on large population-based data (Hoefsloot et al., 2013). A sharp increase has also been observed in Tuscany, Italy between 2004 to 2014 with NTM isolates of *M. gordonae M. intracellulare* and *M. avium* and showing larger prevalence in people older than 60 years (Rindi et al., 2016). In selected TB endemic African Countries, the prevalence among presumed TB patients were as high as 4.2 to 15% and upto 18 to 20% among chronic MDR-TB patients (Prevots and Marras, 2015). While increase in AIDS patients is known to proportionally increase NTM infections, high cases of AIDS patients but very low NTM infections in Africa has been assumed to be due to deaths before NTM establishes in the host (Von Reyn et al.,1993).

The Indian scenario

There have been an increasing number of reports from India since the beginning of this century. However the true prevalence rate in the country is not deducible since the data available are mostly isolated cases and do not represent the data of the country as a whole. The increasing frequency of NTM reports from India may be due to the availability and accessibility of better techniques and improved laboratories and also well sensitized clinicians.

A large scale study from central India, became available recently where 4620 clinical samples from probable pulmonary and extrapulmonary tuberculosis (ETB) patients were processed for mycobacterial culture between 2013 to 2015. A total of 263 (29%) NTM isolates were obtained with 13 different mycobacterial species. Two of the most common isolates were *M. fortuitum* and *M. intracellulare* (Umrao et al., 2016). An earlier report from the same region was also reported where samples from suspected ETB were analyzed accounting to an isolation rate as high as 27.4% (Maurya et al., 2015). The most common species isolated were same in both the studies. A research based hospital in Mumbai analyzed 14,627 samples between 2005 to 2008 where 127 NTM were isolated. Of the total NTM isolation, 58 (46%) were definite NTM cases fulfilling the microbiological, radiological and clinical criteria, 26% were probable and 28% uncertain cases. The common etiological agents included MAC, *M. kansasii, M. chelonae, M. kansasii* and *M. haemophilium* from disseminated sites, *M. scrofulaceum* and *M. haemophilium* from lypmphandenitis, MAC, *M. chelonae*, MAC, *M. abscessus*, and *M. kansasii* from pulmonary site; *M. chelonae, M. abscessus, M. fortuitum, M. marinum, M. ulcerans* and *M. terrae* complex from extrapulmonary sites; (Shenai et al., 2010). MAC was a very common isolation in this study. In an earlier case of ETB, Jolly and Mohanty (1994) reported a case of *M. xenopi* causing renal tuberculosis. A study from CMC Vellore, South India still remains significant till date where between 1999- 2004 a large scale study was conducted involving 32,084 specimens received for culture. Among these, 4,473 (13.9%) were acid fast culture positive of which 173 (3.9%) belonged to the NTM group. Only 115 (66.5%) could be identified upto the species level showing the highest susceptibility (99.2%) to

amikacin. *M. chelonae* (46%) and *M. fortuitum* (41%) were the most common isolation from pus, biopsy and sputum specimens. Repeated isolations of NTM were observed from these patients with 6 isolations from AIDS patients and 5 in post-operative infections (Jesudason and Gladstone, 2005). Several post-operative wound infections, occupationally acquired NTM infections and hospital outbreaks have been reported from India and more are being continuously available some of which are discussed as follows.

Some of the earliest studies from India comes from Karnataka dating back to 1981 where 6,829 relapsed cases of pulmonary TB cases were examined and 13.7% were atypical mycobacteria isolated (Kotian et al., 1981). The study observed that NTM cases were higher in TB relapsed cases, some of which were suspected to be of zoonotic origin. Other early studies during the time include NTM infection from human cases with major isolation of *M. avium, M. fortuitum, M. chelonae*. A retrospective approach in nature, it was conducted at Tuberculosis Research Centre (TRC), Chennai, India, where out of 4,943 TB patients, 20 were NTM of which 10 patients died (Ramakrishnan et al., 1981). Shanker et al. (1989) analysed 4,554 sputum samples of 3,943 patients and obtained 604 (13.3%) TB positive culture from which 48 cultures (7.9%) were obtained. 47 were positive for NTM of which only 02 were considered as probable cases and in 45 patients, the bacteria appeared to be colonizers in the lungs without bringing out any disease.

Clinical isolates from a study in Guwahati from Northeastern India, by Kalita et al, (2005) were identified as *M. fortuitum-chelonae* complex from post-operative non-healing wound. One of the few reports from the region, this study indicated the occurrence of NTM infections in the area. Wound infections post-surgery and other invasive procedures seems to be commonly reported in the country as indicated from the literature with isolations of varying mycobacterial species (Kalita et al., 2005; Shah et al., 2010; Ghosh et al., 2017). Commonly isolated from the environment, *M. fortuitum* was reported by Wali et al. (1996) from an infection of the endometrium as a rare cause of infertility. Mohite et al. (2001) reported pulmonary infection of *M. fortuitum Chelonae* complex after bone marrow transplantation in an patient. Following laparoscopic inguinal herniorrhaphy another *M. chelonae* infection was reported by Sethi et al. (2003). Multiple infections after commercial renal transplantation in India, isolation of *M. fortuitum* has also been observed by Tomazic et al. (2006). Rajini et al. (2007) observed postoperative infection of laparoscopic surgery wound due to *M. chelonae. M. fortuitum* has also been isolated from a case of conjuctival necrosis of a human patient (Rao et al., 2013). In another patient, *M. chelonae* was isolated from a non-healing post-operative site causing chronic wound infection and abdominal incisional hernia (Verghese et al., 2014). Infections by *M. massiliense* leading to infection by pulmonary TB has been reported by Mitra et al. (2012), while Sadanandan et al. (2013) observed two cases of *M. massiliense* in cardiovascular infections. One of the patients had undergone aortic valve replacement with a porcine bioprosthetic valve from where NTM was reported. Chadha et al. (1998) reported an outbreak of post-surgical wound infections due to *M. abscessus*, a highly resistant species and frequently isolated in developed countries. Alexander et al. (2007) encountered *M. chelonae, M. fortuitum, M. abscessus* and *M. terrae* in renal transplant recipients.

NTM isolation from patients with failed Catergory-II TB treatment course has also been reported from the country with species isolations of *M. chelonae* and *M. fortuitum*

(Dholakia et al., 2015). Aggarwal et al. (2001) isolated two cultures of *M. fortuitum chelonae* complex out of 138 lymphadenitis patients in a study of peripheral tuberculous lymphadenitis. Several reports from NTM isolations from AIDS patients have been documented from India and *M. avium* complex (MAC) seem to be a comparatively frequent isolation. Shah et al. (2012) also isolated MAC from an AIDS patient. In another study among AIDS patients *M. avium* (6.6%) and *M. fortuitum* (5.7%) was isolated and among different species, isolates like *M. austroafricanum, M. flavescence, M. celatum, M. intracellulare M. duvalii M. phlei* and *M. terrae* were observed (Singh et al., 2007). Saravanakumar et al. (2000) reported NTM from leprosy and TB patients. Among leprosy patients and their contacts, very high isolation rate of 78% belonged to the species of *M. fortuitum* among the NTM isolations, while other species isolated were *M. smegmatis, M. scrofulaceum, M. kansasii* and MAC.

M. marinum infections has been seen in humans from eastern part of India involving mycobacteriosis-like infections on the hands of fish handlers, majority male (Ajithkumar et al., 2010; Sengupta et al., 2012). Several other cutaneous infections in human have been reported to be caused *M. fortuitum* in the country (Fogla et al., 2003; Sarma and Thakur, 2008). Mukhopadhya et al. (2003) reported perianal fistulae caused by *M. fortuitum*. Muthusami et al. (2004) reported *M. fortuitum* infection associated with chronic soft tissue from 23 patients over a 12-year period from 1991 to 2002. Even non-immunocompromised individuals are known to be infected by these pathogens and in India, Sivasankari et al. (2006) reported NTM infections among HIV seronegative patients in Pondicherry. Even ocular infections (Srinivasan et al., 2005), spinal tuberculosis due to disseminative infection of NTM (Duttaroy et al., 2004), drug resistant NTM (Joseph and Shah 2014) have been noted in the country including nosocomial outbreak of NTM infection in sites of laparoscopic surgery (Vijayaraghavan et al., 2006). Hospital outbreak due to *M. abscessus*, known to be an "antibiotic nightmare", was also reported from a pediatrics unit due to contamination in the water distribution (Raman et al., 2000).

Species diversity among the NTM isolations within India itself are variable and with the increasing members of the genus, species identification becomes crucial for diagnosis and treatment. Huge number of reports from India illustrates the potential of NTM infections in susceptible individuals and calls for cautious diagnosis and treatment especially while managing tuberculosis cases in this TB laden country.

2.4. Identification, differentiation and speciation of Mycobacteria: Diagnostics

Various identification approaches from conventional, molecular, hybridization techniques to proteomic methods have been described in the following sections along with their advantages and disadvantages keeping in mind the rural and modestly funded laboratories.

2.4.1. Solid culture followed by biochemical test- Conventional method

Smear microscopy is still widely used in all RNTCP centre and rural set-ups for detection of mycobacteria in sputum or any other specimen. The conventional method of identifying mycobacteria by acidfast staining and culture growth on media along with biochemical tests is still considered as a gold standard method. Fluorescent microscopy staining by auramine staining is also employed due to higher sensitivity (Davis et al., 2013). Both NTM and

MTBC members however have equal chances of detection by microscopy. Mycobacterial isolation by culture is necessary to establish a disease which can be supplemented with histopathology and other clinical presentations such as signs, symptoms, radiological scans, cytolopathology, smear microscopy, personal or family history and antibiotic susceptibility (Duan et al., 2016). Specific media containing malachite green like the widely used egg-based Lowenstein Jensen (LJ) and agar-based media such as Middlebrook are used for isolation (Metchock et al., 1999). Culture of mycobacteria is labour intensive and involves high biosafety risk, due to which many laboratories no not opt for *Mycobacterium* culture. Growth temp (25, 37, and 45°C), growth time required to form mature colonies are also factors required to be checked which are tedious and error prone. A series of biochemical tests are involved for differentiating the species viz. Arylsulfatase test, Catalase test (68°C), Detection of Pyrazinamidase activity, Iron uptake, Niacin test, Nitrate reduction test, Sodium Chloride Tolerance, Susceptibility to thiophen-2-carboxylic acid hydrazide (T2CH), Tellurite reduction test. Nocardia could also be mistaken for mycobacteria during laboratory diagnosis. They are capable of growing in specific media used for mycobacterial grown and are known to be partially acid-fast and display features similar to rapidly growing mycobacteria like beaded branching. These properties present confusion while conventionally differentiating the two (Muricy et al., 2014). The long incubation period, safety issues and ambiguous results in the biochemical tests results make conventional methods a less favourable approach.

The genus *Mycobacterium* comprises a noncultivable species (*Mycobacterium leprae*), fast-growing species (eg. *M. fortuitum, M. chelonae, M. abscessus*) which shows visible colonies in less than seven days of culture and slow-growing species giving visible colonies in more than seven days of culture, or in routine practice, between 3 and 8 weeks of culture. Solid media requires prolonged incubation but provides an inexpensive approach. The importance of skilled laboratory technician and experienced staff cannot be ignored. Contamination rates have been noted to be high when inexperienced and untrained staffs have used these culture systems. Culture-based detection of mycobacteria in samples is time consuming and can be subject to overgrowth by non-mycobacterial bacteria. Colony morphology characteristics can be used to distinguish between NTM and MTBC members who however would require skilled observation. Pigmentation (dark and photoinduction conditions), growth temperatures (25, 37, and 45°C), growth time required to form mature colonies are also factors required to be checked which are tedious and error prone. These factors could provide a presumptive idea for the mycobacterial species only for highly experienced technician which may be limited to a minor group only. Isolation is also cumbersome and require considerable biosafety preparation followed by other tests, subculture, DNA extraction, a series of biochemical test for identifying species for more than 184 species which is a real challenging job and only reference laboratory of national repute can opt for such facilities. Further, though the number of new species has been increasing over the years, database of biochemical speciation has not been updated for years which increases the difficulty level further (Hall et al., 2006). Conventional approaches are highly time consuming and consequently several researchers prefer the more rapid and reliable molecular methods like PCR and sequencing.

Detection and differentiation of M. tuberculosis and M. bovis

At least eight different cultural and biochemical tests are used extensively for differentiation of the two isolates from each other and some of these important tests have also been included in WHO guidelines for differentiation and speciation (Grange et al., 1996). Selected discriminatory phenotypic characteristics of *M. tuberculosis* and *M. bovis* are used which may include colony morphology, growth in presence of T2CH and biochemical tests like Pyrazinamidase, Nitrate reduction, Niacin accumulation and change in colour of bromocresol medium (Niemann *et. al.*, 2000).

2.4.2. Instrumentation based culture and detection

Various companies have come up with rapid automatic systems for identification of mycobacteria like BACTEC 460 TB radiometric system and SeptiChek AFB biphasic system which are both products of BD systems, Cockeysville, Md., but although known to be rapid, these two instruments requires a large investment. Moreover, the BACTEC 460 equipment leaves behind large amount of radioactive waste and poses high risk to the technician performing the experiment for needle punctures and compromises other safety issues. The newer model, BD BACTEC™ MGIT™, was developed to avoid these challenges based on quenching of fluorescence and is able to perform antibiotic susceptibility tests as well. Other liquid based instruments for culture also include VersaTREK system (Trek Diagnostics, Cleveland) and MB BacT (bioMérieux, France). Another rapid diagnostic equipment for direct culture testing by nucleic acid amplification is the catridge based Gene-Xpert, which is a technique recommended by WHO for early detection of tuberculosis and drug resistance (WHO, 2017). Though detection of *M. tuberculosis* is relatively rapid, NTM identification is limited in these instruments as differentiation between the two is not possible. Apart from confirmation of the disease and presumptive idea of mycobacterial origin, solid culture provide additional information through colony morphology, growth of time-pattern and limit the confusion of mixed culture. However, it is also crucial that isolation of NTM during mycobacterial culture needs to be clinically correlated and adjuncts of rapid culture and detection techniques are recommended when financial assistance are accessible (Griffith et al., 2007).

2.4.3. Liquid culture followed by rapid immunochromatogenic techniques/ DNA probe or DNA hybridization assay

Some reliable immunochromatogenic techniques based on *mpb64* gene have been developed and kits are available, like the capilia Tb-Neo kit (TAUNS, Japan) and the more popular SD Bioline test kit (Standard Diagnostics, Korea). These kits provide a very rapid and easy approach to identify *M. tuberculosis* depending on the detection of identification bands in the strip provided which would show coloured bands depending on the binding or non-binding of the target antigen from the specimen (Arora et al., 2015; Ramos et al., 2016). Commercially available rapid liquid-based culture systems can be directly used as adjunct to this kit based immunochromatogenic identification. The rapid instrumentation based culture system or solid/liquid culture confirming the isolate to be of *Mycobacterium* genus can be used in conjunction with MPT64 rapid test to detect the presence of MTBC and negative MTBC test can be tagged as NTM if acid fast staining and other previous culture technique confirm them for genus *Mycobacterium*.

Line Probe assay (LPA) is another molecular based technique which permits rapid detection of *M. tuberculosis* and clinically important NTM like *M. avium, M. intracellulare* and common contaminant in sputum such as, *M. kansasii* and *M. malmoense*. The initial method to proceed include a quick protocol on DNA extraction directly from the sputum. Following the amplification of this gene, the amplicons are hybridized into nitrocellulose membranes after which species identification is done using different probes. This method is a highly recommended approach even endorsed by WHO for *M.tuberculosis* identification.

2.4.4. PCR-based Approaches

The limitations of conventional methods and high end instrumentation based methods for identification of *Mycobacterium* spp. eventually demands for search of other techniques and molecular based approaches, specifically nucleic acid based amplifications, shows their potential to overcome this drawback. The amplification based methods are not only sensitive and specific but in due course of time is getting cost effective. Several laboratories have designed PCR based approaches for identification and differentiation of MTBC and NTM from clinical samples. Techniques such as multiplex PCR approaches involving amplification of multiple genes in a single tube or multiple primers for a single gene as in duplex PCR have largely been developed to be used in conjunction with the already available approaches. Human resource and infrastructure development in this direction will defiantly make huge impact and many laboratories can slowly adopt these nucleic based detection tests. In case of tuberculosis, direct detection from clinical specimen are available as discussed earlier, but altogether solid media based isolation followed by application of nucleic acid based technique is a good approach for the diagnostic mycobacterial laboratories. Although there are a number of well proven sophisticated nucleic acid based techniques, only those methods which are relatively simple to perform, interpret and are cost effective, in terms of MTBC and NTM differentiation, have been discussed in length.

Culture followed by PCR: Simplex and Multiplex

Targeting genes like *hsp65, rpoB, 16S rRNA, dnaJ,* insertional sequences and several other genes for amplification is commonly applied for accurate and relatively rapid identification of mycobacteria (Soini and Musser, 2001). Multiplexing of primers targeting regions for genus confirmation as well as identification of species or complex in a single tube reaction are also some good approaches for NTM and MTBC differentiation. Some of the methods involve mycobacterial genus-specific *hsp65* PCR in conjunction with IS*6110* PCR- which is specific for MTBC (Bensi et al., 2013); or *hsp65* amplification for genus confirmation followed by RD9 PCR which can differentiate *M. tuberculosis* and *M. bovis*. These approaches not only detect organisms of mycobacterial origin but also differentiate MTBC and NTM. Detection in this type of approach can be done by either amplification or non-amplification of the gene targets and also by visualization of different amplicon sizes representing each of the groups. Sometimes error like non-specific amplification could also occur, as in *hsp65* PCR where some of the species of non-mycobacterial origin like *Nocardia, Propionibacterium* could also be amplified which then would require additional conformation tests. Isolation of mycobacterial DNA from clinical sample and further use for downstream PCR amplification need ample standardization therefore molecular

methods are mostly limited to reference laboratories and to adopt in mid-level laboratories, the accuracy needs to be tested with many other existing techniques and standardized.

Duplexing or multiplexing of PCR for MTBC and NTM differentiation is usually done by amplification of discriminating genes which can be accurately predicted on the basis of different band sizes obtained or according to the presence or absence of the amplified bands. Several modified PCR approaches have been reported which have been implemented in diagnostic and research laboratories. Kim et al. (2004) developed an *rpoB* gene-based duplex PCR method for differentiating mycobacteria directly from sputum with a positive acid-fast bacilli and compared with culture based identification approach. The two sets of primers produced 195-bp DNA fragments of *M. tuberculosis* and 515-bp fragments of NTM. Though a higher sensitivity and specificity was observed in culture-based approach, direct sputum analysis by PCR, supplemented with sequencing shows a relatively rapid approach. Another duplex PCR assay followed by restriction fragment analysis was developed to differentiate MTBC and NTM based on the *rpoB* gene (Kim et al., 2001). This PCR amplifies two bands, one each for MTBC and NTM. The MTBC specific band is of 235 bp, while the NTM specific band is 136bp. Further, this 136 bp amplicon can be further subjected to restriction digestion by *Hae*III and *Msp*I. Combination of the methods such as the duplex *rpoB* PCR and *rpoB* PCR based restriction digestion have also been used by other authors for identification and differentiation of mycobacterial species from clinical samples (Huang et al., 2012). Other approaches are also available where multiplexing has been performed using the generic *16S rRNA* and *MPB70* gene specific to the MTBC. Multiplex PCR employing *IS6110, MTP40* and 32 KDa is also promising which can detect MTBC, *M. tuberculosis* and NTM at one go. This method has the ability to detect mixed infection from clinical or broth cultures also. Further, Sinha et al. (2016) presented a nested multiplex, an approach where more specific identification is done using the PCR products of the previous reaction which also increases the detection ability. Several other multiplex and nested PCR have been developed targeting the *rpoB* gene, *oxyR*-a*hpC, pncA*, MTP40, antigen intergenic region, insertional sequences (Mustafa et al., 1999; Bamaga et al., 2003; Mokkadas et al., 2007; Sinha et al., 2016). PCR based differentiation of MTBC members is also important for right treatment. Speciation of MTBC is routinely not done but could be differentiated for right treatment. PCR targeting regions like RD4, RD9, 500bp, oxyR, pnc and others can be used (de los Monteros et al., 1998; Sales et al., 2014). 500bp gene has been known to be specific to *M. bovis* with an amplification just 500bp helping in differentiation of the closely related members of the MTBC complex (Rodriguez et al., 1995). However 500bp gene is specific for *M. bovis* but its specificity is debated owing to amplification of some of the strains of *M.tuberculosis* (Metaxa-Mariatou et al., 2004). Other authors have also used this primer for identification of *M. bovis* from both clinical and animal sources and observed 100% correlation between conventional and this approach (Das et al., 2007).

Nucleic acid based identification also has several drawbacks like human error, specific reagent use, technical issues, contaminations, space constraints. These limitations however have been significantly reduced with technology advancement. Amplifying genes in a PCR reaction tube has become more convenient where optimized reagents like 'MasterMix' which just require addition of DNA, target primers and nuclease free water (commercial or autoclaved triple distilled water). These reagents are again optimised containing more

robust DNA polymerase that can complete a reaction within 40 to 50 minutes as contrast to the typical 2 hours running time. The costs of thermal cyclers have also dropped with even pocket size thermal cyclers becoming available, although the latter still at a higher cost. The DNA isolation of mycobacteria may however require more effort compared to other bacteria as the cell wall makes the organism difficult to disperse the clumps and lyse. For long term use and storage, dedicated mycobacterial DNA isolation kits can aid in this task as any many companies have come out with *Mycobacterium*-dedicated DNA isolation kits. Snap-chill or hot-cold lysis approaches is enough for routine detection when culture is performed and sufficient starting material is available for further downstream processes (Hosek et al., 2006). Involvement of a biotechnology or bioinformatics support structure is required to oversee such diagnosis, as proper handling of instruments, accurate analysis, correct interpretation, reduced contamination and error will be reduced to greater extent.

Culture followed by PRA (PCR Restriction Analysis)

The 60 kDa heat-shock protein is one of the important mycobacterial protein of conserved nature and had been used in many identification approaches. Successful amplifications has been achieved from culture isolates by several authors mainly employing the most commonly used primer (Telenti et al., 1993) for amplification of partial fragment, sometimes called the 'Telenti fragment'. Several modifications to this *hsp65*-based approach for mycobacterial speciation have also been developed (Kim et al., 2005; Chimara et al., 2008; Varma-Basil et al., 2013). A molecular method (Telenti et al., 1993) referred to as PRA (PCR Restriction Analysis) is being described due to its well-deserved popularity. Following *hsp65* gene amplification, the method employs *Bst*EII and *Hae*III restriction enzymes for amplicon digestion to detect various mycobacterial species. However, because the algorithms for representing different banding pattern was a limitation in identifying the rare species, an extension of this approach was made by Chimera et al. (2008) who updated several banding pattern of other species and strains which is useful in identification of mycobacterial species. Several other authors have also modified this approached by amplifying other regions of the *hsp65* gene and using different restriction enzymes. Modification of such PRA approach based on *hsp65* has been developed employing enzymes *Nru*I and *Bam*HI but with limited band pattern (Devallois et al., 1997; Hafner et al., 2005; Kim et al., 2005; Varma-Basil et al 2013). Although highly reliable, PRA-*hsp65* could be limited in being unable to identify newly described or novel species due to unavailability of fragmentation pattern. Even within commonly encountered species sometimes more than one distinct PRA patterns exist leading to uninterruptable results. Kim et al. (2005) designed a primer set amplifying a 644 bp sequence of the *hsp65* gene which could also be digested using *Xho*I enzyme. These methods provide a cheaper alternative for quick differentiation of NTM species along with differentiation of NTM and MTBC due to their different band pattern.

Following the amplification of beta subunit of the *rpoB* gene, restriction digestion methods have also been developed. The use of restriction enzymes *Ava*II and *Hae*III on the amplified 360bp *rpoB* gene for speciation of NTM are also available (Kim et al., 2001). More enzymes have been used for digestion of the *rpoB* gene like the one described by Kim et al. (2004) employing enzymes *Msp*I and *Hae*III. Restriction maps have also been generated for other housekeeping genes like 16S rRNA using a large set of enzymes, *AluI*,

HaeIII, HhaI, MboI, MnlI, MspI, RsaI, TaqI. Among these enzymes *ThaI* with *HhaI* proved to be the most discriminating enzyme (Huges et al.,1993).

Although several restriction digestion of other genes are also available, the *hsp65* based PRA method is one of the most followed methods through the world laboratories and is able to identify most of the clinically relevant isolates of MTBC and NTM. Laboratories with limited funding can slowly adopt this technique which could significantly reduce treatment expense, agony of patient with irrelevant treatment exercise. PRA offers an inexpensive alternative to commercial probe tests for clinical and veterinary laboratories with expertise in PCR and band pattern analysis.

2.4.5. Culture, PCR and Sequencing

All mycobacteria, unless unique or novel can be identified by sequencing of one of the many genes like the *rpoB, dnaJ, secA*, 16S rRNA, ITS regions etc. This approach is rapid and reliable but requires a skilled worker to analyse the sequencing results. In the present era where the cost of sequencing have also reduced greatly, Sanger sequencing can be commercially performed under 500 rupees per reaction for bulk samples. Some of the promising genes that can be used for identification through sequencing is discussed.

hsp65 based sequencing

hsp65 based sequencing and its analysis has several advantages. Due to its relatively high identification power within a small size amplicon of just 441bp, this partial gene can be easily sequenced for species identification compared to other gene targets. Sanger sequencing gives good base reading for upto 600-700 bp, and *hsp65* producing an amplicon size of 441 bp falls under the range where Sanger sequencing could cover in a single read. This approach is one of the simplest approach for mycobacterial genus identification as the product is convenient and reliable which can also be easily searched for homology through the BLAST analysis database of NCBI, National Library of Medicine (NLM). Identification of the species is also possible for most of the commonly observed NTM in clinical setting in addition to MTBC. Though, *hsp65* was commonly used for slow growing NTM, identification of rapid growers are also equally possible. Having the support of a biotechnologist or a bioinformatics specialist can make such molecular detection methods for diagnostic laboratories easily accessible for several laboratories. Quick species identification also helps the physician to initiate the correct treatment course in a timely manner.

rpoB based sequencing

The *rpoB* gene is known to be highly conserved and a good target to differentiate intraspecies differences of mycobacteria and has been popularly used in mycobacterial identification scheme (Adekambi et al., 2003). Resistance to rifampicin is known to occur when mutation occurs in this gene. This method is also a good choice especially of rapidly growing NTM. A partial sequence of *rpoB* gene of about 752bp as per Adekambi et al. (2003) is a commonly used approach. A bidirectional sequencing may be required as single reading in Sangers do not give reliable result for further analysis. There are other partial *rpoB* primers available which could also be sequenced for identification purposes. A

product size of 360bp which works well for Sanger platform is also a good choice for rapid grower NTM (Lee et al., 2003).

16S rRNA based sequencing

The complete 1.5 kb region of 16S rRNA is being used for identification of almost all bacterial species, including mycobacteria (Pascual et al., 1995). A popular platform that consists of 16S rRNA database, ezbiocloud (earlier RIDOM) project, exploits the use of partial 16s rRNA sequences for species identification (Yoon et al., 2017). The limitation of 16sRNA for mid-level laboratory is that the amplicon size is long which would also require bi-directional sequencing. Assembling of these two reads require sound technical knowledge and may also involve cloning for some studies. Kit-based sequencing for bacterial identification like the Microseq 500 employs the 16S rDNA bacterial which is also commonly used in diagnostic laboratories.

2.4.6. Culture and Matrix Assisted Laser Desorption/Ionisation Time-Of-Flight Mass Spectrometry (MALDI-TOF MS) identification

This instrument is an important tool introduced for bacterial and fungal identification in a very short time. A varying size of database for bacterial and fungal identification is available to compare the reference spectrum for confirming majority of the species. This instruments detects the abundant ribosomal proteins of m/z range of 2,000-20,000. Wattal et al. (2016) demonstrated the reliability and higher sensitivity of this instrument to even 16S rRNA sequencing. Two variations of this instrument are available in the market, one introduced by Brucker, Germany called as MALDI Biotyper and the other introduced by Shimazu, Japan called the SARAMIS. Identification of mycobacteria, mainly the unique and rare strains however remains highly restricted due to limited availability of spectral database in both these instruments (Hoshino & Suzuki, 2015; Kodana et al., 2016). Possibility also exists during the use of this equipment that the specimens would get mixed with the egg-based medium to the target plate which could suppress material ionization (Annesley et al., 2003) and a mixed culture with similar concentration may also be difficult to get identified as two separate species (Kodana et al., 2016). The quality of spectra are worse by direct use from liquid medium than solid medium and it is more difficult to identify species (Lotz et al., 2010; Mediavilla-Gradolph et al., 2015). Hence sometimes an additional solid culture step is required to obtain a conclusive result which may be time consuming. Sample preparation of mycobacteria to disrupt clumps and improve protein extraction require little modification than other bacteria (Saleeb et al., 2011; Ahlstrom et al., 2014). There have been immense interest for identification of bacteria through MALDI-TOF MS and has attracted because it is rapid, accurate, and has lower operational costs (Seng et al., 2010). An estimated cost for MALDI-TOF MS identification of bacteria was found to be only 17 to 32% (around rupees 109 per sample) of the costs of conventional identification methods (Seng et al., 2010) which was supported by two other authors (Bizzini et al., 2010; Cherkaoui et al., 2010). MALDI however has the limitation of not being able to identify the unique isolates and the numerous strain diversity of mycobacteria due to non-availability of reference spectrums and difficulty in lysing the thick waxy wall of the cells. Hence adjuncts of PCR and DNA-DNA- Hybridization (DDH) are required. MALDI is also a high end instrumentation based technique and so it is better diagnostic option for well-funded hospitals as running cost is relatively cheaper.

2.5 Drug resistance in NTM

Apart from able to overcome the barrier of many disinfectants, the NTM are known to be intrinsically resistant to several drugs including some of the drugs used for tuberculosis treatment. Macrolides are commonly used for treatment of the diseases caused by this group. One of these drugs, clarithromycin is known to be an effective drug to work against the NTM. However species like *M. avium* and *M. intracellulare* are clinicians 'nightmare' and *M. abscessus* is known to show rigid resistance to this drug. A large number of studies have been done to study the acquiring of resistance in NTM (Meier et al., 1996; Nash et al., 2006; Maurer et al., 2012). Two mechanisms to clarithromycin resistance have been known— The single base mutation in the 23S rRNA at positions 2058 and 2059 of the mycobacteria, known as the *rrl* gene which confers resistance to this drug; and resistance conferred by the inducible gene Erm (erythromycin ribosomal methylases). Macrolide resistance in *M. fortuitum* and *M. smegmatis* is also known to be conferred by the inducible *erm* gene and hence clarithromycin susceptibility reports are sought for *M. fortuitum* infections. Significant risk in macrolide monotherapy of MAC infections are also observed since this complex is known to have a single copy of the ribosome. Hence a base mutation at 2058 or 2059 position of the 23S rRNA gene is expectedly known to confer clarithromycin resistance even in MAC (Meier et al., 1996). *M. kansasii* is occasionally resistant to isoniazid and ethambutol, and mostly known to be resistant to rifampicin due to which treatment failures have been noted. Thereby MIC determination is usually sought for rifampicin (Griffith et al., 2007). However for *M. marinum* no significant risk of mutational resistance have been reported for antimycobacterial drugs. Except ethambutol, *M. haemophilum* a fastidious species is known to be susceptible to all catergory-I antituberculosis drugs including sulphonilamides and macrolide agent clarithromycin. Susceptibility or resistance pattern are unavailable for several other slow growers like *M. ulcerans*, *M. avium* subsp. *paratuberculosis* and *M. genavense* as they do not thrive in susceptibility tests media devoid of supplements (Cocito et al., 1994; Realini et al., 1998). Recommendations for many newly introduced, less frequently isolated and other fastidious organisms are unavailable. The CLSI guideline recommends microbroth dilution for MIC determination of selected NTM. When an isolate during MIC determination shows intermediate or resistant, management is best dealt by an expert and verified with another test (Griffith et al., 2007). Several other virulence genes of NTM such as Twin-arginine translocase (Tat) export system, Sec-dependent general secretion system, ESX export systems have been explored including resistance of MAC to killing by host macrophages (Li et al., 2005; Bottai et al., 2014; Viale et al., 2014; Fedrizzi et al., 2017). Conserved in many pathogenic and non-pathogenic mycobacteria are other genes like *lprG* and *P55* are known to confer resistance to several drugs. The LprG is a lipoprotein known to modulate the host's immune response against mycobacterial infections, and functioning as an efflux pump is the integral membrane protein *P55*. Given the significance of these two genes in *M. tuberculosis* virulence, their presence and implications have also been explored in MAC (Viale et al., 2014). Whole genome analysis of large number of NTM species have now been analysed to identify virulence and drug resistance determinants and to understand the transmission dynamics of this group (Fedrizzi et al., 2017). Such studies remain crucial for clinical diagnosis and driving the right treatment course for infection by NTM which otherwise remains highly unsettled.

CHAPTER 3

HYPOTHESIS

Tuberculosis (TB) remains to be an economic and public health burden globally despite immense attention and funds poured in by governments and philanthropic individuals. While tuberculosis grabs wide attention, the other members of the same genus *Mycobacterium*, commonly called the nontuberculous mycobacteria (NTM) is creeping into the limelight with increasing reports of clinical significance and misdiagnosis with tuberculosis. Considered a rising concern in the developed countries where TB has been controlled, these group of mycobacteria are being identified as emerging pathogens clinically indistinguishable from TB. In resource constrained settings, TB is usually diagnosed by sputum smear microscopy and extrapulmonary TB by clinical examination. The hitch in this approach is mistaking *M. tuberculosis* with NTM bacilli— the two being identical under microscopic view but requiring distinct anti-mycobacterial therapies. Sometimes a primary misdiagnosis leads to wrong treatment exposing patients to harsh medication causing hardship and often leading to death. Usually for a clinician, a regular assumption would be *M. tuberculosis* infection if a patient presents with persistent cough more than three weeks, lung consolidation, nodules and positive acid fast sputum or other biopsy specimen. The patient then goes on to receive a therapy of 06 months for first line anti-tuberculosis drugs. In such cases if the patient relapses, they possess the risk of being misdiagnosed with drug resistant TB and treatment is initiated in some cases. Such flawed treatment line by well-intentioned physicians often leads to unfortunate events. Basically, the clinical presentation of mainly the pulmonary infection by MTBC or NTM is hard to distinguish unless the entity is correctly speciated by adjuncts of culture, biochemical, molecular methods and then clinically correlated.

There are around 184 *Mycobacterium* species, of which *M. tuberculosis* is among the most common cause for tuberculosis in human among the cultivable mycobacteria while the rest are also capable of causing infections in human. There is a need to recognize the most common mycobacterial species besides *M. tuberculosis* which can infect human for epidemiological understanding and public health importance. Exploring the infectious cycle of these pathogens in animal and their products which are sources of human food and occurrence in our surrounding environment could aid in the understanding of maintenance and transmission of this pathogen. An increasing number of NTM are being constantly designated as human and/or animal pathogens making it crucial for determination and discrimination of mycobacteria. Despite their clinical, zoonotic and environmental significance, the identification of mycobacteria has always been challenging. Timely detection and treatment of these atypical infections are difficult in weak resource settings and often underestimated. Studying these emerging pathogens will augment our understanding of the distribution, frequency and interaction of this emerging opportunistic pathogen. The reference laboratory of national level for tuberculosis may have all the required instrumentation and techniques to detect and differentiate MTBC and NTM but it still remains highly limited in modest resource setting. There is need to adopt and document the simplest techniques which can fill this gap so that many more laboratories can be enabled for rapid and accurate detection of NTM and MTBC. It is also essential to study the phylogenetic relationship between the isolates of man, animal and environment to recognize the transmission pattern and the evolutionary relationship of these pathogens in different hosts..

CHAPTER 4

OBJECTIVES

1. Isolation of mycobacteria, with special interest on Nontuberculous Mycobacteria (NTM), from human, animal and environmental samples.

2. To study the presence of drug-resistant genes of clinically important mycobacterial isolates.

3. Correlate animal and human mycobacterial isolates with the environment isolates.

4. To estimate the evolutionary relationships and genetic diversity of the isolates by phylogenetic study.

CHAPTER 5

MATERIALS AND METHODS

5.1 MATERIALS

5.1.1 Glassware. All glasswares used in the study were procured from the following manufacturers:

Borosil Glass Works Ltd, Gujarat, India

Corning Inc., New York, United States

Merck Lifescience, New Jersey, United States

Riviera, Mumbai, India

5.1.2 Plasticware. Centrifuge tubes, microfuge tubes, membrane filters, filtration unit, micro pipettes, deep well plates and other plasticwares were of the following make:

Corning Inc., New York, United States

Genaxy, New Delhi, India

Sartorius, Bangalore, India

Tarson Product Pvt. Ltd, India

Thermo Fischer Scientific, Massachusetts, United States

5.1.3 Chemicals. Molecular biology and analytical grade chemicals and reagents were used according to the requirement in the study. The sources of various chemicals and reagents were as follows:

Applied Biosystems, California, United States

BD BBL & Difco, New Jersey, United States

Bioserve, Hyderabad, India

FINAR, Ahmedabad, India

Genetix Biotech Asia Pvt. Ltd , New Delhi, India

Himedia laboratories private Limited, Mumbai, India

Imperial Lifesciences, India

Merck Lifescience, Mumbai, India

Promega, Wisconsin, United States

Sigma Aldrich, Missouri, United States

SRL SISCO Research laboratories Pvt. Ltd, India

Takara, Japan, New Delhi, India

ThermoFischer Scientific, Mumbai India

5.1.4 Kits

Biochemical test kits	Himedia, India
DNeasy® Blood & Tissue	Qiagen, USA
QIAquick PCR Purification Kit	Qiagen, USA
QIAquick Gel Extraction Kit	Qiagen, USA
InstAclone PCR cloning kit	ThermosScientific, USA

5.1.5 Equipment

Following equipment were used for various purposes during the course of study

Autoclave	Medica Instrument Mfg co. (Equitron), Mumbai, India
Biosafety cabinet (Pathogen level 3)	ESCO, Singapore
Computerized gel documentation	Cleaver Scientific (UVITEC), Warwickshire, United Kingdom
Cooling Centrifuge	REMI Laboratory instruments, Maharashtra, India; HERMLE Labortechnik GmbH, Germany
Cooling microcentrifuge	Eppendorf, Hamburg, Germany
Deep freeze (-20^{o}C)	Blue Star Engineering & Electronics Ltd, Mumbai, India
Electronic weighing balance	Eppendorf, Hamburg
	Kern, Germany
Hot plate	Riviera Glass Pvt. Ltd, Mumbai, India
Incubator	Borg Scientific, Chennai, India
Laminar Air-flow	NEO Equipments, Kolkata, India; Narang Scientific Works Pvt. Ltd, New Delhi, India
Microwave oven	Samsung, Seoul, South Korea
Oil immersion microscope	Olympus, Tokyo, Japan
PCR thermal cyclers	Eppendorf, Hamburg, Germany; BioRad, California, United States; Takara, Japan;

	ThermoScientific, Massachusetts, United States
Refrigerators	Samsung, Seoul, South Korea; LG, Seoul, South Korea
Shaker incubator	Borg Scientific, Chennai, India
Vortex mixture	Tarson Product Pvt. Ltd (Spinix), India
Water bath	Vision Scientific Co., Ltd, Korea
Water purification system	Merck Milipore, Massachusetts, United States

5.1.6 Reference strains

1. *Mycobacterium bovis (AN-5)* {Courtsey- Indian Veterinary Research Institute (IVRI), Kolkata}
2. *Mycobacterium tuberculosis* {Institute of Microbial Technology (IMTECH), Chandigarh, MTCC300}
3. *Mycobacterium avium* (IMTECH, Chandigarh, MTCC1723)
4. *Mycobacterium phlei* (IMTECH, Chandigarh, MTCC1724)
5. *Mycobacterium smegmatis* (Microbiologics, Minnesota, ATCC607)
6. *Mycobacterium fortitum* (IMTECH, Chandigarh, MTCC1023)
7. *Mycobacterium vaccae* (IMTECH, Chandigarh, MTCC272)
8. *Mycobacterium kansasii* (IMTECH, Chandigarh, MTCC3058)
9. *Mycobacterium tuberculosis* (Microbiologics, Minnesota, ATCC25177)
10. *Mycobacterium smegmatis* (Microbiologics, Minnesota, ATCC607)
11. *Mycobacterium fortitum* (Microbiologics, Minnesota, ATCC6841)
12. *Mycobacterium kansasii* (Microbiologics, Minnesota, ATCC12478)
13. *Staphylococcus aureus* (Microbiologics, Minnesota, ATCC25923)

5.2 GROWTH MEDIA FOR MYCOBACTERIA ISOLATION

Both solid media and broth were used in this study to ensure maximum retrieval of cultivable mycobacteria. Solid media comprised of egg based as well as agar based, the description of which are as follows:

5.2.1 Lowenstein Jensen (LJ) slant with glycerol (Lowenstein, 1931; Jensen, 1955)

The original formulation of this egg based medium, called Lowenstein Jensen-glycerol (LJ-G) was developed by Lowenstein (Lowenstein, 1931) which was used in the study for growth of almost all types of mycobacteria from all sources. The medium was modified by Jensen (Jensen, 1955) contains mineral salts, growth stimulants and replaced congo red with a dye called malachite green which turns the look of the medium greenish making it easy to distinguish colony appearences and also inhibits other undesirable bacterial contaminants. Glycerol was used as a source of carbon for the tubercle bacillus growth.

5.2.2 Lowenstein Jensen pyruvate (LJ-P) slant (Stonebrink et al., 1969)

This medium is the same as LJ-G but with a minor modification of pyruvate addition instead of glycerol. Some mycobacterial species of bovine origin are glycerophobic (Schaeffer, 1952) and do not grow in LJ-G. We used this medium with the aim to isolate all forms of mycobacteria from bovine origin as well. LJ medium with pyruvate was thus prepared as per the formulation of Stonebrink et al., 1969.

5.2.3 Middlebrook 7H10 (Middlebrook and Cohn, 1958)

This agar based media developed by Middlebrook and Cohn (1958) have been used to avoid contamination prone to egg based media and for obtaining early growth of *M. tuberculosis*.

5.2.4 Middlebrook 7H11 (Cohn et al., 1968)

A modification of M7H10 by addition of 1 gm/l pancreatic digest of casein makes up Middlebrook 7H11 which was developed by Cohn et al. (1968). Alternatively we used this media for isolation as it known to enhance growth of fastidious organisms. Antibiotics like polymixin, carbenicilin and antifungal like Amphotericin B is used in this medium for making it selective by inhibiting growth of many other bacteria. In this study a combination of all these antibiotics called ‘penta mix’ was supplemented in the medium (Appendix).

5.2.5 Middlebrook 7H9 (Middlebrook and Cohn, 1958)

Developed by Middlebrook and his co-workers, this broth provides supplements to enhance faster growth and have been used in the study for mainly subculture, performing drug susceptibility tests and biochemical tests. In this broth, glycerol supplies carbon and energy. Middlebrook ADC Growth Supplement, is used in the medium containing bovine albumin, dextrose and catalase. Dextrose is known to supply energy, while albumin protects mycobacteria from toxic fatty acids and catalase splits toxic radicals. Mycobacteria are known to grow more rapidly in broth media but gross contamination is a constant disadvantage.

5.2.6 Lowenstein Jensen medium preparation

Both egg based Lowenstein Jensen media (LJ-G and LJ-P) as well as agar based Middlebrook 7H10 and Middlebrook 7H11 were used for isolation as per requirement. Samples of human and animal origin were inoculated on both LJ-G and LJ-P so as not to miss out on certain mycobacterial species such as *M. bovis* which is selective of pyruvate for its carbon source. Water samples were inoculated only on Middlebrook 7H10, while

soil samples were inoculated both in LJ and Middlebrook 7H10. Broth based Middlebrook 7H9 was used for recovery and storage of the mycobacterial isolates.

All the media were prepared following the manufacturer's protocol (Difco and BBL; Hi-Media) and therefore have not been discussed in detail. However preparation of the egg-based LJ medium used in this study is discussed below give its technical significance.

a. The mineral salt solution (prepared according to manufacturer's protocol) and either glycerol or pyruvate was dissolved in distilled water and autoclaved at 121 degree Celsius for 15 minutes at 15 psi.

b. To prepare the egg emulsion, use fresh laid eggs (not more than 5 days old) from recognized hatchery.

c. Rinse the eggs with soap and water and wipe with 70% alcohol.

d. Under sterile condition, firstly break the eggs into a beaker to check its integrity. Then transfer into a flask containing the glass beads and mix vigorously to break the yolk and homogenize the material (Fig 3).

Fig 3. Preparation of egg emulsion

e. Mix well to make a homogenized solution and sieve in a sterilized cloth/gauze through a funnel.

f. Measure the required amount in a graduated cylinder and add to the sterilized and cooled mineral salt solution.

g. Dispense about 7-10 ml of the medium in 30 ml screw-capped bottles, place on sloped racks and inspissated at 80-85 ^{o}C until hard (about 50-90 minutes) for 3 consecutive days in slanting position, Fig 4.

Fig 4. Lowenstein Jensen (LJ)-Pyruvate media after inspissation

5.3 SAMPLING STRATEGY

5.3.1 Geographical area under study

Our study was concentrated on selected locations of two states in the Northeastern Region (NER), India (Fig 5), which is rather isolated from the main land and data availability on scholarly articles of NTM is also infrequent.

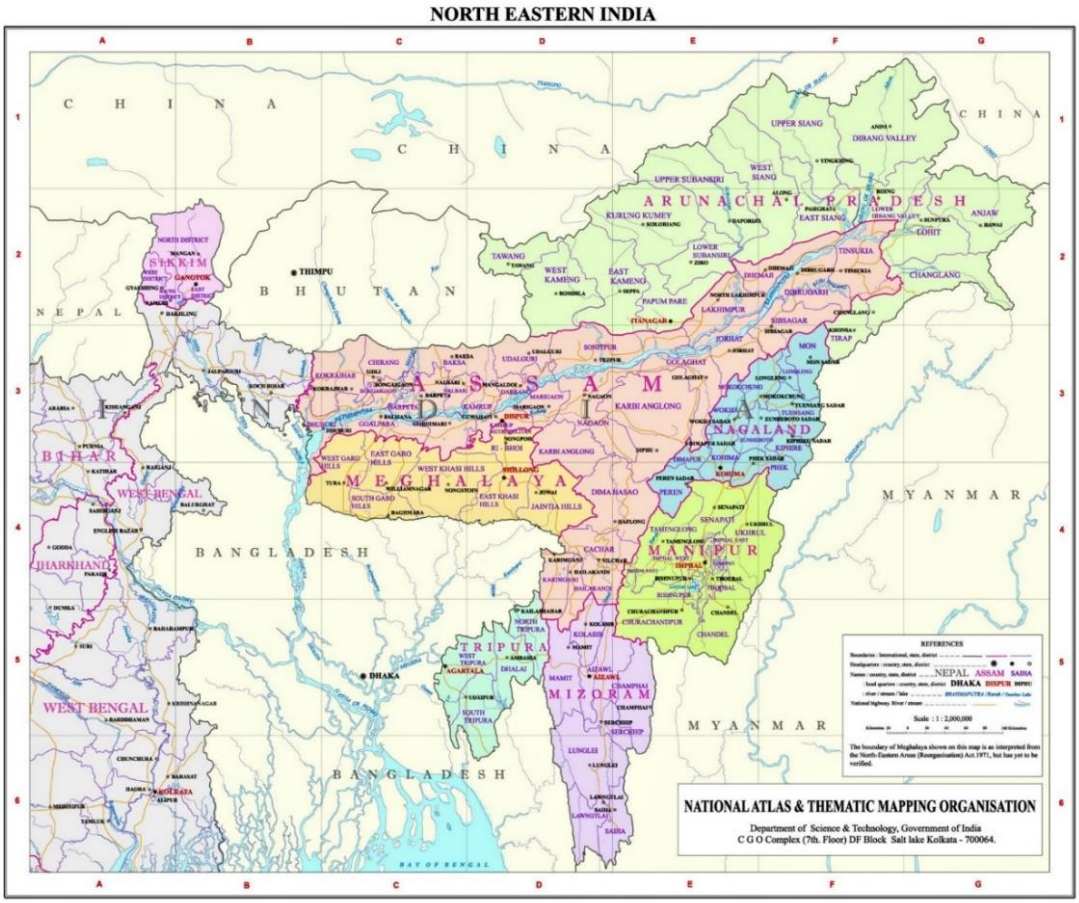


Fig 5. The Northeastern Region (NER) of India. Image source: Ministry of Development of North Eastern Region (DONER)

A ground level study on the presence and distribution of NTM mainly was required as rise in infections with NTM along with the existing burden of *M. tuberculosis* needs to be addressed. The NER is located in the eastern-most part of India sharing international border by China, Myanmar, Nepal, Bhutan and Bangladesh.

a. *Khasi Hills of Meghalaya*

The Meghalaya state (Fig 6) comprises of 11 districts and Shillong is the capital city of state. Our sampling sites included locations in Ri-Bhoi, East Khasi Hills and West Khasi Hills which covers the Khasi Hill region. In our study these three sites would be clubbed and broadly addressed as the Khasi Hills.

Fig 6. Map of Meghalaya State. Image source: Ministry of Development of North Eastern Region (DONER)

b. *Dimapur district of Nagaland*

The Nagaland state comprises of 11 districts, capital city being Kohima and Dimapur as the largest city (Fig 7). Depending on the accessibility and collaborative opportunity, samples were collected from Dimapur district of the state from where all samples were transported with stringent biosafety measures and processed within the stipulated time.

Fig 7. Map of Nagaland State. Image source: Ministry of Development of North Eastern Region (DONER)

5.4 IDENTIFICATION SCHEME DESIGNED FOR THE STUDY

A flowchart of the scheme used in the study is presented in Fig 8 beginning from sample collection to species identification.

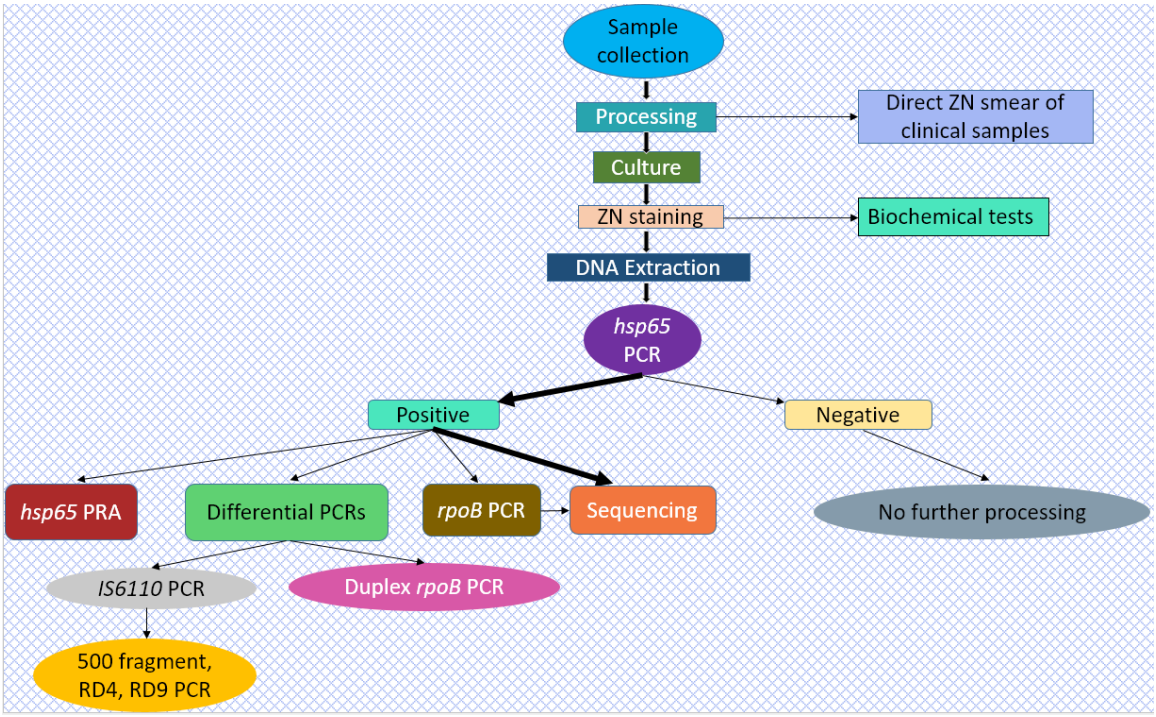


Fig 8. Flowchart of the identification scheme designed for the study.

5.5 SAMPLES TARGETED FOR ISOLATION OF MYCOBACTERIA

In our study a total of 555 samples were collected from geographically dispersed sites in and around Khasi Hills of Meghalaya and the Dimapur district of Nagaland. Samples were collected throughout the year irrespective of the season. These were from three different sources— human, animal and environment, which have been discussed in detail below.

A variety of samples from the above three said sources were processed for isolating mycobacteria from these region. Sampling approach included both targeted and random depending upon the applicability and availability of sample targeted.

Samples targeted for isolation of mycobacteria were as follows:

a) Human samples (Sputum sample, Urine, Tissue, Biopsy, Aspirate, and other body discharges)

b) Animal samples (Milk, Lymph node, Nasal swabs, Dermal scrap)

c) Environmental samples (Water and Soil; mainly concentrated on farms and slaughter houses)

Although a wide variety of samples were desired for mycobacterial isolation, only limited sources could be accessed. Details of samples processed in the study is given in the pie chart (Fig 9).

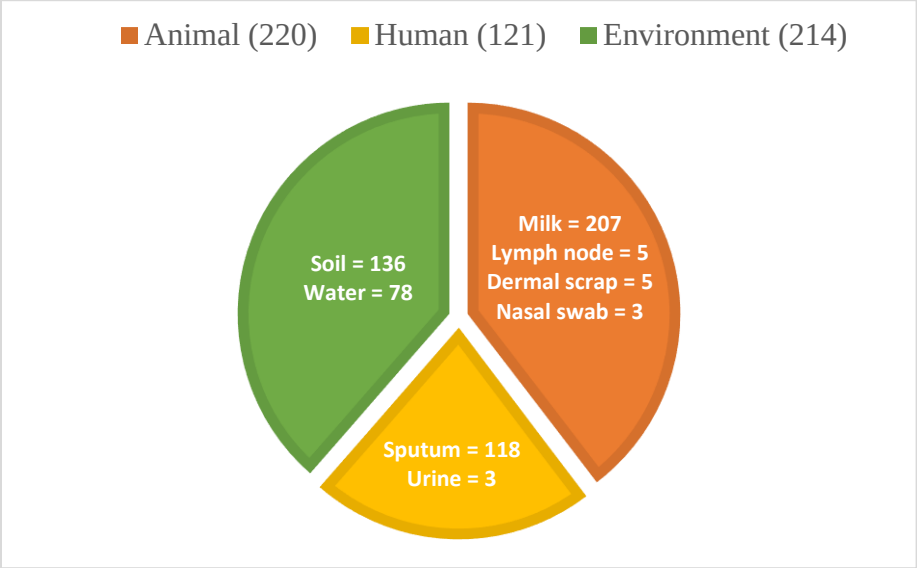


Fig 9. Details of samples collected from the three sources

5.5.1 Human

In total, 121 samples from suspected tuberculosis patients were collected from Revised National Tuberculosis Control Programme (RNTCP) centre of Dimapur, Nagaland following official consent from the physician in charge, and a few samples (12) were obtained from Khasi Hills of Meghalaya state which were voluntarily submitted to our laboratory. Abiding by the ethical guidelines, consent was acquired from these individuals only after which the samples were used for our routine research. These 121 samples consisted of 118 sputum sample and 3 urine samples .

Sputum and Urine

Sputum samples were deposited by the patients themselves at the Revised National Tuberculosis Program (RNTCP) unit following the instructions given. After the samples were tested for Acid-fast bacilli examination at the centre, collection vials were sealed with parafilm, transported to the laboratory in a sample box maintaining 4 degree Celsius and processed immediately.

Urine sample (early morning and spot) was also deposited by the patient in sterile wide-mouthed container after being directed to follow the standard procedures. Following ethical guidelines, consent forms, when required, were explained and signatures acquired.

All samples were sealed and transported to laboratory under refrigerated condition which were processed immediately or within 24 hours of collection under strict biosafety measures.

Approval from the Institutional Biosafety committee (IBSC), ICAR RC for NEH Region, Meghalaya, consisting an external examinee was obtained for mycobacterial research in the department.

5.5.2 Animal

Suspected tuberculous tissues samples of animals, largely bovine and swine (Fig 10), were collected by veterinary practitioner in sterile containers which were transported and processed immediately. Samples were stored in 4°C when not analysed immediately.

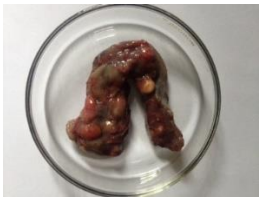


Fig 10. A porcine lymphnode processed for mycobacterial isolation

Bovine Milk

During the sampling period from 2012-2016, 207 raw milk samples from small dairy herds were collected from two states of NER of India comprising four districts from two states. Thirty one (31) samples of individual cow and 1 pooled sample were collected from Dimapur district of Nagaland State, and 175 samples from the Khasi Hills of the state of Meghalaya. The sampling locations were selected on the basis of significant dairy farms present in the area and accessibility to the institute where the samples could be transferred in cold chain within 24 hours. The practice of milking the animals in this region is hand milking. Raw milk samples were transported to the laboratory located in Meghalaya state under refrigerated condition and were processed within 24 hours of collection.

Canine

A total of 08 canine samples were collected with the assistance of a veterinary practitioner, of which 05 were nasal swabs and 03 were dermal scrapings of domesticated dogs exhibiting tuberculosis-like symptoms.

Swine

Five madibular lymphnodes of suspected tuberculosis and NTM were also analysed for detection of mycobacteria.

5.5.3 Environmental

Water

Samples from each site were collected according to standard methods given by American Public Health Association (1995). Water samples were collected from sources which was usually not connected to a water treatment system and were representative of the water source utilized in the area for drinking, household chores, farming or for livestock.

1. All samples were collected in 500ml pre-cleaned sample bottles. The collection sites were carefully examined and sample bottles clearly marked to avoid error during evaluation.

2. When collected from taps, the taps were allowed to run clear for upto 2 minutes.

Samples were transported to the laboratory and stored at $4^{o}C$ until analysis. Sodium thiosulfate was not used for storing the samples, since the samples were used within 24 to 48 hrs of collection and also because it is known to interfere with the growth of mycobacterial growth (Thomson et al., 2008).

Soil

The top soil of the sampling site was dug out and collected in pre-sterilised sampling vials. Upto 50 gm of each sample was taken and processed within 12 hours of collection.

5.6 SAMPLE PROCESSING AND CULTURE

5.6.1 Human

The samples were decontaminated and digested as per Petroff's method (1915), inoculated on freshly prepared or readymade LJ-G media (Hi-media, India). Freshly prepared LJ-P media was simultaneously used for isolation of glycerophobic mycobacteria mainly of bovine origin. The inoculated slants were further incubated at $37^{o}C$ for two weeks upto four months with regular interval checking. Tubes with screw caps were kept loose for the first 03 weeks to permit circulation of carbon dioxide for initiation of growth. The caps were tightened after 03 weeks to prevent dehydration. The visible primary growths on solid media was examined by acid fast staining and further sub-cultured or stored for confirmation and molecular characterization.

Sputum (Petroff, 1915)

Protocol

1. Approximately 3ml of the sputum sample was taken in a sterile centrifuge tube to which was added 3 ml 6% sulphuric acid.
2. The mixture was mixed well and allowed to act for 3 minutes.
3. The tubes were centrifuged for 15 mins upto 3000 rpm.
4. The pellet was washed twice with NSS followed by centrifugation again for 15 mins at 3000rpm.
5. The sediment was then mixed with 0.2 ml of sterile distill water and one to three loopful inoculated on LJ-G and LJ-P slants.
6. The slants were incubated for 37°C for upto 3 months.

The supernatant and all waste was discarded in 5% Phenol solution.

Extrapulmonary fluids and other body discharge (Kent et al., 1985; Gopinath and Singh, 2009)

Protocol

1. Upto 50ml urine samples (Fig 11) were centrifuged at 3000Xg for 20 minutes.

Fig 11. Urine samples from TB follow-up patient

2. An equal amount of 4% NaOH was added to the resulting pellet and for 15 minutes it was decontaminated.

3. The suspension was neutralized in phosphate-buffered saline (PBS) and centrifuged for 20 minutes at 10,000 rpm.

4. The pellets were then re-suspended in PBS and inoculated after preparing smears for Ziehl-Neelson staining.

5. Re-suspended pellets were also inoculated on LJ-G and LJ-P and incubated at 37°C for upto 03 months.

5.6.2 Animal

Milk (Dundee et al., 2001)

Protocol

1. The centrifuge vials containing the milk samples (approximately 50ml) were centrifuged at 2500g for 15 minutes before decontamination.

2. The pellet were then decontaminated with hexadecylpyridinium chloride (HPC), avoiding the cream layer, by re-suspending in 10 ml 0.75% (w/v) of the HPC and incubated at room temperature (21°C) for 05 hours

3. Following decontamination and incubation, the samples were centrifuged for 15 minutes at 3000 rpm.

4. The pellets were then again re-suspended in 1 ml PBS and inoculated for isolation onto LJ-G and LJ-P slants.

5. The slants were incubated at 37^{o}C for 3 months and examined weekly for growth.

6. Visible primary growths on the slants were checked for their colony morphology and subjected to acid-fast staining. Probable mycobacterial colonies were further

sub-cultured in respective media and were processed for DNA extraction and molecular characterization.

lymph node (van Ingen et al., 2010)

Protocol

1. The excised lymph nodes were decontaminated by rinsing in boiling water for 5 to 8 seconds.
2. The tissue was then cut aseptically into fine pieces and triturated using sterile mortar and pestle.
3. The homogenized tissue was decontaminated with 3 volume parts of 6% H_2SO_4 for 10 minutes at room temperature.
4. Following decontamination, this solution was centrifuged at 12000 rpm for 10 minutes.
5. The supernatant was discarded and the sediment inoculated in LJ-G and LJ-P slants after following two times wash in PBS.
6. The inoculated slants were incubated at 37^{o}C for 3 months and examined at regular intervals.

Nasal swab and Dermal scrap (Petroff , 1915)— Same as sputum sample processing

5.6.3 Environment

Water (Thomson et al., 2008)

Water samples were processed and inoculated with slight modification from the referred manuscript. Decontamination by cetylpyridinium chloride (CPC) is known to control contamination is also reduces mycobacterial yeild to a certain extend. The applicability of this decontamination method however would depend on the origin of the samples. The use of CPC was found to effectively replace the alkali decontamination procedures of specimens for mycobacterial analysis and was found suitable for decontamination of large volumes of water (Thomson et al., 2008). CPC has also been recommended as a means of eliminating non-acid-fast organisms from sputum and several other clinical samples (Stottmeier et al., 1970).

Protocol

1. The samples were first decontaminated for 30 minutes with 0.005% CPC.
2. Following decontamination samples were carefully vacuum-filtered through 0.4μm filters.

After subsequent filtration, the filter was rinsed with sterile distilled water to remove any residual contaminants.

3. Filters were picked with a sterile pointed forcep and smeared both on Middlebrook 7H10 and 7H11 agar plates and covered immediately to avoid any exposure to contaminants.

4. The inoculated slants were incubated at 37^{o}C till growths were observed.

Soil (Thorel et al., 2004)

Protocol

1. 2gm each of the samples were suspended in 20ml sterile distilled water (DW) and ground with sterile sand.

2. The mixture was allowed to stand for 1 hour to let the sediments settle.

3. The supernatant was then collected carefully in a sterile centrifuge tube, mixed with approximately 20ml of 0.75% (w/v) hexadecylpyridinium chloride (HPC) and incubated at room temperature for upto 18 hours.

4. Following incubation the tubes were spinned for 20 minutes at 5000 g.

5. The pellets were dissolved in sterile DW and inoculated on the LJ slants and Middlebrook 9H11 agar plates.

6. The slants and plates were incubated at 37 ^{o}C and examined at regular intervals.

5.7 CONVENTIONAL CHARACTERIZATION OF ISOLATES

5.7.1 Cultural characteristics and ziehl-neelson staining

Colony morphology was examined and documented if typical rough-tough-buff colonies were apparent as in *M. tuberculosis*, *M. smegmatis* etc. or smooth colonies as of *M. chelonae*, *M. avium* etc. Attention was also given on which media was the preferred choice for growth (LJ-Glycerol/LJ-Pyruvate). These observations substantiated in differentiating mycobacteria from other contaminants.

Microscopic examination by ziehl-neelsen staining/acid fast staining (Baron et al., 1994)

Primary identification involved examination of the colony characteristic followed by ZN staining and subsequently microscopic examination.

Principle

As visible colonies were observed on the slants, smears were made after looping out a single colony, ZN staining was performed, and observed under microscope for detection of acidfast bacilli (AFB). Due to the presence of high lipid content in the cell wall known as the mycolic acids, mycobacteria resist staining and give confusing results to Gram's stain (Morello et al., 2006) and are responsible for the staining pattern of poor absorption followed by high retention and successful penetration by the aqueous-based staining solutions (such as Gram's) is prevented. The lipoid capsule of the mycobacteria is waxy at room temperature due to their high molecular weight. The staining reagents include the

primary stain ZN carbolfuchsin, decolourizer acid alcohol and counterstain methylene blue. Carbolfuchsin is the primary stain which is heated enough so that the stain is taken up by the cells. Washing with acid-alcohol (95% HCl- 95% Ethanol) solution decolourizes the non-acid-fast cells and counterstain methylene blue is taken up, thus showing blue coloured cells. Other bacteria which are readily decolorized by acid-alcohol are called nonacid-fast. The acid fast bacilli under the microscope appears brightly stained bacilli in pink or red against a blue background. Other bacteria like *Nocardia* can also be stained by this method.

Protocol

1. Distilled water was added on the slide to help the cells adhere and cells were inoculated using a microbiological loop to make a proper film.
2. The smear was air-dried completely and heat-fixed by passing it over the flame 7-8 times.
3. The slide was heated over a spirit lamp from below until a mild steam was observed. The slide should not be boiled or overheated. The steaming and moist slide was allowed to stand for five minutes with repeated heating whenever required.
4. The slide was rinsed gently in running tap water until the slide runs clear.
5. The slide was then rinsed with the decolourizer solution and immediately washed with tap water to turn it faintly pink.
6. The slide was then flooded with the counterstain, and allowed to stand for 20 to 30 seconds, gently rinsed with slow running tap water.
7. The slide was then blotted and left for air drying.
8. The prepared slide was examined under oil immersion.
9. Acidfast bacilli appear red while debris and other non-acid-fast bacteria blue.

Acid-fast staining was done for all the presumed mycobacterial colonies as the preliminary test to check any positive samples. The cultures showing acid fastness were further processed for molecular confirmation and subcultured into the respective media when required.

5.7.2 Biochemical Tests (Koneman, 1997; Isenberg, 1992; Tille, 2015)

A long list of biochemical tests are commonly employed for differentiation of mycobacterial species such as, Arylsulfatase test, Catalase test (68°C), Detection of Pyrazinamidase activity, Iron uptake, Niacin test, Nitrate reduction test, Sodium Chloride Tolerance, Susceptibility to thiophen-2-carboxylic acid hydrazide (T2CH), Tellurite reduction test. However only selected tests were performed when required due to unconclusive results and gruelling long hours in this approach. Growth time required to form mature colonies are hugely time-consuming and require considerable biosafety preparation and risk exposure to the technicians. Further a series of 8-10 biochemical tests are required for identifying up to species level for over 184 species is a real challenging job

and only reference laboratory of national repute can perform these tests. Adding to this is the continous increase in the number of new species and with lack of updated database for biochemical speciation, the difficulty level increase to further extent (Hall et al., 2006). Citing the above reasons, molecular method was alternatively preferred and employed in the study.

Only the tests performed are discussed below. Essential biochemical tests have been performed such as Nitrate Reduction, Niacin detection, Catalase test, Pyrazinamidase and T2CH for selected isolates when required.

A. Niacin test

Somes strains of mycobacteria like *M. chelonae, M. tuberculosis* and *M. simiae* have a blocking in the scavenging pathway of nicotinamide adenine dinucleotide (NAD) due to which they lack the enzymes to further convert niacin to NAD. The niacin which do not get metabolized gets accumulated on the agar or slants. Using 4% aniline and 10% cyanogen bromide the excreted niacin in the media is detected which is indicated by yellow colour formation. Differentiation of *M. bovis* and *M. tuberculosis* is commonly done by this test. *M. bovis* is niacin negative while *M. tuberculosis* is niacin positive. Certain isolations from animal origin grew colonies representing features to NTM. To rule out the identity of any zoonotic species or bovine tubercle bacilli, this test was performed on selected strains using the niacin detection kit by Himedia, India, according to the manufacturer's protocol as discussed.

Protocol

1. 2 ml of sterile distilled water was dispensed to the slant.
2. Using sterile scalpel or needle the slant was cut to expose the excreted niacin from the media.
3. Slant were then kept horizontally for 20 minutes at room temperature to allow niacin and distilled water to dissolve.
4. The slant was allowed to stand in an upright position for 5 minutes and this solution was used as the test sample.
5. Part A (1ml) reagent provided in the kit was added to Part B (1 ml) to which 1 ml of the above solution was mixed.
6. Development of yellow colour within 5 minutes indicates positive reaction while no development of colour indicated negative result.

Controls: Negative and positive controls were provided in the kit otherwise, an un-inoculated reagent can be used as negative control and *M. tuberculosis* can be inoculated for positive control.

B. Nitrate reduction test

This test is most sensitive to detect nitrate-reducing mycobacteria. Mycobacteria vary in their ability in reduction of nitrate to nitrite. In this test sodium nitrate detects the presence of nitrate reductase. Reduction of nitrate to nitrite is detected by the enzyme nitrate reductase which is observed by addition of dihydrochloride –N- naphtyl ethylendiamine and sulfalinamide that forms a complex of diazonium chloride with a fuchsia color. Species that reduce nitrate include *M. tuberculosis,* the *M. terrae complex, M. kansasii* etc. and most rapid growers except *M. chelonae.* The nitrate reduction test was used to distinguish between organism with similar characteristics. Nitrate reduction test kit by Himedia, India was used and the manufacturer's protocol is given below.

Protocol

1. A loopful of vigorously growing culture was mixed in the nitrate buffer provided.
2. The tubes were incubated for upto 2 hours at 37˚C.
3. This was followed by acidifying the buffer by adding 2 drops of 0.1N HCI.
4. The nitrate reagent in the kit was rehydrate in 5 ml sterile distilled water and 2-3 drops added to the above test tube.

Within 30 to 60 seconds formation of red colour was noted. Positive test is interpreted by formation of red colour. No change within this period was interpreted negative.

Control: A reagent control without culture was used as negative control while standard *M. tuberculosis* culture was used as a positive control.

C. Catalase test

Two classes of catalase are present in mycobacteria- the thermolabile and thermostable. The antioxidant enzyme catalase is responsible for getting rid of H2O2 molecules from the cells that are produced during respiration. In *M. bovis* and *M. tuberculosis* activity is inhibited at 68°C while in the rest of the mycobacteria maintains the activity even at the increased temperature. The test was performed on selected strains to differentiate between MTBC and NTM. When the test is performed, oxygen bubbles are formed which is caused by enzyme activity in the 10% Tween 80 and 30% perhydrol. The enzymatic activity can be measured by the height of the bubbles formed in the column. Kit based catalase test (Himedia, India) for both semiquantitative and heat stable catalase was performed.

Protocol

Firstly, Part A and Part B reagents containing 30% H_2O_2 and (10%) Tween 80 was mixed in equal volume before use.

i. Semiquantitative Test

1. The LJ medium was inoculated with one week old test liquid culture (1ml) and incubate at 37˚C for upto two weeks.

The caps of the tubes should is instructed to be kept slightly loose to permit adequate exchange of air.

2. Freshly prepared catalase reagent is then added (1ml) to the tubes and allowed to stand upright for 05 minutes.

The height of bubbles or the effervescence over the surface of culture medium was recorded.

Bubbles from 05-50mm were considered weakly positive; >50mm as strongly positive and lack of bubbles interpreted negative.

ii. Heat stable catalase

1. Several colonies of test organism was emulsified in 0.5ml Catalase buffer.
2. The tubes were carefully wiped to decontaminate the surface and kept in waterbath at 68˚C for 20 minutes.
3. Following incubation, the tubes were cooled 21 degrees. And 0.5ml of freshly prepared catalase reagent was added to the tubes.

Formation of bubbles on the surface of the fluid was noted upto 20 minutes. Bubble development was inferred positive while lack of bubbles interpreted as negative.

Controls: *M. tuberculosis* was used as negative control while *M. smegmatis* was used as a positive control.

D. Thiophen-2-carboxylic acid hydrazide susceptibility test (T2CH or TCH)

The bovine species, *M. bovis*, is susceptible to low concentration of T2CH, *M. tuberculosis* is not inhibited by the presence of T2CH. This allows differentiation of these two species, *M. bovis* and *M. tuberculosis*. Selected samples of mainly animal and human origin, differing in the typical rough-tough-buff colony characteristics displayed by *M. tuberculosis* were tested. TCH test kit for mycobacteria by Himedia, India was used for performing this test.

Protocol

1. A turbid broth culture of the test organism was prepared using 03 week old cultures grown in LJ.
2. 10 µl of the turbid culture was inoculated on every quadrant.
3. Plates were incubated at 37˚C for 14 to 21 days and examined for growth at regular intervals.

Concentration of T2CH— Quadrant I: 0µg/ml; Quadrant II: 1µg/ml; Quadrant III: 5µg/ml; Quadrant IV: control quadrant.

Growth when observed in quadrant without drug and no growth in presence of the drug were sensitive and were designated as positive. While samples showing growth even in the presence of the drug were designated as negative.

Control: The negative control was served by *M. tuberculosis*.

E. Detection of Pyrazinamidase activity

Using freshly prepared ferrous ammonium sulphate ($Fe(NH_4)_2(SO_4)_2$, 6H2O) this enzymatic activity is usually used to distinguish between *M. bovis* and *M. tuberculosis* where pyrazinamide susceptible strains hydrolyse pyrazinamide. *M. bovis* is resistant to pyrazinamide while *M. tuberculosis* is susceptible.

Protocol

1. The agar medium with containing 02-03 week old test culture was heavily inoculated (1 ml).
2. The PYZ Reagent provided in the kit was mixed with 10 ml sterile distilled water. 1 ml of this solution was added to all the tubes.
3. The tubes were carefully allowed to stand on a rack and stored at 2 to 8˚C for 04 hours.
4. Following incubation, tubes were observed under normal room light against a white background.

Formation of a pink or red coloured band between the reagent layer and agar surface indicates a positive test.

Control: *M. tuberculosis* served as the positive control and negative control was a tube inoculated with sterile distilled H2O. Alternatively, *M. bovis* can also be used. A few strains of *M. bovis* and some other species may produce a very faint pink band, which can be scored as negative.

5.7.3 Phenotypic test (Mshana et al., 1998; Sankar et al., 2008)

The WHO and Centre for Disease Control (CDC), Atlanta, USA, has laid down specific recommendations and guidelines concerning anti-tubercular drug susceptibility methods for *M. tuberculosis* but hardly any guidelines is available for NTM. For prescribing the correct therapeutic drug, anti-mycobacterial susceptibility testing is crucial for management of the NTM disease. However routine drug susceptibility testing of NTM are usually not done though susceptibility testing of selected NTM species is endorsed by the American Thoracic Society (ATS) (Griffith et al., 2007). Infections due to NTM is difficult to treat due to their intrinsic resistance to the major classes of drugs, which may be attributed to their variable habitat (Philalay et al., 2004; Nash et al., 2006).

Employing a dye based technique with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), colorimetric assay using oxidation-reduction indicator was performed in deep-well microplate to determine the minimum inhibitory concentration

(MIC) for our NTM isolates, focusing on *M. abscessus* isolates. Mitochondrial dehydrogenase is known to reduce the yellow dye MTT by living cells to produce insoluble purple MTT formazan crystals (Mosmann, 1983; Thom et al., 1993). This colorimetric assay indicates the number of viable bacteria and the ability of these live cells to reduce MTT. Dead mycobacteria are unable to reduce MTT, which after solubilization can be measured spectrophotometrically and even visualized with unaided eyes. In our study, we used the MTT dye assay to determine the MIC by detecting the viability of NTM isolates after treating with clarithromycin and other anti-mycobacterial drugs (Mshana et al., 1998; Sankar et al., 2008).

Drug susceptibility testing for the drug clarithromycin was performed for samples which were of clinical significance. Antibiotic therapy of most of the pulmonary NTM infections includes a combination treatment including clarithromycin. This macrolide, clarithromycin is considered an important component in antimicrobial chemotherapy of pulmonary *M. abscessus* infections (Griffith et al., 2007), being the only drug that can be administered orally and with proven efficacy.

In our study, the clinically relevant samples were tested phenotypically by this MTT assay for susceptibility to clarithromycin. The results obtained were then simultaneously verified by a genotypic approach presented in the results section. In brief, studies in the highly resistant *M. abscessus* shows that the amplified and sequenced *rrl* gene with an inducible *erm* gene can deliberate resistance to clarithromycin by mutation of a single base. The sequenced gene was inspected for any base mutation and compared with the resulting MTT assay output of the isolate. Minimum inhibitory concentration (MIC) for drugs like isoniazide and ethambutol were tested for selected isolates as these are also used in the chemotherapy of certain NTM infections (Esteban et al., 2009). For standardization of the protocol, the drug isoniazide and clarithromycin was used in *M. smegmatis* culture.

Preparation of Drugs

The drugs to be used were reconstituted in their appropriate solvents suggested by the manufacturer (Sigma-Aldrich, USA). Accordingly, Clarithromycin was dissolved in Acetone (at 50mg/ml), ethambutol in H_2O (50 mg/ml), and Isoniazide in H_2O followed by filtration through 0.22µm.

The concentrations used are as follows—

Clarithromycin	Stock- 10mg/ml; Working-200µg/ml
Isoniazide	Stock- 10mg/ml; Working-200µg/ml
Ethambutol	Stock- 10mg/ml; Working-200µg/ml

Preparation of the MTT dye

The dye, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide, was reconstituted following the manufacturer's protocol (5 mg/ml of H_2O). The working stock (1mg/ml) was prepared from the main stock (10mg/ml) by diluting in the media broth viz., Middlebrook 7H9 broth.

Protocol (As per Sankar et al., 2008 with minor modifications)

1. 100µl of Middlebrook 7H9 broth were added to well numbers 2 to 12 of the 96 well microtiter plate (Fig 12).

2. 200µl Middlebrook 7H9 broth and 200µl of drug from the working stock were added to well number 1. The concentration of the drug here now being 100 µg/ml.

3. After thorough mixing in well number 1, 200µl of the mixture was mix well and transfered to the consecutive wells upto well number 11 (two-fold dilution).

The drug concentrations from well no. 1 to 11 were as follows: 100/50/25/12.5/6.25/3.125/1.563/0.781/0.39/0.195/0.0975µg/ml

4. Following serial dilution, 100 µl McFarland No. 1 bacterial culture was added from well number 2 to 12.

The wells in column number 1 act as a negative control with only the drug and broth, while the wells in column number 12 with no drugs served as inoculum-growth control. Depending on the organism tested the plates were then incubated for upto 4 days at 37°C.

5. After incubation, except for row number 1 containing Middlebrook 7H9 broth and drug, 50 µl of the MTT dye (1mg/ml) was added to all the wells.

6. The plates were then incubated at 37°C for upto 24 hours.

Visual interpretation of the MIC was done by observing the colour change from yellow to purple. This colour conversion indicated growth of bacteria.

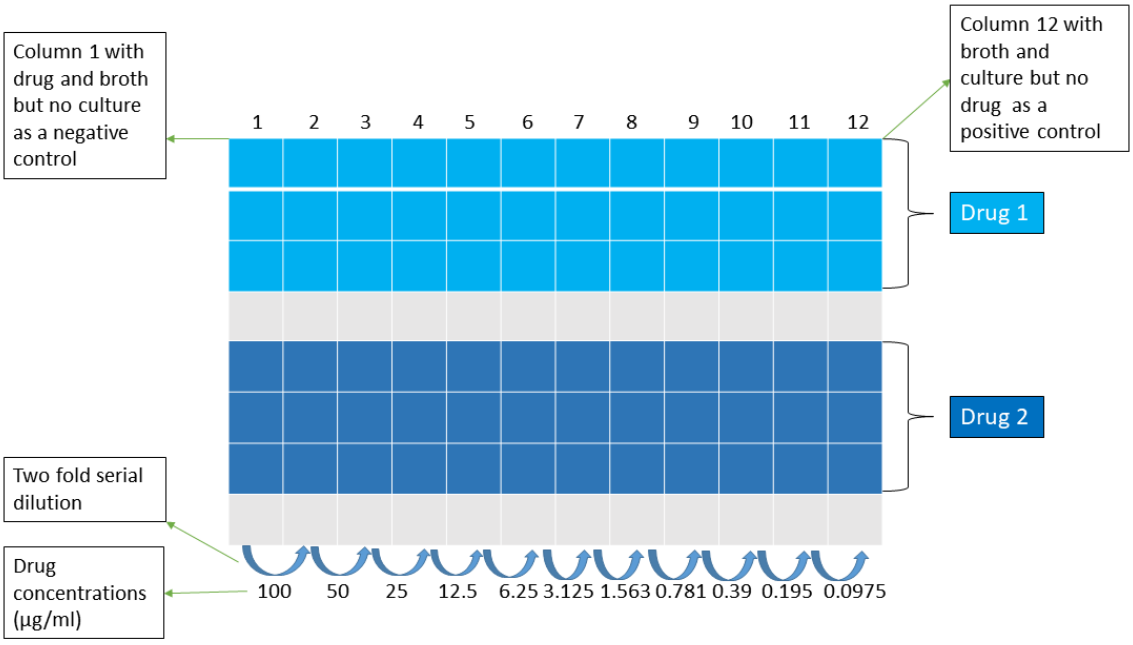


Fig 12. Phenotypic drug susceptibility test by MTT assay in 96 deep well plate. Image adapted with minor modification from Sankar et al. (2008)

5.8 SPECIES IDENTIFICATION BY MATRIX ASSISTED LASER DESORPTION IONIZATION- TIME OF FLIGHT MASS SPECTROMETRY (MALDI-TOF MS)

MALDI-TOF MS is a form of mass spectrometry that can be directly applied to microbial cells fixed in a matrix that results in spectra which are analyzed by the system. This technology is used for detection structural, soluble and ribosomal proteins which are present in high abundance, and also other macromolecules (Fig 13).

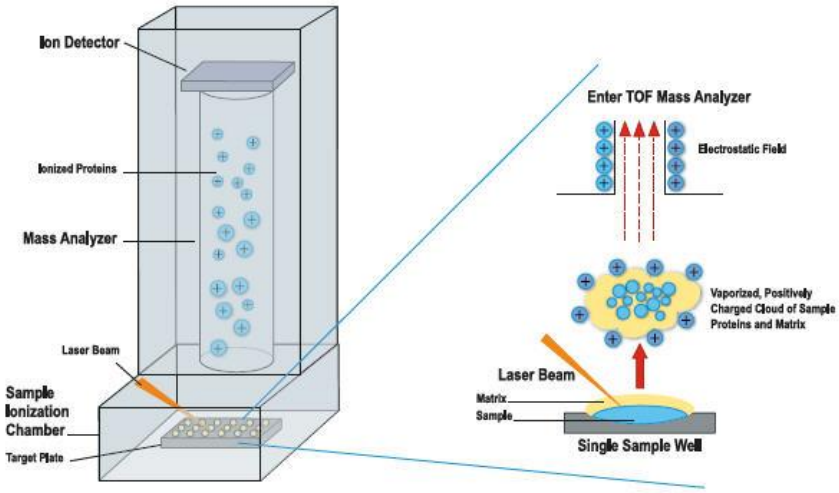


Fig 13. Sample identification by MALDI-TOF MS. Image source: MYO CLINIC, Myo Medical Laboratories. https://news.mayomedicallaboratories.com/2015/04/13/maldi-tof-ms-for-the-diagnosis-of-infectious-diseases-2/

For mycobacteria, total microbial proteins are analyzed by MALDI. Samples are fixed in a matrix solution and the charged particles are accelerated by a laser. Proteins and peptides are separated by increasing mass and are detected at the top of the TOF (time of flight) analyzer. The time taken for the ionized particles to passage through the mass analyzer is proportional to the ion's mass. The detected molecules are presented with a pattern of characteristic peaks called "spectrum" which are analyzed by the software installed in the system which analyzes the mass spectra against the library of stored spectra.

The VITEK® MS Plus system which was used in our study contains a broad range of database of different bacterial species and fungal species which are clinically relevant and can permit the identification of organisms (Fig 14). The instrument is attached to an 'Acquisition Station' which displays the status of the instrument real time and acquires the sample spectra. The spectra gets transferred to be analyzed in the system software, called the MYLA® software which also provides integration and presentation of data in a single window for viewing. The VITEK® MS Plus system provides two software platforms: VITEK® MS for routine patient (IVD) testing and the VITEK® MS RUO (Research Use Only). The former software allows detection of clinically relevant species while the latter contains broad research database for microorganisms mainly to be employed for research use and not clinical diagnosis. The manufacturer claims the database to have been created by accumulating the mass spectra of multiple isolates per species from geographically distinct strains, varied sample origins, and different media use for culture under different incubation duration with 1286 spectra for mycobacteria in the library.

Fig 14. BioMerieux Vitek MS in Nazareth Hospital, Shillong, Meghalaya.

As compared to conventional, phenotypic or PCR-based identification, the MALDI-TOF MS requires low sample quantity, allows rapid turnaround time (1-2 minutes per sample), relatively cheaper reagents and fewer technical support. The MALDI-TOF MS method has attracted interest for identification of bacteria because it is rapid, accurate, and has low operational costs (Seng et al., 2013; Bilecen et al., 2015).

Three presumed NTM isolates viz., PK504, FU239 and FU362, from follow-up tuberculosis patients were shared with us by Nazareth Hospital, Shillong, Meghalaya, India for molecular studies. Samples were processed in their mycobacteriology laboratory and cultured in liquid media using Mycobacterial Growth Indicator Tube (MGIT) instrument. All these isolates were sub-cultured in solid LJ medium for pure preserving and obtaining pure colonies in our laboratory. Using freshly grown cultures the isolate was processed for MALDI identification according to the manufacturer's protocol with *Escherichia coli* ATCC 8739 as control. Due to their pathogenicity and high lipid content cell wall, sample preparation of mycobacteria differ from other organism and require some modifications for inactivation, improved protein extraction and clump disruption methods (Saleeb et al., 2011; Deol et al., 2013). The procedure for sample processing and slide preparation was performed according to the manufacturer's protocol, given in the chart flow below (Fig 15).

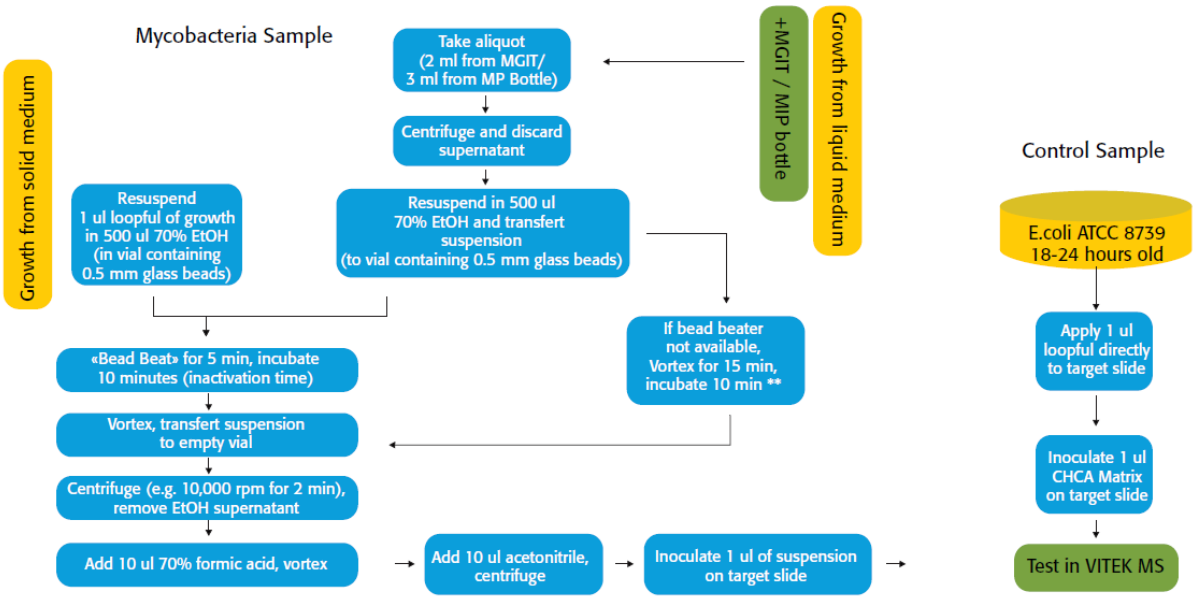


Fig 15. Flow chart for sample preparation of mycobacteria for processing in VITEK® MS Plus system. Image source Deol et al. (2013).

Protocol

Sample processing for Mycobacteria from solid culture (LJ and Middlebrook)

1. In vial containing 0.5 mm glass beads and 500 µl 70% ethanol, one micro litre loopful of growth was re-suspended.
2. The vials were vortexed in a bead beater vigorously for upto five minutes and for inactivation incubated at 10 minutes (room temperature).
3. This was followed by carefully transferring the suspension into an empty vial and centrifuged at 10,000 rpm for two minutes.
4. Not to disrupt the pellet the ethanol supernatant was carefully discarded.
5. 10 µl of 70% formic acid was added after drying the pellet.
6. The tubes were vortex for 15 minutes and 10 µl acetonitrile was added followed by centrifugation at 10,000 rpm for 02 minutes.
7. This suspension (1 µl) was fixed on the spot of the target slide by mixing with a matrix made of 1 µl of CHCA (α-Cyano-4-hydroxycinnamic acid), and taken for test.

Sample processing for Mycobacteria from liquid culture

1. 2 ml aliquot was used from MGIT or 03 ml of any broth culture.
2. The tubes were centrifuge and the supernatant discarded in an effective disinfectant (5% phenol)

3. The pellet was dissolved in 70% ethanol (500 µl) transferred to vial containing 0.5 mm glass beads and followed the same procedure as in solid culture (steps 2 to 7).

Control Sample

Escherichia coli ATCC 8739 of 18-24 hours old is used as the control.

1. 1 µl loopful of the culture was directly applied to target slide
2. 1 µl of CHCA Matrix was added on the spot containing the test organism
3. Tested in VITEK MS

5.9 MOLECULAR IDENTIFICATION

Studies on mycobacteria depend heavily upon the discrimination value of identification methods. Conventional identification tests are time consuming and at times may be inefficient in characterizing and distinguishing various mycobacteria (Steed et al., 2006). Available data show that molecular methods have greatly improved the taxonomic knowledge, which have surpassed HPLC for mycolic acid content or biochemical tests, and instead allows better differentiation of different mycobacterial organisms (Selvaraju et al., 2005). A culture growth or a positive AFB smear does not always confirm infection of the entity isolated. Further analysis is required for detection of infection by correlating with clinical features or repeat isolations.

For identification and differentiation of our isolates, several mycobacterial genus and species-specific genes have been targeted. Sequencing of these genes were done for exploring the lineage and comparing with native and global isolates with the aid of phylogenetic analysis. The work flow for molecular studies was initiated by isolating the double stranded hereditary material— the deoxyribonucleic acid (DNA), followed by *in vitro* amplification of the targeted gene, cloning, sequencing and phylogenetic studies, details of which are discussed in the following pages.

5.9.1 Genomic DNA extraction

DNA extraction was performed both by conventional approach as well as kit-based methods irrespective of the isolate type and media where they were grown. Conventional method was used due to low cost and higher DNA yield, although the yield is highly variable. In crude extraction methods, it was not easy to remove the cell or other debris and the contaminating reagents which may inhibit downstream applications. Kits which were silica-based were the preferred alternative as the time taken was shorter, simple to perform, optimum DNA recovery was achieved with high purity and better scope to handle larger sample quantity. The disadvantage of using kits was their high cost. Both methods were used in this study which are described below.

I. Conventional methods

Boiling followed by Snap-chill method (Surendran et al.*, 2002; Arora* et al.*,2006)*

In this method, DNA isolation was done by boiling followed by snap chill.

1. A loopful of the cultures grown in solid media were directly inoculated in PBS and centrifuged at 3000 rpm for 5 minutes.
2. 100 ml PBS was used for dissolving the pellets.
3. This pellet suspension was subjected to vigorous boiling at 100°C in a heating block or water bath upto 30 minutes and then immediately snap chilled by any one of the following.
    - Placing in ice for 5-10 minutes
    - Freezing at -20°C for 5 minutes (Kuske et al., 1998)
    - Placing in the refrigerator until they reached 4°C (Kuske et al., 1998)
4. Following freezing, rapid and intense thawing of the sample was done by placing the tubes immediately in a heating block (98°C) or boiling water bath.
5. This process was repeated 3-5 times.

This lysate was used as the template for molecular detection..

Cetyltrimethylammonium bromide (CTAB) lysis method (Murray and Thompson, 1980)

The original CTAB method described by *Murray and Thompson, 1980* was adopted as the reagents required were readily available and obtained good DNA yield.

A. Murray and Thompson, 1980

1. In an eppendorf tube containing 400µl of 1X TE buffer (1mM EDTA /10mM Tris-HCl, pH 8.0) 2 to 3 loops of growth from freshly grown mycobacterial culture on Middlebrook 7H10 and 7H11 were transferred.
1. The cells were killed at 80^{o}C for 20 min in a heating block and cooled at 21^{o}C.
2. 50µl of 10mg/ml lysozyme was added to the above tubes and vortexed briefly followed by incubation at 37^{o}C for 1h.
3. 5µl of 10mg/ml proteinase-K and 70µl of 10% SDS solution were added and vortexed.
4. For disruption of the bacterial cell membranes, tubes were incubated at 65^{o}C for 10 mins.
5. Following incubation, 100µl each of pre-warmed CTAB-NaCl and 5M NaCl solution was added. The mixture was vortexed until the liquid became whitish and then was incubated at 65^{o}C for 10 min.
6. Subsequently, 750µl of phenol:chloroform:isoamyl alcohol (25:24:1) was added and vortexed.

7. To release the DNA into the solution the tubes were centrifuged for 8 min at 8000 rpm.

8. A fresh microfuge tube was kept ready where the aqueous phase which contains the extracted DNA was carefully transferred

9. From this aqueous phase, DNA was precipitated by addition of 0.6 volume of isopropanol.

10. The tubes were then kept at –20^{o}C for 30 min followed by centrifugation for 15 min at 11,000g.

11. Leaving just about 20 µl above the pellet, the resulting supernatant was discarded

12. The pellet was gently washed with 1ml of chilled 70% ethanol followed by centrifugation at 11,000g for 5 minutes.

13. The supernatant was discarded, leaving about 20µl above the pellet and centrifuged again for 1 min at 11,000g.

14. The supernatant was discarded by careful pipetting and the tubes were left inverted to dry the pellets at room temperature for upto 30 mins or until complete evaporation of ethanol.

15. Dried pellet was finally dissolved in 20 to 30µl of 1x TE buffer, aliquoted in small quantities and except for one tube to be used immediately, the rest were was stored at -20^{o}C for further use.

II. Kit-based method (DNeasy® Blood & Tissue, Qiagen, USA)

DNeasy® Blood & Tissue have been majorly used for genomic DNA extraction. The isolated DNA could be used for molecular process. The time for DNA isolation was significantly reduced involving a few ready-to-use reagents and giving high purity DNA even though the yield was compromised which may be due to elution of DNA from column-based silica membrane. Majority of the samples, irrespective of growth medium used, were isolated by this kit following the manufacturer's 'cultured cells' protocol, and the DNA was used for all the downstream molecular studies.

5.9.1a DNA quantification

Assessing the quantity and purity of samples prior to downstream analysis is important not only to avoid time and financial loss wasted in failed experiments but verify the suitability for downstream testing. We used the Thermo Scientific NanoDrop 2000 spectrometer for assessing the quality of our isolated DNA by following the manufacturer's protocol. The NanoDrop 2000 spectrophotometer is known to measure 2 to 15,000 ng/µL of double stranded DNA.

<u>Protocol</u>

1. The Nucleic Acid application installed in the system was opened in which the "Nucleic Acid" option was chosen.

2. A blank measurement was done which was saved as the reference spectrum.

This blank solution used for any solution should be of a similar ionic strength as the sample solution and the same pH. For nucleic acid samples, blank buffers are generally TE (Tris-ETDA) or distilled water, depending on what the DNA has been dissolved.

3. 1- 2µL of the blank was pipetted directly onto the measurement pedestal followed by lowering the "measuring arm'.

4. The ID of the test sample was entered and the concentration measured. The reading gets stored and the next measurement can be done.

Before taking another measurement, both the measurement surfaces was wiped with a lint-free tissue.

5. The same procedure was followed for the next test samples and the tips were changed for every measurement.

Purity ratio as given by the manufacturer for 260/280 is generally ~ 1.8 to be accepted as "pure" for DNA. Although purity ratios are important indicators of sample quality, the manufacturer's suggest that the best indicator of DNA or RNA quality is functionality in the downstream application of interest like real-time PCR. A vivid description on the working of a nanodrop can be viewed in the video (Desjardins et al., 2010) available in this website: www.jove.com/video/2565/nanodrop-microvolume-quantitation-of-nucleic-acids

5.9.2 Polymerase chain reaction (PCR)

For achieving the aim of this study, combinations of previously described molecular techniques have been used which are discussed in detail in the following sections. As a consequence of the close genomic relationship and the high antigenic cross-reactivity among mycobacteria, it is not an easy task for identification of a species-specific sequence. Experiments were designed so as to detect all the culturable mycobacteria, keenly the NTM, from the three sample sources we targeted. Amplification of our gene-of-interests were achieved by involving several primers which are listed in Table1.

Sl. No	Region or gene targeted	Primer seqeunce (5'-3')	Product size (bp)	Reference
1.	*hsp65*	ACCAACGATGGTGTGTCCAT CTTGTCGAACCGCATACCCT	439	Telenti et al. (1993)
2.	*IS*6110	CCTGCGAGCGTAGGCGTCGG CTCGTCCAGCGCCGCTTCGG	123	Eisenach et al. (1990)
3.	500bp	TCGTCCGCTGATGCAAGTGC CGTCCGCTGACCTCAAGAAG	500	Rodriguez et al. (1995)
4.	RD9	GTGTAGGTCAGCCCCATCC CAATGTTTGTTGCGCTGC GCTACCCTCGACCAAGTGTT	306 (*M.tuber-culosis*) 206 (*M. bovis*)	Parsons et al. 2002
5.	RD4	AACGCGACGACCTCATATTC AAGGCGAACAGATTCAGCAT	400	Sales et al. (2014)
6.	*rpoB* (Duplex)	CGTACGGTCGGCGAGCTGATCCAA CCACCAGTCGGCGCTTGTGGGTCAA GGAGCGGATGACCACCCAGGACGTC CAGCGGGTTGTTCTGGTCCATGAAC	235 (MTBC) 136 (NTM)	Kim et al. (2004)
7.	*rpoB*	GGCAAGGTCACCCCGAAGGG AGCGGCTGCTGGGTGATCATC	723	Adekambi et al. (2003)
8.	*rpoB*	TCAAGGAGAAGCGCTACGA GGATGTTGATCAGGGTCTGC	360	Lee et al. (2003)
9.	*16srRNA*	GAGTTTGATCCTGGCTCAG AAGGAGGTGATCCAGCCGCA	1500	Pascual et al. (1995)
10	*rrl* (23S rRNA)	CCTGCACGAATGGCGTAACG CACCAGAGGTTCGTCCGTC	700	Maurer et al. (2012)

Table 1. List of all the primers used in the study.

The isolates identified as mycobacteria by conventional methods were firstly subjected to *Mycobacterium* genus specific *hsp65* PCR (Telenti et al., 1993). Isolates showing amplifications of *hsp65* confirms to be a *Mycobacterium*. Subsequently, these amplicons were purified and subjected to restriction enzyme digestion using the enzymes *Bst*EII and *Hae*III. Though this approach categorised majority of the isolates into MTBC and NTM, identification of several strains were restricted due to species with overlapping or multiple patterns within a single species and limited number of patterns available. *hsp65* PCR positive isolates were simultaneously processed for sequencing. Isolates now confirmed to be mycobacteria were also checked for amplification of the insertional sequence IS*6110*. This was done for detection of MTBC members, which identify the MTBC members

among the isolates. Isolates amplified in IS*6110* PCR was then subjected to species-specific PCR detection of *M. tuberculosis* and *M. bovis* using 500bp fragment, RD9 and RD4 regions. Parallel to this, a highly intra-species discriminating conserved region known as the *rpoB* gene which encodes the β-subunit of the RNA polymerase in mycobacteria was also amplilfied and sequenced for identifying our NTM isolates.

Two genes were targeted for identification and the obtained sequences were used for phylogenetic studies. One is the genus-specific *hsp65* gene and the other being *rpoB* gene. Two sets of *rpoB* gene primers were used (Adekambi et al., 2003; Lee et al., 2003) due to repeated sequencing failure and non-specific amplifications despite repeated attempts through the Adekambi et al. (2003) primers. Another approach was adopted for categorizing the MTBC and the NTM. A duplex PCR based on *rpoB* as the gene target was also used in the study which amplified two different bands sizes for MTBC and NTM thereby differentiating the two. It is also claimed to be able to differentiate MTBC and NTM in a single reaction containing. Species which indicated to be unique and rare were also subjected to amplification by the housekeeping gene- 16S rRNA. These two gene amplicons were either directly purified and sequenced or cloned and sequenced depending upon the requirement. Nucleotide sequences were examined for any misreading before processing for further studies. Phylogenetic analysis of the isolates was carried out using individual tree based analysis on the sequenced gene with reference strains of MTBC and best matches. All sequences were aligned using Clustal W algorithm and the dendrogram was inferred using the freely available software MEGA7. Sequence study was again initiated with BLAST analysis in NCBI for identification. The isolate's sequence was put on blast analysis in National Center for Biotechnology Information (NCBI), U.S. National Library of Medicine for identification of the species. Next, Sequence alignment for each gene was performed using CLUSTAL W program in MEGA 7 (Kumar et al., 2016) after which all the three genes were individually subjected to phylogenetic analysis together with the NCBI database reference ATCC sequences of the closest BLAST hits. For rare and important isolates phylogenetic analysis by concatenation of two to three genes were performed. Analysed sequences were also submitted to GenBank, NCBI for record.

5.9.2a Reaction preparation

All reactions were carried out in a thin-walled 200 µl PCR tube. A Thermo Scientific DreamTaq Green PCR Master Mix (2X) ready-to-use solution was used in the study containing DreamTaq DNA Polymerase, dNTPs, $MgCl_2$ and optimized buffer. The master mix is capable of producing amplification up to 6 kb from genomic DNA. The specification of the master mix is given below and the details of the components and reaction preparation is given in table 2.

Polymerase: DreamTaq DNA Polymerase
Hot Start: No
Fidelity (vs. Taq): 1 X
GC-Rich PCR Performance: Low
Reaction Format: SuperMix or Master Mix
Product Overhang:3'-A

Sl. No.	Component		Concentration	Amount per reaction
1.	Master mix	Buffer (DreamTaq Green)	10X	10µl
		$MgCl_2$	20 mm	
		dNTP mix	2 mM each	
		DreamTaq DNA Polymerase	1.25U	
2.	Forward primer		10 pm/µl	1 µl
3.	Reverse primer		10 pm/µl	1 µl
4.	Template DNA		20ng/ µl-2µg/µl	2µl
5.	Nuclease free water			6 µl
Total reaction volume				20 µl

Table 2. Components and reaction preparation of PCR

All PCR reactions were prepared according to table 2 and optimization was done for each primer, given in the following pages. Positive and negative controls were used in each experiment and the reaction mixtures prepared in the dedicated molecular work bench. Thermal cyclers of various make were used in the study.

5.9.2b PCR standardization and gene amplification

1. *hsp65* (Telenti et al., 1993): This 60 kDa heat-shock protein family due to their highly conserved primary structures and ubiquity have been considered for wide applications. Isolates showing amplifications of *hsp65* confirms to be a *Mycobacterium*. The standardized protocol is given in Table 3.

Table 3. Optimized PCR conditions for *hsp65*

Step No.	Process	Temperature	Time	
1.	Initial denaturation	95°C	4 minutes	
2.	Cycle denaturation	95°C	30 seconds	35 cycles
3.	Annealing	59°C	30 seconds	
4.	Extension	72°C	35 seconds	
5.	Final Extension	72°C	7 minutes	
6.	Hold at 4°C			

Polymerized Chain Reaction-Restriction Enzyme Analysis (PCR-REA)

Restriction digestion of *hsp65* for speciation of mycobacterial species was done for isolates showing amplification of *hsp65*. The isolates were subjected to restriction digestion using the enzymes *Bst*EII and *Hae*III. This restriction enzyme (RE) digestion was done to identify the species of the confirmed mycobacteria. Published algorithms (Telenti et al., 1993; Chimara et al., 2008) of the band fragmentation pattern produced by the enzymatic treatment was inferred to identify the species of the isolates.

The enzymes were procured from ThermoFisher Scientific, USA. The *Hae*III restriction enzyme recognizes GG^CC sites while *Bst*EII restriction enzyme recognizes G^GTNACC sites. RE digestion was performed as per the recommended protocol by the manufacturer for samples directly used after amplification.

Protocol

1. In a thin walled 200 µl tube the following mixtures were added:

PCR reaction mixture	10 µl (~0.1-0.5 µg of DNA)
nuclease-free water	9 µl
10X Buffer	2 µl (Buffer R for *Hae*III; Buffer O for *BstE*II)
Enzyme	2 µl

2. The tubes were mixed gently and mildly centrifuged down.
3. Both the digestions were carried out by incubation at 37°C for 1 upto 16 hours.
4. The digested products were checked in by agarose gel electrophoresis as described in section 5.9.2c.

2. IS6110 (Eisenach et al., 1990)**:** IS*6110* is 1,361 bp long containing 28-bp imperfect inverted repeats at its extremities with three mismatches and 3-bp direct repeats. This insertional sequence was found to be specific to mycobacteria belonging to the MTBC. Primers have been designed from this sequence to detect *M. tuberculosis* in clinical specimens. The optimized PCR conditions are as follows (Table 4).

Table 4. Optimized PCR conditions for *IS6110*

Step No.	Process	Temperature	Time	
1.	Initial denaturation	95°C	5 minutes	
2.	Cycle denaturation	95°C	30 seconds	35 cycles
3.	Annealing	70°C	35 seconds	
4.	Extension	72°C	30 seconds	
5.	Final Extension	72°C	5 minutes	
6.	Hold at 4°C			

3. 500bp (Rodriguez et al., 1995): Given its zoonotic potential the 500bp fragment of *M. bovis* was targeted for the species identification (Table 5). Studies have shown the amplification of an approximately 500 bp fragment present in the genome of 15 different *M. bovis* strains, including *M. bovis* BCG. Based on a partial nucleotide sequence of the 500 bp fragment, oligonucleotide primers were designed which have been used in the study. Although 500bp gene was reported to be found only in *M. bovis*, its specificity is debated owing to amplification of some of the strains of *M. tuberculosis* (Metaxa-Mariatou et al.**,** 2004) and the same has also been observed in our study.

Table 5. Optimized PCR conditions for 500bp fragment

Step No.	Process	Temperature	Time	
1.	Initial denaturation	95°C	4 minutes	
2.	Cycle denaturation	95°C	30 seconds	35 cycles
3.	Annealing	59°C	30 seconds	
4.	Extension	72°C	35 seconds	
5.	Final Extension	72°C	7 minutes	
6.	Hold at 4°C			

4. RD4 (Sales et al., 2014): Primers designed flanking the region of RD4 were optimized for identification of *M. bovis* isolates. PCR conditions are given in Table 6.

Table 6. Optimized PCR conditions for RD4

Step No.	Process	Temperature	Time	
1.	Initial denaturation	95°C	4 minutes	
2.	Cycle denaturation	95°C	30 seconds	35 cycles
3.	Annealing	59°C	35 seconds	
4.	Extension	72°C	35 seconds	
5.	Final Extension	72°C	7 minutes	
6.	Hold at 4°C			

5. RD9 (Parsons et al., 2002)**:** Members of the MTBC are closely related and speciation of the members require targeting different regions which might show a deletion or an insertion in specific regions. Comparative genomic analyses with the genome of *M. tuberculosis* H37Rv has provided information on regions of difference (RD 1 to RD 16) some of which were deleted in *M. bovis* and *M. bovis* BCG and other members of the complex (Parsons et al., 2002). Deletion analyses suggested the presence or the absence of six regions (RD 1, RD 3, RD 5, RD 9, RD 10, and RD 11) originally described as being deleted in the genomes of BCG isolates relative to the sequence of *M. tuberculosis* H37Rv. The region of differention 9 (RD9) region is present in *M. tuberculosis* but absent in *M. bovis*. The RD9 PCR conditions are same as that of RD4. This PCR helped in differentiation of *M. bovis* and *M. tuberculosis* in a single reaction tube. PCR conditions are same as given in Table 4.

6. Duplex *rpoB* (Kim et al., 2004): A duplex PCR assay, to differentiate MTBC and NTM by using a single gene, the RNA polymerase betasubunit-encoding gene (*rpoB*). Even the coexistence of *M. tuberculosis* and NTM can be detected by the presence of two different PCR products in a single reaction mixture.PCR involving two different sets of primers were used to differentiate MTBC and NTM by amplifying the 235- and 136-bp *rpoB* DNAs of respectively. The optimized PCR conditions are given in Table 7.

Table 7. Optimized PCR conditions for duplex *rpoB*

Step No.	Process	Temperature	Time	
1.	Initial denaturation	95°C	5 minutes	
2.	Cycle denaturation	95°C	30 seconds	35 cycles
3.	Annealing	60°C	35 seconds	
4.	Extension	72°C	30 seconds	
5.	Final Extension	72°C	5 minutes	
6.	Hold at 4°C			

7. *rpoB* (Adekambi et al., 2003): The *rpoB* gene sequence is known to be a highly discriminating with the capacity to characterize emerging rapid growers and detect mycobacterial infections. This gene provides valuable help in differentiating the rapidly growing mycobacteria at both the intra- and interspecies level. This gene was used for phylogenetic analysis of the isolates and when clear distinction of closely related species within a complex could not be made. PCR amplification of the *rpoB* gene was carried out using all the presumed mycobacterial genomic DNA as template. Partial *rpoB* sequence was amplified using primer sets reported by Adekambi et al. (2003) and Lee et al. (2003). The former primer set produced a product size of 764 bp while a smaller amplicon size of 360 bp was obtained by the latter primer. Amplification using primers of Adekambi et al., (2003) was possible only for a limited number of species due to high non-specific amplifications and repeated sequencing failure. Therefore for these isolates, a shorter amplicon size targeting another region of the *rpoB* gene reported by Lee et al. (2003) was used. This primer was chosen due to its discriminatory capacity within a short amplicon size as claimed by the author and cost effectiveness for sequencing. The optimized PCR conditions are given in Table 8.

Table 8. Optimized PCR conditions for *rpoB* (Adekambi et al., 2003)

Step No.	Process	Temperature	Time	
1.	Initial denaturation	95°C	4 minutes	
2.	Cycle denaturation	95°C	35 seconds	35 cycles
3.	Annealing	57°C	35 seconds	
4.	Extension	72°C	40 seconds	
5.	Final Extension	72°C	7 minutes	
6.	Hold at 4°C			

8. *rpoB* (Lee et al., 2003): The 360-bp region of the *rpoB* gene (bases 902 to 1261 and codons 302 to 420) belonging to *M. tuberculosis*; was found to be useful in the differentiation of more than 50 species of mycobacteria by restriction fragment length polymorphism (RFLP) studies. A primer was designed against this 360-bp region of the *rpoB* gene which contains highly informative sequences. The optimized PCR conditions are given in Table 9.

Table 9. Optimized PCR conditions for *rpoB* (Lee et al., 2003)

Step No.	Process	Temperature	Time	
1.	Initial denaturation	95°C	5 minutes	
2.	Cycle denaturation	95°C	30 seconds	35 cycles
3.	Annealing	60°C	35 seconds	
4.	Extension	72°C	30 seconds	
5.	Final Extension	72°C	5 minutes	
6.	Hold at 4°C			

9. *16s rRNA* (Eisenach et al., 1990): *16s* rRNA is encoded by the 16S ribosomal DNA (rDNA). Genetic investigation of mycobacterial taxonomy found the 16S rRNA to represent nucleic acid sequence stretches which were suitable for identification of mycobacteria. These hypervariable sequences are known as region A and B, which correspond to the *Escherichia coli* positions around 130 to 210 and 430 to 500 respectively. The complete 1,500 bp of the gene was amplified and sequenced for isolates which were unique and unusual to substantiate the complete characterization of those rare isolates. Standardized conditions given in Table 10.

Table 10. Optimized PCR conditions for *16S rRNA*

Step No.	Process	Temperature	Time	
1.	Initial denaturation	95°C	4 minutes	
2.	Cycle denaturation	95°C	35 seconds	40 cycles
3.	Annealing	59°C	35 seconds	
4.	Extension	72°C	1 minute, 20 seconds	
5.	Final Extension	72°C	7 minutes	
6.	Hold at 4°C			

10. *rrl* (Maurer et al., 2012): Many rapidly growing mycobacteria (RGM) are macrolide resistant due to bacterial genes that encode proteins which methylate ribosomal DNA in a critical region of the large subunit (50S) ribosome. This ribosomal region, in the 23S component region of the 50S ribosome is termed "domain V". Domain V is the region in

which macrolides bind to the ribosome, and a single methylation or mutation of critical bases can result in a conformational change resulting in macrolide resistance. Methylation of base 2058 or 2059 is the most common mechanism of resistance in the RGM. Erythromycin ribosomal methylase ("*erm*" gene) methylates 2058, resulting in macrolide resistance. Although it does not occur with any recognizable incidence in other bacterial species, mutations in domain V, like A2058G or A2059G results in macrolide resistance. These mutations are known as "*rrl*" mutations, where *rrl* is the gene encoding a 23S peptidyl transferase in the large ribosomal subunit. Species like *M. abscessus* commonly acquires clarithromycin resistance mutations in this *rrl* gene. In our study, the *rrl* gene was amplified and sequenced for clinically relevant samples to inspect any resistance to clarithromycin (Maurer et al., 2012). Following sequencing, acquisition of a 2058AG mutation in the *rrl* gene was checked and alternatively correlated with the phenotypic test described in section 5.7.3. The optimized PCR conditions are given in Table 11.

Table 11. Optimized PCR conditions for *rrl*

Step No.	Process	Temperature	Time	
1.	Initial denaturation	95°C	4 minutes	
2.	Cycle denaturation	95°C	35 seconds	35 cycles
3.	Annealing	59°C	35 seconds	
4.	Extension	72°C	40 seconds	
5.	Final Extension	72°C	5 minutes	
6.	Hold at 4°C			

5.9.2c Visualization of amplified PCR product

Varying concentration of 1-2.5 % agarose gel was prepared depending upon the product size to be detected. The required amount of agarose was weighed and dissolved in 1X TAE (Tris-acetate-EDTA) by heating. After cooling the mixture to RT, ethidium bromide (3 µl/100 ml) was added and poured into a casting tray. When the gel was cooled and solidified, the combs were removed and the tray placed in the tank containing the TAE buffer. 3-7 µl of the PCR products were loaded directly into the wells. A DNA marker/ ladder was also loaded beside the PCR samples to measure and interpret the band sizes. The loaded gel was given a run at 90 V/60 mA for 30-40 minutes. Amplified products were visualized under UV transilluminator and the gels were photographed using the gel documentation system for analysis.

5.9.2d PCR product purification

For single specific bands

Purification of PCR products are required to remove the salts and unused primers or uncorporated nucleotides that might affect the downstream processes. Following the recommended manufacturer's protocol, we used Applied Biosystems™ CleanSweep™ PCR Purification Reagent and QIAquick PCR Purification Kit Protocol (Qiagen, USA)

depending upon the requirement for product purification. The protocol for 'CleanSweep' PCR clean-up is given below.

Protocol

1. The PCR purification reagent called the 'CleanSweep' is placed on ice or a minicooler.

2. To 5 µl of the PCR product, 2 µl of the reagent is added.

For PCR product volumes greater than 5 µl, the volume of the reagent is added proportionally.

3. In the thermal cycler, the option for 'incubation' was chosen and the following conditions are set:

- 37°C for 15 minutes to hydrolyze primers and dephosphorylate dNTPs.

- 80°C for 15 minutes to inactivate the 'CleanSweep' reagent.

These cleaned up PCR products were stored at -20°C until use.

For non-specific multiple bands

Samples with multiple bands were excised with QIAquick Gel Extraction Kit, Qiagen, following the manufacturer's protocol.

Protocol

1. The gel with non-specific bands were placed in a UV illuminator and the mode for excision was selected.

Care was taken to cover all exposed surfaces by using gloves, eye safety goggles, lab coat and a face shield while performing this task.

2. Specific single band was incised with a clean scalpel and transferred into a 1.5 ml micro centrifuge tube

3. 750 µl of buffer QG was added to the tube and incubated at 50°C for 10 min with vortexing every 3 min to help dissolve the gel.

4. After the gel has completely dissolved, colour of the mixture was examined to check if it was similar to buffer QG without dissolved agarose. If the color of the mixture is changed, 10 µl of 3 M sodium acetate, pH 5.0 was added till the mixture turns yellow.

5. Following this, 250 µl of isopropanol was added to the sample and mixed by inverting

6. Samples were then transferred to a 2 ml collection tube containing a spin column

7. The tubes were centrifuged at 13,000 rpm for 1 min.

8. The flow-through was discarded and the spin column was placed back into the same collection tube and the remaining samples were centrifuged again.

9. 500 µl of buffer QG was added to the spin column and centrifuged for 1 min at 13,000 rpm

10. The filtrate was discarded and 750 µl buffer PE was added to the column and centrifuged at 13,000 rpm for 1 min.

11. The flow-through was discarded and the spin column was placed back into the tube for dry spin.

12. The spin column was then placed on a 1.5 mL tube and 15 µl buffer EB was added to the center of the column and allowed to stand for 1 min.

13. The tubes were centrifuged at 13,000 rpm for 1 min and the purified DNA in the tube was stored at 4°C for further use.

5.9.3 Cloning and transformation

Sequences of only selected unique isolates were cloned and sequenced. Cloning was done by InstAclone PCR cloning kit (ThermosScientific, USA) which is a TA system for cloning PCR products with 3'-dA overhangs generated by *Taq* DNA polymerase and other thermostable DNA polymerases which lacks proofreading activity. The TA cloning vector used is pTZ57R/T whose restriction map and multiple cloning site (MCS) is given in Fig 16 & 17). Short DNA fragments (<1 kb) were cloned with a much higher efficiency compared to long ones. Therefore, long PCR products must be purified well to remove any interfering smaller fragments from the PCR reaction solution.

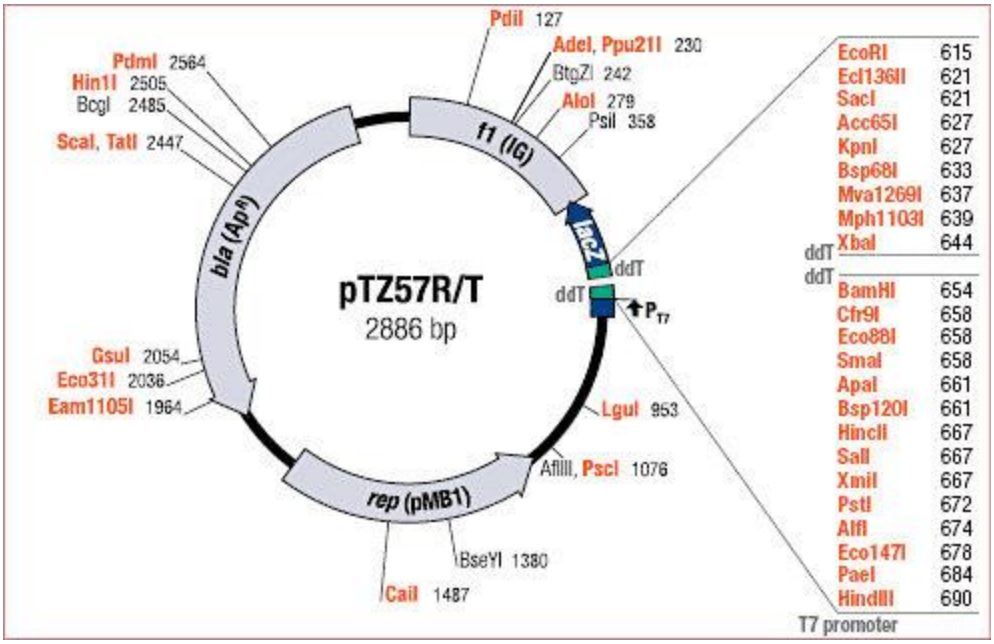


Figure 16. Restriction map of vector pTZ57R/T. Image source: ThermoFisher Scientific website. https://www.thermofisher.com/order/catalog/product/K1213

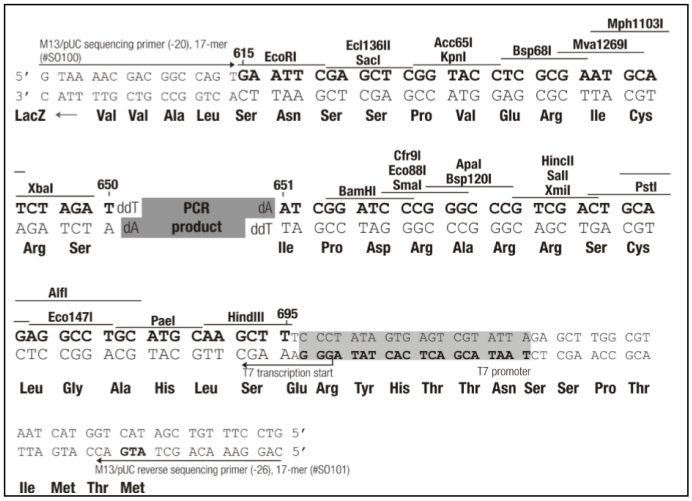


Fig 17. The sequence of multiple cloning site (MCS) of the vector pTZ57R/T. Image source. Thermoscientific website.
https://www.thermofisher.com/order/catalog/product/K1213

Preparation of competent cells (Sambrook et al.*, 1989)*

1. Revival of the host for competent cells (DH5α) was performed for transformation.
2. Lyophilized vials were broken and 100 µl of sterile LB (Luria-Bertani) broth was added.
3. A loopful of suspension was streaked on LB plate and incubated at 37ºC till visible growths were observed.
4. A single colony was inoculated into 5 ml of LB medium and incubated at 37ºC overnight.
5. 1 ml of overnight culture was inoculated into 100 mL of LB media in a 100ml conical flask and incubated at 37ºC in a shaker for 2-3 hours.
6. Culture flask was chilled on ice for 10-20 min.
7. Cultures were transferred aseptically into a sterile centrifuge tubes and spun at 6000 rpm for 8 min at 4ºC.
8. Supernatant was discarded and to the cell pellet, approximately 15 ml of cold 0.1 M CaCl2 solution was added aseptically.
9. Tube was placed on ice for 30 min followed by centrifugation at 6000 rpm for 8 min at 4ºC.
10. Supernatant was discarded and resuspended into 0.6 ml of cold 0.1 M $CaCl_2$ (calcium chloride) solution.
11. Aseptically, aliquots of 100 µl competent cell were prepared on 6 prepared chilled tubes.

12. This was done without removing centrifuge tubes from the ice. Prepared cells were stored at -80ºC until further use.

Ligation

With a view for transformation, ligation was performed as follows according to the manufacturer's protocol.

5x Ligation buffer	3 µl
pTZ57R/T Vector	0.5 µl
T4 DNA ligase at 1000 CEU/µl	0.5 µl
Template	4 µl
Nuclease free water	7 µl

The reaction was stored overnight at 4ºC.

Transformation

For the competent cells to uptake the exogenous DNA ligated into the vector, transformation was done as follows:

1. 5 µl of plasmid DNA was added to 5 aliquots of 100 µl of competent cells.
2. After gentle tapping, it was placed on ice for 20ºC.
3. Cells were heat-shocked by placing the vials in water bath at 49ºC for 2 min and placed on ice to chill for 5 minutes.
4. One ml of LB broth was added aseptically to the vials and incubated at 37ºC in a shaker for an hour to allow the bacteria to recover and express the antibiotic resistance.
5. Following this, 100 µl of LB broth was pipetted and spread thoroughly using a loop on LB plate containing Ampicillin, X-gal and IPTG.
6. Hundred microlitre of competent cells that has not been transformed was streaked on the plates as control.
7. Plates were incubated at 37ºC overnight.
8. On completion of incubation, applying blue white selection procedure, white colonies were selected that indicated successful transformation of the cells.
9. Selected colonies were inoculated in fresh LB broth and incubated overnight at 37ºC.
10. On completion of incubation, loopful of transformed cells were cultured in semi-solid nutrient agar stabs and stored at 4ºC until sequencing.

5.9.4 Sequencing

The purified PCR samples of *hsp65, rpoB,* selected *rrl*, *16s rRNA,* and cloned samples were sequenced by Sanger's method. Products of *rpoB,* and selected *rrl* and *16s rRNA* genes were sequenced bidirectionally by the respective primers on requirement. The cloned products were sequenced using the using M13/pUC and T7 standard primers. Sequencing was performed both by outsourcing and in the institute (Indian Council of Agricultural research RC for NEH Region, Meghalaya, India). Some of the sequencing was assigned to Eurofins Scientific, Luxembourg, Chromous Biotech, Bangalore, India, Xcelris genomics, Ahmedabad, India. A larger number of the samples were sequenced by us in the ABI Prism 3500 genetic analyzer (Applied Biosystems), using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA). The sequencing procedure in 24 capillary applied biosystem sequencer is discussed below.

5.9.4a Reaction preparation

Every single reaction was prepared using the following reagents in separate wells. A 96-well plate was used.

Terminator Ready Reaction Mix	8 µl
Template	1 µl
Primer	0.5 µl
Deionized water	11.5 µl
Total volume	20 µl

The plate was mixed well and spun briefly.

5.9.4b Cycle Sequencing

Amplification of the purified PCR products was performed in one of the thermal cyclers available in the laboratory with 96 well slot to fit in the plate. The reaction plate was placed in a thermal cycler and initial denaturation was performed in rapid thermal ramp at 96°C for 1 min. After initial denaturation, the reaction was set for a 25 cycles with the given conditions as— rapid thermal ramp at 96°C for 10 seconds; rapid thermal ramp at 50°C for 5 seconds and rapid thermal ramp at 60°C for 4 min. After the completion of the cycle, the rapid thermal ramp was set for 4°C and held until ready to purify.

5.9.4c Product purification by Ethanol/EDTA/Sodium Acetate precipitation method

Following amplification, the products were purified before it was subjected to electrophoresis for the product analysis as given below:

1. The 96-well reaction plate from the thermal cycler was removed and briefly spun and 2 µl of 125 mM EDTA was added to each well.

2. This was followed by 2 µl of 3 M sodium acetate and 50 µl of 100% ethanol.

3. The plate was sealed with aluminum tape, mixed by inverting 4 times and incubated at room temperature for 15 min.

4. Using a plate adapter, the plate was spun at the maximum speed of 2000–3000 × *g* for 30 min.

5. The plate was inverted and spun up to 185 × *g*, then removed from the centrifuge and 70 µl of 70% ethanol was added to each well containing samples.

6. With the centrifuge set to 4°C, the plate was spun at 1650 × *g* for 15 min.

7. The plate was one again inverted and spun up to 185 × *g* for very briefly for few seconds min and removed from the centrifuge.

8. The samples were resuspended in injection buffer until further use.

5.9.4d Sample run; Electrophoresis on ABI PRISM 3500 DNA Analyzer

Electrophoresis was performed using POP-7™ polymer, 50-cm, with the desired run module and dye module described below.

Run module: Seq1_1POP7DefaultModule; Seq1_2POP7DefaultModule

Dye Set/Primer (Mobility) File: DT3500POP7{BDv3}v1.mob

After completion of sequencing reactions, trace files were obtained from the sequencer and chromatograms were read using Trace Viewer available at http://www.technelysium.com.au/chromas.html. Sequences were converted to FASTA format and were processed for phylogenetic / relatedness analysis.

5.9.5 Sequence homology exploration in NCBI BLASTn

All the sequences once obtained were checked for chromatogram quality, trimmed at the starting and the ending bases, followed by homology search. All sequences obtained through sequencing were searched for homologous database sequences in Basic Local Alignment Search Tool (BLASTn) (Altschul et al., 1990) available at http://blast.ncbi.nlm.nih.gov/Blast.cgi with default algorithm parameters for establishing sequence identity through homology search. The output data were recorded for further analysis.

Through BLAST search, sequenced genes were used for mainly species identification. Specific identity value for each gene was used for confirmation of species. Cut-off values with the corresponding reference sequences employed for species confirmation were, ≥ 99.3% for *rpoB* (Salah, 2008), 97.0% for 16S rRNA (Stackebrandt and Goebel, 1994) and ≥ 97% for *hsp65* (Kim et al., 2013).

5.10 PHYLOGENETIC ANALYSIS

As the base calling in Sanger sequencing is poor at the beginning of the sequence read and after 600-700bp read, it is important that these regions be removed before any analysis. Due to the same reason sequences of our study have been reduced to a considerable extend, although analysis was not altered. Trimming was done parallel to visually checking the chromatograms, comparing the signal intensities in the chromatogram and the database sequences. Comparison of sequences in BLAST with the database and references was done to check for mismatches and gaps and correlated with the obtained chromatogram of our sequences. If large "noise" is observed and the base calling is weak, sequencing was repeated for such isolates.

After analysis of the sequences, all files were saved in fasta format and rechecked for homology in BLASTn. Trimming off weak regions significantly increased the chances of more accurate similarity hits. When sequences were found to be of high quality, they were submitted to GenBank NCBI through the BankIt portal where sequence sizes 200 bp and above can be deposited. Sequences were then submitted when they were correctly identified, checked for the correct reading frame and all isolate sequence details noted. These sequences when processed and confirmed by the GenBank curators to be correctly represented, accession numbers were provided and the sequences were also processed for phylogenetic analysis.

Phylogenetic analysis for dendrogram construction was initiated following a BLAST homology search. From the resultant homology search, representative reference sequences, a few sequences of the closest hits and sequences of clinical origin were downloaded from GenBank and stored for subsequent analysis for comparisons. Phylogenetic study was carried out for representing the BLAST output in an illustrative manner and to explore the evolutionary relatedness and genetic diversity of the isolates. All the database sequences including our sequences to be analysed were saved as fasta files. These files were aligned in MEGA or any of the suitable software that can read a fast format. In our study we have alternatively used tools like the commercially available DNASTAR Lasergene v6 and freely accessible online Bioedit 7.0.4.1 software (Hall, 1999). The alignment was checked if some sequences were too divergent or too long. Longer sequences were trimmed to a fair length to avoid ambiguous clustering.

Following alignment, all the files were transported to MEGA 7 (Kumar et al., 2016) and the tree sessions were initiated in the software using the appropriate statistical methods and clustering approaches. If MEGA was used for alignment, multi-sequence alignment and percentages of similarity between sequences were determined using the build-in CLUSTALW program, version 1.6 from the MEGA version 7 software package. All the molecular evolutionary analyses in this study were conducted using MEGA software.

Phylogenetic trees were obtained from DNA sequences by either Neighbour-Joining (NJ) or Maximum Likelihood (ML) method, using the suitable distance correction model available in MEGA software package. Usually, a bootstrap analysis (1000 repeats) was performed to evaluate the robustness of the phylogenetic tree. Maximum Composite Likelihood (MCL) method is used for estimating evolutionary distances between DNA sequences, which MEGA users frequently employ for inferring phylogenetic trees,

divergence times, and average sequence divergences between and within groups of sequences (Tamura et al., 2007). The advantage of the maximum likelihood approach is that these methods not only take into consideration the differences between the sequences in question but also accounts the differences in them.

The neighbour-joining method was proposed by Saitou and Nei (1987) for reconstructing phylogenetic trees from evolutionary distance data displaying tress to be generally better than the other methods and quite efficient in obtaining the correct tree topology. It was found applicable to any type of evolutionary distance data. NJ according to genetic distances considers relationship between sequences without considering an evolutionary approach. Therefore this approach clusters similar sequences in the matrix with lower differences to a reliable extend but if a divergent database needs to be analysed, other approaches is more applicable. It should be noted that evolution can go at a different rate in different tree branches which would impact the topology of the trees to a great extent. Hence it is important to employ the most suitable distance methods.

All trees was inferred using the applicable statistical methods and tests. When the individual trees constructed based on single genes were indecisive, the conclusive credentials of the isolate was judged for by constructing a phylogenetic tree based on consensus of all the sequenced genes by concatenation, where combination of any of the three genes-16s rRNA, *rpoB* and *hsp65*, whichever available, were aligned head-to-tail to form a more resolved phylogeny (Gadagkar et al., 2005). For this database reference sequences were downloaded from NCBI database. Sequences from reliable sources were selected for all the genes to be merged. It was carefully considered that the different gene sequences were obtained from the same depositor if available and were of similar length. Reference sequences were given the preference for concatenated approach and others were considered if not available. The trees obtained by this approach provided better discrimination of even the unique and rare isolates. The subsequent speciation of the isolates obtained during the study and their distinctness among the 184 reported mycobacterial isolates were recognised by exploring their dominance in the global and Indian context through comparison with published literatures. To a larger extend, analysing the phylogenetic tree profiles obtained for various genes advanced our understanding on the relatedness and diversity of the isolate from man, animal and environment.

5.11 MUTATION CHECK IN THE *rrl* GENE

Among the macrolides, clarithromycin is one of the main drugs used for NTM infections, mainly *M. abscessus*. There are two main mechanisms well established. Encoding the inducible erythromycin ribosomal methylases (Erm) are a set of genes which is known to confer resistance to clarithromycin through methylation of the adenine at position 2058 of the 23S rRNA. The second mechanism is mutations in the drug-binding pocket of the bacterial 23S rRNA gene at positions 2058 and 2059 (Maurer et al., 2012). High quality bi-directionally sequenced *rrl* gene sequences were obtained for checking the single base mutation that confers clarithromycin resistance in mycobacteria. This acquired resistance is associated with point mutations at positions A2058 and A2059 in a region of the *rrl* gene encoding the peptidyltransferase domain of the *23S rRNA* gene (Tebas et al., 1995; Nash et al.,2006).

To identify these base mutations, the *rrl* gene sequences of our isolate sequences which were trimmed of low quality were again aligned with the published reference sequence of *M. abscessus* (GenBank accession number NC_010397) and *E. coli* (GenBank accession number AE014075). This exercise was performed in DNASTAR Lasergene v6 due to its better representation of the required analysis. All sequences saved in fasta format were transferred to the software and following CLUSTALW analysis the base position(s) were checked manually according to the *E. coli* sequence numbering. Electropherograms of the *rrl* genes of our sequences were checked for any double peaks at position 2058 according to numbering in the *rrl* gene of *E.coli.* The results obtained were simultaneously correlated with the MIC determined through the phenotypic test described in section 5.7.3.

CHAPTER 6

RESULTS AND DISCUSSIONS

6.1 Overall sampling result

In the present study, a total of 555 samples from three sources comprising of 08 different types of samples were analysed for isolation of mycobacteria. These samples were collected under three broad categories viz., Animal, Human and Environment. The 08 sample types included milk, lymph node, dermal scraping and nasal swab from animal sources; sputum and urine from clinical sources; water and soil from environmental samples. The sampling strategy for collection of samples was convenient sampling, of which animal and environmental samples were random collection while clinical sampling were targeted towards tuberculosis suspected group.

Convenient sampling approach for animal and environmental samples were adopted based on several factors like distribution and availability of farms in the selected geographical study sites, access to locations from where samples could be transported and processed within the stipulated time. Targeted sampling was adopted for human clinical samples and we gained access to a Revised National TB control Program (RNTCP) unit in Dimapur district, Nagaland. This explains the majority of our samples analysed in the study from this region and fewer suspected TB and extrapulmonary TB cases obtained from the other geographical site under study, Khasi Hill region of Meghalaya. RNTCP is a large scale implementation by the government of India for TB control in the country where initiatives have been taken to address HIV/TB co-infection and MDR-TB.

Details of the samples collected from Dimapur district of Nagaland and the Khasi Hills of Meghalaya are given in Table 12. A total of 229 samples were collected from Dimapur, of which 32 were animal samples, 88 from environment, and 109 of clinical origin. A total of 326 samples were collected from Khasi Hills comprising 188 animal samples, 126 environmental samples and 12 of clinical origin.

Sl. No.	**Location**	**Sample**		**Total samples collected**
1.	Khasi hills, Meghalaya	Animal (188)	Milk	175
			Lymphnode	5
			Dermal scraping	3
			Nasal swab	5
		Human clinical (12)	Sputum	9
			Urine	3
		Environmental (126)	Soil	86
			Water	40
			Total	**326**
2.	Dimapur, Nagaland	Animal (32)	Milk	32
		Human clinical (109)	Sputum	109
		Environmental (88)	Soil	50
			Water	38
			Total	**229**

Table 12. Location wise sampling details

An overall total of 555 samples were processed for mycobacterial isolation from animal (220), human (121) and environmental sources (214). A sum of 62 (11.17%) isolates were positive for *Mycobacterium* genus identified mainly by genus specific *hsp65* PCR and speciated majorly through sequencing.

Sl. No.	**Sample Type**		**Total Samples pro-cessed**	**Total culture & PCR positive**	**Organism identified through sequencing**		
					NTM	**MTBC**	**Others**
1.	Animal samples	Milk	207	28 (13.53%)	26 (12.56%)	1 (0.48%)	1 (0.48)
		Lymph node	5	-	-	-	-
		Dermal scrap	3	1 (33.33%)	1 (33.33%)	-	-
		Nasal swab	5	1 (20%)	1 (20%)	-	-
Total			**220**	**30 (13.64%)**	**28 (12.73%)**	**1 (0.45%)**	**1 (0.45%)**
2.	Human samples	Sputum	118	28 (23.72%)	2 (1.69%)	23 (19.49)	3 (2.54%)
		Urine	3	-	-	-	-
Total			**121**	**28 (23.14%)**	**2 (1.65%)**	**23 (19%)**	**3 (2.48%)**
3.	Environ-mental samples	Soil	136	7(5.15%)	5(3.68%)	-	2(1.47%)
		Water	78	4(5.13%)	3(3.84%)	-	1 (1.28%)
Total			**214**	**11 (5.14%)**	**8 (3.74%)**	**-**	**3 (1.40%)**
Sum Total			**555**	**69 (12.43%)**	**38 (6.85%)**	**24 (4.32%)**	**7 (1.26%)**

Table 13. Overall sampling and isolation of mycobacteria from the study

Other supportive gene targets like *rpoB* and series of molecular identification approaches were also used for confirmation of the isolates. Out of 69 (12.43%) isolates sequenced, 38 (6.85%) were NTM, 24 (4.32%) belonged to MTBC and 07 (1.26%) were non-mycobacteria. The details of samples, species identified are given in Table 13, and the details of how the results have been achieved would be discussed herewith.

From the animal source a total isolation rate of 13.63% was noted comprising of 30 isolates. From which 28 (93.33%) isolates were NTM, 01 (3.33%) belonged to the MTBC member and 01 (3.33%) was a non-mycobacteria. From the total 29 (13.18%) isolates of mycobacteria, 27 (93.10%) isolates were from milk and 02 (6.89%) isolates were of animal tissue samples. Mycobacteria has been isolated from raw milk of cattle from the country but no recent data is available and global isolation from milk is variable. Appuswamy et al. (1980) at a rate of 5.8% isolated mycobacteria from cattle milk including bulk and individual samples. Isolates included *M. fortuitum, M. aquae, M. intracellulare, M. phlei* and *M. kansasii*. From northern India, members of MTBC were also obtained from milk samples including *M. tuberculosis* (28.5%) and *M. bovis* (15%) (Srivastava et al., 2008). Large isolations of mycobacteria from milk has been reported from Brazil with varying species. Bezerra et al. (2015) reported close to 17% isolation from Brazil of mainly MTBC members, while out of an isolation rate of 4.1% total mycobacterial isolation rate from Turkey, larger isolates belonged to NTM species (Aydin et al., 2012).

The clinical sources involved targeted sampling and an isolation rate of 21.19% was noted with 25 mycobacterial isolates out of total 118 clinical samples. Of these presumed and confirmed cases, 23 (92%) isolates were *M. tuberculosis* and 02 (8%) were NTM. The isolation rate from India varies among studies reported and a collective data of the whole country is unavailable (Narang et al., 2017). A large scale study from central India involved 4620 clinical samples from probable pulmonary and extrapulmonary tuberculosis (ETB) patients between 2013 to 2015. A total of 263 (29%) NTM isolates were obtained with 13 different mycobacterial species. Two of the most common isolates were *M. fortuitum* and *M. intracellulare* (Umrao et al., 2016). An earlier report from the same region was also reported where samples from suspected ETB were analyzed accounting to an isolation rate of 27.4% (Maurya et al., 2015). In Asia isolation of varied NTM species has been observed which is different from the common isolations of Europe and North America. The most common isolations of NTM include *M. chelonae* and *M. fortuitum* in India and China, *M. fortuitum* and *M. kansasii* in Iran, *M. abscessus* in Saudi Arabia and MAC being the most common in South Korea, Taiwan, Hong Kong and Singapore (Lai and Hsueh, 2014). A wide scale literature survey of NTM epidemiology in Asia presents a data differing from the developed countries with a wide distribution of species within the region and also variation in antibiotic resistance pattern (Lai and Hsueh, 2014). Data from Iran on NTM prevalence have been reported through a large scale data review spanning between 1992 to 2014. An upsurge of NTM prevalence in clinical settings from 8% in 1995 to 18% in 2014 was noted in Iran (Velayati et al., 2015). Between 1995 to 2005, the NTM infections have been known to rise in the European region covering Northern Ireland, England and Wales. While in 1996 it was noted to be 0.9 per 1 lakh population, in 2005 a steep rise to 2.9% per 1 lakh population has been noted (Moore et al., 2010). Prevalence of NTM infections in Australia and North America is higher than the reported prevalence in Europe falling under 3.2 to 9.8 per 1 lakh population. In selected TB endemic African Countries, the prevalence

among presumed TB patients were as high as 4.2 to 15% and upto 18 to 20% among chronic MDR-TB patients (Prevots and Marras, 2015).

The isolation rate from environmental sources in our study stands at just 3.74% with 08 NTM isolates. Environmental isolation rate in the study is significantly reduced which we believe could be due to the processing protocol employed. We suppose our previously standardized protocol could not decontaminate the presence of the larger bacterial flora of the samples from the study region. Due to the high contamination, plates had to be discarded before the growth of fastidious mycobacteria. A wide variety of mycobacterial species are being isolated from environment, of which *M. avium* complex (MAC) and *M. fortuitum*, are commonly isolated in both developing and developed countries from varied water distribution systems (Gopinath et al., 2010; Thomson et al., 2013; Velayati et al., 2015; Donohue et al., 2015). In India, NTM isolation from environment have also been reported (Kamala et al., 1994; Bannalikar et al., 2006; Lavania et al., 2008; Narang et al., 2009). Of the total 08 isolates from environment, 04 of the species were identified as *M. chelonae* (50%). It may be noted that *M. chelonae* was also the major species isolated (75%) from the animal sources. Although no human cases due to this species were noted in the study, major human isolations have been attributed to *M. fortuitum* complex and *M. chelonae* in the country (Jesudason and Gladstone, 2004; Umrao et al., 2016). Nosocomial infections involving contamination through water distribution system and through contamination of invasive instruments like bronchoscopes and catheters have also been reported (Raman et al., 2000; Simmons et al., 2011; Lai and Hsueh, 2014).

We isolated 03 very unique isolates, 02 from animal and 01 from human, which did not correspond to any of the reported isolates. But to designate these isolates there is a need to perform all the conventional, cultural, biochemical, molecular and proteomics profile, which could not be attained due to limited available resources. We followed the multiple gene concatenated approach to determine its phylogenetic position based on *hsp65*, *rpoB* and 16sRNA genes and substantiated with *hsp65*-PRA profiles which could clearly distinguish the novelty of the isolates.

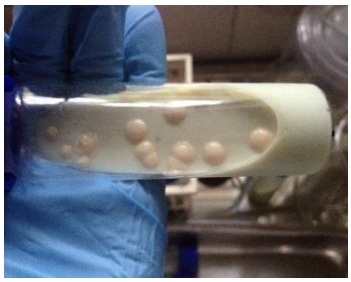
Further details and significant findings of the study is discussed under three broad sections source wise. “Case studies” have also been presented under these sections with detail description of the significant findings in the study.

6.2 Culture and Ziehl-Neelson (ZN) staining

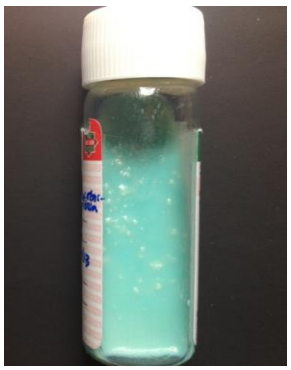
Of the total 555 samples processed, 69 isolates were presumed to be mycobacteria on the basis of acid fast bacilli observed after Ziehl-Neelson staining. All these presumed isolates were then checked by genus-specific PCR. The images of culture slants of representative isolates and the acid fast bacilli observed under the microscope at 1000X magnification is given in the following pages (Fig 18 & 19).

A. *M. lentiflavum* (PK504)

B. *M. peregrinum* (1AG)



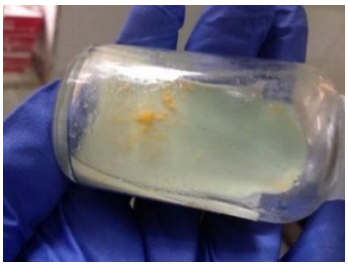
C. *M. chelonae* (M4)



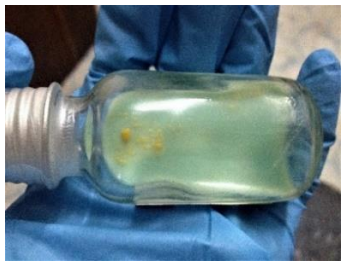
D. *M. chelonae* (AH4)

E. *M. lentiflavum* (M140)

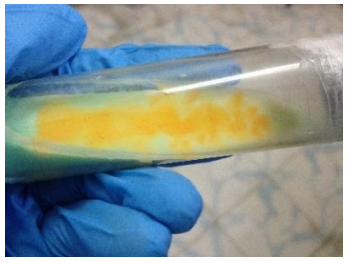
F. *M. lentiflavum* (M8)



G.Isolate 2837

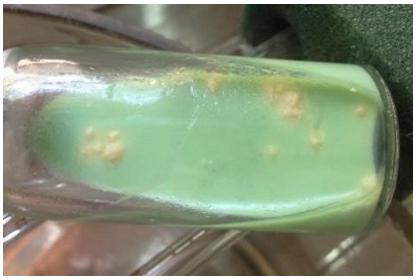


H. Isolate HDG



I. *M. novocastrense* (2824)

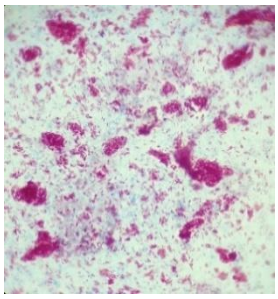
J. *M. chelonae* (M43)



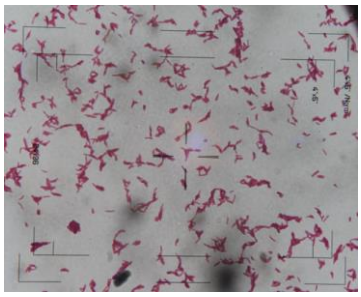
K. *M. abscessus* (FU362)

L. *M. tuberculosis* (SERO)

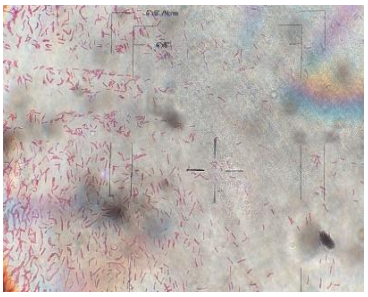
Fig 18. Culture Growth of Mycobacteria on LJ medium and M7H9 broth. A. From Sputum B. From Milk C. From Milk D. From Soil E. From Milk F. From Milk G. From Sputum H. From dermal scrap I. From Sputum. J. From Milk K. From Sputum L. From Sputum (in Middlebrook 7H9 broth)



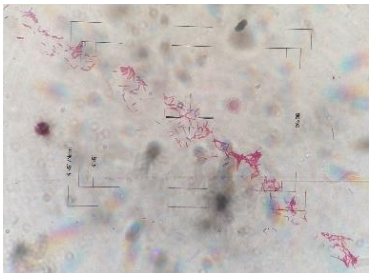
A. *M. novocastrense* (2824)



B. *M. fortuitum* (W34)

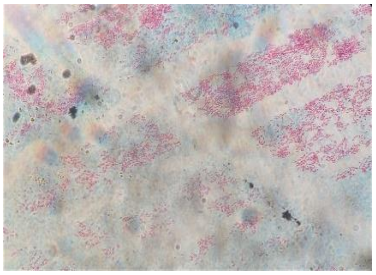


C. *M. chelonae* (M43G)

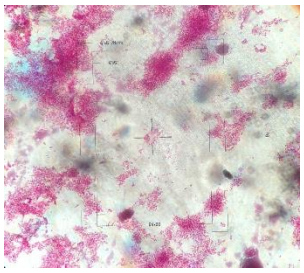


D. *M. chelonae* (M23P)

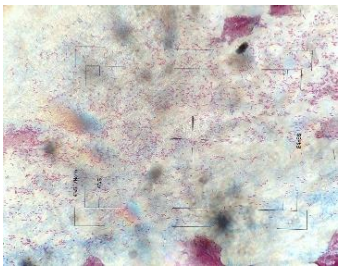
E. *M. lentiflavum* (PK504)



F. Unspeciated (HDG)

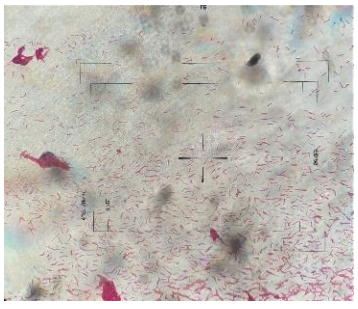


G. *M. tuberculosis* (2833A)

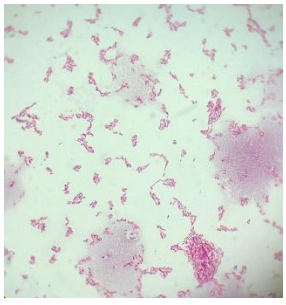


H. *M. tuberculosis* (12A)

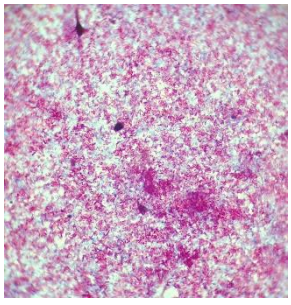
I. *M. tuberculosis* (02A)



J. *M. tuberculosis* (2992G)



K. *M. lentiflavum* (1AG)



L. Unspeciated (RF)

Fig 19. Microscopic view of mycobacterial bacilli following Ziehl Neelsen staining. A. Sputum isolate B. Water isolate C. Milk isolate D. Milk isolate E. Sputum isolate F. Animal isolate G. Sputum isolate H. Sputum isolate I. Sputum isolate J. Sputum isolate K. Milk isolate L. Soil isolate.

6.3 Biochemical tests

A few important biochemical test has been performed on selected isolates. Although several biochemical tests are used for identification of mycobacteria, these methods are tedious, error prone, poses risk to the handler due to long exposures of highly communicable airborne pathogens and very often exhibit inconclusive results (Hall et al., 2006). Moreover, since freshly grown cultures are required for most of these tests, sub cultures are required to be performed leading to multiplication of pathogenic isolates requiring long incubation period and large space consumption. In addition, sub culturing of the mycobacterial isolates did not always give optimum growth for performing these tests in the required multiple sets. Biochemical tests are known gold standards for bacterial identification but in an era where molecular approaches are widely available providing rapid, cost effective and accurate identification, molecular approach was adopted for definitive identification and differentiation of our isolates. Following are some of the tests performed on selected isolates and the results are shown below.

A. Niacin test

Two clinical isolates 2837 and PK504, and 01 milk isolate (M77) were tested for release of niacin in the media alongwith standard mycobacterial isolates of *M. tuberculosis* and *M. smegmatis* (Fig 20).

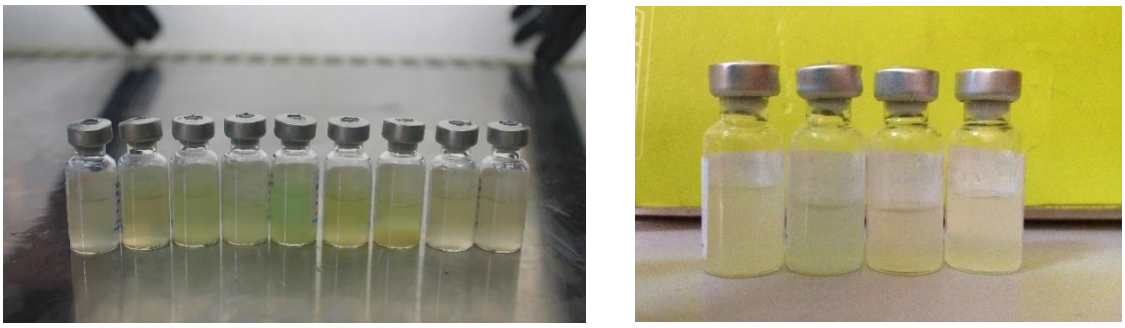


Fig 20. Test for presence of niacin. Yellow coloration indicates a positive reaction.

All the isolates were negative showing no yellow coloration. The results are given in table 14.

Sl. No.	Sample ID	Source	Niacin test result
1.	Ngl 2837	Clinical	Negative
2.	PK504	Clinical	Negative
3.	M77G	Milk	Negative
4.	*M. tuberculosis* (MTCC300)	Standard	Unconclusive
5.	*M. smegmatis* (ATCC607)	Standard	Negative

Table 14. Niacin detection test result and sample source

Three tests are usually used for differentiation of NTM and MTBC which comprises of Niacin, Nitrate and growth on p-nitrobenzoic acid (PNB). We performed Niacin test for a few of the isolates which were intended to be distinguished. Niacin can be either positive or negative for NTM while being positive for MTBC. Three test isolates tested negative including standard *M. smegmatis* (ATCC607) while result of *M. tuberculosis* (MTCC300) was unconclusive. The results could not be interpreted accurately due to poor color formation.

B. Nitrate reduction test

Nitrate reduction test is another test used for differentiation of NTM and MTBC. *M. tuberculosis* is known to be positive while *M. bovis* and some *M. africanum* are negative. For the NTM niacin test is usually negative as in *M. asiaticum* and *M. haemophilum.* While species like *M simiae* are positive Fig 21.

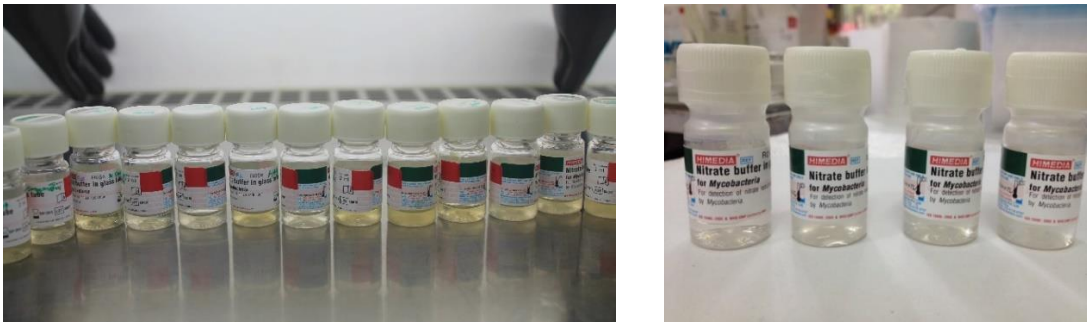


Fig 21. Nitrate reduction test. All samples negative

Sl. No.	Sample ID	Source	Nitrate Reduction test result
1.	Ngl 2837	Clinical	Negative
2.	Ngl 2374P	Clinical	Negative
3.	PK504	Clinical	Negative
4.	M43G	Animal	Negative
5.	M140G	Animal	Negative
6.	HNP	Animal	Negative
7.	HDG	Animal	Negative
8.	AH4	Environment	Negative
9.	*M. smegmatis* (ATCC607)	Standard	Negative

Table 15. Nitrate reduction test result and sample source

Eight test isolates comprising of 03 clinical isolates, 01 environmental isolate and 04 animal isolates were tested for reduction of nitrate along with a standard strain of *M. smegmatis* for comparison (Fig 15). Cultures not more than 05 weeks old were used and all the 09 samples were negative for the test. All the test isolates were correctly nitrate negative

except for isolate HDG from animal source which showed homology to *M. simiae* complex in BLAST. The *M. simiae* complex is usually positive for nitrate test. However given the novel nature, this variation may be expected.

C. Catalase test

In most of the *Mycobacterium* spp. catalase enzyme is stable to heat at pH 7.0. However in some NTM species like *M. haemophilum, M. marinum, M. chelonae* etc. including the tubercle bacilli, the enzyme is deactivated due to which generation of oxygen bubbles in water due to conversion of hydrogen peroxide is not observed. In the semiquantitative test, results were interpreted as strong and weak positives depending on the height of bubbles formed.

i. Semiquantitative catalase test

Seven test samples were included for semiquantitative catalase test (Fig 22) for differentiation of MTBC and NTM including a standard strain of *M. smegmatis* (ATCC607) as a positive control, the results of which are presented in Table 16.

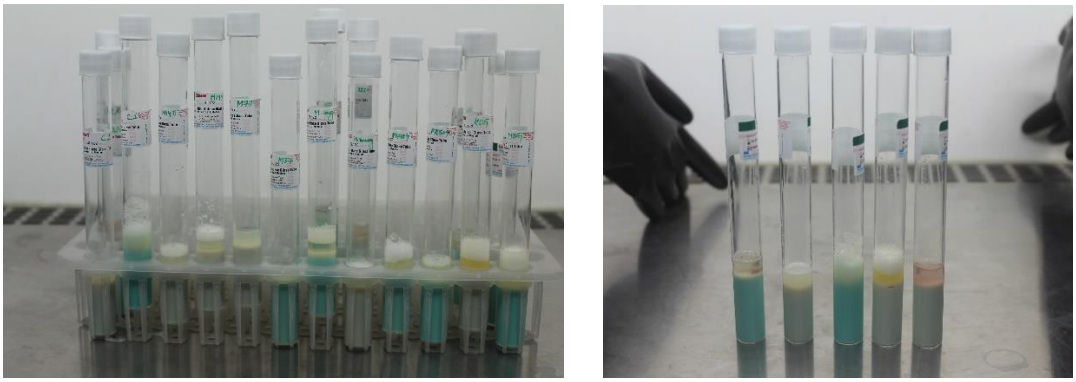


Fig 22. Bubble formation in semiquantitative catalase test

Sl. No.	Sample ID	Source	Semiquantitative catalase
1.	Ngl 12A	Clinical	Negative
2.	2830	Clinical	Weakly positive
3.	Ngl 2824	Clinical	Weakly positive
4.	PK504	Clinical	Weakly positive
5.	HDG	Animal	Weakly positive
6.	M77G	Animal	Negative
7.	M140G	Animal	Weakly positive
8.	*M. smegmatis* (ATCC607)	Standard	Weakly positive

Table 16. Semiquantitative catalase test result and sample source

Including the positive control, 04 clinical and 01 animal isolate were weakly positive and 02 isolates, 01 each of clinical and animal were negative. On comparing the result following molecular identification, all isolates were correctly interpreted. Isolate M77G which was

identified as *M. chelonae* and isolate Ngl 12A, a *M tuberculosis* isolate was correctly negative. The isolate 2830 which was weakly positive was later identified as *Nocardia*.

ii. Heat stable catalase test

For differentiation of MTBC and NTM, more isolates exhibiting optimum growth were tested for catalase activity inhibition at 68°C (Fig 23).

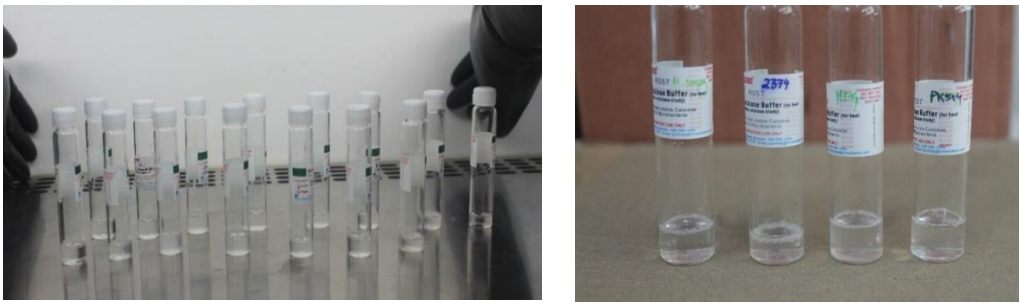


Fig 23. **Heat stable catalase test**. Four tubes showing positive reaction by bubble formation.

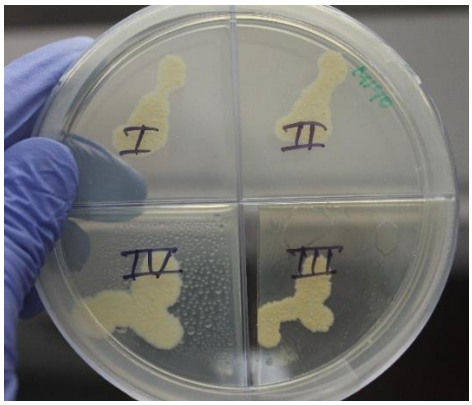
Sl. No.	Sample ID	Source	Heat stable catalase
1.	Ngl 2374P	Clinical	Positive
2.	PK504	Clinical	Positive
3.	Ngl 2837	Clinical	Negative
4.	M43G	Animal	Negative
5.	HDG	Animal	Positive
6.	HNP	Animal	Negative
7.	AH4	Environmental	Negative
8.	*M. smegmatis* (ATCC607)	Standard	Positive

Table 17. Heat stable catalase test result and sample source

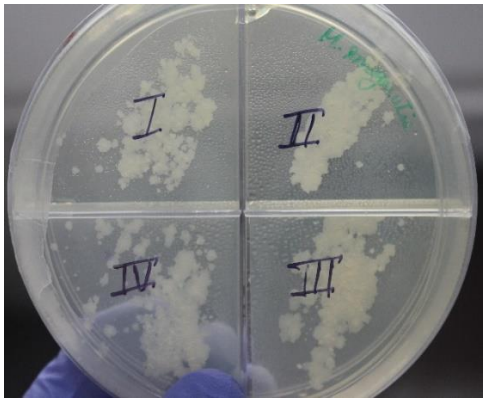
Among the 07 isolates tested, the *M. chelonae* isolates (M43G, AH4) expectedly showed inhibition of catalase activity at 68°C and interpreted as negative but the *M. tuberculosis* isolate Ngl 2374P isolate was false positive. Results of 03 unique isolates (Ngl 2837, HNP, HDG) could not be correlated as they were expected to be probably novel. Control positive *M. smegmatis* isolate could be interpreted correctly (Table 17).

D. Susceptibility to thiophen-2-carboxylic acid hydrazide (T2CH or TCH)

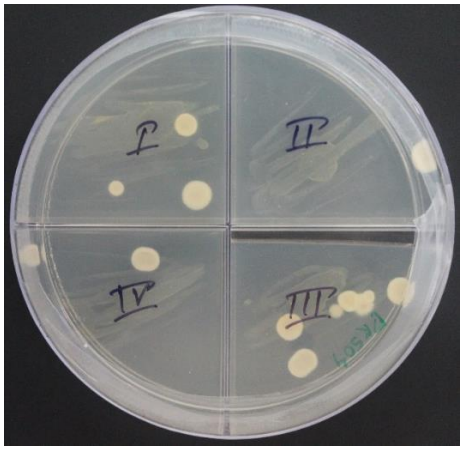
This test is used to differentiate *M. bovis* from *M. tuberculosis* as the former is susceptible to low concentrations of T2CH. Clinical and animal isolates were mainly checked for T2CH susceptibility and *M. tuberculosis* was used as a negative control (Fig 24). Six isolates were tested. Contamination of the plates despite precautionary measures made interpretation of the results challenging.



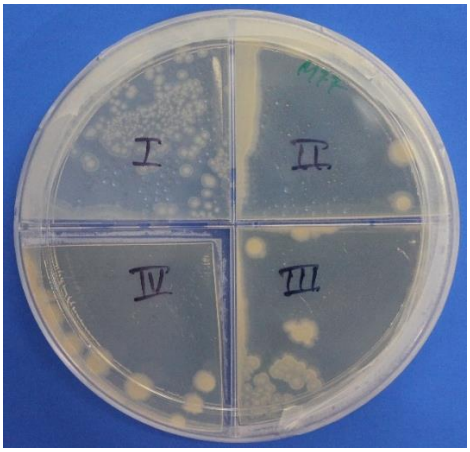
A. Animal isolate (M140)



B. *M. smegmatis* (ATCC 607)



C. Animal isolate (HDG)



D. Clinical isolate (PK504)

Fig 24. T2CH test plates. Plates A and B shows growth in all quadrants in the presence of T2CH showing their resistance. Plates B and C also shows colony growth but with contamination.

Although T2CH testing is known to be the only single test that assigned isolates to any specific member of the MTBC, the Asian strain of *M. tuberculosis* and all other members of the MTBC are reported to be susceptible to TCH. Therefore in our study the test was performed to understand the outcome. The details and the results of the samples tested are given in Table 18.

Sl. No.	Sample ID	Source	T2CH test
1.	PK504	Clinical	Resistant
2.	Ngl 12A	Clinical	Growth failed
3.	Ngl 2824	Clinical	Growth failed
4.	HDG	Animal	Resistant
5.	M140G	Animal	Resistant
6.	*M. smegmatis* (ATCC607)	Standard	Resistant

Table 18. T2CH test results and sample source

Three isolates (PK504, HDG and M140) were resistant showing growth in all the quadrants including the ones with the drug (quadrant II & III). Standard *M. smegmatis* isolate also showed growth in all quadrants. The interpretation of the results for 02 of the clinical isolates could not be done due to failure of growth.

E. Detection of Pyrazinamidase activity

Isolates from all three sources were tested for pyrazinamidase activity (Fig 25) to distinguish between bovine and human origin as *M. bovis* is resistant to pyrazinamide while *M. tuberculosis* is susceptible. Species like *M. chelonae*, *M. fortuitum* complex, and some photochromogens like *M. simiae* and *M. marinum* are also known to be mostly positive for which selected isolates were tested.

A. Strongly positive tubes B. Weakly positive and negative tubes

Fig 25. Pyrazinamidase test. Presence of pyrazinamidase detected by addition of ferrous ammonium sulfate.

Sl. No.	Sample ID	Source	Pyrazinamidase test.
1.	Ngl 2837	Clinical	Negative
2.	PK504	Clinical	Weakly positive
3.	Ngl 2824	Clinical	Negative
4.	2830	Clinical	Negative
5.	Ngl 12A	Clinical	Negative
6.	M140G	Animal	Strongly positive
7.	M77G	Animal	Negative
8.	HDG	Animal	Strongly positive
9.	*M. smegmatis* (ATCC607)	Standard	Strongly positive

Table 19. Pyrazinamidase test results and sample source

The details of the sample sources and the results are presented in Table 19. Three isolates, two of animal origin and the standard *M. smegmatis* were strongly positive, and a slow grower PK504 was weakly positive. Three clinical isolates tested negative of which animal isolate M77G, later identified as *M. chelonae* was tested false negative. Isolate 2830 of clinical origin was of non-mycobacterial origin on further molecular tests.

The selected biochemical tests performed above could help us distinguish only to a very limited range and gave confusing outputs. Frequent contamination of the plates, as in T2CH test plates made interpretation difficult during this experiment leading premature disposal of plates before desired growths were observed. Obtaining large fresh inoculums for the test, exposure to long term toxic reagents, aerosol generating procedures added majorly to the inconvenience.

6.4 DNA quantification

The DNA extracted both by conventional and kit based methods were quantified in Thermo Scientific NanoDrop 2000 spectrometer for assessing the quality of our isolated DNA by following the manufacturer's protocol. DNA concentration was acquired under the range of 13ng/µl to 95 ng/µl which were accordingly used for further molecular processes. Some low concentration of DNA yield could be attributed to the low inoculum used as the starting material. The very few number of colonies available during primary isolations and limited colonies obtained after subculture was a constant snag during the study. However, the purity of the DNA obtained through kit based extraction, was good with 260/280 ratio of ~1.8 which is generally accepted as "pure" for DNA. Most of the extraction was done by the Qiagen kit discussed in section 5.91 given factors like high purity DNA yield which avoided any inhibitions during further downstream processes and ability to handle larger sample quantity due to the simple protocol.

6.5 Molecular identification by PCR

The study employed adjuncts of several molecular approaches for identifying and differentiating the isolates. Of the total 555 samples processed in the study, a total of 69 probable growths were obtained out of which 62 (11.17%) samples were PCR positive for *Mycobacterium* genus-specific *hsp65* gene amplification. These amplified isolates were further subjected to further PCR amplifications for differentiation of NTM and MTBC by *hsp65*-PRA, IS6110 amplification (specific for MTBC) and a few species by duplex PCR. MTBC was subjected to species identification of *M. bovis* and *M. tuberculosis* by 500bp fragment, RD4 and RD9 PCR. 16S rRNA gene amplification was done for unique isolates and *rrl* amplification for clinically important isolates. Sequencing was performed for all PCR positive isolates for identification and for lineage studies through phylogenetic analysis. Discussion on PCR amplification of each gene and the results obtained are presented in the following sections.

Non-specific amplifications were also observed for the genes used in the study. A total of 7 (1.26%) isolates which were amplified in PCR were non-mycobacteria. *hsp65* gene primers showed 03 false positive results amplifying *Streptomyces sampsonii*, *Rhodococcus equi* and *N*ocardia. *rpoB* also wrongly amplified *Rhodococcus equi, Nocardia and Propionibacterium acnes*. 500bp fragment also showed a non-specific amplification of *M. tuberculosis*.

6.5.1 *Mycobacterium* genus identification by *hsp65* gene amplification

Isolates showing acid fast bacilli after ZN staining were processed for DNA extraction and tested for *hsp65* gene amplification using previously described primers (Telenti et al., 1993). Amplicon sizes of 441bp was obtained for a total of 62 isolates which were supposed

as confirmation of *Mycobacterium* genus. All the positive isolates were further analysed for species identification.

The *hsp65* gene, during our study proved to be a very suitable choice for identification of mycobacterial isolates. All our isolates, were primarily identified by amplification of this gene and no ZN positive isolates were missed during this identification. The relatively small amplicon size of just 441bp was easily amplified in the PCR reaction which was also convenient for performing further downstream analysis.

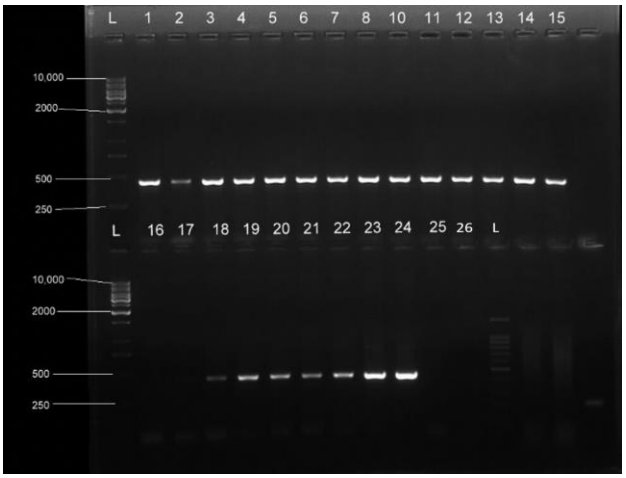


Fig 26. Amplified 441bp of the partial *hsp65* gene product of positive samples. L-Ladder (Thermo Scientific GeneRuler 1 kb DNA Ladder); Lane 1- *M. tuberculosis* MTCC 300; Lane 2- *M. smegmatis* MTCC 607; Lane 3 to 24-isolates from the study; Lane 25-Negative control (*Staphylococcus aureus* ATCC25923); Lane 26-NTC.

The *hsp65* PCR was standardized with standard strains and tested for amplification of other isolates from the study. The amplification of the test isolates along with the standard strains are given in Fig 26.

Polymerase Chain Reaction-Restriction Enzyme Analysis (PCR-REA)

The amplified partial 441bp *hsp65* gene products of the isolates were subjected to restriction enzyme digestion with *Hae*III and *Bst*EII enzymes. Various fragments were obtained for different species. The band patterns were analysed for identification of NTM and MTBC employing the available database (Telenti et al., 1993; Chimara et al., 2008). Sufficiently reliable rapid results were obtained using this PCR-REA technique (Fig 27 & Fig 28). However in several cases, due to the strain diversity of our isolates, undefined banding patterns were observed, as also reported by other authors (Chimara et al., 2008), due to which further identification approaches were required even for speciation.

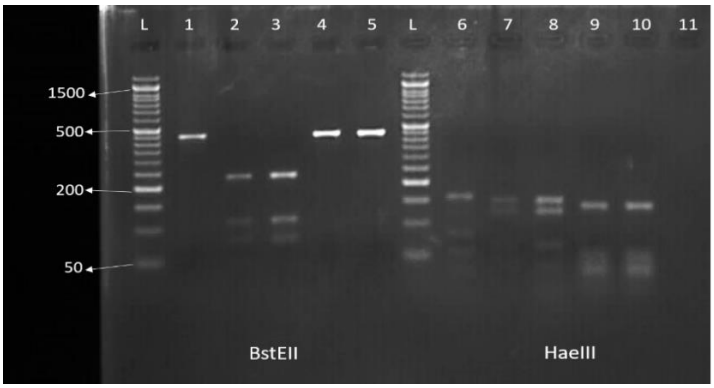


Fig 27. PCR-REA for partial *hsp65* gene optimized using standard NTM and *M. tuberculosis* cultures. Lane L-50bp ladder; Lane 1- *M. vaccae* MTCC272 (*BstE*II digestion); Lane 6- *M. vaccae* MTCC272 (*Hae*III digestion); Lane 2- *M. tuberculosis* MTCC300 (*BstE*II digestion); Lane 7-*M. tuberculosis* MTCC300 (*Hae*III digestion); Lane 3,4,5 -Test samples digested with enzyme *BstE*II; Lane 8,9,10-Test samples digested with enzyme *Hae*III.

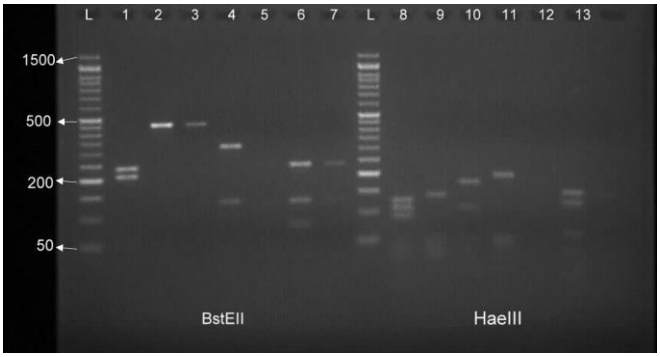


Fig 28. PCR-REA for partial *hsp65* gene performed on *hsp65* positive samples employing the standardized protocol. Lane L-50bp ladder; Lane 1 to 7-Test samples digested with enzyme *BstE*II; Lane 8 to 13-Test samples digested with *Hae*III.

6.5.2 Identification of MTBC isolates by IS6110 amplification

The insertional sequence IS6110 is widely used for identification of *M. tuberculosis* in clinical setups. All 25 clinical isolations and most of the isolates from environmental and animal sources were tested for amplification of this gene to identify the MTBC members of our isolations. This sequence, however, cannot differentiate between the members of the complex due to which three other primers were used for differentiating *M. bovis*, *M. tuberculosis* and also the NTM. Fig. 29 shows the amplification of the standard *M. tuberculosis* isolate and test samples detected by this gene.

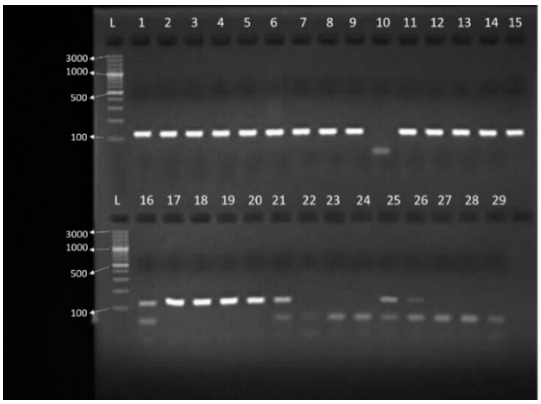


Fig 29. The 123bp sequence amplification of *IS6110*. Lane L-Ladder (Thermo Scientific GeneRuler 1 kb DNA Ladder); Lane 1- *M. tuberculosis* MTCC 300; Lane 2 to 27- Test isolates from the study; Lane 10, 22, 23, 24 & 27- NTM isolates; Lane 28- *M. smegmatis* MTCC 607; Lane 29- NTC.

IS6110 primarily distinguished the total 62 (11.17%) mycobacterial isolates into 24 (38.71%) MTBC and the rest 38 (61.3%) belonging to other mycobacterial species depending on the amplification and non-amplification of 123bp sequence. Out of 24 MTBC isolates identified by this PCR, 23 were of clinical origin and 01 of Animal source.

6.5.3 Amplification of 500bp fragment for differentiating *M. bovis*

A total of 24 MTBC isolates that were positive for IS6110 were subjected to 500bp fragment amplification to test if any isolates of bovine origin were present i.e. *M. bovis* (Fig 30).

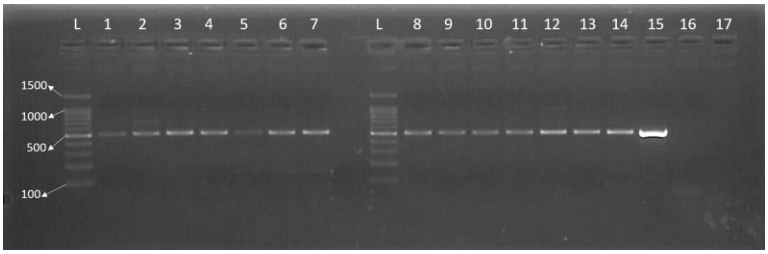


Fig 30. Amplified 500 base pair fragment. Lane L- Ladder (GelPilot 100bp plus, Qiagen). Lane 1to14-Test samples showing false positive amplification; Lane 15- *M. bovis* (AN5); Lane 16- *M. vaccae* MTCC272; Lane 17-NTC.

In this PCR amplification test, we observed very large positive results which was in general unlikely given the source of isolation. Blood samples were used in this test, which is not an ideal choice for direct molecular identification and not included in the study. Test with culture isolates also gave ambiguous result. Therefore to confirm the outcome of this PCR result, all the isolates were again tested for other *M. bovis* specific primer which is described below.

6.5.4 Detection of *M. bovis* by RD4 region amplification

This gene was included in the study to reconfirm and also to dispense the doubt of the numerous amplifications observed in 500bp fragment PCR. The PCR was standardized using several standard isolates available with us and the results obtained are showed in Fig 31.

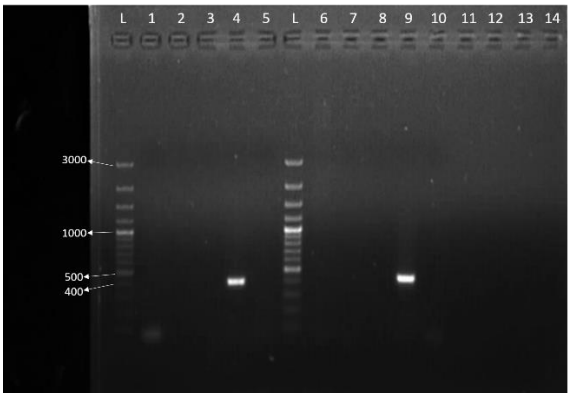


Fig 31. Amplification of RD4 region of *M. bovis* (AN5). Lane L- Ladder (GeneRuler 100 bp Plus DNA Ladder). Lane 1-*M. smegmatis* ATCC607; Lane 2- *M. vaccae* MTCC272; Lane 3-*M. tuberculosis* MTCC300; Lane 4&9- *M. bovis* (AN5); Lane 6,7,8,10,11,12,13-test isolates not amplifying 500bp fragment; Lane5&14-NTC.

When some isolates were tested with RD4 region primers specific for *M. bovis* (Sales et al., 2014) none of the isolates including the standard MTBC and NTM cultures showed amplification. The only isolate amplifying the RD4 region was standard *M. bovis* (AN5) strain. This indicated a significant false positivity of 500bp PCR. The same were also reported by several other authors acknowledging the same (Metaxa-Mariatou et al., 2004; Shah et al., 2004; Sales et al., 2014). As noted by the author (Sales et al., 2014) from where the RD4 primers were adopted, RD4 was a good choice for detecting *M. bovis* showing high specificity. This primer set was used for detecting any probable *M. bovis* during the rest of the study without any false positive results.

6.5.5 RD9 region amplification for *M. tuberculosis*

RD9 region was included in the study to detect the *M. tuberculosis* isolates. RD9 is present in *M. tuberculosis* and therefore amplified a 333bp, while in those species where it is deleted, as in *M. bovis*, a 206 bp was amplified, while no amplification was observed in NTM. This differentiation PCR was useful in identifying both *M. tuberculosis* and *M. bovis*

RD9 based PCR were standardized with standard strains of *M. tuberculosis* (MTCC300), *M. bovis* (AN5), *M. vaccae* MTCC272 and *Staphylococcus aureus* ATCC25923 as negative control (Fig 32).

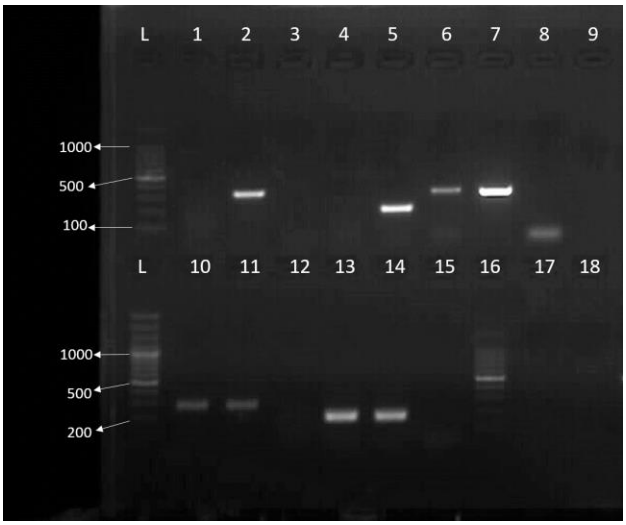


Fig 32. RD9 region amplification. Lane L - Ladder (GeneRuler 100 bp Plus DNA Ladder). Lane 1- *Staphylococcus aureus* ATCC25923 (Negative control) ; Lane 2- *M. tuberculosis* MTCC300; Lane 3, 4 - *M. smegmatis* ATCC607; Lane 5- *M. bovis* (AN5); Lane 6 to14 - test samples; Lane 15-NTC.

6.5.6 Duplex PCR for differentiation of NTM and MTBC

The *rpoB* based duplex PCR was used in our study with the aim to distinctly differentiate the MTBC and the NTM. The PCR was optimised with standard strains of *Staphylococcus aureus*, NTM and *M. tuberculosis*. All the isolates that amplified for *rpoB* gene was tested for this differentiating PCR (Fig 33).

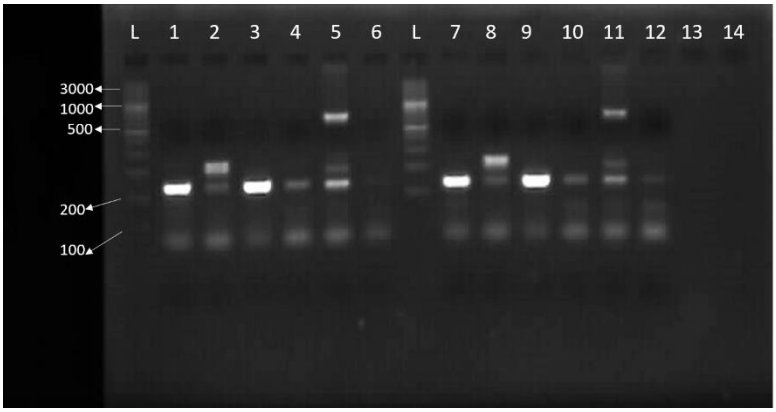


Fig 33. Amplification of mycobacteria by duplex *rpoB*. Lane L- Ladder (GeneRuler 100 bp Plus DNA Ladder). Lane 1,5,7,11,12- test samples; Lane 2&8- *M. vaccae* MTCC272; Lane 3&9- *M. avium* MTCC1723; Lane 4&10- S. *aureus* ATCC25923; Lane 6, 13 -NTC.

This duplex PCR despite repeated optimization with standard strains did not provide satisfying results when applied to our field isolates. It can be observed in the representative gel image (Fig 33) that lane 5,11 and12 with test isolates and the standard strain of *M. vaccae* in lane 2&8 shows distinct non-specific bands even under optimized conditions making interpretation of the results ambiguous. The results are difficult to be interpreted

by untrained observers and to interpret without good exposure to band pattern analysis. Similar observations can be noticed in the gel images presented in published literatures with multiple non-specific bands even under optimized conditions with standard strains while multiplexing primers (Cousins et al., 1996; Bamaga et al., 2003; Lima et al., 2013) and even when DNA concentrations were varied (Kim et al., 2006). This approach thereby could not be engaged as a convenient approach for primary speciation of mycobacteria in this study.

6.5.7 *rpoB* gene (Adekambi et al., 2003) amplification

rpoB gene shows high heterogeneity between species of mycobacteria which is why this gene has been successfully employed in conjunction with other housekeeping genes even in describing new species in the *Mycobacterium* genus since its introduction (Ben Salah et al., 2009; Hennessee et al., 2009; Ingen et al., 2012; Kim et al., 2013; Costa et al., 2015). This region was also a supportive target gene and for reaffirmation of the isolates. The PCR conditions of the *rpoB* gene was optimized with standard strains and is presented in Fig 34 along with the isolates showing specific amplification.

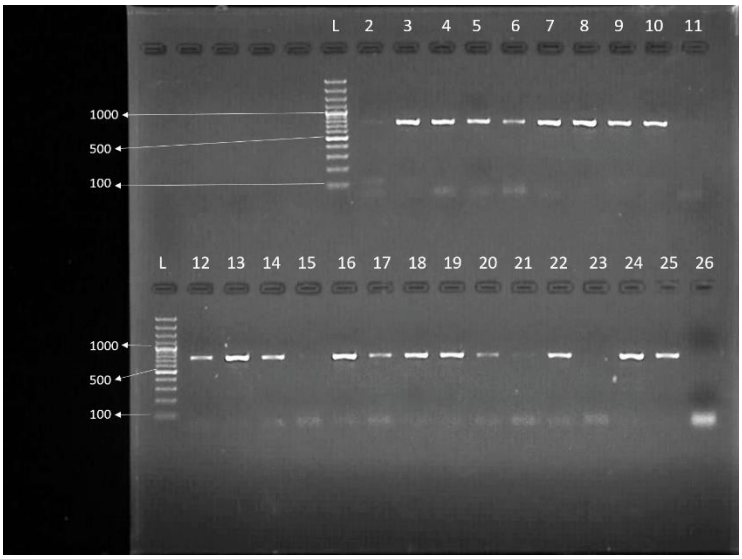


Fig 34. Amplified 723bp partial *rpoB* gene product. Lane L- Ladder (GeneRuler 100 bp Plus DNA Ladder). Lane 2 to 23- test isolates; Lane 24- *M. vaccae* MTCC272; Lane 25- *M. tuberculosis* MTCC300; Lane 26- NTC.

It was attempted to amplify this gene of all the isolates but due to the diverse nature of our isolates from this region, the outcome was inadequate (Fig 35). The multiple binding site of the primer chosen was clearly visible in the chromatograms following sequencing and also with the primer BLAST platform in NCBI. While through excision of the specific bands from the gel some isolates could be included for further analysis, we had to divert into using a different primer set (Lee et al., 2003) for several of our isolates which was of a smaller amplicon size (360bp) that presented no non-specific amplification and could be phylogenetically useful. The primer is discussed in the following sections However, we amplified and cloned the former primer for some of our unique and clinically significant isolates.

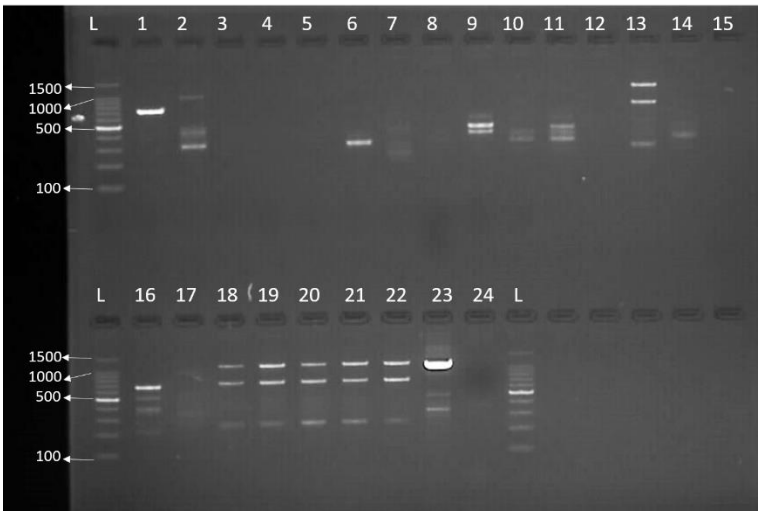


Fig 35. Multiple non-specific bands observed in optimized conditions for *rpoB* primers (Adekambi et al., 2003). Lane L- Ladder (GelPilot 100bp plus, Qiagen). Lane 1 to 23-Test samples; Lane 24- NTC.

6.5.8 *rpoB* gene (Lee et al., 2003) amplification

Isolates which could not be amplified or be used for further studies were amplified by this gene (Fig 36). Due to its smaller amplicon size, amplification and sequencing was more convenient with satisfying results. Reports are available of this gene being successfully used in phylogenetic analysis (Cooksey et al., 2004; Zhang et al., 2013) and atleast one report of the former *rpoB* primer of Adekambi et al. (2003) failing to amplify leading to adopting this primer for the study (Pagnier et al., 2009).

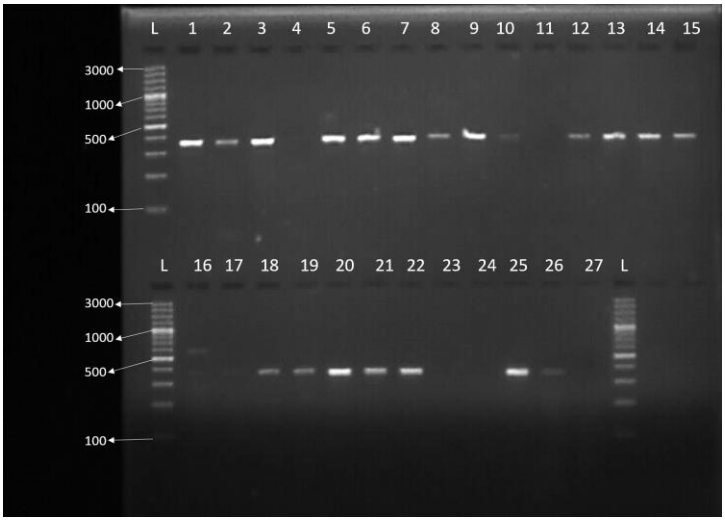


Fig 36. Amplification of 360bp partial *rpoB* gene product. Lane L- Ladder (GeneRuler 100 bp Plus DNA Ladder). Lane 1 to 24- test isolates; Lane 25- *M. smegmatis* ATCC607; Lane 26-*M. tuberculosis* MTCC300; Lane 27- NTC.

6.5.9 16s rRNA gene amplification of unique isolates

The gold standard for molecular identification and differentiation of bacteria, the 16s rRNA, was amplified for speciation of unique isolates (Ngl 2837, HDG, HNP) in the study. Along with *hsp65* and *rpoB* gene amplification, comparison of more than one gene is known to provide a more promising discriminatory tool for exploring unique and novel isolates (Ben Salah et al., 2009; Van Ingen et al., 2012; Kim et al., 2013). Sequences of the 16S rRNA were not only checked for similarity in NCBI BLAST but additionally through an identification scheme called EzBioCloud, available online (Yoon et al., 2017). This interface contains a database of 16S rRNA and whole genome assemblies which performs a similarity search likely to BLAST but based solely on the 16S rRNA gene. The 16s rRNA PCR was optimized using standard strains of *M. tuberculosis* and NTM along with the isolates from the study (Fig 37).

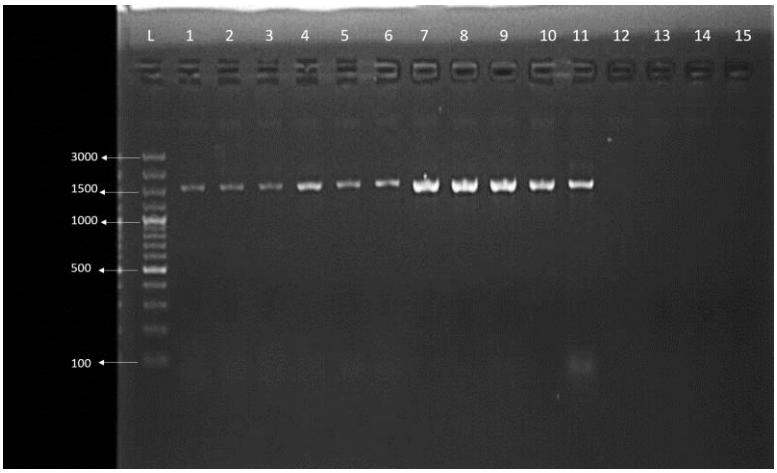


Fig 37. Amplification of 1500bp partial 16S rRNA gene of unique isolates. Lane L- Ladder (GeneRuler 100 bp Plus DNA Ladder). Lane 1 to 6- test isolates; Lane 7- *M. smegmatis* ATCC607; Lane 8- *M. vaccae* MTCC272; Lane 9- *M. bovis* AN5; Lane 10-*M. tuberculosis* MTCC300; Lane 11- Ngl 2837; Lane 12- NTC.

6.5.10 *rrl* gene amplification

A total of 25 isolates were also amplified to check for single base mutations at positions A2058 and A2059 in the *rrl* gene. Some of the clinically relevant species and atleast one representative of a species from the study were checked for this point mutation. Isolates which could be correlated with the phenotypic drug susceptibility tests included *M. chelonae*, from animal source, *M. fortuitum* from environmental source, *M. lentiflavum* from animal and clinical sources (Fig 38).

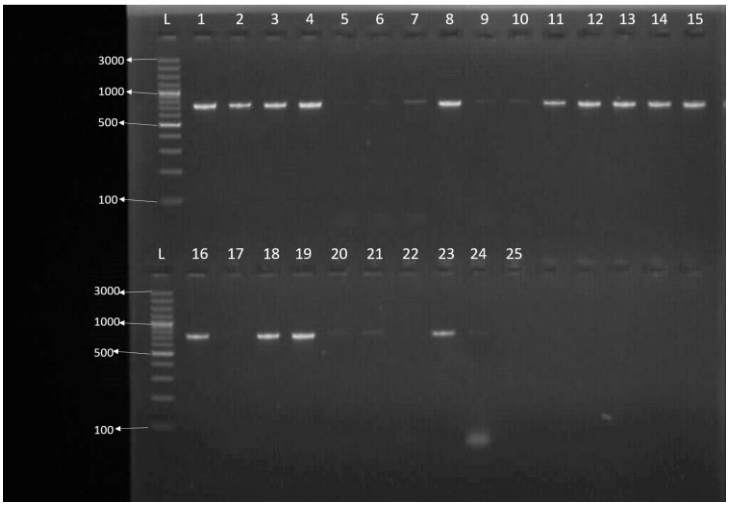
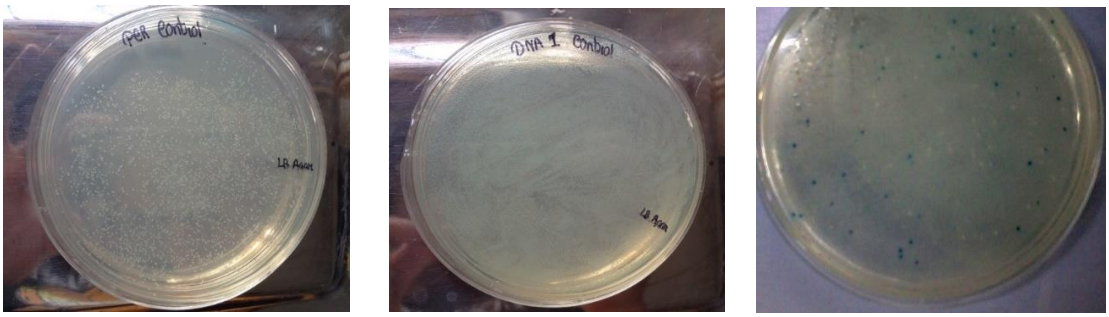


Fig 38. Amplified 700bp *rrl* gene. Lane L-Ladder (GelPilot 100bp plus, Qiagen); Lane 1- *M. tuberculosis* MTCC300; Lane 2- *M. vaccae* MTCC272; Lane 3 to 22, 24- test isolates; Lane 23 *M. smegmatis* ATCC607; Lane 25- NTC.

6.6 CLONING OF SELECTED SIGNIFICANT ISOLATES

Cloning of amplified PCR products was performed for 16S rRNA, *rpoB* and a few *hsp65* gene. As discussed in the previous sections, only rare isolates and unique ones which could not be speciated by multiple identification PCR and BLAST analysis were cloned and sequenced. Three genes of four isolates in total were cloned and sequenced to obtain the complete targeted genes (Fig 39). The isolates include, clinical isolates Ngl 2837 and Ngl 2824, animal isolate HDG and HNP. After amplifying the desired target genes of our isolates, the PCR products were purified and ligated into the Vector pTZ57R/T. Competent cells using DH5-Alpha were prepared by calcium chloride treatment discussed in section 5.9.3. Transformed colonies were selected based on blue/white screening. Recombinant clones were checked for product insert by colony PCR and finally sequenced using standard primers T7 promoter or M13/pUC.



A. PCR control plate B. DNA control plate C. Test plate

Fig 39. Control and transformed plates in LB agar. A: PCR control plate for checking cloning efficiency. White colonies indicate product insert into the vector pTZ57R/T. B: DNA control plate without insert for checking transformation efficiency of competent cells. C: Distinct blue/white colonies on an overnight LB agar plate with ampicillin.

6.7 GENE SEQUENCING BY SANGER DIDEOXY GENE SEQUENCING METHOD

6.7.1 Overall species identification by *hsp65* and *rpoB* genes

For *Mycobacterium* species identification, a total of 69 (12.43%) PCR positive isolates were sequenced, of which 30 (13.63%) were from animal source, 28 (23.14%) of clinical origin, and 11 (5.13%) were from environmental samples (Table 13). All the 69 isolates were amplified and could be identified upto the species level by *hsp*65. The purified amplicons of the amplified isolates were sequenced by Sanger's dideoxy chain termination method in Applied Biosystems 3500xl Genetic Analyzer, Germany. Nucleotide sequences were examined for any misreading and trimmed of low quality before processing for further studies. Sequence study was initiated with BLAST analysis in online server of National Center for Biotechnology Information (NCBI), U.S. National Library of Medicine. A total of 10 different species were identified and 03 isoates have been desisgnated as probale novel or unique isolates (Table 20).

Sl. No.	Sample ID	Source	Location	Species identified by *hsp65* sequencing	BLAST homology
1.	M2G	Animal (milk)	KH, Mgh*	*M. chelonae*	100%
2.	M4P	-do-	-do-	*M. chelonae*	99%
3.	M5P	-do-	-do-	*M. chelonae*	100%
4.	M11G	-do-	-do-	*M. chelonae*	100%
5.	M13	-do-	-do-	*M. chelonae*	100%
6.	M15P	-do-	-do-	*M. chelonae*	100%
7.	M21P	-do-	-do-	*M. chelonae*	100%
8.	M23P	-do-	-do-	*M. chelonae*	100%
9.	M25G	-do-	-do-	*M. chelonae*	100%
10.	M26P	-do-	-do-	*M. chelonae*	100%
11.	M29	-do-	-do-	*M. chelonae*	99%
12.	M38G	-do-	-do-	*M. chelonae*	100%
13.	M43G	-do-	-do-	*M. chelonae*	100%
14.	M48G	-do-	-do-	*M. chelonae*	100%

Sl. No.	Sample ID	Source	Location	Species identified by *hsp65* sequencing	BLAST homology
15.	M67G	-do-	-do-	*M. chelonae*	100%
16.	M68G	-do-	-do-	*M. chelonae*	100%
17.	M71G	-do-	-do-	*M. chelonae*	99%
18.	M74P	-do-	-do-	*M. chelonae*	100%
19.	M75	-do-	-do-	*M. chelonae*	99%
20.	M77G	-do-	-do-	*M. chelonae*	100%
21.	M148G	-do-	-do-	*M. chelonae*	100%
22.	1AG	-do-	-do-	*M. lentiflavum*	100%
23.	M8G	-do-	-do-	*M. lentiflavum*	100%
24.	M12G	-do-	-do-	*M. lentiflavum*	100%
25.	M140G	-do-	-do-	*M. peregrinum*	99%
26.	M20	-do-	-do-	*M. goodii*	100%
27.	M116G	-do-	Dmp, Ngl**	*M. tuberculosis*	100%
28.	HDG	Animal (Skin scrap)	KH, Mgh	*Mycobacterium* sp.	99%
29.	HNP	Animal (Nasal swab)	-do-	*M. genavense*	97%
30.	Mgh SERO	Clinical (Sputum)	KH, Mgh	*M. tuberculosis*	100%
31.	Ngl 12A	-do-	Dmp, Ngl	*M. tuberculosis*	100%
32.	Ngl 2832	-do-	-do-	*M. tuberculosis*	100%
33.	Ngl 1125P	-do-	-do-	*M. tuberculosis*	100%
34.	Ngl 2384P	-do-	-do-	*M. tuberculosis*	100%
35.	Ngl 2831	-do-	-do-	*M. tuberculosis*	100%
36.	Ngl 2833A	-do-	-do-	*M. tuberculosis*	100%

Sl. No.	Sample ID	Source	Location	Species identified by *hsp65* sequencing	BLAST homology
37.	Ngl 1115	-do-	-do-	*M. tuberculosis*	100%
38.	Ngl 1120P	-do-	-do-	*M. tuberculosis*	100%
39.	Ngl 2995	-do-	-do-	*M. tuberculosis*	100%
40.	Ngl 1136P	-do-	-do-	*M. tuberculosis*	100%
41.	Ngl 1140G	-do-	-do-	*M. tuberculosis*	100%
42.	Ngl 2127G	-do-	-do-	*M. tuberculosis*	100%
43.	Ngl 2992G	-do-	-do-	*M. tuberculosis*	100%
44.	Ngl 2995	-do-	-do-	*M. tuberculosis*	100%
45.	Ngl 2123G	-do-	-do-	*M. tuberculosis*	100%
46.	Ngl 2374P	-do-	-do-	*M. tuberculosis*	99%
47.	Ngl 7A	-do-	-do-	*M. tuberculosis*	100%
48.	Ngl 3262P	-do-	-do-	*M. tuberculosis*	100%
49.	Ngl 1129G	-do-	-do-	*M. tuberculosis*	100%
50.	Ngl 1135	-do-	-do-	*M. tuberculosis*	100%
51.	Ngl 05	-do-	-do-	*M. tuberculosis*	100%
52.	Ngl 02	-do-	-do-	*M. tuberculosis*	100%
53.	Ngl 2824	-do-	-do-	*M. novocastrense*	100%
54.	Ngl 2837	-do-	-do-	*Mycobacterium* sp.	98%
55.	AH4	Environmental (Soil)	KH, Mgh	*M. chelonae*	100%
56.	S7	-do-	-do-	*M. chelonae*	100%
57.	S9	-do-	-do-	*M. immunogenum*	98%
58.	S12	-do-	-do-	*M. saopaulense*	96%

Sl. No.	Sample ID	Source	Location	Species identified by *hsp65* sequencing	BLAST homology
59.	RF	-do-	-do-	*M. pyrenivorans*	98%
60.	W11	Environmental (Water)	-do-	*M. chelonae*	100%
61.	W12	-do-	-do-	*M. chelonae*	99%
62.	W34	-do-	Dmp, Ngl	*M. fortuitum*	95%

Table 20. Species identification of mycobacterial isolates from the study.

Note: * Khasi Hills, Meghalaya; **Dimapur, Nagaland

In this study, 10 different types of *Mycobacterium* species and 03 probable novel species were isolated from different locations and sources indicating the diversity of mycobacteria present in this region of the country. The details of these isolates have been presented in Table 21.

Sl. No.	Species identified	Total number of isolates
1.	*M. tuberculosis*	24
2.	*M. chelonae*	25
3.	*M. lentiflavum*	3
4.	*M. peregrinum*	1
5.	*M. fortuitum*	1
6.	*M. immunogenum*	1*
7.	*M. saopaulense*	1*
8.	*M. goodii*	1*
9.	*M. novocastrense*	1*
10.	*M. pyrenivorans*	1*
11.	Unspeciated	3
	Total	62

Table 21. Different types of species identified in the study.

*Support with other gene sequencing required for further confirmation due to low BLAST similarity cut-off.

6.7.2 16s rRNA similarity check in EzBioCloud

Another platform is available online called the EzBioCloud where curated database of only the 16S rRNA gene of prokaryotes are stored (Yoon et al., 2017). Functioning akin to NCBI BLAST, a similarity search on the basis of the said gene can be performed to find the closest species. The EzTaxon database, is known to also represent phylotypes other than the formal nomenclature system. The 03 unique isolates which showed non-concordant results or low similarity cut-off in *hsp65* and *rpoB* gene BLAST were subjected to sequencing of the *16S rRNA*. These sequences were explored in both NCBI and EzTaxon databases. Similarity search of the isolate HNP yielded the exact species identification and similarity cut-off in both the identification portals- the isolate was designated as *M. yongonense* strain 05-1390 with 99.79% similarity, which is the type strain for the species. However the results obtained for the other two isolates (Ngl 2837 and HDG) varied significantly from BLAST and did not provide any conclusive output. Both results from BLAST and EzBioCloud are presented in Table 22.

Sl. No.	Isolate ID	Source	EzBioCloud (Percent similarity)	BLAST (Percent similarity)
1.	Ngl 2837	Clinical	*M. malmesburyense* WCM 7299(T) (99.04%)	*Mycobactrium* sp. G2Z43 (99%)
2.	HNP	Animal	*M. yongonense* 05-1390(T) (99.79%)	*M. yongonense* 05-1390 (99.79%)
3.	HDG	Animal	*M. sulgai* ATCC 35799(T) (77.61%)	*Mycobactrium* sp. 668 (97%)

Table 22. 16S rRNA based species identification by EzBioCloud and NCBI BLAST.

6.8 Drug Susceptibility Test

Micro-titre plate assay for Drug Susceptibility Test (DST) was performed by detection of cell viability using the dye Tetrazolium bromide (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide). The method was standardized using the model organism of NTM, *M. smegmatis* ATCC607 (Fig 41). Drugs isoniazide and clarithromycin was initially used for standardizing the protocol with drug concentration ranging from 100mg/ml to 0.3mg/ml with 2 fold dilution in each consecutive well. Other drugs used in treatment of NTM infections like ethambutol and isoniazide were also tested simultaneously although they could not be correlated genotypically with base mutation in the *rrl* gene. Interpretation of MIC and use of drug concentration was followed according to the approved standard of CLSI for susceptibility testing of mycobacteria (NCCLS, 2003).

Addition of the indicator dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) allows distinction between live and dead cells and clear visualization through colour change. After 24 hours incubation at 37°C the live cells reduces the MTT

dye due to which the yellow color of the dye turns to blue/violet dye. The plate before (Fig 40) and after (Fig 41) addition of the dye is shown below.

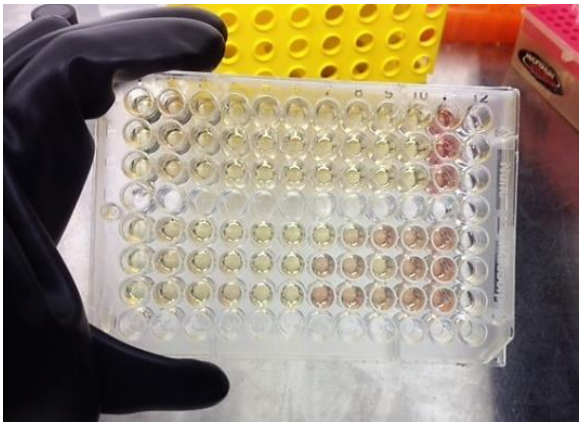


Fig 40. View of a 96 well micro-titre plate just before addition of the MTT dye for viability check.

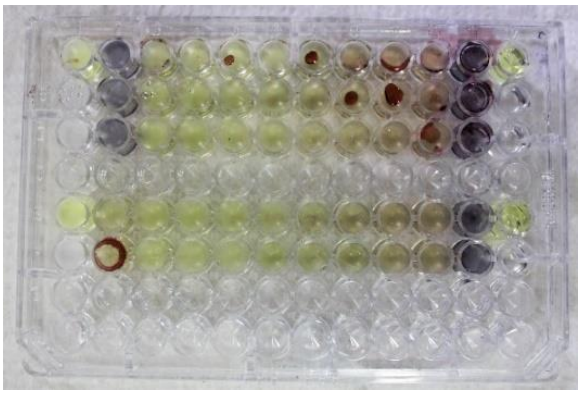
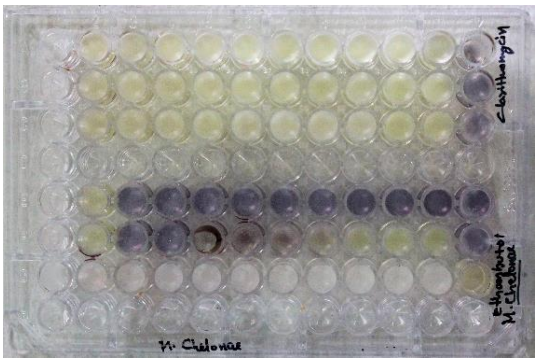
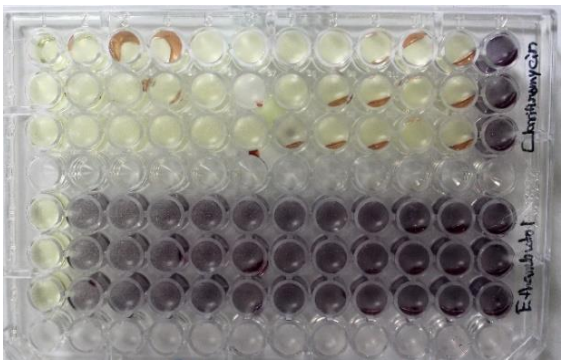


Fig 41. DST test standardized using *M. smegmatis* ATCC607 tested for clarithromycin. MTT dye added.

DST was performed on 08 isolates which were clinically relevant and represent most species from the study. One *M. fortuitum* isolate (C1) from environment is from outside the study and was tested for comparison. It was observed that 06 of the isolates tested were susceptible to clarithromycin, one of the main drugs for NTM infection. The breakpoint of ethambutol for *M. chelonae* isolate (M77) could not be interpreted due to error in the experiment (Fig 42A). *M. fortuitum* from environmental sample depicted resistance to ethambutol showing growth from drug concentration 100 to 0.19 µg/ml. The unspeciated isolate HDG from animal source, which was predicted to belong to the *M. avium intracellulare* complex (MAIC) was resistant to isoniazide (Fig 43A). *M. peregrinum, M. lentiflavum* and *M. novocastrense* were all susceptible to isoniazide (Fig 43 B,C,D).

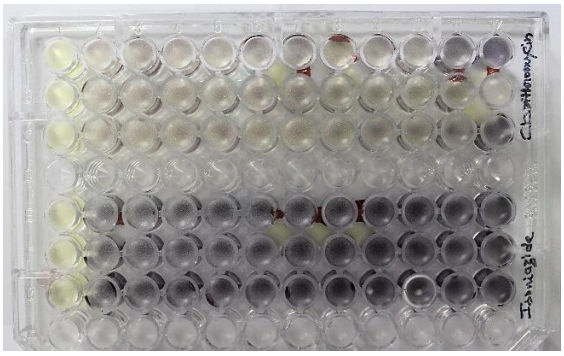


A. *M. chelonae* (M77) from milk

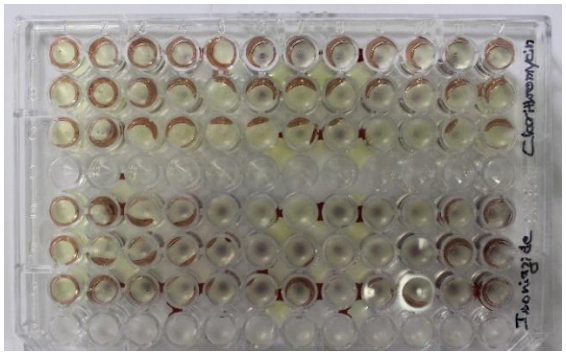


B. *M. fortuitum* (C1) from environment

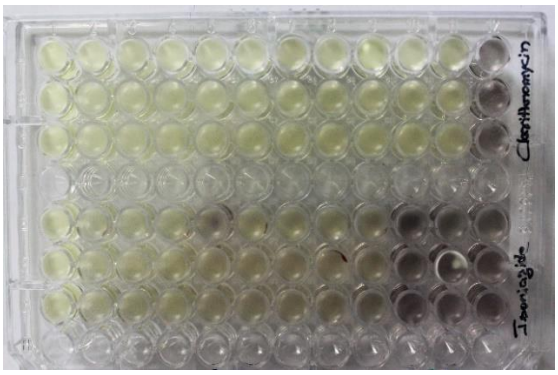
Fig 42. Isolates from milk and environment tested for clarithromycin and ethambutol drug.



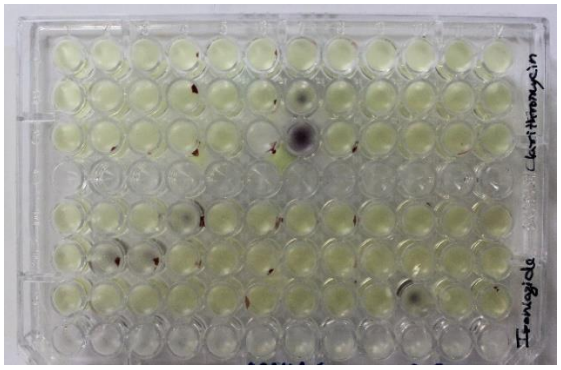
A. Unspeciated animal isolate (HDG)



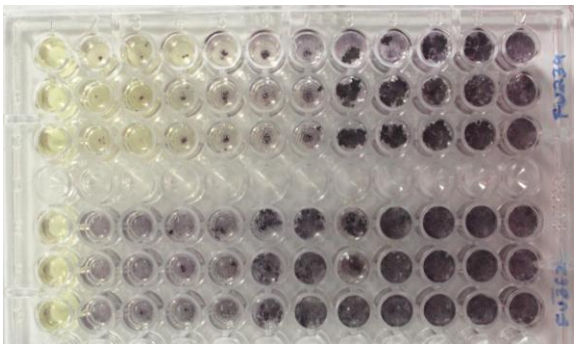
B. *M. peregrinum* (M140) from milk



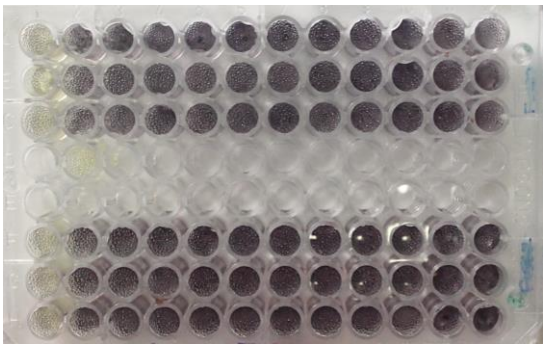
C. *M. lentiflavum* (PK504) of clinical origin



D. Rare clinical isolate *M. novocastrense* (2824)



E. *M. abscessus**-Clarithromycin



F *M. abscessus**-Isoniazid

*(FU239-top three rows & FU362-bottom three rows)

Fig 43. Clinically important isolates from animal and clinical origin tested for clarithromycin and isoniazide.

Two isolates of *M. abscessus* were available to us which was shared from the mycobacteriology laboratory of Nazareth Hospital, Meghalaya. The isolates were isolated from patients undergoing tuberculosis treatment. Parallel to molecular identification, these isolates were tested for clarithromycin and isoniazid resistance as these entities are known to exhibit high resistance to clarithromycin (Griffith et al., 2007; Howard, 2013). These isolates showed resistance to clarithromycin and isoniazide with growth observed in the wells from drug concentrations 100 to 0.09% µg/ml. The minimum inhibitory concentration (MIC) for rapidly growing mycobacteria like *M. abscessus* stands at ≥8 µg/ml (NCCLS, 2003). The report was presented to the treating physician to clinically correlate the observation. Correlation between *in vitro* testing of this drug with skin and soft tissue infections have been observed due to which MIC determination have been recommended. Determination of MIC for isoniazide of this species has not been recommended. However, it was measured since the patients were under this antituberculosis drug regime. Breakpoint was unavailable for RGM but growth was observed from concentration 100 µg/ml onwards. In the US, *M. abscessus* remains the most common isolated species from NTM lung infections accounting for almost 80% of the total cases (Griffith et al., 1993). This test was again compared genotypically with the acquisition of mutation at the *rrl* gene which is discussed in the following section 6.9.

6.9 Mutation check following sequencing of *rrl* gene

A total of 25 amplified *rrl* genes were sequenced for selected isolates from all the three sources. The sequences were trimmed at the beginning and end to remove low quality bases. These sequences from each source were separately aligned with the *E. coli* (AE014075) and *M. abscessus* (NR_077010) sequences. Separate alignment were done for the three sources for clarity and the results are depicted from Fig 44-47.

From Fig 44-46 it can be observed after alignment that at position 2058 of *E. coli* (AE014075) numbering, there was no base mutation of A to G indicating susceptibility to clarithromycin. Genotypically, the susceptible isolates from environment include two isolates of *M. chelonae* (AH4 and RF) and a water isolate of *M. fortuitum* (Cl) from outside

our study. Results of *M. chelonae* isolates (AH4 and RF) could not be correlated with phenotypic tests as the experiment could not be performed due to unavailability of optimum growth. Nine animal isolates comprising of 07 *M. chelonae* and 01 each of *M. lentiflavum* (M12) and *M. tuberculosis* (M140) isolates were aligned to check for base pair mutation. All the isolates were genotypicaly sensitive to clarithromycin.

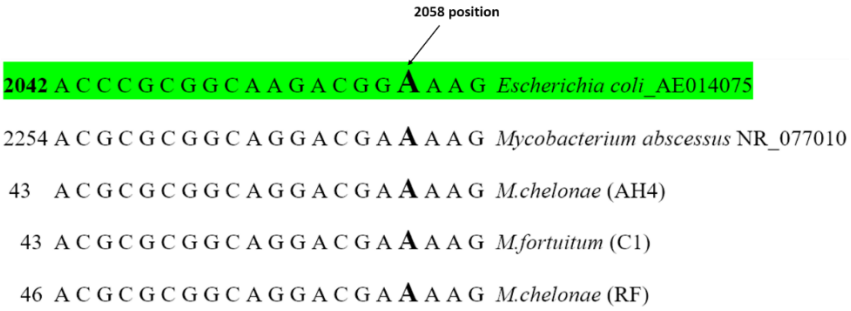


Fig 44. Analysing single base pair change at position 2058 of three environmental isolates. The *E. coli* sequence referred for numbering has been highlighted in green and the position where the mutation check is to be done is shown in bold.

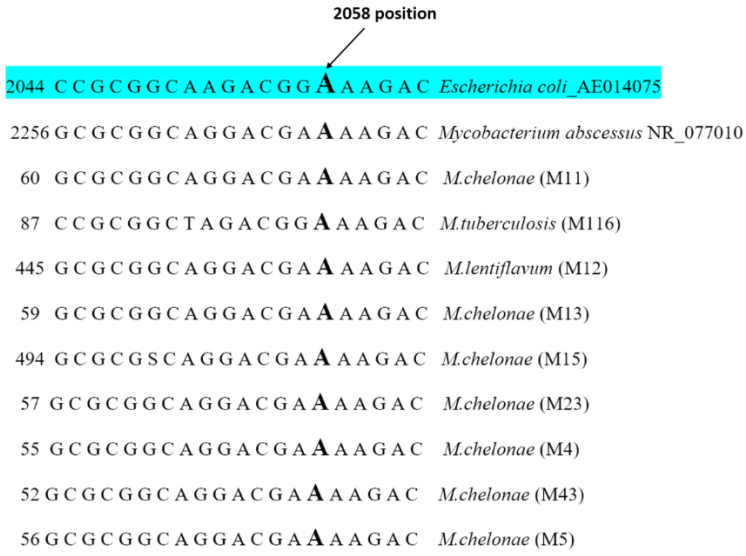


Fig 45. Analysing single base pair change at position 2058 of nine animal isolates. The *E. coli* sequence referred for numbering has been highlighted in blue and the position where the mutation check is to be done is shown in bold.

Ten clinical isolates comprising of 05 *M. tuberculosis* isolates, one *M. novocastrense* (2824), 01 probably novel species (2837G), 01 *M. lentiflavum* (PK504) and 02 *M. abscessus* isolates were sequenced to check for mutation. Except for the 02 *M. abscessus* isolates (Mgh FU362, Mgh FU239), rest 08 of the clinical isolates were sensitive. For isolates PK504 and 2824, susceptibility test correlation of genotypic and phenotypic results of clarithromycin shows that both the results were concordant with each other. The plates can be referred to section 6.8.

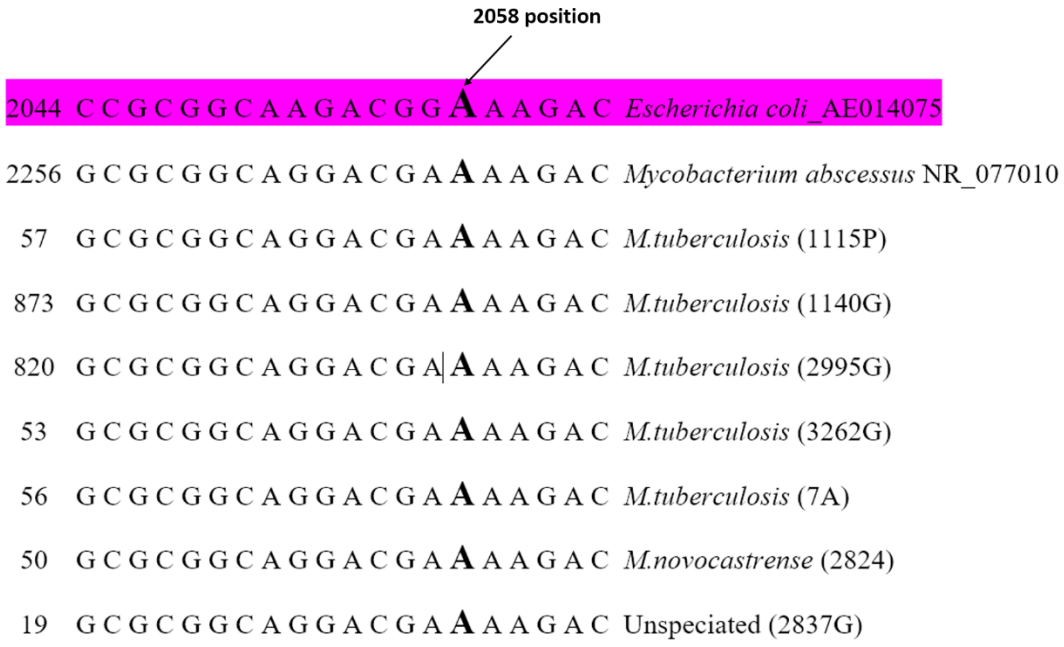


Fig 46. Alignment of seven clinical isolates for *rrl* mutation check at position 2058 bp. The *E. coli* sequence referred for numbering has been highlighted in pink and the position where the mutation check is to be done is shown in bold.

In two *M. abscessus* isolates (Mgh FU362 and Mgh FU239) a point mutation was observed at 2058 bp of the 23s rRNA (Fig 47). The results when correlated were found to be concordant. Selected isolates of all three sources were aligned with these *M. abscessus* isolates and acquisition of mutation at the 2058 bp of the *rrl* gene was noted. To reaffirm the mutation, multiple sequencing of the gene to rule out any possible error was done. All the results showed the mutation. These results were correlated with the DST performed for clarithromycin (section 6.8) where concordant results were observed. Both the isolates showed resistance to clarithromycin genotypically and phenotypically.

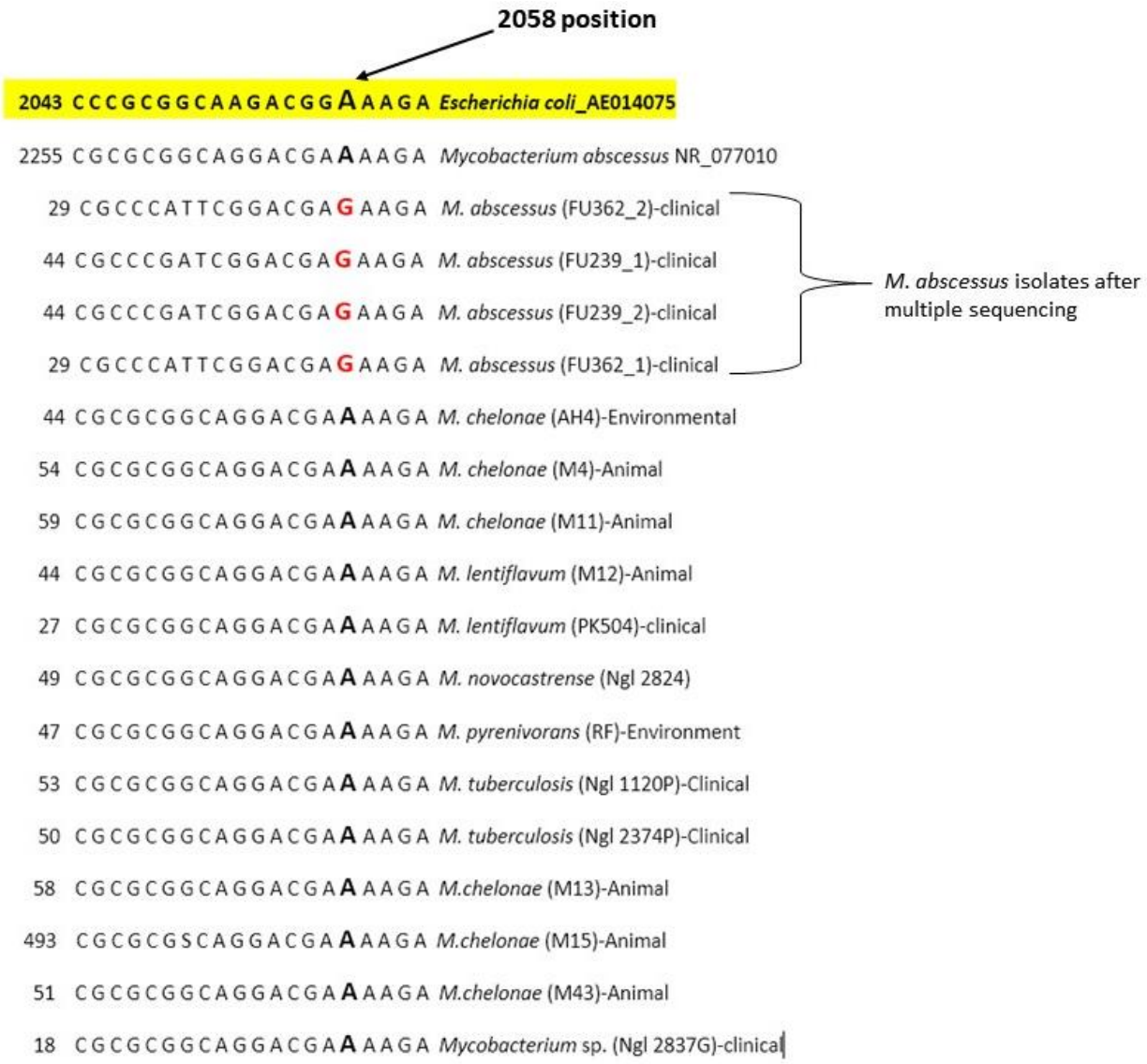


Fig 47. Alignment of *M. abscessus* isolates for *rrl* mutation check at position 2058. The *E. coli* sequence referred for numbering has been highlighted in yellow and the position where the mutation check is observed has been made in bold red. The bases without mutation at position 2058 is made bold in black.

6.10 PHYLOGENETIC STUDY FOR EXPLORING EVOLUTIONARY RELATIONSHIPS AND GENETIC DIVERSITY OF THE ISOLATES

Both *hsp65* and *rpoB* genes were used for phylogenetic analysis of the isolates from the three sources. However for probable novel NTM isolates of clinical origin (animal and human), due to their significance, in addition to both *hsp65* and *rpoB* genes, 16S rRNA gene was also used for more thorough description. Both *hsp65* and *rpoB* genes were helpful for species identification. The isolates could be conveniently identified by *hsp65*, while in addition, *rpoB* proved to be a useful gene for understanding inter and intraspecies diversity and for reaffirmation of species. The uniqueness of an isolate however could be identified when both these genes were used. Low similarity cut-off in BLAST and non-concordant species identification were indication of an isolate's uniqueness. The complete 16S rRNA (1.5kb) was important in characterization of these isolates.

Phylogenetic studies were performed by creating a large database of the nearest BLAST hits and global isolates for all the isolates. Concatenation of three genes- *hsp65*, *rpoB* and 16S rRNA were used successfully for human clinical isolate 2837 for assigning its unique stand in the dendrogram. All phylogenetic studies were performed in the freely available software MEGA software version 7.0 (Kumar et al., 2016). Evolutionary relationships and species distinction has been presented in three categories depending to the source of isolation, with two tress each based on *hsp65* and *rpoB*. Concatenated trees were made only on specific requirement for exploring novelty.

The dendrograms are presented from Fig 48 to Fig 68 which were constructed to represent the species identification through BLAST in a defined manner by placing all the isolates under a single tree along with their respective global and standard isolates. Phylogenetic clustering allowed specific differentiation of closely related species and clearer representation in an illustrative format.

6.10.1 Dendrogram construction for representation of Animal isolates based on *hsp65* and *rpoB* genes

Out of 220 samples screened for mycobacterial isolation, 30 yielded positive for *hsp65* genus specific PCR which when sequenced confirmed 29 (96.67%) isolates as mycobacteria and one (3.33%) to be of non-mycobacterial origin (Table 13) . Further, molecular tests such as *hsp65*-PRA, IS6110, RD4 and RD9 indicated 28 isolates to be NTM and 01 as a member of MTBC isolates. The lone MTBC isolate obtained from milk was subjected to a series of molecular tests which proved the isolate to be *M. tuberculsis* (RD9 positive, 500bp false positive, RD4 negative, *hsp65* PRA).

A case of dog presenting with chronic cough and skin disease had yielded 02 mycobacterial isolate, one each from nasal swab and skin scraping. Both the isolates were NTM and appeared to be unique in nature. The details for which have been discussed as a case report in section 6.10.1a.

Analysis of these 30 isolate sequences in BLAST could identify 28 isolates as NTM and 01 to MTBC. Among these, 23 (82.14%) isolates showed closest BLAST hits with *M. chelonae* all of which had the best match to clinical strains from China and 99% identity with reference strain of *M. chelonae* (KP017250). Showing best match with clinical

isolations, 03 (10.71%) of the isolates (M8G, M12G, 1AG) were identified as *M. lentiflavum* and 01 (0.45%) isolate as *M. peregrinum* (M140G). An isolate (M116G) could only be identified up to complex level i.e MTBC with 100% match with both *M. tuberculosis* and *M. bovis* since partial *hsp65* is unable to individually speciate these members as they share high degree of similarity within the complex. The MTBC isolate was confirmed to be *M. tuberculosis* through a series of differentiation PCR primers of RD9, RD4 and 500bp. The RD9 primers for region of difference 9 which is known to be specific only in *M. tuberculosis* showed 333bp amplification of our isolate in PCR. Primers for the region of difference 4 (RD4) amplified a 400bp amplicon of the positive controls of *M. bovis* AN5 while isolate M116G did not. The 500bp fragment specific only to *M. bovis* presented a false positive 500bp PCR amplification of our isolate. In addition to these two primers, colony morphology and media preference of the isolate to grow in LJ glycerol helped in differentiating the isolate as *M. tuberculosis*.

The *hsp65*-based phylogenetic tree was constructed to give a clarity in distinguishing the isolation of mycobacteria of animal origin. The isolations were majorly of milk origin. Taking the best BLAST hits from NCBI these database sequences were downloaded and aligned to make the resultant tree (Fig 48). Our milk isolates depicted clearly that species identification was accurate by presenting clusters respective to each species. The 21 isolates of *M. chelonae* formed a tight cluster with Chinese isolations from farmed Sturgeon fish (KC684916, KC684918), which is also another isolation from a food source. Also closely associated isolates are *M. chelonae* isolations from tuberculosis patients of TB reference laboratories of China (KF432760, KF432755, KF432759). The isolates are also reaffirmed to be *M. chelonae* by grouping with standard ATCC isolate of *M. chelonae* subsp. *chelonae* (AJ310226). The M20 isolate in the tree forms a loose cluster with the standard strains of ATCC *M. godii* (AY458071, AF547839, DQ523522) and *M. smegmatis* isolates (CP009494, CP009496, AY299161, KP017251). This isolate is expected to be unusual due to the low similarity cutoff in BLAST homology search. By extending a little further from both the *M. smegmatis* and *M. goodii* isolates in the tree, it again indicates a possibility of variation from these isolates. The single *M. peregrinum* isolate (M140- KX232666) correctly identified itself by way of clustering with the standard *M. peregrinum* isolates (AF547865, AY458069) and human (AM902953) database isolates. Adjacent to this cluster is the *M. arceuilense* cluster which were some of the closest BLAST hits in the NCBI database. This is a relatively newly introduced species and similar tree topology with *M. peregrinum* has also been noted (Konjek et al., 2016). The three *M. lentiflavum* isolates (1AG- KX232669, M8G- KX232667 and M12G- KX232668) also formed a close collection with the species-specific reference isolates from the database and sputum samples of TB patients from Greece (GU433895) and clinical isolate from China (KF432476). The isolates however deviated slightly from the standard strain of *M. lentiflavum* (AF547851). The single isolate of MTBC from milk (M116G- KX232670) also settled tightly with the reference strains of *M. tuberculosis*, *M. bovis*, *M. microti* and *M. caprae* as observed in the tree.

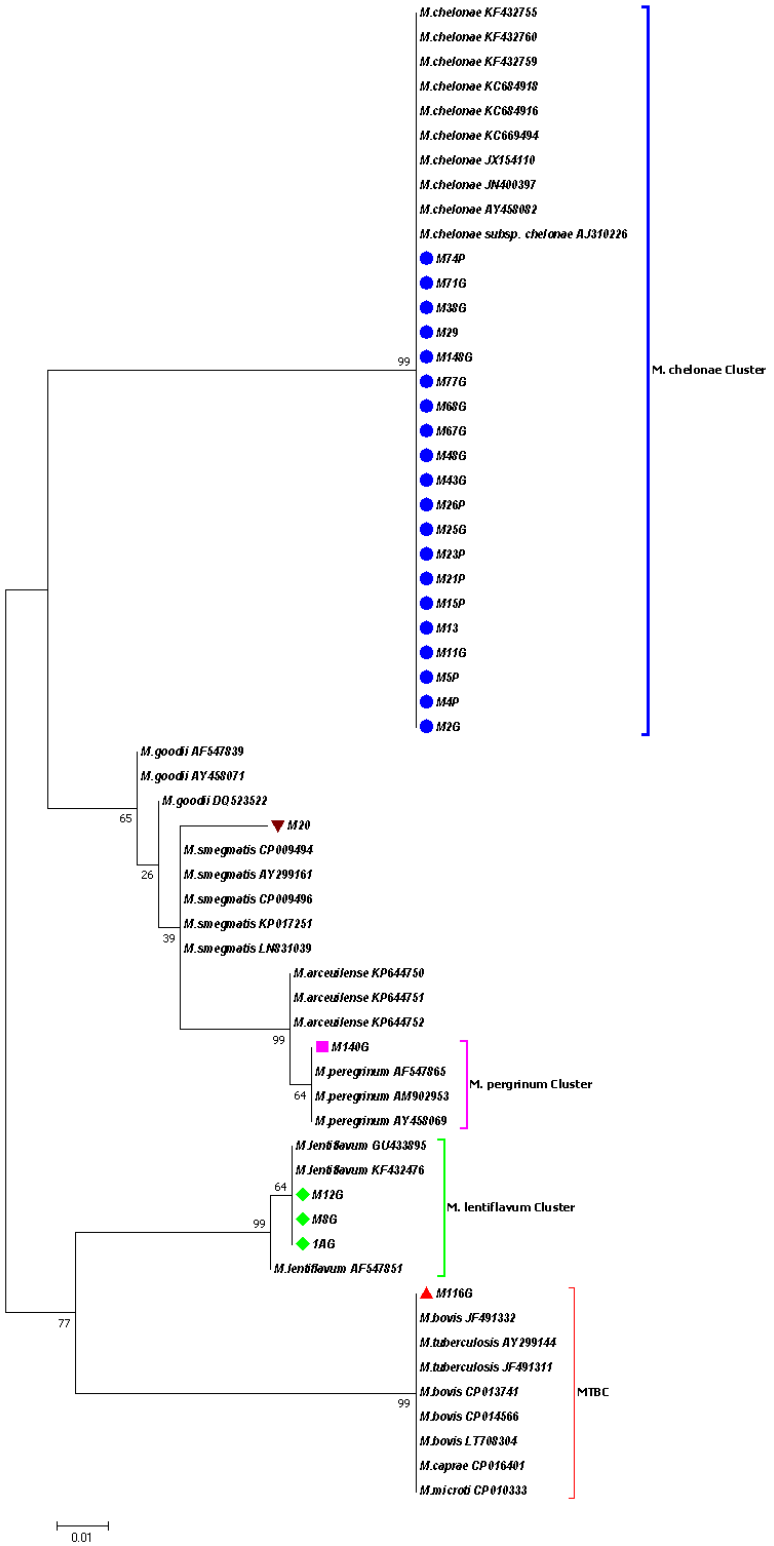


Fig 48. *hsp65* based phylogenetic tree for milk isolates. Phylogenetic tree of the milk isolates with reference and clinical strains was inferred using the Maximum Likelihood method based on the Tamura-Nei model. Percentage of trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.

As compared to the *hsp65*-based tree, the *rpoB*-based ML tree of the milk isolates showed several clusters placing the isolates in more variable positions. The *rpoB* gene used in the study is highly discriminating, especially among rapid growers and the tree in Fig 49 depicts the same by intra-species categorization while all the species have been placed correctly. Majority of the *M. chelonae* isolates (marked in triangles) formed a large cluster along with surface water isolates from Brazil (KT779889, KT779888, KT779882, KT779881), another tap water from Iran (KU861859) and zebra fish from US (DQ866798). Standard strain in this cluster include EU770577. Nine isolates formed another subtree in this large cluster although in the BLAST homology search, the best similarity were obtained from the environmental isolates from Brazil and Iran. The tree indicates a slight variation of these nine isolates from the other four milk isolates. Isolate M68 formed a separate phyletic line adjacent to the large cluster depicting variation from the Brazil and Iran isolates to which majority of the isolates showed similarity. However one isolate (M13) formed another separate cluster with standard strains showing large variation from all the clusters in the tree (*M. chelonae* cluster 2). The BLAST homology showed closest with environmental Iran and clinical Brazil tissues which do not correlate in the tree. Nevertheless the isolate is confirmed as *M. chelonae*. Isolate M20 (yellow circle) was again placed closely with *M. smegmetis* and *M. goodii* indicating again its special position (M20 cluter). High sequence homology in BLAST of this isolate was observed with the Chinese strain of *M. goodii* (CP012150) from soil, but the result of which did not correlate in any of the trees (*hsp*- and *rpoB*-based). It may be noted that a considerable number of insertions were observed in the *rpoB* gene sequence of this isolate thereby producing a comparatively low similarity indent in BLAST (95%). The *M. goodii* isolate (KU861847) is another water isolate from Iran while the *M. smegmatis* (EU597590) isolate is tissue sample from US. Moreover, the *rpoB* sequence similarity search in BLAST gave very low similarity value of 95% indent with both type strains of *M. goodii* (AY262736) and *M. smegmatis* (LN831039). The unconclusive result output in BLAST could be attributed to the close relatedness between these two species. As documented in the first description of *M. goodii*, this species was earlier assembled under the *M. smegmatis* group 2 due to the heterogeneity among this species. With a taxonomic study (Brown et al., 1999) involving several biochemical tests, antibiograms, RFLP and other genomic and proteomic studies the *M. smegmatis* group 2 was described into a new species- *M. goodii*. The source of M20 isolate lies in a farm from Mawlyngot falling under the district of East Khasi Hills from where several other *M. chelonae* species have been isolated. This is the lone *M. goodii* species isolated in the study. *M. peregrinum* isolate (M140) formed a loose cluster with the standard strains (AY147166, JF712876) and a clinical isolate from Italy (HM807425). The three *M. lentiflavum* isolates (1AG, M8, M12G) formed a separate tight cluster with majority of clinical samples from (JX294409, KU362985, KM507936) and a standard *M. lentiflavum* isolate (*M. lentiflavum* cluster). The variations in *hsp65* and *rpoB* identification requires close observation to ratify the discrepancies by further comparisons with more global isolates and more stringent tree building approaches like concatenation of genes which has been discussed in the following sections.

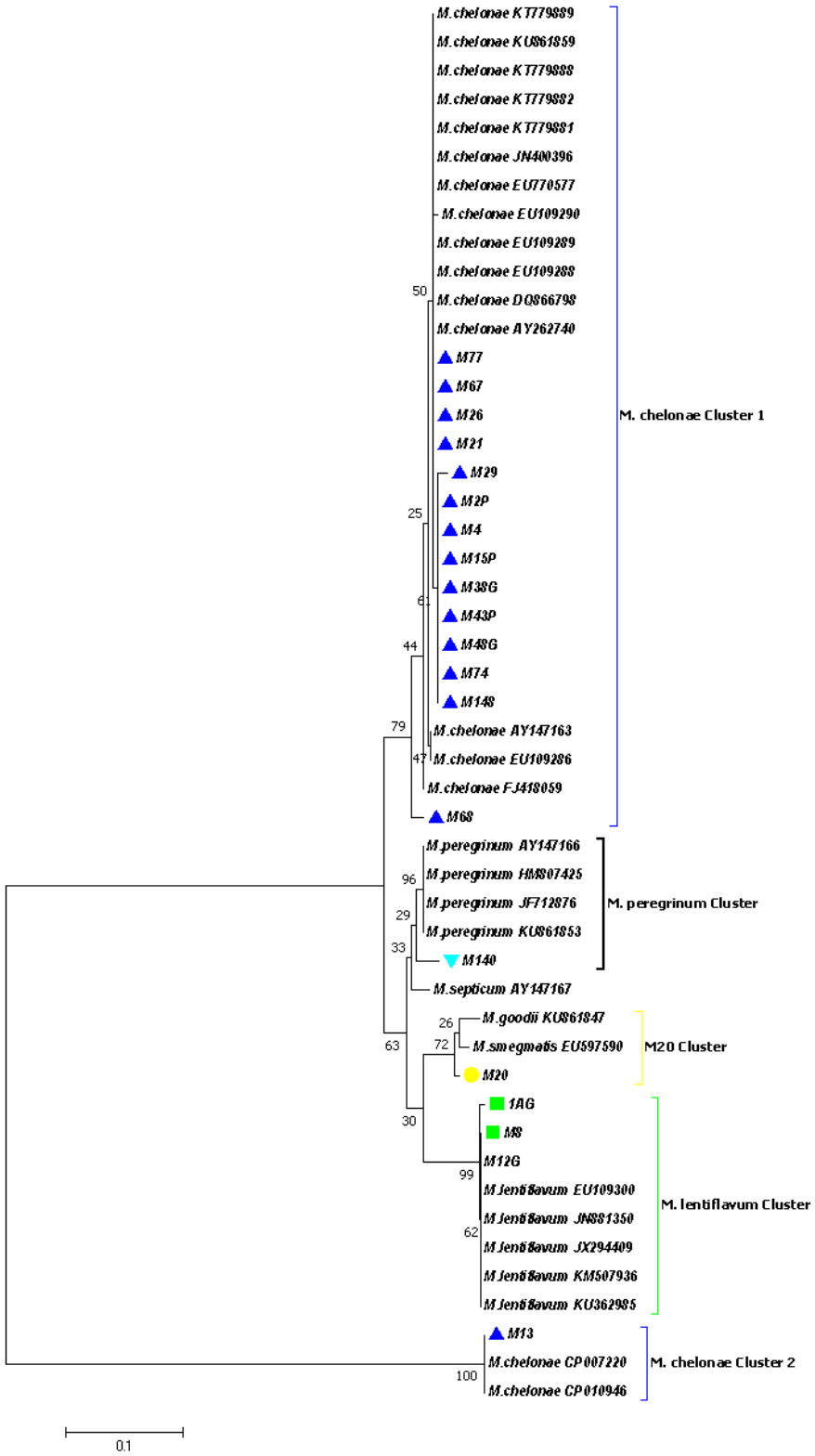


Fig 49. *rpoB* (Adekambi et al., 2003) based phylogenetic tree for milk isolates. Phylogenetic tree of the milk isolates with reference and clinical strains was inferred using the Maximum Likelihood method.

A separate *rpoB* gene-based tree was constructed from another primer (Lee et al., 2003) for those isolates which could not be sequenced by the primer planned to be used in the study (Adekambi et al., 2003). It is likely that due to high polymorphism observed in several of our isolates, the *rpoB* gene described by the latter author could not be implemented extensively for the large number of the isolates in the study. Moreover, given the high heterogenic nature of the *rpoB* gene (Adekambi et al., 2003; Lee et al., 2003), this observation is probable.

A *rpoB* (Lee et al., 2003) based maximum likelihood tree was constructed for five of our isolates (M23P, M25G, M5P, M11G and M71G). The blast similarity hits of this gene were varying from the *hsp65* BLAST homology and the same is depicted in the tree (Fig 50). Two isolates, M71G and M11G were identified as *M. immunogenum* contrastingly from the *M. chelonae* identification through *hsp65* BLAST search. The *M. immunogenum* cluster formed by this isolates fell close to only the standard strains (AY262739). *M. lentiflavum*, *M. peregrinum*, *M arceuilense* and *M. septicum* have all been added in the tree for comparison. This unsettled similarity or species identification through Lee et al. (2003) primers could be also due to the small sequence targeting a highly polymorphic region. Irrespective of which gene was used for identification, most of our *M. chelonae* and *M. lentiflavum* isolates were depicting closest similarity with the global clinical isolations.

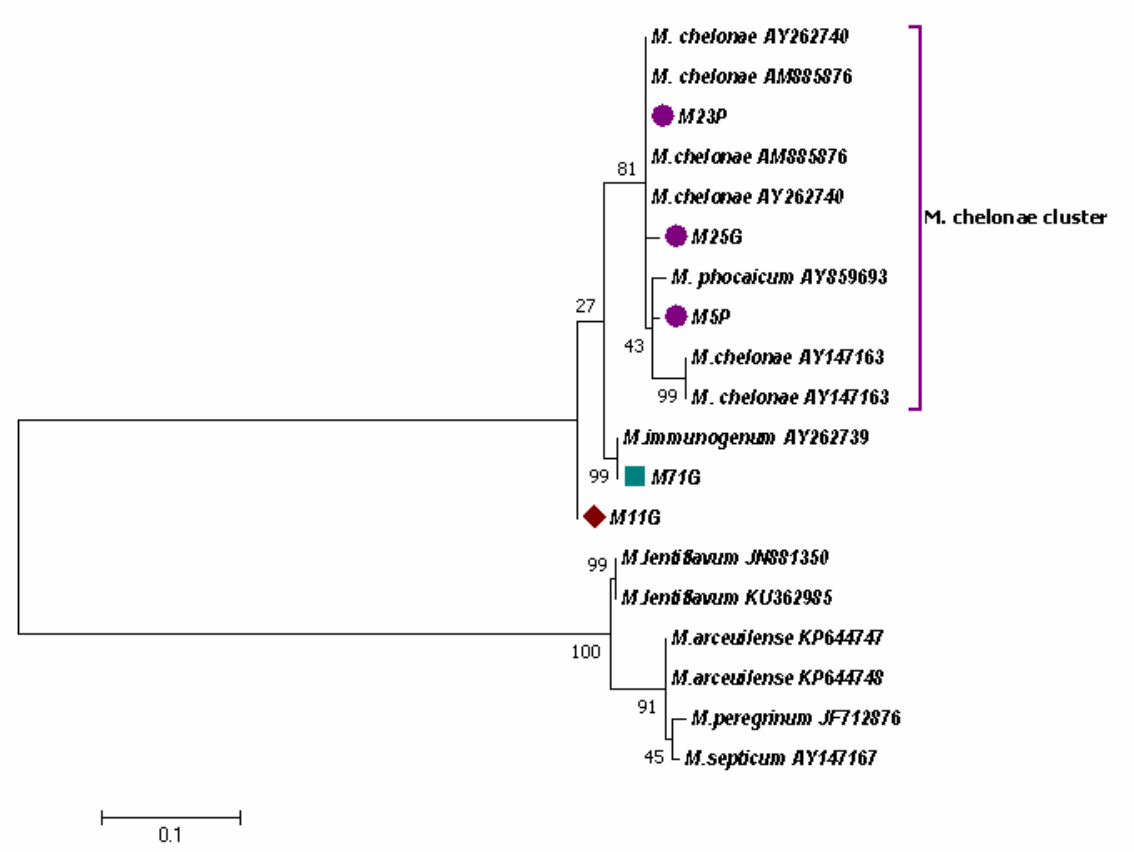


Fig 50. *rpoB* (Lee et al., 2003) based dendrogram for five milk isolates. The tree was constructed through the Maximum Likelihood method with 1000 replicates.

Among the 29 total isolation from milk, species belonging to the nontuberculous mycobacteria have been predominant among our milk isolations, 28 (96.55%) with one isolation from the member of MTBC (3.45%) and among the 28 NTM isolations, 21 were *M. chelonae* species. Such observation would require a careful consideration as most of the human infection of NTM globally, including India (Kalita et al., 2005; Kannaiyan et al., 2015; Umrao et al., 2016), is attributed to this species and reports of major isolation of *Mycobacterium fortuitum-chelonae* complex from environmental samples in India is also being well documented (Lavania et al., 2008). Other NTM isolated in the study— *M. peregrinum* and *M. lentiflavum* are also opportunistic pathogens and are occasionally reported to being recovered from human clinical cases (Jimenez-Montero et al., 2014; Wachholz et al., 2016). The Northeastern region of the country shows a high prevalence of AIDS which predisposes the population to such opportunistic infections.

The possibility of mycobacterial infections through milk is likely given that *M. chelonae* and *M. peregrinum* are among the heat tolerant species of NTM (Schulze-Röbbecke et al., 1992). The infective dose of *M. tuberculosis* is less than 10 bacilli for human, while the infective dose of NTM is unavailable given its opportunistic nature. Clustered isolation was observed from a single village which may be due to a series of factors like environmental contamination, unhygienic diary activities or the climatic conditions favoring these species with ambient conditions to flourish.

In India, especially during occasions of offerings to the deity, raw milk is the integral part of the offering. Practice of household preparations of fermented products with raw milk like *dahi* (curd) or sweet dishes is also common. Significant isolation of these clinically important *Mycobacterium* sp. from raw milk could lead to unforeseen events of mycobacterial infection. With such entities circulating in food nutriments, the public becomes susceptible by intake of both raw, and sometimes pasteurized milk, if the initial dose of these NTM are high and sufficient number of bacilli survives pasteurization. Farmers in India are not required to cull the old and sick herds due to religious sentiments and in consequence the healthy and infirm cattle are stocked under the same shade providing a favorable ground for potential infections. Moreover, remotely situated towns with limited access to medical assistance and poor hygiene may aggravate the transmission. While significant isolation of opportunistic pathogen like *M. chelonae* from a widely consumed dietary nutriment is notable, educating the farmers on the importance of hygienic standards in dairy farms and also sensitizing the health workers will ultimately equip us from any possible unpreparedness of grim consequence arising from the evitable mycobacterial menace.

Report of significant number of *M. chelonae* isolations has been reported as an original paper in '*Proceedings of the National Academy of Sciences, India, Section B.*' The Abstract is given below:

"*Owing to the fact that raw milk intake is still practiced in selected communities globally, milk borne diseases need to be addressed as some mycobacterial species use milk as a ploy for disseminating the disease. An approach was made in this study for screening the mycobacterial species in raw cow milk. For mycobacterial isolation, a total of 200 milk samples were collected from three districts of Northeast India- Dimapur district of*

Nagaland state; East Khasi Hills and Ri-Bhoi district of Meghalaya state. Samples were collected by trained personnel and transported in cold chain. Following decontamination, samples were inoculated in both Lowenstein Jensen (LJ)-glycerol and LJ-pyruvate and incubated up to 12 weeks with regular interval check. Colonies were examined for acid-fastness and confirmed by genus-specific hsp65 PCR. Amplicons were sequenced, analyzed in Basic Local Alignment Search Tool (BLAST) and phylogenetically explored. A total of 22 (11%) isolates were recovered. Sequencing of genus-specific hsp65 gene identified 17 (80.95%) isolates to be Mycobacterium chelonae, 03 (14.28%) as M.lentiflavum, 01 (4.76%) M.peregrinum and 01 (0.5%) as a member of Mycobacterium tuberculosis bacillus complex (MTBC). The dendrogram presented vivid species distinction as the isolates clustered into their respective phyletic line. Significant isolation of M.chelonae from raw milk in this study is noteworthy as it is an opportunistic human pathogen and one of the most dominant species isolated from human nontuberculous mycobacteria (NTM) infections."

6.10.1a Case study- Molecular diagnosis of Non tuberculous Mycobacterial infections in dog (Abstract published in the *International Journal of Infectious Disease*, 2016 as a supplementary)

We investigated the cause for chronic cough and skin infection in a senile pet dog and could isolate two NTM species from its nasal track and skin lesions. This six year old non-descript male pet dog (Fig 51) from the Khasi Hill region of Meghalaya, India was persistently coughing for more than six months with loss of body weight. On examination we found the dog had distress in respiration and had one skin lesion on the body. Owning a mycobacterial laboratory we took up to process its sample and collected both the nasal swab and skin scrap aseptically by a trained veterinarian after harnessing the pet dog. Samples were transported to the laboratory and processed immediately.

Fig 51. The Pet dog, Meghalaya. An immunologically compromised semi pet dog from where two mycobacterial isolates were obtained

Growth was observed on solid media after three months of incubation in both the samples. The nasal isolate grew a few days ahead of the dermal isolate. ZN staining showed very tiny acid fast bacilli which differed in morphology both the samples. The slides examined under the microscope showed strongly acid fast tiny bacilli with non-cord forming distribution of cells indicative of NTM (Monteiro et al., 2003). Genus confirmation was done on the basis of *hsp*65 gene amplification which was then subjected to further

speciation of the isolate. PRA-*hsp65* profiles could only infer the isolate to be a Non-tuberculous Mycobacteria (NTM). The dog was instantly prescribed anti-tuberculosis drug and was put on therapy. BLAST analysis in NCBI exhibited no homology in the three genes with comparatively low similarity cut-offs. BLAST hit of *hsp65* for HNP was *M. genevense* with 97% similarity indent, and HDG showed closest homologies with *M. simiae* complex. Both the isolates showed closest similarity with *M. chimaera* with 97% homology. For HNP isolate the 16S rRNA gene depicted 99% closeness to the reference strain of *M. yongonense*. Two genes, i.e. *hsp65* and *rpoB* were individually subjected to phylogenetic analysis in MEGA7 taking together the reference ATCC sequences of MAIC and the closest BLAST hits which also presented similar ambiguous output with no conclusion on the isolates' species. Concatenation of the sequences ultimately presented higher lucid discrimination and determined the isolates (HNP and HDG) as novel entities (Figure 52). The tree was built through maximum likelihood approach with 1000 replications in MEGA. Frequently isolated in clinical settings and environmental sources, members of the *Mycobacterium avium-intracellulare* complex (MAIC) comprising genetically distinct species and subtypes holds significant agents responsible for opportunistic infections. Phylogenetic analysis indicate the isolate as a distinct new member within MAIC with a unique clinical manifestation in the present dog's case by way of being less lethal as compared to other reported cases.

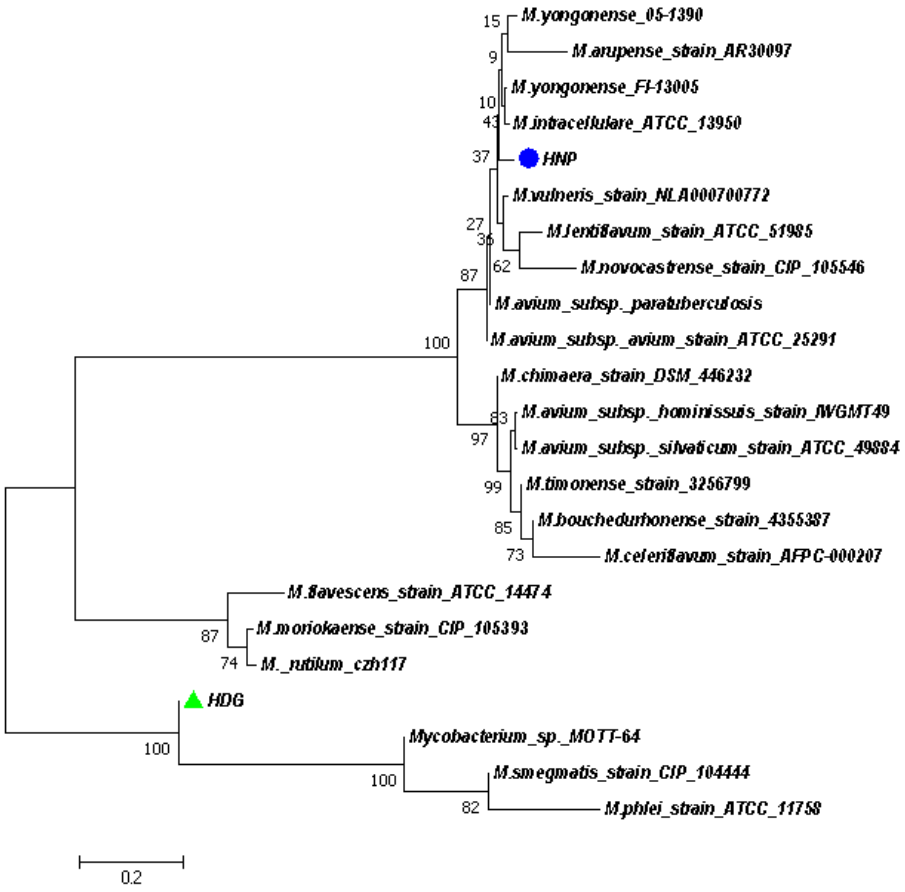


Fig 52. Concatenated phylogenetic analysis of HNP and HDG isolates based on *hsp65* and *rpoB*.

6.10.2 Dendrogram construction for representation of Clinical isolates based on *hsp65* and *rpoB* genes

Hundred and twenty one (121) human samples were processed for isolation consisting of 11 sputum and 03 urine samples (Table 21). Urine samples did not yield any *Mycobacterium* whereas sputum samples yielded 25 (20.66%) mycobacterial isolates of which 23 (92%) were confirmed to be of *M. tuberculosis* and 02 (8%) as NTM. The 25 *Mycobacterium* isolates were presumed to be of genus *Mycobacterium* through their growth resembling to *Mycobacterium* in Lowenstein Jensen media (glycerol/or pyruvate or both). The acid fast staining confirmed them to be acid fast bacilli and later on application of *hsp65* gene PCR they yielded desired 441bp corresponding to be *Mycobacterium* genus. Further differentiation PCR for MTBC and NTM was perfomed by IS6110, RD4 and RD9 which confirmed that 23 isolates belongs to MTBC and 02 to be NTM. *hsp65* RE yielded band patterns which could not be correlated with the reported banding pattern for the 02 NTM species. The 23 MTBC suspected isolates were rechecked by species specific differentiating PCR for *M. tuberculosis* and *M. bovis* viz., RD9, RD4 and 500bp. All the 23 isolates amplified RD9 and did not amplify RD4 and 500bp confirming the isolates to be *M. tuberculosis*. *hsp65* sequencing and BLAST analysis of all these 23 isolates of *M. tuberculosis* could lead us to identification of these isolates as MTBC as *hsp65* cannot differentiate between the members of this group.

hsp65 sequencing of the suspected NTM was able to assign the species as NTM of which 01 was identified *M. novocastrense* (Ngl 2824) but one of the isolate Ngl 2837 could not be identified by *hsp65* sequencing. These 02 NTM were again subjected to *rpoB* PCR which again showed isolate Ngl 2824 as *M. novocastrense* but isolate Ngl 2837 speciation was inconclusive. Hence, isolate Ngl 2837 was targeted for further identification with 16s rRNA sequencing. Concatenation of all these three genes- *hsp65, rpoB* and 16s rRNA assigned this isolate in the category of novel NTM. The case details of which is discussed further in section 6.10.2b.

An ML tree was constructed for all the clinical isolates irrespective of whether they were MTBC or NTM. Fig 53 shows the clear distinction of two clads, The MTBC and the NTM cluster clearly depicting the species identified through BLAST similarity search. Each of the NTM also formed distinct phyletic line as identified through BLAST. In the large MTBC cluster, several global isolates of *M. tuberculosis* have been added for relating the diversity. However all the isolates irrespective of geographical isolations formed a tight cluster. Some other members of the complex have also been added from the database for comparison. The global isolates include Beijing-like strain (CP017598), isolate from Malaysia (CP010968), US (CP009195) and Vietnam (AP018033). *M. bovis* isolates included isolates from South Korea (CP012095), a standard isolate (CP009449) and a BCG strain (CP014566). Two *M. tuberculosis* isolates from the study (Ngl 2832 and Ngl 2374P) extended into a distinct subtree in this large cluster indicating a variation from the rest of the isolates. All of our isolates (marked in blue triangles) showed the best homology with one isolate from India (KX232670) whose whole genome as *M. bovis* has been submitted recently. Two *M. abscessus* isolates (Mgh FU239 and Mgh FU362), formed a loose cluster with standard reference sequences of *M. abscessus* and one isolate from China (KF432764). Forming a cluster closely adjacent to these standard *M. abscessus* isolates, the two isolates

seem to be varying in view that they were placed in a separate phyletic line. The *M. lentiflavum* isolate (PK504) formed a tight cluster with its standard strains. Isolate Ngl 2824 identified as *M. novocastrense* placed itself closely with a human isolate of *M. novocastrense* from Mexico (JX154123). The Ngl 2837 isolate could not cluster with any reported species of *Mycobacterium* as it was assumed to be a novel strain. In the phylogenetic tree, it came under the cluster of *M. novocastrense, M. moriokense, M. brassiae, M. flavescense* and *M. rutilum* but relatively making a phyletic line very distinct from the rest affirming its unique stand in the lineage. As per our study objectives, emphasis have been given to the NTM isolates and those obtained from clinical source have been explored critically through case studies. Individual dendrograms for each of the clinical NTM isolate have also been constructed and discussed in the following sections.

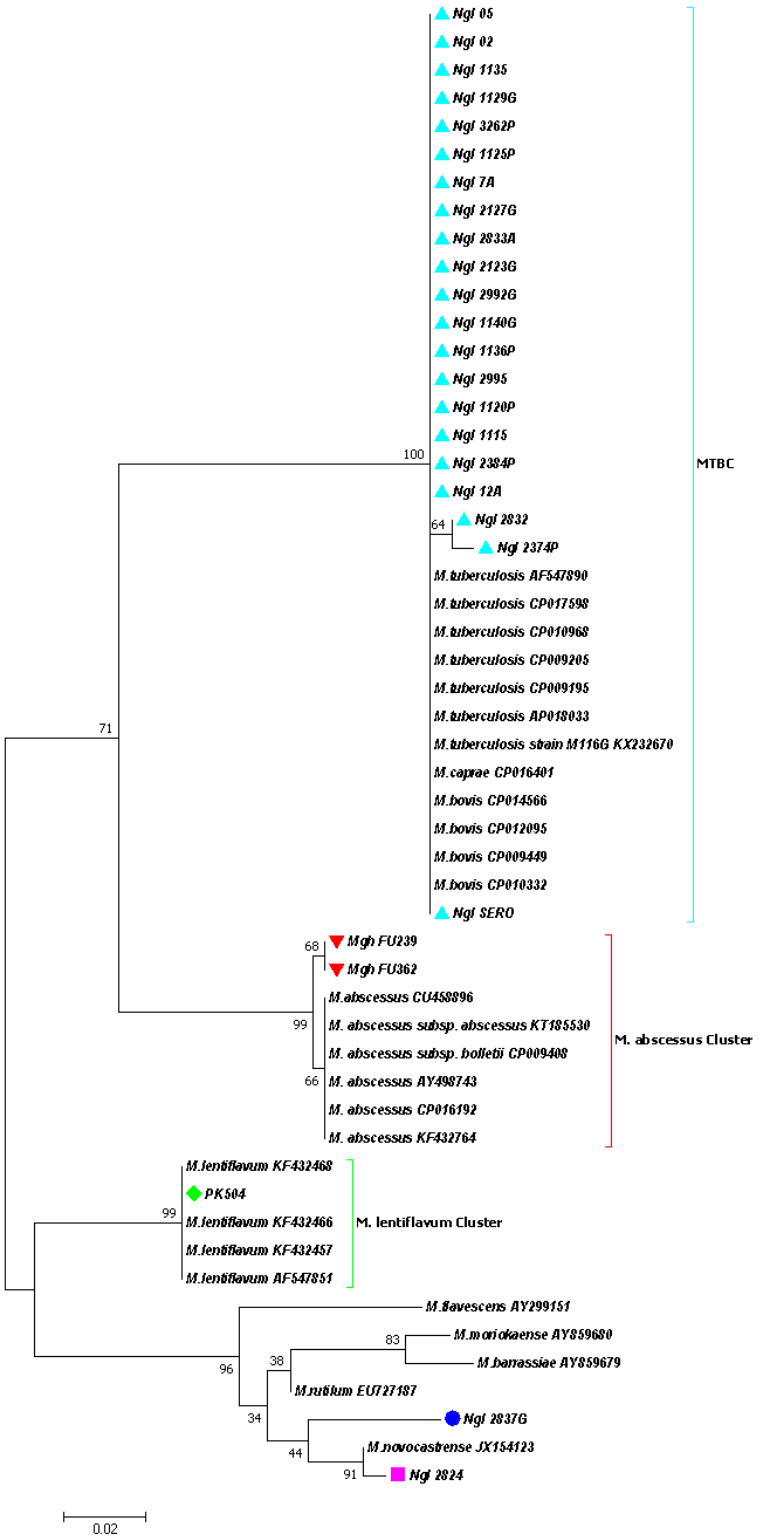


Fig 53. *hsp65* based phylogenetic tree of clinical isolates. The phylogenetic tree of the clinical isolates with reference and clinical strains was inferred using the Maximum Likelihood method. Isolate 2837 formed a distended plyletic line given its uniqueness.

Two trees of *rpoB* has been constructed for clinical isolates depending on good quality sequences available for either of the genes. The *rpoB*-based tree of Adekambi et al., 2003 was made for those isolates for which sequences were available (Fig 54). The tree depicts four distinct clusters representing each species. The *M. abscessus* isolates (Mgh FU239, Mgh FU362) formed a tight cluster with, clinical isolate from US (JN400390), France (KC352792) and Spain (KT185552). Close to this cluster but separated in a phyletic line are *M. abscessus* standard strains (KC352778, AY147164, AY262741) and clinical isolate from Iran (KU362972). The *M. lentiflavum* isolate (PK504) isolates from TB reference laboratories from Iran (KU362985, KU861880, KM507936) and several standard strains (JN881350, EU109300). One *M. tuberculosis* isolate (Mgh SERO) also formed a close cluster with several isolates of Peru (CP023635, CP23640, CP023639, CP023617, CP23606) as well as other species of the complex such as *M. microti, M. africanum*, and *M. pinnipedii*. The probable unique isolate (Ngl 2837) came close to an recently submitted unspeciated isolate from UK (MF774024) and formed a dispersed cluster with *M. arupense, M. moriokaense* and *M. barrassiae*.

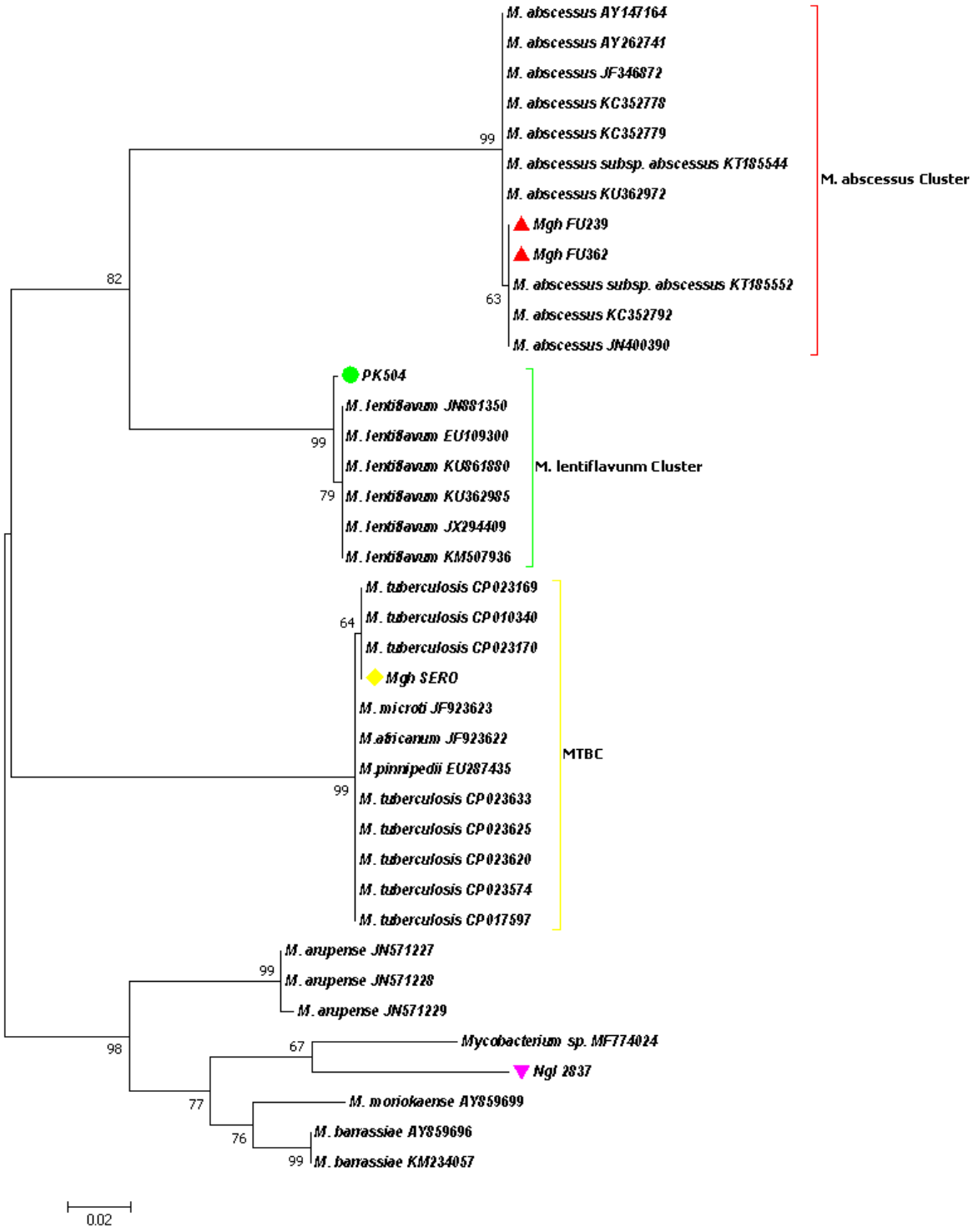


Fig 54. ***rpoB*** **(Adekambi et al., 2003) based phylogenetic tree of five clinical isolates.** The phylogenetic tree of the clinical isolates with reference and clinical strains was inferred using the Maximum Likelihood method.

rpoB gene amplification for majority of the *M. tuberculosis* isolates were done by Lee et al. (2003) primer and good quality sequences of approximately 250 bp were obtained for phylogenetic analysis. All isolates, as in *hsp65* gene BLAST, showed the best homology with the Indian isolate (CP023708). A large cluster of *M. tuberculosis* was obtained where all our isolates also clustered together (Fig 55). The global isolates in this cluster include isolates from US (CP009196, CP009183) and Peru (CP023635, CP23640, CP023639, CP023617, CP23606). One isolate (Ngl 2824) was identified with high indent value in BLAST (100%) as *M. novocastrense* through *hsp65* gene homology search. However, *rpoB* showed the closest homology with *M. phlei* and other species like *M. neoaurum., M. barrassiae*, and *M. chubuense*. Addition of database *M. novocastrense rpoB* sequence in the tree did not cluster the related species.

The three trees (Fig 53, 54 & 55) indicate that our isolates are closely similar to other global isolates showing close clustering with isolates from UK, Peru, Spain, US and Iran. In the BLAST, irrespective of whichever gene was used for homology search, one isolate recently submitted from India (KX23267) showed the best homology for our *M. tuberculosis* isolate.

The purpose of the study was to know the other circulating mycobacterial species in human in addition to the major *M. tuberculosis* cases. The isolation was oriented to cover the all the mycobacterial species except *M. lepare* and *M. avium* subsp. *paratuberculosis* as one is non-cultvable and as for the latter, growth is not supported by LJ based glycerol or pyruvate media. In our small scale study from samples of primary TB centre or sample submitted by underprivileged individuals, we could find that although the majority of isolation goes around *M. tuberculosis*, we could still isolate two NTM, one of which is rare and another novel. This indicates that the population will have NTM cases which are not able to get detected owing to limited diagnostic facility in these primary centre and area. Both the NTM cases could not be detected at primary care centre and eventually succumbed without getting the required treatment. The underlying condition could be worse as even though our study is very limited it indicates the number of missed NTM cases which cannot be overlooked. In coming days these species may be dominant species among the tuberculosis cases as observed in other developed countries. These NTM need to be given attention especially in the relapsed TB cases which in all time are not drug resistant *M. tuberculosis* cases. NTM infections are increasingly reported from India in recent years identifying itself as an emerging pathogen. Understanding of this pathogen will lead to effective multidisciplinary research between eminent research institutes and health care set ups. Due to the high burden of TB cases in the country, opportunistic pathogens like NTM do not receive the deserved attention especially in resource limited set ups. Encounter of rare and unusual species from this part of the country indicates presence of these opportunistic pathogens which cannot be overlooked. This also points out the need for collaborative effort of constrained settings with better equipped institutions so as not to miss such underreported cases.

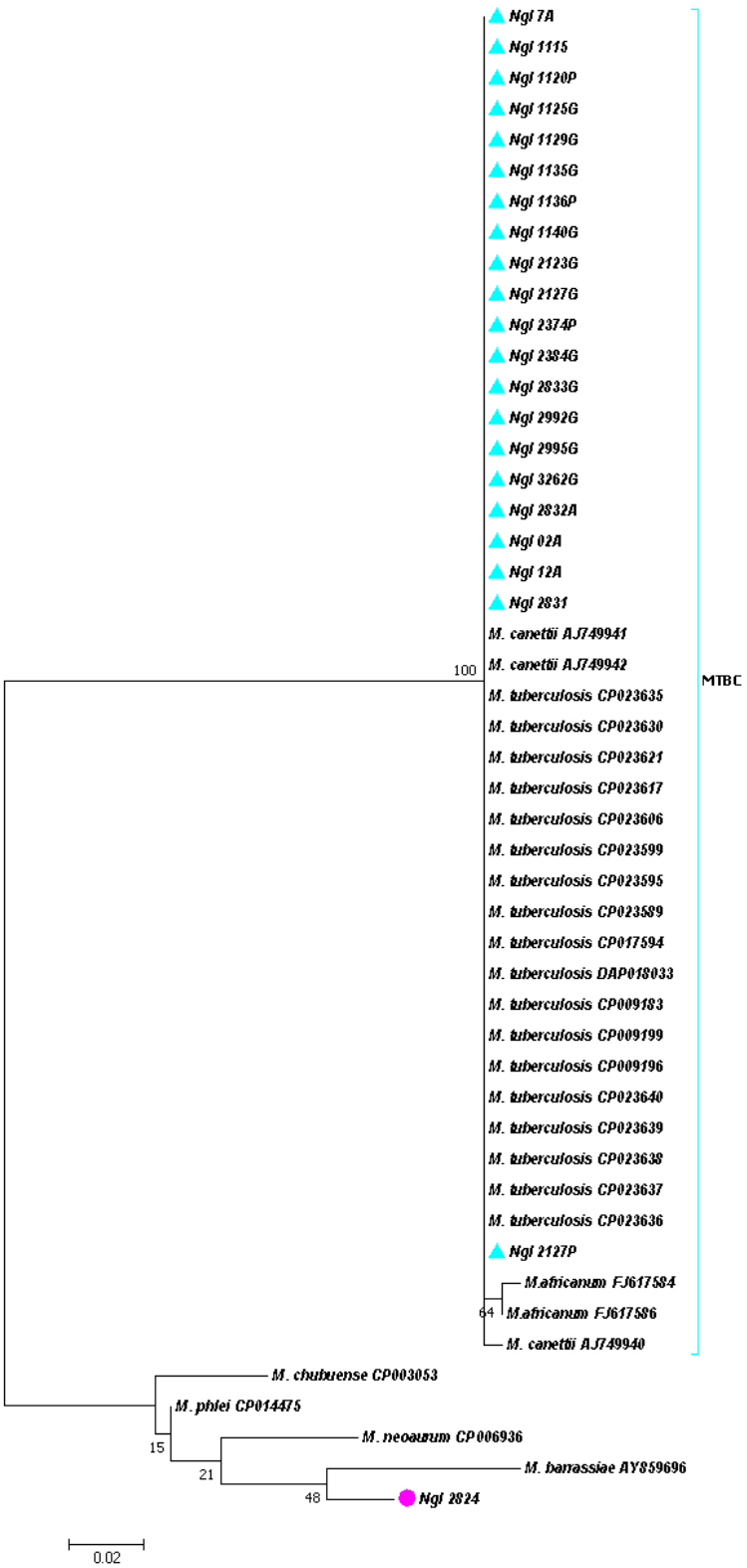


Fig 55 . ***rpoB*** **(Lee et al., 2003) based phylogenetic tree of clinical isolates.** The phylogenetic tree of the clinical isolates with reference and clinical strains was inferred using the Maximum Likelihood method.

Shared isolates from Nazareth Hospital, Meghalaya

In a collaborative effort, besides the isolation done through sampling, 03 NTM isolates were shared with our laboratory where assistance was given for isolation on solid media and further molecular identification with *hsp65, IS6110, RD9, RD4,hp65* PRA, sequencing of *hsp65* and *rpoB* and phylogenetic studies were done. The details of these isolates are presented in case discussion in the following sections.

a. Two relapsed cases of tuberculosis who were not responding to usual TB therapy for first line anti-tuberculosis drugs being treated in Nazareth Hospital, Meghalaya. MALDI-TOF was performed on 01 isolate liquid growth from MGIT which was identified as *M. absecessus* and the other could not be processed for MALDI detection due to turbidity in broth culture. Both the isolates were shared to our lab where isolation on solid media and further molecular identification with *hsp65, IS6110, RD9, RD4,hp65* PRA and sequencing of *hsp65* and *rpoB* were performed. Both the isolates was confirmd to be *M. absecessus* through sequencing.

b. Another isolate was shared for molecular speciation which was identified as *M. lentiflavum* through a series of PCR and sequencing approaches. The detail of this is also discussed as case report in the following sections.

6.10.2a Case study 1- *Mycobacterium lentiflavum* infection in a boy from Meghalaya, India (Published in the International Journal of Mycobacteriology, 2017)

During routine research on Non-tuberculous Mycobacteria we were approached for assistance by a staff whose daughter was suffering from tuberculosis. The young lady was a farm worker with three teenage children. Before collecting any of her sample for analysis, she expired in the hospital. During the same time her 13 year old son was also suffering from undiagnosed illness and could not be cared for due to the mother's critical health. It was only after his mother succumbed to the disease he reported to the local government block level dispensary complaining of scrotal inflammation and pain. The health worker at the dispensary observed symptoms of weight loss, lymphadenitis, intermittent fever, ascitis and hydrocole. He was clinically suspected of suffering from extra-pulmonary tuberculosis-cervical Koch lymphadenitis/ scrofula, and was referred to better equipped hospitals in the city where his chest X-ray, mantoux skin test, blood profile, abdominal ultrasound, FNAC of cervical lymph node for acid fast staining was performed. He was positive for Mauntoux test, and lymph node biopsy test showed acid fast bacilli, his Erythrocyte sedimentation rate was raised (20mm in 1st hour), lung X-ray showed prominent mediastinal lymph nodes and lower abdominal ultrasound reveled moderate ascitis with few mesenteric lymph node in the pre-umblical region. Hydrocoele and scrotal inflamation was observed and accordingly the patient was advised an operative procedure for hernia and hydroceole. The operation could not be done for economic reasons but the child was started on category I drugs through the RNTCP block dispensary from where the case was initially referred.

During the same period, we also collected the urine sample of the child following the ethical guidelines. Sample was transported to our laboratory under refrigerated condition and processed within 12 hours of collection under strict biosafety measures. All the above results were negative and no growth observed. The full 06 month course of treatment was

completed and during the follow-up, his ascites and cervical lymph node swelling had subsided, but he was not gaining substantial weight. To confirm his full recovery, the boy was again referred to city based hospitals where his re-investigation started with blood profiling and X-ray. This time his ESR was even higher (34mm/hr), alkaline phosphatase was on the upper limit (202 U/L) and lung X-ray showed left pleural effusion with underlying consolidation. The case was then referred to Nazareth Hospital Shillong, Meghalaya having an RNTCP unit with better-equipped identification facility for *Mycobacterium* species. The child's bronchial lavage was acid fast smear negative but was flagged as positive for *Mycobacterium* species when inoculated in BACTEC MGIT^{TM} 320 (Becton, Dickinson and Company, Franklin Lakes, New Jersey). Further the isolate (PK504) was assumed as NTM on the basis of a negative result in MPT64 protein detection-based immunochomatographic test kit (SD MPT64TB Ag Kit, Standard Diagnostics, Inc. Gyeonggi-do, South Korea). This followed the change of child's course of treatment to NTM treatment regime. In between these two mycobacterial treatment he has been prescribed two short courses of conventional antibiotics (cefuroxime).

He received treatment with with rifampicin, ethambutol and clarithromycin and improved in health with weight gain, enlargement of cervical lymph node had subsided, fully recovered from ascitis and scrotal inflammation reduced although not cured. Further, the isolate was shared with us and was later confirmed to be *M. lentiflavum* with the help of culture and molecular tests. PCR amplification for *Mycobacterium* genus specific *hsp65* gene which amplified a band of 441 bp confirming it to be of *Mycobacterium* genus. The species-specific PCR for *M. tuberculosis* and *M. bovis* were also tested viz., RD9, RD4 and 500bp primers which were all negative for MTBC suggesting the isolate to be NTM. *hsp65*-PRA displayed band patterns (*Bst*EII-440bp; *Hae*III-145/130bp) which directed the isolate to three NTM species namely- *M. lentiflavum*, *M. florentinum*, *M. simiae*.

This report of *M. lentiflavum* isolated from a child with lymphadenitis is possibly only the second report of *M. lentiflavum* isolation from human source and typical of its kind from child tuberculosis case in India. The first isolation was communicated from AIIMS with a sequence submission of *16S rRNA* gene sequence (GenBank Accession number JQ037846). Isolation of this NTM species illustrates the potential of NTM infections in susceptible individuals and calls for cautious diagnosis and treatment while managing tuberculosis cases in this TB laden country.

The isolate's *hsp65* sequence was used for blast analysis in NCBI for identification which depicted 100% match to *M. lentiflavum.* Phylogenetic analysis of the isolate was also carried out using the sequenced *hsp65* genes with reference strains of *M. lentiflavum*, other closely related species of *M. lentiflavum*, the best matches of BLAST results and outgroups (Fig 56). The isolate (PK504) formed a clad with strains of *M. lentiflavum* placing itself under the *M. lentiflavum* cluster of type strain (AF547851), Greece isolate (GU433895) and several Chinese isolates (KF432466, KF432468, KF432457) from tuberculosis patients. However, the standard ATCC sequence of *M. lentiflavum* (AY373453) fell under a separate phyletic line. Slow growers like *M. rutilum*, *M. tuberculosis*, *M. bovis,* and rapid growers like *M. phlei* and *M. novocastrense* were used for comparison. Later, the isolate was confirmed with MALDI-TOF which also showed the isolate to be *M. lentiflavum*. Further, the purified PCR product of *hsp65* was submitted to GenBank, NCBI (KX431212).

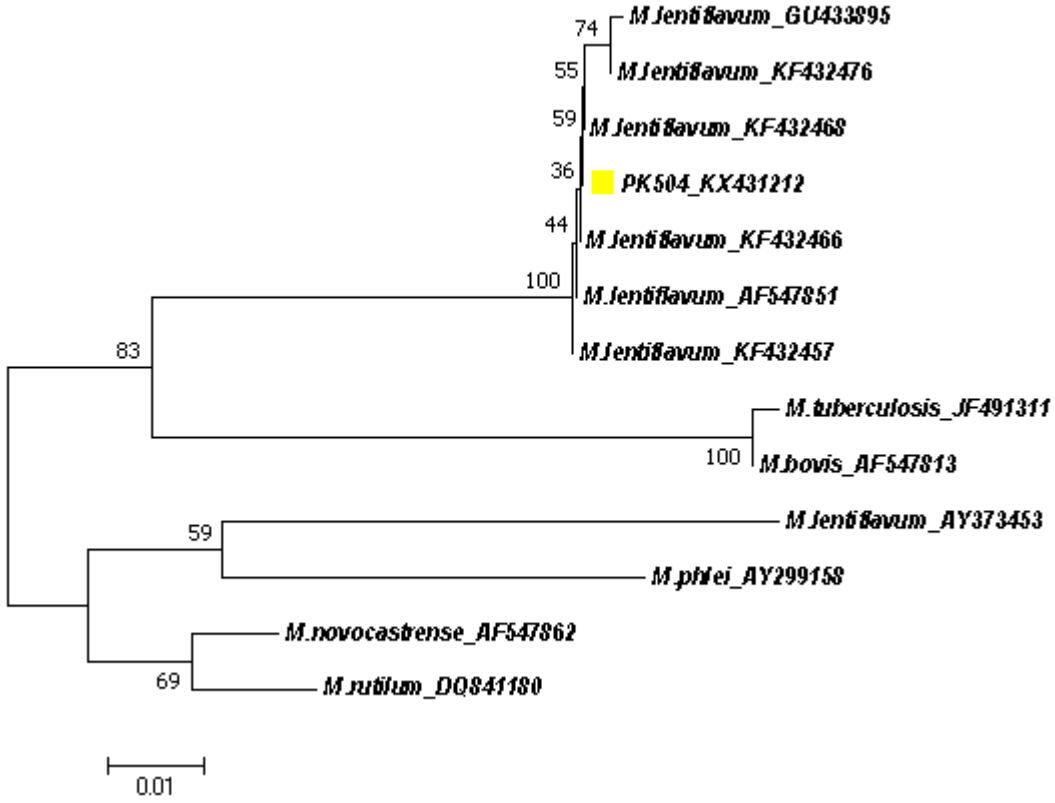


Fig 56. *hsp65* based phylogenetic tree for PK504. The evolutionary history was inferred by using the Neibour joining clustering approach employing *hsp65* gene sequences.

6.10.2b Case study 2- Detailed presentation of 2837 case as unique case and novel isolate (Manuscript under preparation)

Sputum sample from a 69 year old male having long term cough was collected from RNTCP centre of Dimapur district, Nagaland for routine research on nontuberculous mycobacteria. Following the ethical guidelines sample was transported to laboratory under refrigerated condition and was processed within 24 hours of collection under strict biosafety measures. It showed growth on LJ media with typical yellow mycobacterial colonies after three weeks of incubation. The sample was processed for acid fast staining and isolation studies on solid culture media for isolation and identification of the organism. Molecular identification of this isolate was done by amplification and sequencing of *hsp65*, *rpoB* and 16sRNA. PCR product of *hsp65* was then digested with *Hae*III and *Bst*II restriction enzyme and the band pattern was noted and analyzed for species identification. RE digestion of *hsp65* PCR product showed a band pattern of- *Bst*II 441 (no digestion) and *Hae*III- 140/60/55 which was corresponding to *M. novocastrense* type 1 (Lima et al., 2013) which is also a RGM. BLAST result of *hsp65* gene obtained from NCBI showed its nearest match to *M. novocastrense* but *rpoB* best match of 91% to *M. rhodesie* and 16sRNA matches 98% to a group of *Mycobacterium* species of *M. brasillensis*, *M. monokense*, *M.*

holsaticum, M. barrassiae. With ezbiocloud it came to be *M. holsaticum* (98.92%). On finding non-concordant result in BLAST search, final confirmation for its unique stand was done with tree based on concatenation of *hsp65*, *rpoB* and 16sRNA of the isolate with the reference strains. Finally, phylogenetic analysis using concatenated genes for *hsp65, rpoB* and *16sRNA* proved the isolate to be a novel one closely related to clinically significant species of *Mycobacterium* viz., *M. holsaticum, M. moriokense, M. rutilum* and *M. barrassiae* (Fig 57). On trace backing this NTM cases from the RNTCP unit, it was found the person have already died after receiving first line anti-tuberculosis drugs and were initiated for second line of drug treatment suspecting for drug resistant tuberculosis.

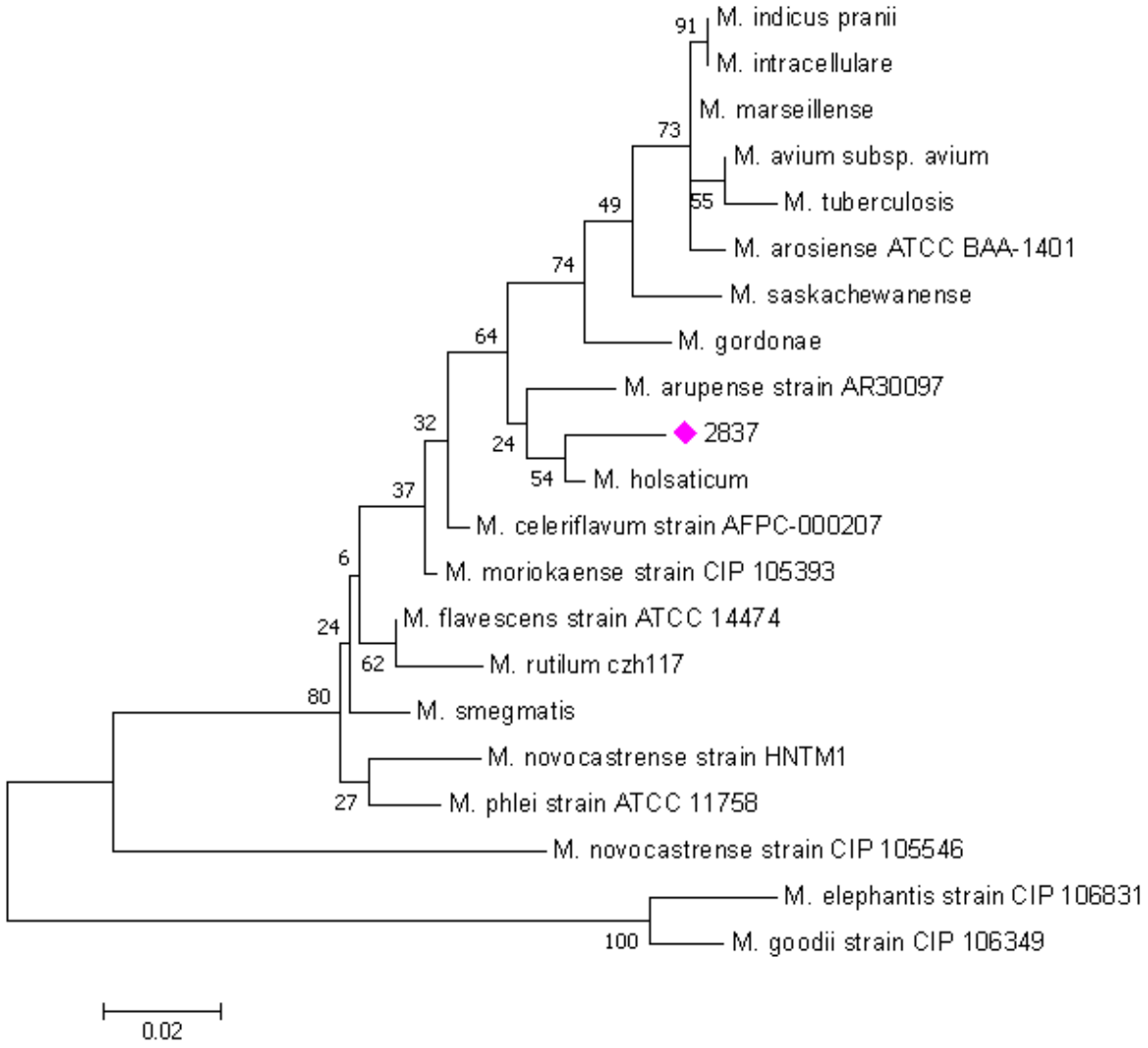


Fig 57. Evolutionary relationships of 2837 presented by concatenated *hsp65, rpoB, 16S* rRNA genes. Phylogenetic tree for the concatenated sequences of 16sRNA, *rpoB, hsp65* for isolate number 2837 with other nearest reference strains of NTM.

6.10.2c Case 3: Detailed case presentation of *M. novocastrense* (isolate 2824) (Manuscript under preparation)

We documented the isolation of a *M. novocastrense* from a probable TB case. The isolate was confirmed to be *M. novocastrense* on the basis of BLAST and phylogenetic analysis of its sequenced *hsp65* gene. This is the first isolation report of *M. novocastrense* from human clinical case in India. Presence of this rare pathogen from human case indicates the graveness of the issue in India where TB is highly endemic. During routine research on mycobacteria we came across a human case suspected from tuberculosis. The patient had chronic cough and was diagnosed to have pulmonary tuberculosis though primary health centre, he had completed the category I drugs for treatment of tuberculosis and was put in the relapsed case category. He was than suspected for MDR and started on category II drugs. During this time we collected the sputum sample of the case following the ethical guidelines and got it transported to our laboratory under refrigerated condition and was processed within 12 hours of collection under strict biosafety measures. Molecular identification was initiated with PCR for *Mycobacterium* genus specific *hsp65* gene. The species-specific PCR for *M. tuberculosis* and *M. bovis* were also tested viz., RD9, RD4 and 500bp primers. PRA was performed on the purified product of *hsp65* amplicon using the enzymes *Bst*EII and *Hae*III to identify the species. *BstE*II did not digest the product and *Hae*III digested it as 140/50/40 bp products. The band patterns directed the isolate to be for two possible NTM species namely- *M. novocastrense* and *M. flavescens*. *Mycobacterium* genus-specific *hsp65* gene PCR amplified a band of 441 bp confirming it to be of *Mycobacterium* genus. The species-specific PCR for *M. tuberculosis* and *M. bovis* with RD9, RD4 and 500bp primers turned out to be negative for MTBC suggesting the isolate to be NTM. Blast analysis in NCBI for identification depicted 100% match to *M. novocastrense*. Phylogenetic tree also depicted the species to be *M. novocastrense* with our isolate forming a clad with Chinese isolates of *M. novocastrense*. Unfortunately, the patient's additional information could not be taken and was not initiated for NTM treatment succumbed while taking the category II tuberculosis drugs.

The evolutionary tree (Fig 58) was constructed using the ML approach based on *hsp65* gene and the isolate was placed among the other three global *M. novocastrense* isolates and closely with an unspecified *Mycobacterium* isolate from UK. The isolate showed slight phyletic line variation from the other *M. novocastrense* isolates as observed in the branch lengths and position. Although several reports of the species isolation have been documented and reported from varied sources (Shojaei et al., 2011; Kyselková et al., 2012; Escobar-Escamilla et al., 2014; Khosravi et al., 2016; Varghese et al., 2017), only three isolates of clinical origin from Iran (HM807382) and Mexico (JX154123), including the type strain (AF547862) could be used in the study due to non-availability of the targeted gene sequence in NCBI database.

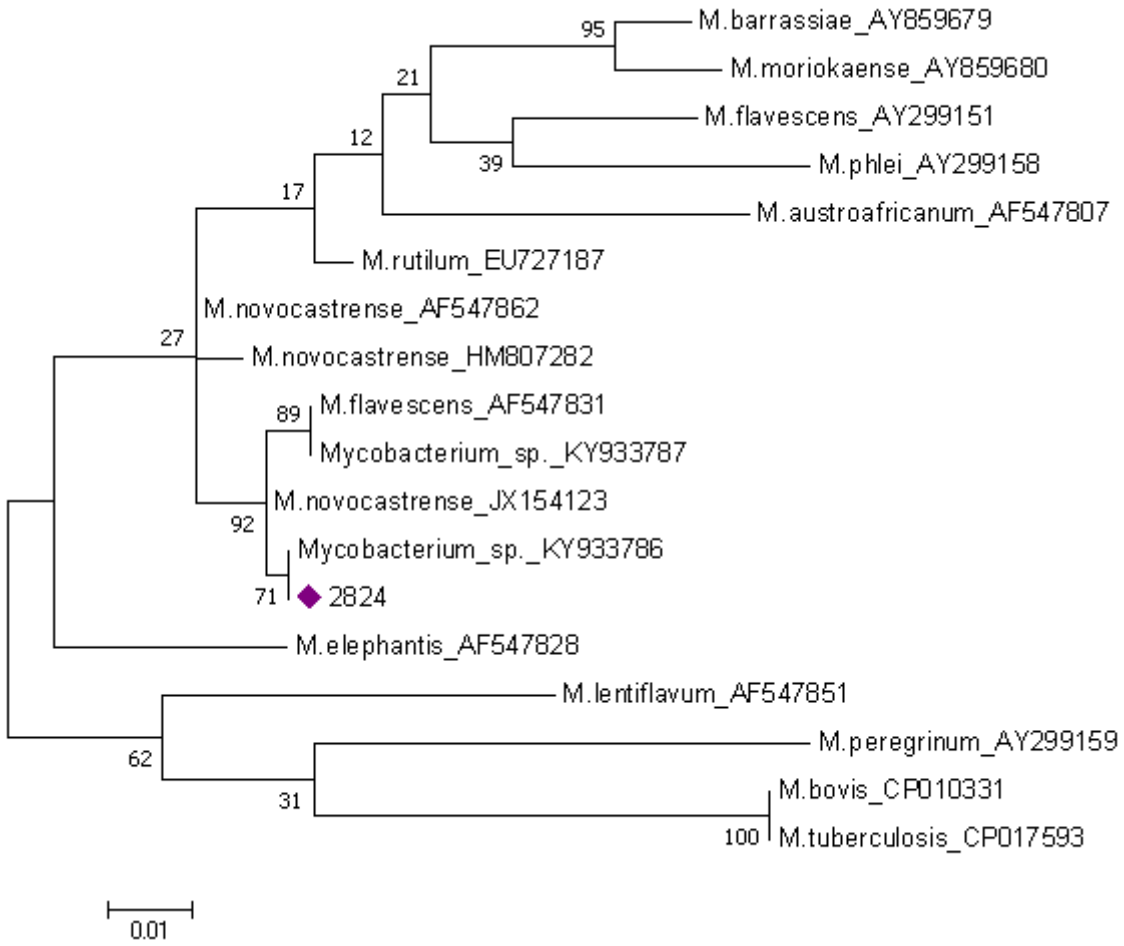


Fig 58. A *hsp65*-gene based tree constructed for isolate 2824 through ML approach in MEGA.

6.10.2d Case study 4- *M. abscessus* cases from Meghalaya (Manuscript under preparation)

Two cases (Mgh FU239, Mgh FU362) being treated for tuberculosis yielded NTM during follow up of the treatment course which were both identified as *M. abscessus*. Isolate Mgh FU239 was obtained from a 19 year old male patient during the 15th month of anti-tuberculosis treatment. The lab smear of sputum was negative and on liquid culture isolation in MGIT turned positive with 1+ grade.

Isolate Mgh FU362 was obtained from a 50 year old female patient during the 6th follow up month of anti-tuberculosis treatment. The sputum smear was negative but yielded NTM on liquid culture. This species of the isolate was initially identified by MALDI. The same could not be performed for isolate Mgh FU239 due to turbidity of the liquid culture.

These isolates were shared for further molecular characterization to our laboratory. Both the culture showed a positive band of 441bp for *hsp65* PCR confirming it for genus *Mycobacterium*. Further applying *IS6110* PCR showed no bands indicating to be an NTM. The initial speciation was done by *hsp65*-PRA which showed the reported pattern of *M. lentiflavum*. These sequences were further confirmed by sequencing of *hsp65* and *rpoB* genes. The BLAST analysis for *hsp65* sequence yielded *M. abscessus* with 99% for both the isolates. *rpoB* also gave the best matches of 99% with *M. abscessus* for both the isolates. The phylogentic tree was constructed for the isolates which well fitted with the ATCC and global clinical isolates of *M. abscessus* gene sequence. Sequences of the two *M. abscessus isolates* from tuberculosis patients were primarily analysed in Bioedit 7.0.4.1 software (Hall, 1999) and aligned with the database sequences of type strains. Phylogeny analyses were conducted to build an ML tree with 1000 bootstrap taking together these two isolates along with other global sequences, to retrieve the most related species (Fig 59). The database sequences from BLAST were downloaded for construction of the ML tree for both these isolates. Tree shows a large cluster of *M. abscessus* and two smaller clusters of *M. fukienense* and *M. massiliense* were added for comparison. In the larger *M. abscessus* cluster, our isolates FU362 and FU239 (marked in green squares) and two clinical isolates from China (KF432719, KF432768) can be seen extending into a separate phyletic line showing its distinctiveness. The large cluster contains clinical isolates from Spain (KT185518, KT185530, KT185531, KT185533) and China (KF432536, KF432624, KF432602, KF432764) and sputum isolates from USA (CP009613, CP009408, CP016192, CP016193). A standard strain is also in the cluster (AY498743). The tree topology clearly re-affirmed the species of these two isolates which were clustered under the *M. abscessus* clad, of which majority of the database sequences are from clinical isolates of China. However it is visible from the tree presentation that within this cluster, the isolates are situated on a highly variable branch distance from the rest of the database sequences of clinical origin and type strains depicting again the diversity of the strains isolated from this region of the country. Even though MALDI-TOF has some limitation for *Mycobacterium* detection such as requirement of a lengthier clump and cell disruption protocol and limited spectral database, it could be successfully employed for rapid confirmation and differention of NTM and MTBC.

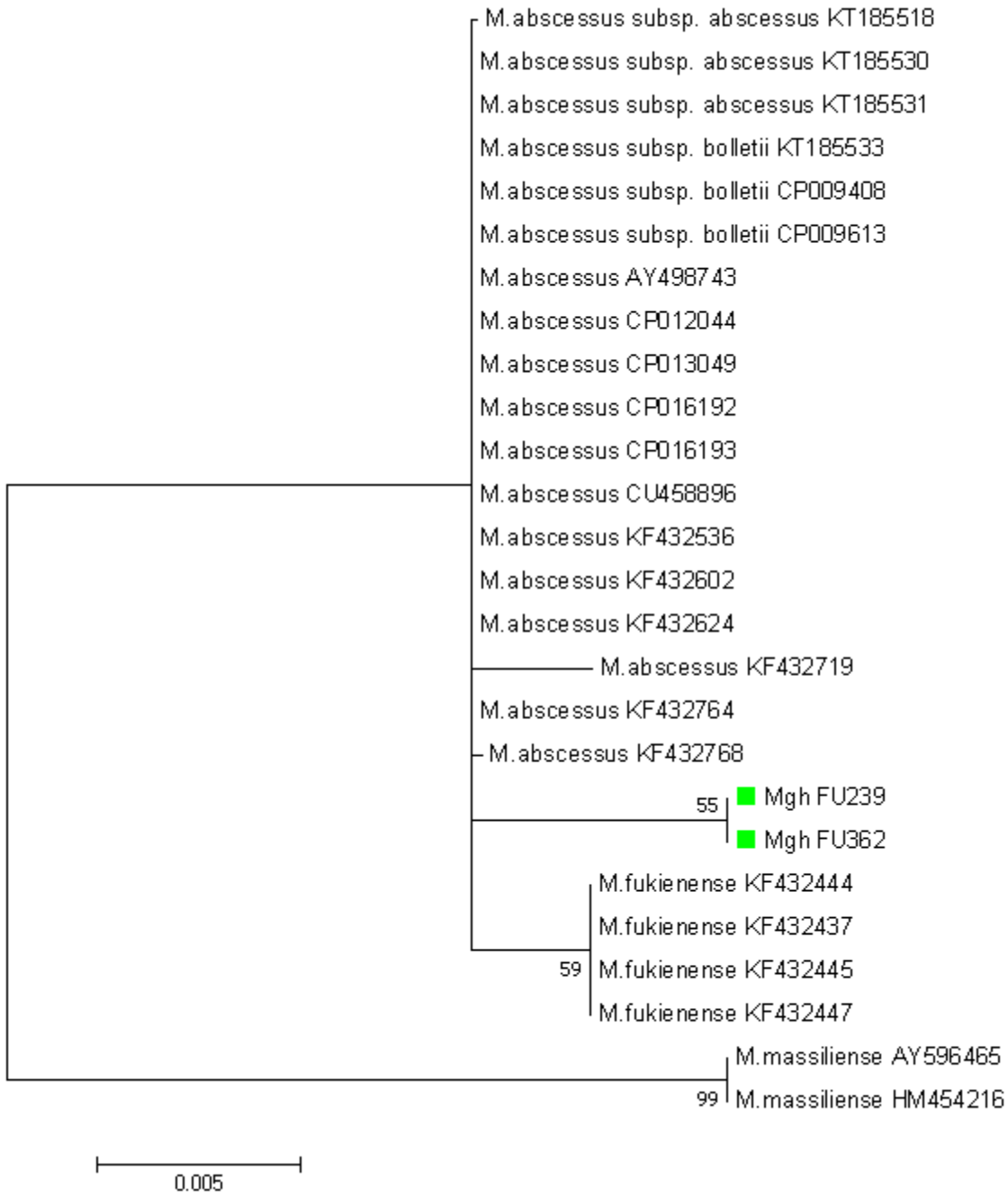


Fig 59. *hsp65* based ML tree for *M. abscessus* isolates (FU362 and FU239).

6.10.3 Representation of environmental isolates in dendrograms based on *hsp65* and *rpoB* genes

From the environmental source 8 (3.74%) NTM isolates were obtained out of a total 214 environmental samples processed (Table 13). The isolates were speciated by *hsp65*-PRA, followed by *hsp65* and *rpoB* sequencing. 11 isolates were *hsp65* PCR positive, and sequencing confirmed only 08 (3.74%) isolates to be mycobacteria, all of which were NTM. Species include *M. chelonae*, *M. immunogenum*, *M. pyrenivorans*, *M. gilvum*, *M. tuberculosis* and *M. fortuitum.* Mycobacteria being ubiquitous in the environment, our isolation rate of (3.74%) is comparatively lower than usually reported. It may have been that the standardized protocol was inefficient for decontaminating the processed samples probably due to the varied microbial flora of the environment (soil and water). The same decontamination protocol with 0.005% CPC was used efficiently in our earlier studies but did not reflect well in this work. Better standardization for environmental isolation of mycobacteria depending on the region and type of soil would be required as isolation from these sources could be challenging (Kyselková et al., 2012).

In the *hsp65* based tree constructed for the environmental isolates (Fig 60), distinct cluster of *M. chelonae* can be visualized under which the water (W11,W12) and soil (AH4,S7) isolates from the study fall under. Both the soil samples were obtained from a cultivated land. The water samples were collected from ponds in an integrated farming system where fishes and ducks were also reared. The database sequences of *M. chelonae* falling under this cluster include standard isolates (CP007220), clinical samples from Greece (GU222633) and China (KF432759). Some of our *M. chelonae* isolates from milk which had been submitted earlier (KX232652, KX231738, KX231739) also clustered with our environment isolates. It is also notable that isolate S7 from soil showed BLAST similarity to a large number of our milk isolates which has been submitted to the NCBI GenBank. Another large number of hits include the rapidly growing mycobacteria known to colonize metalworking fluids. Author Khan et al. (2005) notes that a significant intraspecies variation exist among the isolates of *M. immunogenum* and *M. chelonae* with considerable strain-level genetic diversity which is observed even in our study. *M. fortuitum* (W34) isolation obtained from water tank in a cowshed also assigned itself to the *M. fortuitum* cluster. It formed a firm cluster with other *M. fortuitum* isolates including ornamental fishes from Poland (KX244858, KX231771, KX231769), sputum sample from Greece (GU433894) and another clinical sample from Mexico (JX154098). Soil isolate S12 (blue inverted triangle) showed the closest similarity with a recently described species *M. saopaulense* (Nogueira et al., 2015) in BLAST, isolated from zebra fish but with a relatively lower cut-off (96%). In the tree, isolate S12 forms a distinct phyletic line separated afar from other database sequences but adjacent to *M. saopaulense* (KM973025) of corneal source from Brazil, and other unidentified species of mycobacteria. S9 (purple circle) another soil isolate from a cultivated vegetable farm identified itself closely to standard strains of *M. immunogenum* (AY458081, CP0161898) and *M. franklinii* (KM392059), and a clinical isolate of *M. franklinii* from US (HQ153092). The isolate RF from an animal farm formed loose cluster with *M. pyrenivorans, M. chubuense, M. chlorophenolicum, M. rutilum* and *M. novocastrense*. The BLAST homology showed closest similarity with the standard strain of *M. pyrenivorans* and the tree also placed the two isolates in the same subtree.

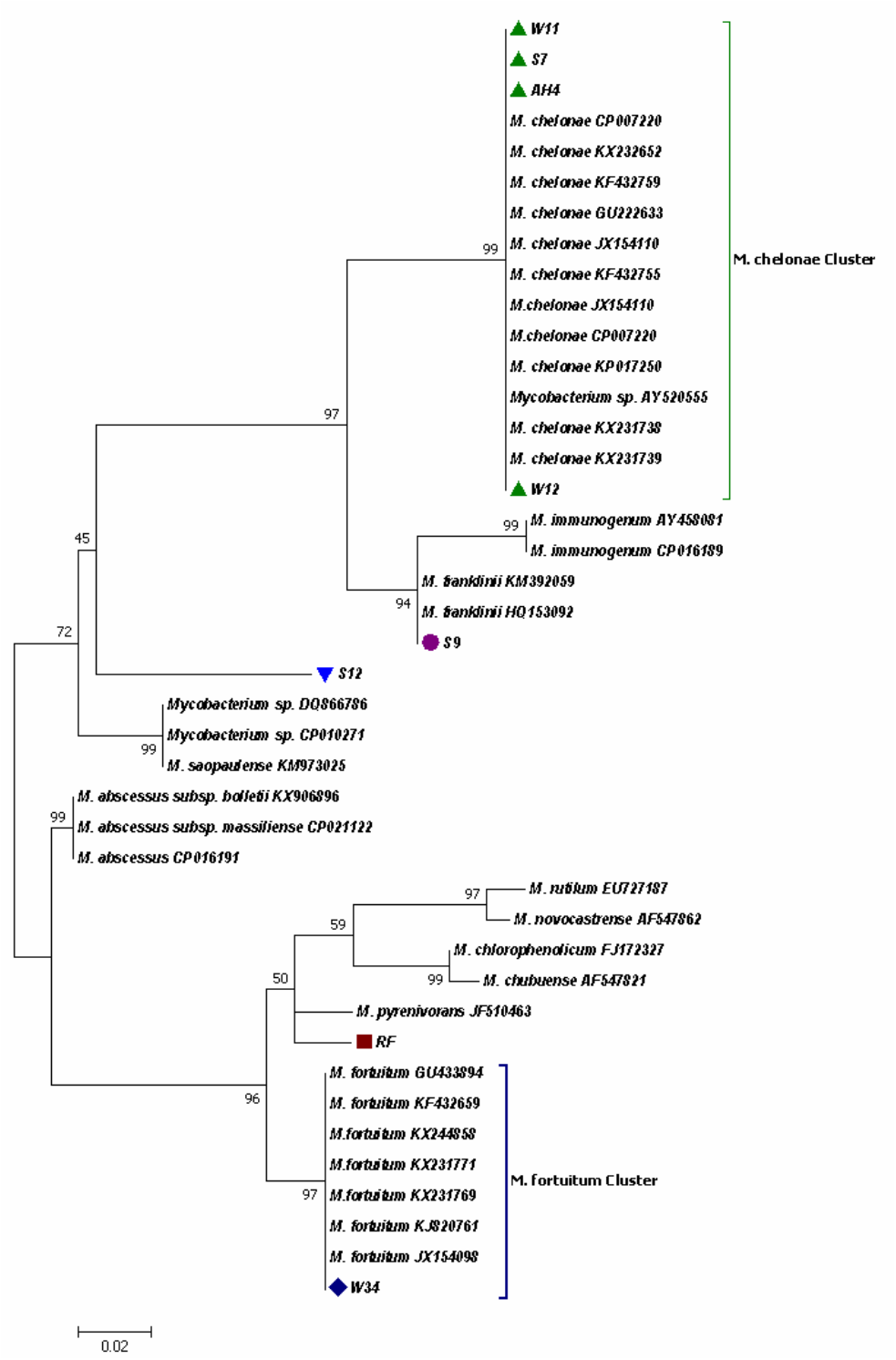


Fig 60. *hsp65* based phylogenetic tree of environmental isolates. The phylogenetic tree of the clinical isolates with reference and clinical strains was inferred using the Maximum Likelihood method.

An *rpoB* based ML tree was built using sequences amplified through the primer described by Lee et al., (2003). Except for two isolates (AH4 and W11), all the other isolates showed sequence homology different from the *hsp65* BLAST homology. The species could not be ascertained through the gene probably due to the small region targeted in the highly polymorphic region. However, an *rpoB*-based tree was constructed for presenting all these isolates as some of them were identified upto atleast the complex level or the homology was observed with the closest similar isolate (Fig 61). *M. chelonae* isolates AH4 and W11 were correctly identified as *M. chelonae* and formed a cluster with other global isolates involving standard strains of (AY147163, AY262740) and aquatic sources from UK (AM88587). Isolate S12 showed closest similarity with *M. abscessus* and in the tree fell adjacent to the same species. Soil samples S7 and S9 collected from cultivated area showed the closest similarity with *M. immunogenum* species in BLAST and the tree depicted similarly. *M. chelonae, M. immunogenum* and *M. abscessus* are all rapid growers and are known to be closely related (Wilson et al., 2001). It may be due to the smaller target size in our study that these closely related group, that they could not be identified up to the species level. Isolate RF formed a very distinct cluster with *M. vaccae, M. vanbaalenii* and *M. chubuense*. The tree indicates the isolate RF for be distantly related to these species. This *rpoB* results of this species also could be correlated with the BLAST homology of *hsp65* where the the best hit was *M. pyrenivorans*, known to be a 16s rRNA subgroup of *M. vaccae* and *M. vanbaalenii* (Derz et al., 2004). Clustering of similar species through phylogenetic analysis is well depicted in *hsp65*-based tree. But intraspecies differentiation has been better observed in *rpoB*-based trees when constructed with Adekambi et al. (2003) but was again limited when primers by Lee et al. (2003) for smaller *rpoB* gene target was used. Although this may sometimes lead to confusion, identification of unique or novel species is well distinguished by this gene due to its higher discriminatory and polymorphic nature.

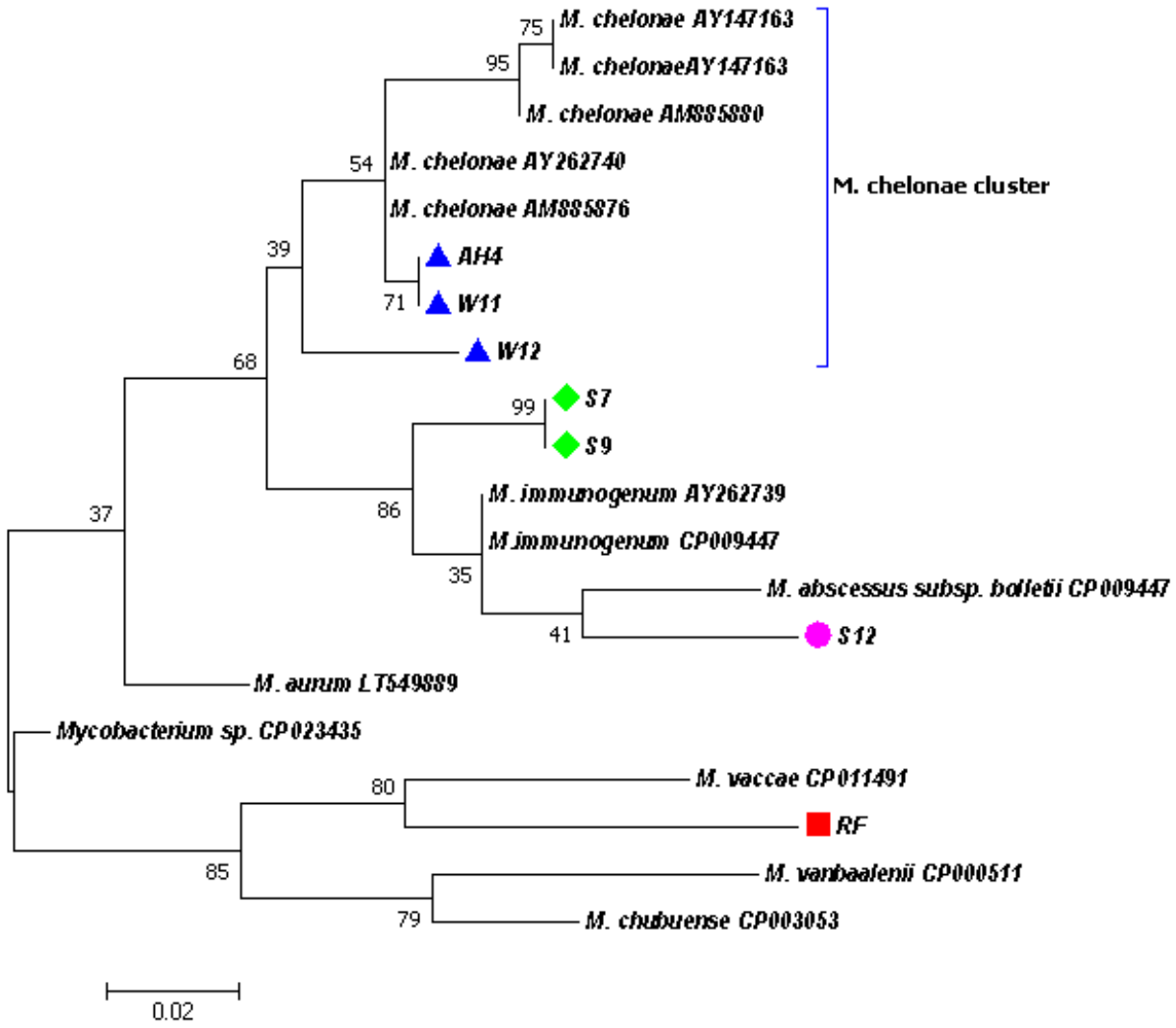


Fig 61. *rpoB* (Lee et al., 2003) based phylogenetic tree of environmental isolates. The phylogenetic tree of the clinical isolates with reference and clinical strains was inferred using the Maximum Likelihood method with 1000 bootstraps.

6.10.4 Comparison trees constructed for isolates from multiple sources

In this exercise, sequence similarity within clusters and diversity between them were analyzed with the aim to explore the diversity of our isolates and attempt to find any correlation from the three sources so as to relate the distribution of these species circulating between the human-animal-environment interface. Evolutionary relationships of these isolates have been studied by analysis of large database sequences of any source isolated and reported globally.

We were able to isolate one *M. lentiflavum* from milk and had one human case also from this region. This shows that the species is present ubiquitously in environment in this region and could be entering animal system. As human infections by this species are noted they can be considered rare but clinically significant. There are 184 species and isolation and

correlation of even one isolate or species is significant as it represents the underlying possibility of trasmission. Similarly *M. chelonae* isolation from both milk and environment could indicate an unexplored cause and the capacity of this species to infect human from this route. *M. tuberculosis* isolation from both animal and human, shows the chance of the entity of shedding into the environment mainly through interaction by infected individuals.

6.10.4a *M. tuberculosis*: Clinical and Animal isolates

An *hsp65*-gene based NJ tree was constructed for all the 24 isolates of *M. tuberculosis* isolates comprising of 23 isolates of human clinical origin and 01 from milk. The dendrogram shows all the isolates closely similar with each other and the global isolates (Fig 62). The isolates of clinical source and one isolate of animal source from milk (M116) also depicted close similarity. All these isolates and the database sequences formed a large clad tightly, indicating close likeness irrespective of the geographical variation of the isolates.

Clinical isolates of *M. tuberculosis* from the study are all isolates from sputum from suspected tuberculosis patients. All the 23 human clinical isolates were isolations from Dimapur district, Nagaland, and one isolate 'SERO', was from a TB suspected cancer patient in Khasi Hills. The 23 smaples from Nagaland were collected from an RNTCP center on monthly basis during the sample collection period. The source of the single *M. tuberculosis* isolate from milk (M116G) was also from a dairy farm in Dimapur district, Nagaland. Being majorly of clinical origin our *M. tuberculosis* isolates showed closest similarity with the sequences of other global clinical strains like sputum samples from USA (CP009205, CP009204, CP009175, CP009184, CP009186, CP009197, CP009199) and China (CP020381); sputum sample of MDR patients from Kazakhstan (CP016888, CP016794, CP012506); bronchial fluid, USA (CP009198, CP009191); clinical strains of Vietnam (AP018033, AP018034, AP018035, AP018036); *M. tuberculosis* Beijing-like strains (CP017598, CP017597, CP017596, CP017595, CP017594, CP017593) and standard strains (JF491311, AY299144). One database sequence CP011510 formed a completely separate phyletic line showing distinctly variable strain. The isolate is a *M. tuberculosis* Beijing strain.

Although *M. tuberculosis* have also been isolated from environmental sources (Velayati et al., 2015) including other members of MTBC which are known to survive in soil and water pipes (Steentoft et al., 2006; Ghodbane et al., 2014), the isolation of *M. tuberculosis* isolation from milk (M116) in this study is very likely to be of human origin. It may be noted that no *M. tuberculosis* isolates of environmental or animal origin could be found during the similarity search in BLAST. This could indicate even from the tree that the isolate from milk (M116) could be of human origin contaminating the milk due to unhygienic diary activities which has also been reported in earlier studies (Srivastava et al., 2008; Ameni et al., 2013). Isolation of MTBC from milk is also commonly reported globally and in India (Appuswamy et al., 1980; Kahla et al., 2011; Agada et al., 2014; Bezerra et al., 2015).

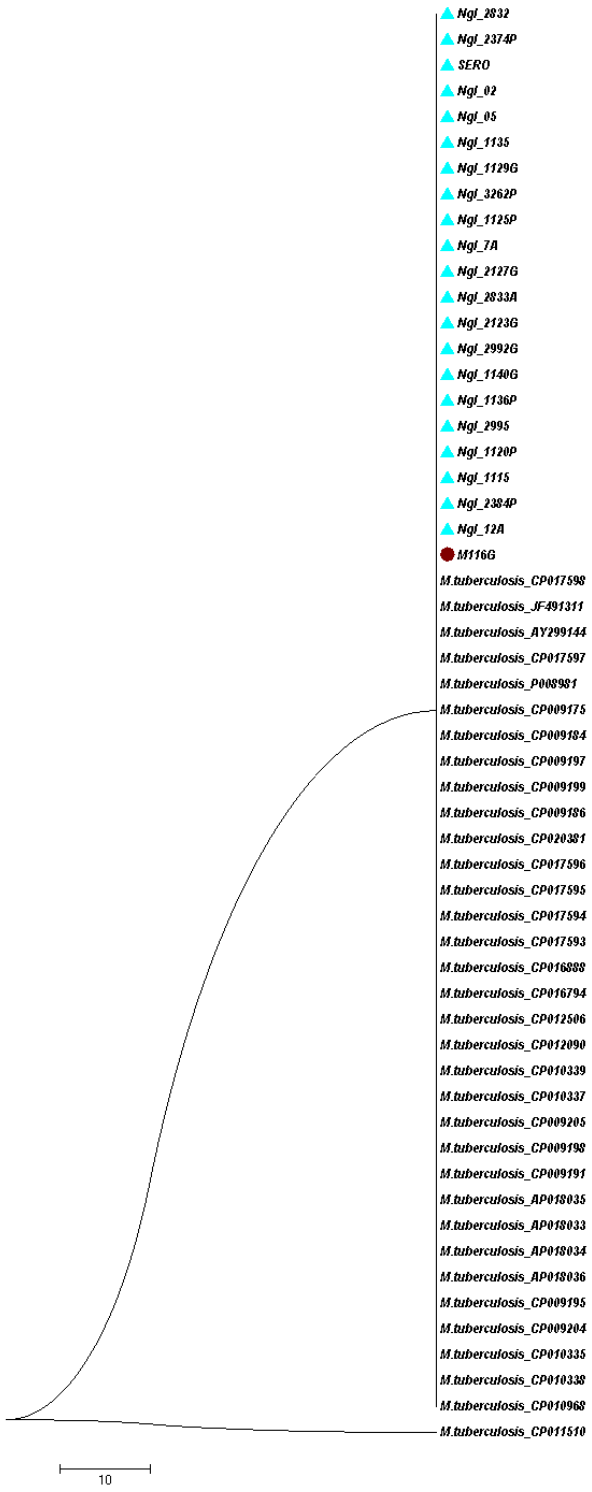


Fig 62. *hsp65*-based NJ tree for *M. tuberculosis*_Clinical and Animal. The evolutionary history was inferred using the Neighbor-Joining method. Percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches

6.10.4b *M. lentiflavum*: Clinical and Animal isolates

There were a total of 04 *M. lentiflavum* isolates from this study, three from milk (1AG, M8, M12) and 01 (PK504) from human. In the *hsp65*-based NJ tree (Fig 63), all these isolates was grouped under one large cluster with minor divergences. Using the 'separate tree view' function in MEGA7, this cluster was zoomed (Fig 64) at the node which has been circled in red to distinctly view each of the phyletic lines.

The tree clearly differentiates the *M. lentiflavum* isolates from milk under a separate cluster while the clinical isolate also remains separated from this tight cluster. *M. lentiflavum* are known to be opportunistic pathogens and are reported from human clinical cases (Jimenez-Montero et al., 2014). Isolation of *M. lentiflavum* from milk is also rare and series of factors could be associated like environmental contamination, unhygienic diary activities or the climatic conditions favoring these species with ambient conditions to flourish in optimum temperature, humidity or less sunshine. However, there was no similarity match of these milk isolates with other milk *M. lentiflavum* isolations or other sources, apart from clinical isolates. All 03 milk isolations have been from separate farms under Khasi Hills, Meghalaya from different locations. The milk isolate showed close sequence similarity with clinical isolates of Turkey (AY379075), Japan (AB362384) and Greece (GU433895). It may be noted that *M. lentiflavum* (AF547851) was a common similarity match in all the BLAST hits of the 03 milk isolates. This isolate shows its origin from China, which was isolated from a sturgeon liver.

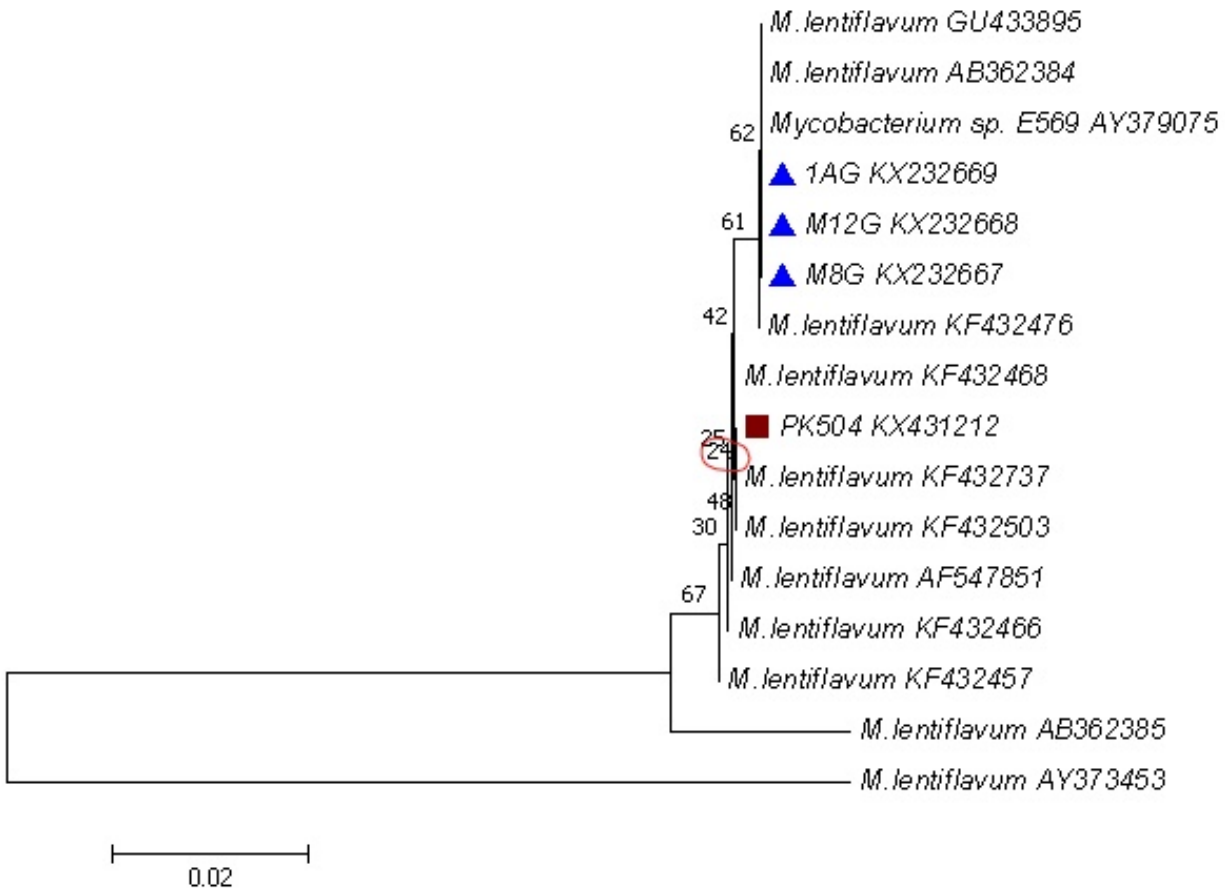


Fig 63. *hsp65* based NJ tree for *M. lentiflavum*_Clinical and Animal isolates. The evolutionary history was inferred using the Neighbor-Joining method. Percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches

Clinical isolate PK504 is also from the Khasi Hills, Meghalaya. The sample was collected from a 14 year old boy suffering from lymphadenitis who had already taken 6 months course of anti-tuberculosis drug regime. Through culture and various molecular diagnostic approaches, the species could finally be identified. On comparison with other global isolates and other *M. lentiflavum* isolates from this study, the isolate showed very close similarity to the Chinese strains (KF432468, KF432737, KF432503) falling under the same phyletic line and subtree. However, the clinical and milk isolates of *M. lentiflavum* did not show much similarity forming separate subtrees depicting variation from the same region.

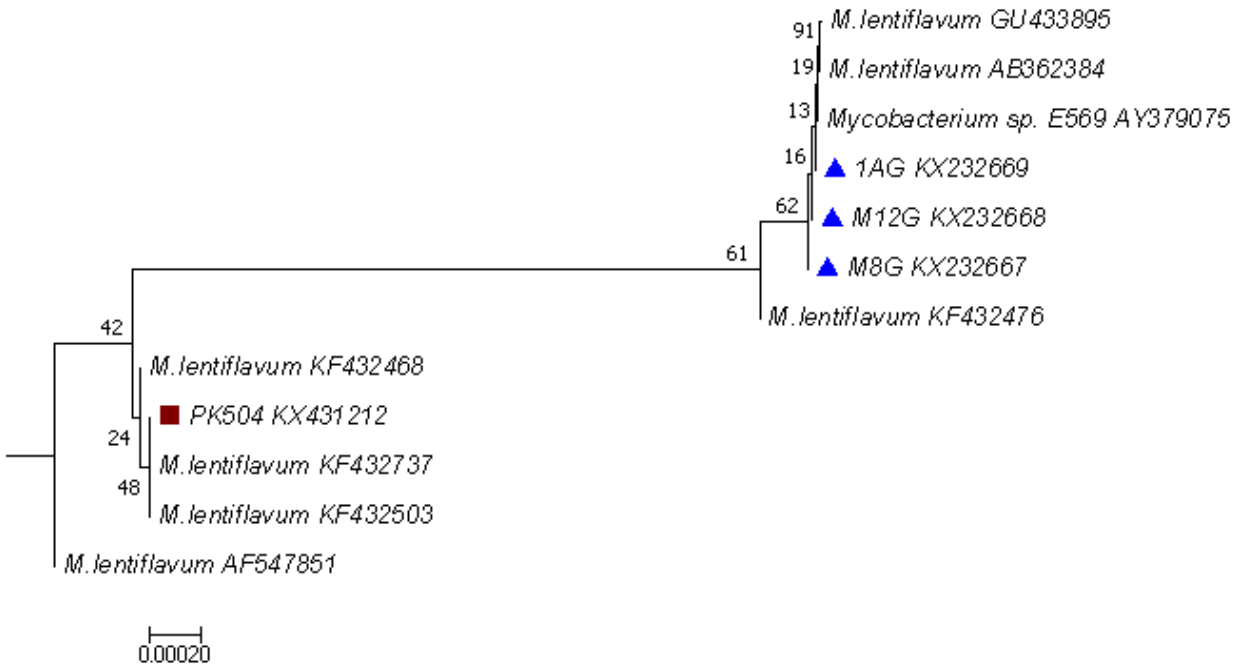


Fig 64. A zoomed sub-tree view of a node. A clear distinction of the isolates can be viewed under two separate clusters.

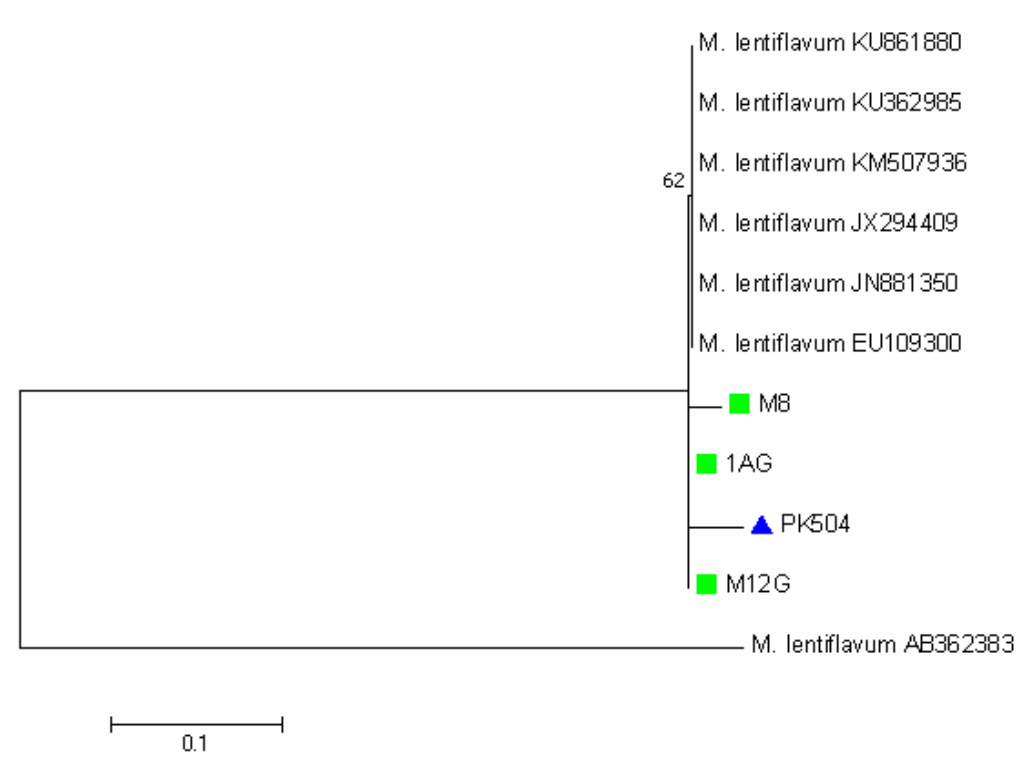


Fig 65. *rpoB*-based (Adekambi et al., 2003) dendrogram representing *M. lentiflavum* from Clinical and Animal origin.

An *rpoB*-based tree was also constructed for all *M. lentiflavum* isolates from the study (Fig 65). Only a limited number of *rpoB* sequences were available in the NCBI database. In total, only seven sequences were available of which one is from tap water source from Iran (KU861880), one sputum sample from TB referenc laboratory from Iran (KU362985) and two other unspecified clinical isolate from Iran (JX294409, KM507936). Two were standard reference sequences (JN881350, AY544939) and a short sequence of 306 bp is a direct submission from Japan AB362383. In the tree, this sequence separates itself from the rest of the global isolates which could be due to the short sequence input for analysis. All our four isolates (marked in triangle and square) fell closely with a large cluster of other global isolates but distinctly identified itself into a separate phyletic line. Even in this new phyletic line, the clinical *M. lentiflavum* isolate (PK504) marked in triangle forms another distinct line separating from the three milk isolates (Marked in squares). Though the tree reaffirms species identification, this *rpoB*-based dendrogram indicates our isolates to be genetically divergent from the reported global isolates.

6.10.4c *M. chelonae*: Animal and Environmental isolates

Twenty one *M. chelonae* isolates were obtained from milk and two each from soil (AH4, S7) and water (W11, W12). All these *M. chelonae* isolates from different sources were compared for their relatedness by building an evolutionary distance dendrogram which was inferred using the Neighbor-Joining (NJ) method. The tree obtained helped in clustering of similar species and also differentiation of closely related species within the available database set that was created by assembling various *M. chelonae* global isolates from NCBI. The tree (Fig 66) presented distinct clustering of milk isolates and environmental isolates. Three *M. chelonae* clusters were obtained where one was a major clade where all 20 milk isolates were grouped along with the global isolates. The global database sequences in this cluster included clinical sources from USA (JN400397) and Mexico (JX154110); drinking water isolates from France (KC669494); Animal isolates like calf tissue from Brazil (KT779819); isolation from fishes from USA (DQ866784) and China (KC684916, KC684918); reference strains (AY299148, AY458082); and majority of the milk isolates were similar to *M. chelonae* isolation from ornamental fish, Poland (KX231727, KX231730, KX231732, KX231733, KX231736, KX231738, KX231739, KX231740). Out of the three clusters obtained, two smaller separate clusters were formed where none of the isolates from the study indicated closeness. These two clusters contained water isolates from both Brazil and France, and isolates from fishes, USA (DQ866784). Of the four environmental isolates, only one of the environmental isolate (S7) from soil formed closest clad with the milk isolates by grouping under this major clad of *M. chelonae*. The rest of the three environmental isolates, one from soil (AH4) and two from water (W11,W12) separated into a very distinct sub-tree under this major clad clearly indicating their deviation from the milk isolates. This sub tree majorily included isolates from reference strains (CP010946,CP007220, KP017250, JF491293 AY458074, AF547818).

The source of isolate S7 (blue triangle) which fell under the same clad of all milk isolate, was from the higher altitude potato cultivated farms under Khasi Hills in Meghalaya. All the 20 *M. chelonae* milk isolates were from the farms located under the same geographical area taken for study. The milk samples are classified as animal source in this study. Presence of *M. chelonae* in this source usually indicates environmental contamination and

less likely through human contamination. Taking inference from this tree (Fig 66), similarity of the soil isolate sequence with the isolate sequences from animal source could be indicative of this species thriving and circulating in the environment of this region and finally gaining access to milk. Significant isolation from both environmental and animal source confirms the presence of environmental mycobacteria and the risk of food contamination. Nevertheless, presence of *M. chelonae* in soil could also be from the animal source, given the farming practice of fertilizing cultivated land using animal manures like cow, goat or pig dung and chicken litters depending upon the availability.

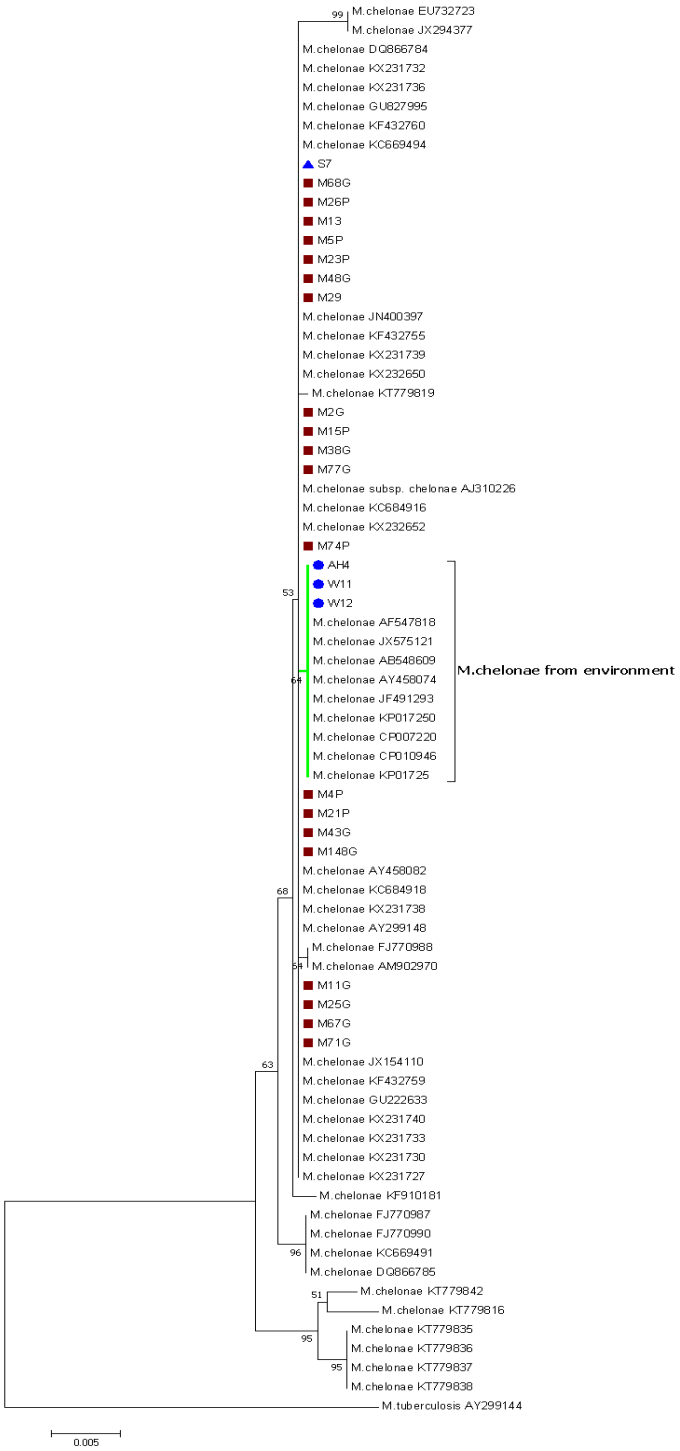


Fig 66. *hsp65* based NJ tree *M. chelonae*_Animal and environmental. Phylogenetic tree based on the nucleotide sequences was constructed by the neighbor-joining method using Kimura-2 parameter. Bootstrap values are indicated at the nodes as a percentage of 1,000 replications, if they were higher than 50%.

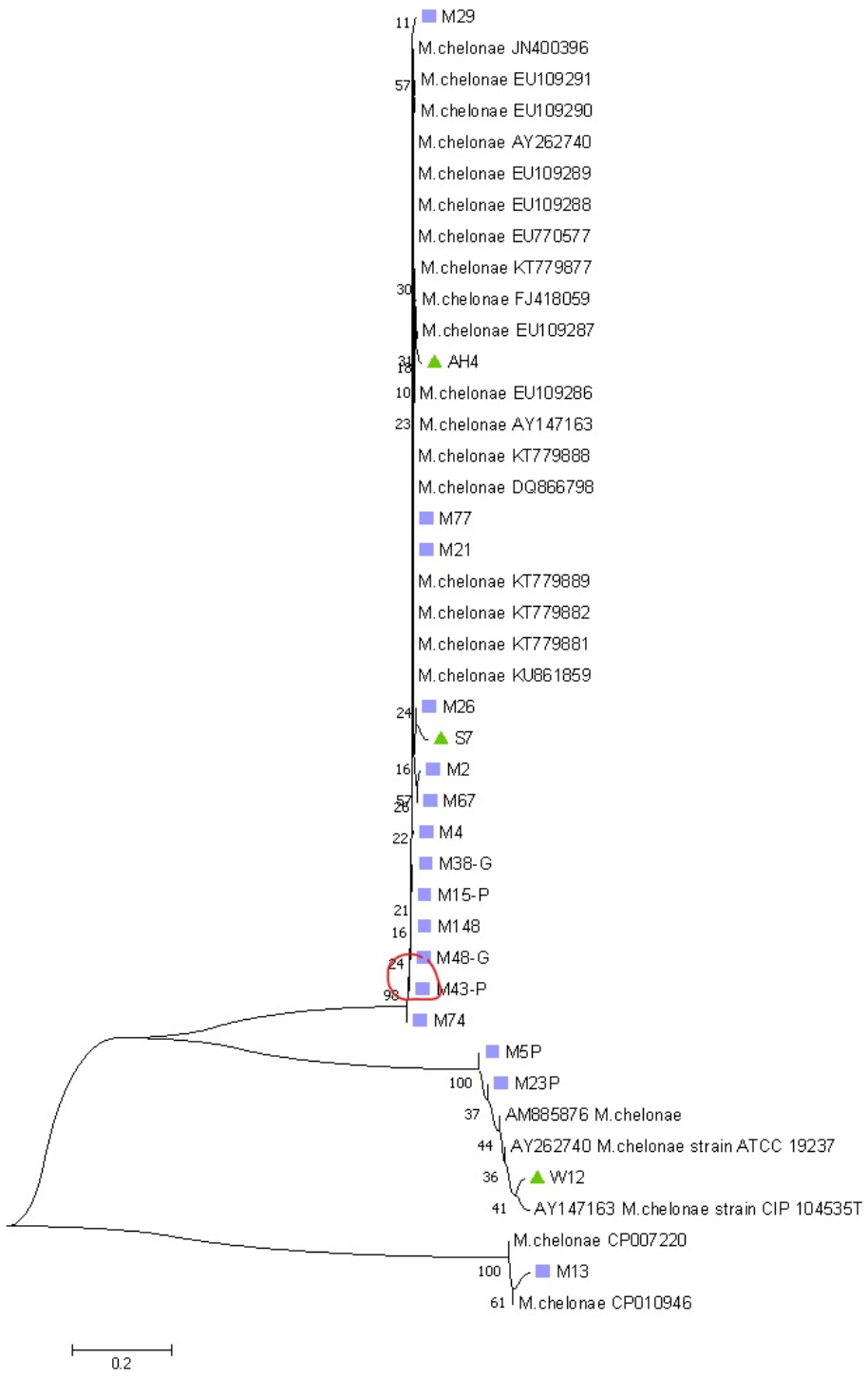


Fig 67. *rpoB* based (Adekambi et al., 2003) NJ tree *M. chelonae*_Animal and Environmental isolates. The evolutionary history was inferred using the Neighbor-Joining method. Percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches.

In the NJ tree based on *rpoB* (Adekambi et al., 2003), the tree output was relatable with the *hsp65* based but with higher discrimination among the isolates which is due to the higher

discriminatory nature of the *rpoB* gene which was used (Fig 67). Three major clusters are visible in this tree where the two smaller clusters comprised mainly of the reference strains of *M. chelonae* (AY262740, AY147163, CP007220, CP010946) under which three milk isolates (M5, M13, M23) and one water isolate (W12) loosely grouped under a sub-tree. The water source and milk collection sites fall under the same region of the Khasi Hills, Meghalaya at distantly located sites.

The larger clad of *M. chelonae* was observed where a large majority of the milk isolates with a total of 12 sequences and 02 soil isolates were grouped together. However it is distinct in this clad that several phyletic lines have merged into this clad. The tree below represents a zoomed view of this subtree by using the function, "Show Subtree Separately", available in the MEGA7 software where the tree was build. The node which have been circled in red have been zoomed to present the tree below (Fig 68). In this expansion of the subtree the intra-species heterogeneity among the isolates can be appreciated. Most of the milk isolates although has fallen under this *M. chelonae* clad, they are predicted to be distinct strains from the other global isolates given their varying branch lengths and positions. Milk isolate M29 (blue triangle) came closest to a clinical isolate from USA (JN400396) while soil isolate AH4 from a potted soil was most similar to clinical strains of France (EU109287) and Brazil (KT779877). The other global isolates in this cluster include isolations from ornamental fish from USA (DQ866784), ATCC strains (EU109289), water (EU770577) and clinical (EU109288) isolates from France, clinical (KT779881, KT779882), animal (KT779877) and environmental (KU861859) isolates from Brazil. Again in this *rpoB* tree, soil isolate S7 from cultivated land, falls under the same cluster of milk isolates from the study (M2, M26, M67) supporting the supposedly environmental-animal cross contamination cycle as discussed earlier.

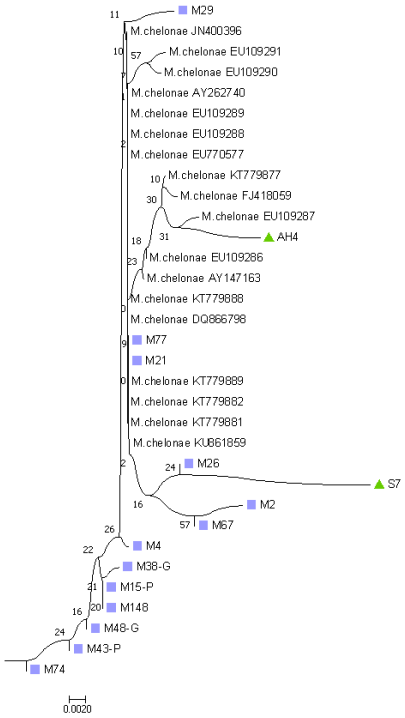


Fig 68. A zoomed view of the *M. chelonae* subtree for clear distinction.

5.11 SEQUENCE ANALYSIS AND NCBI GENBANK SEQUENCE SUBMISSION

A total of 119 good quality sequences have been processed and submitted to National Center for Biotechnology Information (NCBI), U.S. National Library of Medicine and is publically available which can be accessed in the NCBI GenBank. A total of 59 *hsp65* gene sequences, 46 *rpoB*, 13 *rrl*, and one *16s rRNA* genes have been submitted. The accession numbers along with the details of the isolates are given in three separate tables according to the sources Table 23-25 .

Sl. No.	**Sample/ strain ID**	**Species identified by *hsp65***	**Gene**	**Accession No.**	**Gene**	**Accession No.**
1.	M2G	*Mycobacte-rium chelonae*	*hsp65*	KX232650	*rpoB*	MG489979
2.	M4P	*M. chelonae*	*hsp65*	KX232665	*rpoB*	MG489980
3.	M5P	*M. chelonae*	*hsp65*	KX232664	*rpoB*	Not submitted
4.	M11G	*M. chelonae*	*hsp65*	KX232663	*rpoB*	Not submitted
5.	M13	*M. chelonae*	*hsp65*	KX232662	*rpoB*	MG489981
6.	M15P	*M. chelonae*	*hsp65*	KX232652	*rpoB*	MG489982
7.	M21P	*M. chelonae*	*hsp65*	KX232657	*rpoB*	MG489983
8.	M23P	*M. chelonae*	*hsp65*	KX232661	*rpoB*	MG489992
9.	M25G	*M. chelonae*	*hsp65*	KX232649	*rpoB*	Not submitted
10.	M26P	*M. chelonae*	*hsp65*	KX232658	*rpoB*	MG489984
11.	M29	*M. chelonae*	*hsp65*	MG334588	*rpoB*	MG489985
12.	M38G	*M. chelonae*	*hsp65*	KX232656	*rpoB*	MG489986
13.	M43G	*M. chelonae*	*hsp65*	KX232651	*rpoB*	MG489987
14.	M48G	*M. chelonae*	*hsp65*	KX232654	*rpoB*	MG489988
15.	M67G	*M. chelonae*	*hsp65*	KX232659	*rpoB*	MG489989
16.	M68G	*M. chelonae*	*hsp65*	KX232655	*rpoB*	MG489990
17.	M74P	*M. chelonae*	*hsp65*	MG334590	*rpoB*	MG489977
18.	M75	*M. chelonae*	*hsp65*	Not submitted	*rpoB*	Not submitted
19.	M77G	*M. chelonae*	*hsp65*	KX232660	*rpoB*	MG489991
20.	M148G	*M. chelonae*	*hsp65*	KX232653	*rpoB*	MG489976
21.	1AG	*M. lentiflavum*	*hsp65*	KX232669	*rpoB*	MG489975
22.	M8G	*M. lentiflavum*	*hsp65*	KX232667	*rpoB*	Not submitted
23.	M12G	*M. lentiflavum*	*hsp65*	KX232668	*rpoB*	MG489977
24.	M140G	*M. peregrinum*	*hsp65*	KX232666	*rpoB*	Not submitted
25.	M116G	*M. tuberculosis*	*hsp65*	KX232670	*rpoB*	Not submitted

Sl. No.	Sample/ strain ID	Species identified by *hsp65*	Gene	Accession No.	Gene	Accession No.
26.	M20	*M. goodii*	*hsp65*	MG334587	*rpoB*	Not submitted
27.	HDG	*Mycobacterium* sp.	*hsp65*	MG334591	*rpoB*	Not submitted
28.	HNP	*Mycobacterium* sp.	*hsp65*	KT284832	*rpoB*	KT284833
29.	HNP	*Mycobacterium* sp.	*16s rRNA*	KT284834	*rpoB*	Not submitted
30.	M15P	*M. chelonae*	*rrl*	MG461579		
31.	M43G	*M. chelonae*	*rrl*	MG461580		
32.	1AG	*M. lentiflavum*	*rrl*	MG461581		
33.	M12G	*M. lentiflavum*	*rrl*	MG461582		
34.	M8G	*M. lentiflavum*	*rrl*	MG461583		
35.	M4P	*M. chelonae*	*rrl*	MG461584		
36.	M5P	*M. chelonae*	*rrl*	MG461585		
37.	M11G	*M. chelonae*	*rrl*	MG461586		

Table 23. Accession numbers of sequences from animal sources submitted to NCBI.

Sl. No.	Sample/ Strain ID	Species identified	Gene	Accession No.	Gene	Accession No.
1.	Ngl 12A	*M. tuberculosis*	*hsp65*	MG432114	*rpoB*	MG490012
2.	Ngl 2832	*M. tuberculosis*	*hsp65*	MG432115	*rpoB*	MG490010
3.	Ngl 1125P	*M. tuberculosis*	*hsp65*	MG432116	*rpoB*	MG489997
4.	Ngl 2384P	*M. tuberculosis*	*hsp65*	MG432117	*rpoB*	MG490005
5.	Ngl 2831	*M. tuberculosis*	*hsp65*	Not submitted	*rpoB*	MG490013
6.	Ngl 2833A	*M. tuberculosis*	*hsp65*	MG432118	*rpoB*	MG490006
7.	Ngl 1115	*M. tuberculosis*	*hsp65*	MG432119	*rpoB*	MG489995
8.	Ngl 1120P	*M. tuberculosis*	*hsp65*	MG432120	*rpoB*	MG489996
9.	Ngl 2995	*M. tuberculosis*	*hsp65*	MG432121	*rpoB*	MG490008
10.	Ngl 1136P	*M. tuberculosis*	*hsp65*	MG432122	*rpoB*	MG490000
11.	Ngl 1140G	*M. tuberculosis*	*hsp65*	MG432123	*rpoB*	MG490001
12.	Ngl 2127G	*M. tuberculosis*	*hsp65*	MG432124	*rpoB*	MG490003
13.	Ngl 2992G	*M. tuberculosis*	*hsp65*	MG432125	*rpoB*	MG490007

Sl. No.	Sample/ strain ID	Species identified by *hsp65*	Gene	Accession No.	Gene	Accession No.
14.	Ngl 2995	*M. tuberculosis*	*hsp65*	Not submitted	*rpoB*	Not submitted
15.	Ngl 2123G	*M. tuberculosis*	*hsp65*	MG432126	*rpoB*	MG490002
16.	Ngl 2374P	*M. tuberculosis*	*hsp65*	MG432127	*rpoB*	MG490004
17.	Ngl 7A	*M. tuberculosis*	*hsp65*	MG432128	*rpoB*	MG489994
18.	Ngl 3262P	*M. tuberculosis*	*hsp65*	MG432129	*rpoB*	MG490009
19.	Ngl 1129G	*M. tuberculosis*	*hsp65*	MG432130	*rpoB*	MG489998
20.	Ngl 1135	*M. tuberculosis*	*hsp65*	MG432131	*rpoB*	MG489999
21.	Ngl 05	*M. tuberculosis*	*hsp65*	MG432132	*rpoB*	Not submitted
22.	Ngl 02	*M. tuberculosis*	*hsp65*	MG432133	*rpoB*	MG490011
23.	Ngl SERO	*M. tuberculosis*	*hsp65*	MG432134	*rpoB*	MG490016
24.	Mgh FU239	*M. abscessus*	*hsp65*	MG432135	*rpoB*	MG490014
25.	Mgh FU362	*M. abscessus*	*hsp65*	MG432136	*rpoB*	MG490015
26.	PK504	*M. lentiflavum*	*hsp65*	KX431212	*rpoB*	MG490017
27.	Ngl 2824	*M. novocastrense*	*hsp65*	KX815344	*rpoB*	Not submitted
28.	Ngl 2837	*Mycobacterium* sp.	*hsp65*	KX815345	*rpoB*	Not submitted
29.	Ngl 2824	*M. novocastrense*	*rrl*	MG461576		
30.	PK504	*M. lentiflavum*	*rrl*	MG461577		
31.	Ngl 2837	*Mycobacterium* sp.	*rrl*	MG461578		

Table 24. Accession numbers of sequences of clinical origin submitted to NCBI.

Sl. No.	Sample/ strain ID	Species identified	Gene	Accession No.	Gene	Accession No.
1.	AH4	*M. chelonae*	*hsp65*	MG432109	*rpoB*	MG489974
2.	S12	*Mycobacterium* sp.	*hsp65*	MG432110	*rpoB*	Not sbmitted
3.	S7	*M. chelonae*	*hsp65*	MG432111	*rpoB*	MG489973
4.	W11	*M. chelonae*	*hsp65*	MG432112	*rpoB*	MG489993
5.	W12	*M. chelonae*	*hsp65*	MG432113	*rpoB*	Not submitted
6.	S12	*Mycobacterium* sp.	*rrl*	MG461575		
7.	RF	*Mycobacterium* sp.	*rrl*	MG461574		

Table 25. Accession numbers of sequences from environmental isolates submitted to NCBI.

5.12. Gaps observed and future scope of the study

In this study we have attempted to isolate a wide variety of mycobacterial isolates from three different sources- animal, human and environment, keeping the sampling target relatively large. Although 62 isolates were obtained from the study comprising 10 different species, actual epidemiological and prevalence rate in the region could not be made as only two geographical locations were targeted in the study involving convenient sampling depending on the accessibility. Our study could only present a glimpse on the percent positivity and prove the clinical significance of the organism in the region. A multicentre collaborative efforts from the states, clinicians, trained epidemiologists and researchers would be required to conclude on the true prevalence of the organism. Three isolates, one of clinical origin and two from animal sources could not be established as novel species as certain criteria could not be fulfilled like, repeated isolations, battery of biochemical tests, more proteomic and genomic studies. Scope remains for complete description of these unique isolates. Given the source of milk from bovine origin, the isolates were anticipated to be *M. bovis*. However a total of 26 NTM isolates were obtained from this food nutriment without any *M. bovis*. The presence of this opportunistic but clinically significant entities indicates the need to explore their route of transmission which remains for future scope of studies. Our environmental isolation rate of just 3.74% is comparatively low when mycobacteria is known to be ubiquitous in the environment. According to our observation, the poor isolation rate could be because the protocol once standardized for our previous studies was not suitable for the type of soil and water of this region. This also lead to weak correlation studies from different sources aimed in the study. Hence for isolation of mycobacteria from environment, location-specific decontamination protocols should be taken under consideration.

CHAPTER 7

SUMMARY

One of the oldest known diseases of human, Tuberculosis, is caused by the etiological agent *M. tuberculosis* with about one-third of the world's population still suffering from the disease. Tuberculosis remains a major global health problem as infectious disease including India with *M. tuberculosis* as the main causative agent. *M. tuberculosis* is one of the members of MTBC. Its other members are *M. bovis, M. africanum, M. orygis, M. microti, M. pinnipedii* and *M. canetti,* of which *M. bovis* is responsible for bovine tuberculosis with a zoonotic potential. The genus *Mycobacterium* comprises over 184 species including MTBC members, of which *M. lepare* causes leprosy in human and is not cultivable. Other members are ubiquitous in the environment and some are opportunistic pathogens with a wide range of hosts; these are referred to as the NTM. Many of these NTM are responsible for causing infections in human and presents manifestations similar to tuberculosis. In industrialized countries where tuberculosis have been controlled, infections by NTM are more commonly observed than *M. tuberculosis*, mainly among elderly patients. India despite being endemic to *M. tuberculosis* has also been observing an increasing rise of NTM cases. However, hardly any reports are available from the Northeastern region of India on NTM isolation from animal, human or environment. This study was designed to grow all cultivable mycobacteria from human, animal and their interactive environment to understand the distribution of this microorganism and their pathogenic behaviour in different host by establishing the simplest conventional and molecular approach for identification mainly in resource constrained setups.

Overall, 555 samples (121 human, 220 animals and 214 environmental) were collected from geographically dispersed sites in and around Khasi hills of Meghalaya and Dimapur district of Nagaland. The human samples consisted of tuberculosis suspected patients, with relapsed cases being targeted for NTM isolation in mind; animal samples comprised majorly of bovine milk, a few suspected porcine lymph node, and canine samples; and environmental samples included soil and water mainly from the area where animal or human sampling was being carried out. These samples were processed for mycobacterial isolation on solid media after digestion and decontamination. The isolates so obtained were characterized through conventional and molecular methods. Altogether, 62 (11.17%) isolates showing growth on solid media were confirmed to be of genus *Mycobacterium* through molecular identification methods. A series of identification and differentiating PCR were employed before ultimately sequencing specific genes for species identification. The genes for PCR identification include genus specific *hsp65*, *rpoB* and MTBC specific *IS6110*. For differentiation of NTM and MTBC, *hsp65*-PRA was successfully employed and some by the duplex *rpoB*. Sequencing ultimately identified all the species accurately.

Sequencing identified, 38 isolates to be NTM and 24 as MTBC. Out of the 38 (6.85%) NTM, 26 were from milk, 2 from canine origin, 2 from human and 8 from environmental source. Out of 24 MTBC (4.32%), 23 were from human and 01 from bovine milk. These 24 MTBC isolates were differentiated as *M. tuberculosis* and *M. bovis* by differentiation PCR of RD9, RD4 and 500bp fragment PCR. This approach identified all the 24 MTBC as *M. tuberculosis*. The 38 NTM were speciated using *hsp65*-PRA, duplex PCR and *hsp65* sequencing with further BLAST and phylogenetic analysis. Some of the NTM required additional *rpoB* sequencing and BLAST analysis for species confirmation. Out of these 38 NTM, the 08 NTM belonged to environmental species which comprised a diverse range of species such as *M. immunogenum, M. pyrenivorans, M. saopaulense, M. chelonae* and *M.*

fortuitum. Out of the 02 NTM species from human, 01 was identified as the rare *M. novocastrense* and another could not be speciated by either of these two genes. The isolate was presumed a novel species following additional *16s rRNA* sequencing and phylogenetic analysis. There were additional 03 human NTM isolates shared by Nazareth hospital, Meghalaya, for molecular identification which was identified as *M. lentiflavum* (1) and *M. abscessus* (02). From the animal source, 02 NTM of canine origin could not be speciated and were presumed to be novel NTM. However, all 27 NTM isolation from milk were speciated viz. *M.chelonae* (21), *M.lentiflavum* (3), *M.goodii* (1), *M.peregrinum* (1) and *M.tuberculosis* (1) isolate from milk. The three suspected to be novel species through multiple gene study were subjected to further *16s rRNA* gene sequencing. Now with a total of three genes, namely, *hsp65, rpoB* and 16sRNA, concatenated phylogenetic analysis was performed with the closest BLAST hits and ATCC strains. All these isolates were assigned to be unique given their distinct phyletic line within the dendrograms constructed with reported isolates.

The isolation of NTM from all the three sources shows the circulation of this pathogen in this region. The three unique isolates which could not be speciated are all clinical isolates of veterinary and public health significance indicating the graveness of the situation. Our sampling size may be small but indulges in presenting several observations of NTM species circulating in this part of the country for which data is lacking. The human sampling had a targeted approach towards relapsed cases of tuberculosis which either ends up as drug resistant tuberculosis or remains untreated in these constrained settings. Out of the 02 NTM cases in our study, both succumbed to the disease. However the 03 NTM isolates shared by Nazareth hospital with better equipped settings could access the right course of treatment due to timely detection. A large isolation of *M. chelonae* from milk indicates that the higher presence of this species in this belt. NTM infections globally and in India are commonly attributed to this species which calls for careful observation during mycobacterial infections. The isolation of rare and novel NTM from human points to an underlying problem of NTM which could be larger than comprehended from this region.

CHAPTER 8

CONCLUSIONS

A total of 62 (11.17%) mycobacterial isolates were obtained from 555 samples of which the major isolations were from human and animal sources. Of all 38 NTM isolations, major isolations were obtained from animal sources comprising 28 isolates with 73.68% isolation rate. Among all 24 MTBC isolations, 23 were from human sources accounting for major isolation with 95.84% isolation rate. Ten different species were isolated from the study and 03 were probable novel isolates. This indicates high species diversity of mycobacteria present in the Khasi Hills of Meghalaya and Dimapur district of Nagaland. Presence of even 02 NTM isolates from a total of 121 human tuberculosis suspected samples reveals the occurrence of NTM infections in these sites. Isolations from animal and environmental sources strongly points to the risk of such opportunistic infections. Both clinical NTM isolates in the study were obtained from relapsed TB cases who had been unresponsive to first line TB drugs and none of them survived. These outcomes exist due to unavailability of diagnostic facilities in primary health centres for NTM infections and unawareness of the treating physicians. NTM infections are very commonly confused with drug resistant tuberculosis and put on the wrong treatment regimen. The first line TB treatment is for 06 months and treatment of drug resistant tuberculosis is by a different set of drugs for another 18 months while NTM treatment follows a different regimen from the these two treatments for over 18 months. The treatment course is very challenging for the patient and a wrong diagnosis jeopardises the well being of the individual consequently leading to death before diagnosis. In our study, we have been able to isolate one rare NTM i.e., *M. novocastrense* and another novel NTM from human clinical source. Additionally, out of the 03 shared NTM isolates by Nazareth Hospital for molecular confirmation and speciation, one yielded another uncommon species- *M. lentiflavum*, and the other 02 were the notoriously resistant NTM species, *M. abscessus*. These are small but very significant findings which shows that both common and rare NTM species are present in these population, but requires a large scale study to find the wide diversity and true prevalence in this region.

A significant number of NTM have also been isolated from milk of which *M. chelonae* was the dominant species with three *M. lentiflavum* and one each of *M. peregrinum, M. goodii* and *M. tuberculosis* isolations. These finding clearly depicts *M. chelonae* to be one of the dominant NTM in nature and cautions for close observations for human infections by this entity. The dairy sector in the belt need to improve the hygienic practices so that preventable infections such as this are kept on check. Presence of mycobacterial in the raw milk also emphasizes the need to avoid raw milk consumption. Proper heating of milk is important as some species of mycobacteria are known to be heat tolerant and might survive pasteurization if the initial inoculum remains high. It should also be noted that *M. lentiflavum* was observed in milk and a clinical case. Three isolates were obtained from milk and one isolate shared for molecular speciation. This establishes the risk that could be acquired through transmission among the animal-human interface.

The finding of low number of mycobacterial species from environment despite a relatively fair sampling size could be due to the rich microflora in this area with sufficient rains, humidity and varied soil texture and soil microbiome which our standardized isolation technique for water and soil did not hold good but worked in other regions. However, of the total 08 isolates from environment, 04 of the species were identified as *M. chelonae* (50%). In correlation it may be noted that *M. chelonae* was also the major species isolated (75%) from the animal sources. Although no human cases due to this species were noted

in the study, major human isolations in the country have been attributed to *M. fortuitum* complex and *M. chelonae*. A large scale study involving computational genomic studies and genotyping could be implemented to explore the transmission dynamics at the animal-human-environment interface. Although in our study this has been attempted to a minor extend by comparing various database isolates and our isolates through phylogenetic studies, the information still remains murky.

Majority of the laboratory in this regions are technically constrained and access to well established diagnostic laboratories is limited to only a few privileged. We therefore attempted to summarize the best approach for detecting *Mycobacterium* and differentiation for MTBC and NTM in modestly funded clinical setups. Identification by genus specific *hsp65* and MTBC specific *IS6110* was found to be a fairly suitable approach. This could be done in a single tube by duplexing these two genes or separate PCR reactions i.e. *hsp65* followed by *IS6110* PCR. In our opinion resource constrained diagnostic laboratories could follow any of these two approaches, but with a slight favour for the second approach as it is less error prone. Differentiation of MTBC and NTM at the primary level, could avert a high number of misdiagnosed cases of NTM as TB or MDR-TB. MTBC if detected can have two initial treatments in India, one targeted towards *M. tuberculosis* and another for *M. bovis*. Although majority of the cases would be *M. tuberculosis* but a few could be *M. bovis* which is resistant to pyrazinamide, one of the first line treatment drugs for tuberculosis. Hence, if the members of the MTBC is differentiated atleast into these two species, a wrong treatment course could be avoided which otherwise would require a long 6 month course and expose the patient to undesirable drug toxicity. A quick differentiating PCR, RD9 for *M. tuberculosis* and RD4 for *M. bovis* could be fit for this differentiation. There are over 184 mycobacterial species identified so far and when a single species is isolated, it could be any one of these, or at the most could be narrowed down to the 90 species which are considered pathogenic. This presents a real challenge in diagnosis of NTM infections where some drugs in the NTM treatment regimen vary according to the species. The most common approach used for NTM speciation is *hsp65*-RE which we also observed in our study to be a very fine approach. In our opinion, clinical laboratories can now shift to sequencing based identification of bacterial isolates, and strongly suggested for a bulky genus like *Mycobacterium*. Sequencing cost had greatly reduced in the current era, which could be outsourced at just under Rs. 500 per sample or lesser on bulk samples. The relatively small size of 441bp product of *hsp65* obtained by partial amplification could be conveniently sequenced in the Sanger sequencing platform, so a laboratory can easily adopt this rapid and accurate molecular diagnostic approache. Once *hsp65* sequencing is complete, analysis can be carried out in freely available online server NCBI BLAST which can identify all the species reported globally if a trained biotechnologist is available. Culture isolation on either solid or liquid media is still recommended for all mycobacteria diagnosis as direct sample analysis do not give the sensitivity required. New laboratories would require a biosafety cabinet for handling of samples where a quick hot-cold lysis of the culture could be performed for DNA isolation followed by reaction preparation in a PCR set-up area. This can be followed by *hsp65*-PRA or sequencing. Identification of new species still remains challenging for several laboratories globally. But for established pathogens adopting currently available molecular methods and diagnostics according to the suitability and strengthening linkages with national reference laboratories could avert several social and physical distress.

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INDEX

%	percentage
<	less than
>	greater than
≥	greater than or equal to
bp	base pair
g	gram
IU	International units
M	molar
mA	milli-amperes
mg	milligram
min	minute
ml	milliliters
mM	millimolar
ºC	degree Celsius
rpm	revolution per minute
s	seconds
U	unit
V	volts
x g	relative centrifugal force
yr	year
µg	microgram
µL	microliter

ABBREVIATIONS

AFB	acid fast bacilli
ATS	american thoracic society
CDC	centre for disease control
CHCA	α-Cyano-4-hydroxycinnamic acid
CPC	cetylpyridinium chloride
CsCl	caesium chloride or cesium chloride
CTAB	cetyltrimethylammonium bromide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ETB	extrapulmonary tuberculosis
H_2SO_4	sulphuric acid
HCl	hydrochloric acid
HIV	human immune-deficiency virus
HPC	hexadecylpyridinium chloride
IBSC	institutional biosafety committee
IPTG	Isopropyl β-D-1-thiogalactopyranoside
KH	khasi hills
LJ	lowenstein jensen
MAC	*Mycobacterium avium* complex
MAIC	*Mycobacterium avium-intracellulare* complex
MALDI-TOF	matrix assisted laser desorption ionization- time of flight
MDR-TB	multi drug resistant tuberculosis
MEGA	molecular evolutionary genetic analysis
MGIT	mycobacterial growth indicator tube
MIC	minimum inhibitory concentration

ML	maximum likelihood
MTBC	*Mycobacterium tuberculosis* complex
MTT	3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium
NCBI	national centre for biotechnology information
NER	Northeastern region
NJ	neighbour-joining
NSS	normal saline solution
NTM	nontuberculous mycobacteria
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pH	power of hydrogen
PRA	PCR-restriction enzyme pattern analysis
psi	pound-force per square inch
RGM	rapidly growing mycobacteria
RNTCP	revised national tuberculosis control programme
RR-TB	rifampicin-resistant tuberculosis
SDS	sodium dodecyl sulfate
SGM	slow growing mycobacteria
T2CH/TCH	thiophen-2-carboxylic acid hydrazide
TAE	tris-acetate-EDTA
TB	tuberculosis
WHO	world health organization
XDR-TB	extensively drug-resistant TB
X-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactoside
ZN	ziehl-neelsosn

APPENDICES

1. Middlebrook 7H10 Agar

Ammonium Sulfate	0.5 g
Monopotassium Phosphate	1.5 g
Disodium Phosphate	1.5 g
Sodium Citrate	0.4 g
Magnesium Sulfate	25.0 mg
Calcium Chloride	0.5 mg
Zinc Sulfate	1.0 mg
Copper Sulfate	1.0 mg
L-Glutamic Acid (sodium salt)	0.5 g
Ferric Ammonium Citrate	0.04 g
Pyridoxine Hydrochloride	1.0 mg
Biotin	0.5 mg
Malachite Green	250.0
Agar	15.0 g
Oleic Acid 0.6 mL	

Formula approximately per 900ml

2. Middlebrook 7H11 Agar

Pancreatic Digest of Casein	1.0 g
L-Glutamic Acid	0.5 g
Sodium Citrate	0.4 g
Pyridoxine	1.0 mg
Biotin	0.5 mg
Ferric Ammonium Citrate	0.04 g
Ammonium Sulfate	0.5 g
Disodium Phosphate	1.5 g

Monopotassium Phosphate	1.5 g
Magnesium Sulfate	0.05 g
Agar	15.0 g
Malachite Green	1.0 mg

Formula approximately per 900ml

3. Middlebrook OADC Enrichment

Sodium Chloride	8.5 g
Dextrose	20.0 g
Bovine Albumin (Fraction V)	50.0 g
Catalase	0.03 g

Formula approximately per 900ml

4. Middlebrook 7H9 Broth

Approximate Formula Per 900 mL

Ammonium Sulfate	0.5 g
L-Glutamic Acid	0.5 g
Sodium Citrate	0.1 g
Pyridoxine	1.0 mg
Biotin	0.5 mg
Disodium Phosphate	2.5 g
Monopotassium Phosphate	1.0 g
Ferric Ammonium Citrate	0.04 g
Magnesium Sulfate	0.05 g
Calcium Chloride	0.5 mg
Zinc Sulfate	1.0 mg
Copper Sulfate	1.0 mg

Formula approximately per 900ml

5. Middlebrook ADC Enrichment

Approximate Formula Per Liter

Sodium Chloride	8.5 g
Bovine Albumin (Fraction V)	50.0 g
Dextrose	20.0 g
Catalase	0.03 g

Formula approximately per 1000ml

6. Penta mix

Polymyxin B	6000 U
Amphotericin B	600µg
Nalidixic acid	2400µg
Trimethoprim	600µg
Azlocillin	600µg
Water	2.5ml

7. Lowenstein Jensen (LJ) Media

L-Asparagine	3.60 g
Monopotassium phosphate	2.40 g
Magnesium sulphate	0.24 g
Magnesium citrate	0.60 g
Potato starch, soluble	30.0 g
Malachite green	0.40 g

Formula approximately per 600ml

For LJ-glycerol 12 ml glycerol can be added to 600ml of the above mineral solution. For LJ-pyruvate, approximately 4.80 mg pyruvic acid should replace glycerol.

8. Gruft mycobacterial supplement

Penicillin	20000 IU
Nalidixic acid	14mg
Ribonucleic acid	20mg

Water	2.5ml

9. Luria Bertani Broth, Miller (per litre)

Casein enzymic hydrolysate	10g
Yeast extract	5g
Sodium chloride	10g

Final pH 7.5±0.2 (at 25°C)

Luria Bertani Agar Miller (per litre)

Casein enzymic hydrolysate	10g
Yeast extract	5g
Sodium chloride	10g
Agar	15g

Final pH 7.5±0.2 (at 25°C)

10. Reagents for sample processing

a. Sodium hydroxide (NaoH) solution (4%)

NaOH	4g
Distilled water	100ml

Autoclave and store at room temperature.

b. Sulphuric acid (H_2SO_4) solution (6%)

Distilled water	940ml
Con. H_2SO_4	60ml

c. Cetylpyridinium chloride (CPC) (1%)

CPC	1g
Sterile distilled water	100ml

d. Phosphate buffered saline (0.085M, pH7.2)

Sodium Chloride	8g
Disodium hydrogen phosphate	1.16g
Potassium chloride	0.2g

Potassium dihydogen orthophosphate 0.2g

Distilled water 1000ml

11. Reagents for Ziehl-Neelsen staining

a. Carbolfuchsin stain

Basic fuchsin 0.3 g

Ethanol 95% (vol/vol), 10 ml

Phenol, heat-melted crystals 5 ml

Distilled water 95 ml

Basic fuchsin dissolved in the ethanol, followed by addition of phenol, which is then dissolved in the water. Mixed and allowed to stand for several days. Filtered before use.

b. Decolorizing solvent

Ethanol 95% (vol/vol), 97 ml

Hydrochloric acid (concentrated) 3 ml

c. Counterstain

Methylene blue chloride 0.3 g

Distilled water 100 ml

12. Reagents for DNA extraction

a. Ethanol (75%)

Absolute ethanol 75 ml

Distilled water 25 ml

b. Proteinase K (20mg/ml)

Proteinase K powder 20mg

DNAse free water 1000ml

Store at -20 ºC

c. SDS (10%)

sodium dodecyl sulfate 10gm

Nuclease free water 100ml

d. Tris HCl stock, pH 8.0 (1.0 M)

Tris base	121.14gm
Water	1000ml

pH adjusted using concentrated HCl

e. EDTA stock (0.5 M)

EDTA	18.61gm
Distilled water	1000ml

Autoclave

f. 5 M NaCl stock

NaCl	29.2gm
Distilled water	100ml

Autoclave

g. Lysis buffer

1 M Tris	25ml
5M NaCl	70ml
0.5 M EDTA	10ml
CTAB	5g

Bring final volume to 250ml

h. Lysozyme (50 ml/ml)

Store in small aliquots at -20°C. Do not freeze thaw

i. TE buffer(1X)

Tris HCl (pH7.5)	10mM
EDTA(pH8)	1mM

Add water to 100 ml so that the total volume is attained and store at 4ºC

j. CTAB/NaCl

NaCl	29.2 g
CTAB	10 g

Adjust volume to 100ml distilled water

k. TBE (10X)

Tris base	108 g
Boric acid	55 g
0.5 M EDTA (pH 8.0)	20 ml

Deionized water up to 1000 ml.

l. Phenol/chloform/isoamyl alcohol

Phenol	25ml
Chloroform	24ml
Isoamyl alcohol	1ml

13. Solution for agarose gel electrophoresis

a. Tris - acetate - EDTA (TAE) buffer (50x)

Tris base	24.2g
Glacial acetic acid	5.71ml
0.5M EDTA	10ml

Volume made upto 100ml with double distilled water.

Sterilized by autoclaving for 20 min at 15psi on liquid cycle and stored at RT.

b. Ethidium bromide solution (10mg/ml)

Ethidium bromide	10mg
Autoclaved distilled water	1ml

Vortexed and stored protected from light

c. 6x Gel loading dye

Bromophenol blue	0.25%
Xylene cyanol	0.05%
Glycerol	60%
EDTA	60mM

Stored at 4^{o}C.

14. McFarland Standard No.1

1% BaCl2 (ml)	0.1
1% H2SO4 (ml)	9.9

Approximate bacterial suspension per ml $3.0x10^8$

15. Reagents for drug susceptibility test

a. Clarithromycin (10mg/ml)

Drug	10 mg
Acetone	1ml

b. Ethambutol (10mg/ml)

Drug	10 mg
Water	1ml

c. Isoniazide (10mg/ml)

Drug	10 mg
Water	1ml

All drug preparations followed by filtration through 0.22µm

d. MTT dye (5mg/ml)

MTT	5 mg
Water	1ml

16. Reagents for cloning and transformation

a. 1M $CaCl_2$

$CaCl_2$ (anhydrous)	111 g
Distilled water	1000ml

Filter sterilize through a 0.22m filter

b. 0.1M $CaCl_2$

1M $CaCl_2$	100ml
Distilled water	900ml

Filter sterilize through a 0.22m filter

c. 50% Glycerol

Glycerol	50ml
Distilled water	50ml

Autoclave

d. 0.1M $CaCl_2$ + 15% glycerol

1M $CaCl_2$	100ml
50% Glycerol	300 ml
Distilled water	600ml

e. IPTG (24mg/ml)

IPTG	24 mg
Water	1 ml

f. X-gal (20mg/ml)

X-gal	20
DMSO	1ml

g. Ampicillin (100mg/ml)

Drug	100gm
Water	1ml

Filter sterilize