Sequencing Vs MALDI-TOF- tools for accurate identification of Non-Tuberculous Mycobacteria-a Pilot study

Dissertation submitted in partial fulfilment of the rules and regulations for the M.D. (Branch-IV Microbiology) examination of the Tamilnadu Dr. M.G.R. Medical University to be held in May, 2019

CERTIFICATE

This is to certify that the dissertation entitled, "**Sequencing Vs MALDI-TOF- tools for accurate identification of Non-Tuberculous Mycobacteria-a Pilot study**"is the bonafide work of Dr. Ann Susan Sam toward the M.D (Branch – IV Microbiology) Degree examination of the Tamil Nadu Dr.M.G.R.Medical University, to be conducted in May-2019.

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DECLARATION

I hereby declare that this M.D Dissertation entitled "**Sequencing Vs MALDI-TOF- tools for accurate identification of Non-Tuberculous Mycobacteria-a Pilot study**"is the bonafide work done by me under the guidance of Dr. Joy Sarojini Michael, Professor, Department of Clinical Microbiology, Christian Medical College, Vellore. This work has not been submitted to any other university in part or full.

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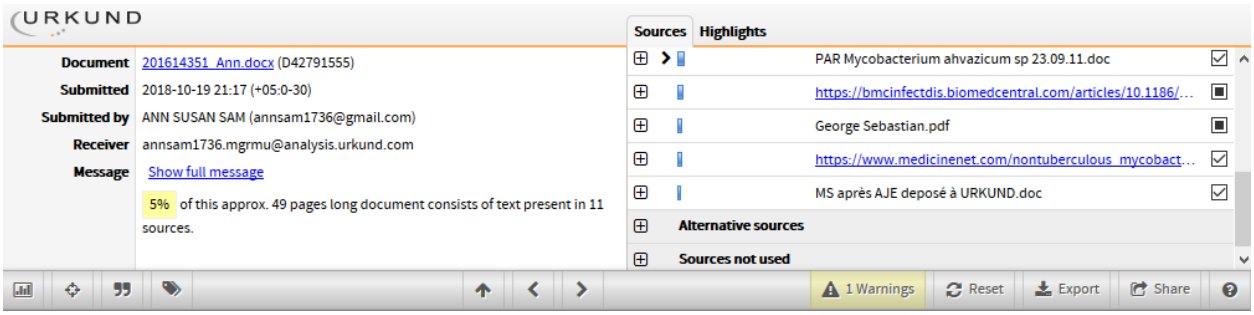

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1.INTRODUCTION

Mycobacteria that are other than *Mycobacterium tuberculosis* (MTB) complex and *Mycobacterium leprae*are known as non-tuberculous mycobacteria (NTM)(1). Nontuberculous mycobacteria have traditionally been seen as environmental organisms of limited clinical relevance, overshadowed by their more aggressive cousin, *Mycobacterium tuberculosis.* It was not until the HIV pandemic which highlighted disseminated *Mycobacterium avium* complex (MAC) as major opportunistic infection syndromes that their significance was recognized by the healthcare community, a role which further cemented by the expansion of iatrogenic immunosuppression.

There are more than 190 species of nontuberculous mycobacteria (NTM)(2). They are rod-shaped organisms that have a thick waxy cell wall and they form biofilms that allow them to grow in nutrient- and oxygen-poor conditions.The diseases caused can be classified into four groups- progressive pulmonary disease, superficial lymphadenitis, skin and soft tissue infections and disseminated infections. Disseminated infections are typically seen only in immunocompromised individuals.

On microscopy, they resist decolourisation by acid due to the mycolic acid present in the cell wall. Isolation of the organism by culture from a sterile site or multiple positive cultures from an unsterile site is necessary for etiological diagnosis. NTM are classified based on the rate of growth and pigment production on solid media, preferred one being Lowenstein Jensen medium. Slowly growing NTM take 7 days or more to produce

mature growth. Rapidly growing NTM grows within 7 days. Slow growers are subdivided into three groups based on pigment production- photochromogens (produce pigment only in the presence of light), scotochromogens (produces pigment in the absence of light) and non-chromogens (does not produce pigment).

Identification of NTM species is extremely important as the treatment is species-specific. Most NTM are intrinsically resistant to or only partially susceptible to the standard anti-tubercular drugs.

Biochemical tests like nitrate reduction, aryl sulfatase production, growth in 5%NaCl and urease production are traditionally being used for species identification. It is useful only for the identification of the most common 10-15 species. It cannot identify most of the new species. Moreover, these tests have a prolonged turnaround time, especially for slowly growing NTM. Hence, newer methods using molecular detection such asHigh-Performance Liquid Chromatography (HPLC),Line Probe Assay (LPA), DNA probes, Matrix Assisted Laser Desorption Ionization- Time of Flight (MALDI-TOF) and sequencingof DNA have been developed which will reduce the turnaround time and can detect novel species.

All Mycobacteria species contain mycolic acids in the cell wall. HPLC uses the principle of identification of NTM based on their chromatographic profile of mycolic acids in their cell wall. LPA uses reverse hybridization technology targeting the conserved segments of DNA like 16S-23S rRNA spacer region and 23s rRNA. PCR amplicons of the target region binds to oligonucleotide probes arranged on a membrane strip giving the species

identification. Commercial DNA probes are available, targeting the highly conservative 16S rRNA region of mycobacteria.

HPLC, LPA and commercial DNA probes have high cost and limited utility as they are restricted to identification of the most frequently isolated NTM species. On the other hand, MALDI-TOF is a rapid, cost effective and reliable method of identification of NTM species.This technology is designed to provide a protein “fingerprint” based on the desorbed ions from the cell surfaces.The instrument software automatically acquires and analyses the data and generates a profile for comparison to a database of reference spectra composed of previously well-characterized isolates giving the species identification of all the common and some of the uncommon NTM species. The method is rapid, with relatively simple technique and associated with significantly lower consumable costs than traditional microbiological identification methods. But the limitation of MALDI-TOF MS is that it requires a larger quantity of organism for analysis because there is no preamplification step, which can affect the identification of slowly growing NTM.

Sequencing of 16SrRNA isimportant in a reference laboratory. It is used as a gold standard confirmatorymethod for speciation of bacteria including NTM as it identifies the newer and unidentified species which are not identified by biochemicalsand other tests. However, closely related species like *M. abscessus* and *M. chelonae; M. houstonense* and *M. senegalense; M. intracellulare and M. chimaera*can have sequence similarity of > 97 % in the 16S rRNA region and hence cannot differentiated. Sequencing of other targets

like16S-23S Internal Transcribed Spacer (ITS), *rpoB* gene, *hsp65*gene will help to overcome the above problem.

This study is the standardization of the molecular methods of NTM identification i.e., DNA sequencing of 16S rRNA and ITS regions and evaluation of MALDI-TOF against this sequencing methods. MALDI-TOF is a rapid and accurate method of proteomics-based identification, which can be a part of the routine diagnostic armamentarium.This will enable early and accurate identification of NTM causing disease and therefore help in appropriate management of patients.

2.AIM & OBJECTIVES

AIM:

- Evaluation of MALDI TOF and molecular sequencing for accurate identification of Non-Tuberculous Mycobacteria

OBJECTIVES:

1. Standardize 16S rRNA and ITS sequencing methods and evaluate Matrix Assisted Laser Desorption/Ionization- Time of Flight(MALDI- TOF)

2. Comparison of MALDI-TOF against the above sequencing techniques

3.REVIEW OF LITERATURE

3.0 HISTORY

Mycobacterium (Greek origin. *myces* - fungus; *bakterion* - small rod) is without doubt one of the most clinically significant and extensively studied organisms of bacterial taxa. Tuberculosis and leprosy, which are the most important diseases caused due to mycobacteria, have been recognized throughout history(3,4). *Mycobacterium leprae* (originally named *Bacterium leprae*) was discovered by Armauer Hansen from unstained material in 1873 and the staining method was developed and observed by Albert Neisser in 1880(5). The clinical interest in mycobacteria began with the work of Robert Koch in the late 1800s, who detected the tubercle bacillus (originally named *Bacterium tuberculosis*) in 1882 from stained infected tissue and cultivated the organism on inspissated serum medium(6).

All members of the genus Mycobacterium other than the *Mycobacterium tuberculosis* complex and *Mycobacterium leprae* are collectively labeled as environmental mycobacteria or nontuberculous mycobacteria (NTM). Before the end of the 19^{th} century, bovine, avian, reptilian, piscine, and saprophytic varieties of mycobacteria had been described(7).

Table 1. Early case reports of NON-TUBERCULOUSMYCOBACTERIA:

1885 - Alvarez and Tavel et al describes Myco*bacterium smegmatis* (8).

1900 - case report by Marzinowski of a rapidly growing AFB, from the tonsillar crypt and sputum of two patients(9).

1904- case report by Lichtenstein with description of thin, elongated AFB in the sputum of a patient who had recurrent attacks of hemoptysis and calls it as “pseudo tubercle bacilli”(10).

1904- the first report of a chronic injection site abscess caused by acid fast bacilli (11).

1918- AFB are noted in the chronic pustular skin lesions of a soldier from England who had been wounded in battle (12).

1926- Aronson identifies the cause of a disease in salt water fish in the Philadelphia aquarium as mycobacterial and named it as *Mycobacterium marinum.* He described the colonies as " a lemon-yellow color which later became a deep orange," (13).

1936- Steenken and Landau make an important observation that some colonies of non-tuberculous mycobacteria changed from white to chromogenic "when left at room temperature and exposed to light”(14).

1938- da Costa Cruz describes acid fast bacilli from the injection site abscess of a 25-year-old woman and namedit as *Mycobacterium fortuitum*(15)

1951 - Linell publishes a paper describing a new mycobacterial disease of man from skin lesions and the causative bacillus was named *Mycobacterium ulcerans*(16).

1943- Feldman and co-workers reported the recovery of an avian like bacillus from a

man with silico-tuberculosis(17). This organism was later shown to be *Mycobacterium avium* by serological tests(18).

1953 - Frerich and Moore describes another novel species *Mycobacterium abscessus* from a patient with subcutaneous abscess-like lesion of the gluteal region(19).

Gibson JB discovers that NTM parasitize the damaged lung(20).Buhler and Pollak publishes 2 cases of pulmonary infection caused by a "yellow bacillus" and the descriptions of the organism now called *Mycobacterium kansasii*(21).

Despite sporadic reports of the isolation of many varieties of NTM from clinical specimens in the early part of the twentieth century, the only mycobacteria that were taken seriously as a cause of human disease were those of *Mycobacterium tuberculosis* and *Mycobacterium bovis*.

However, in the early 1950s, after it had become routine to culture rather than to carry out only staining of specimens for acid-fast bacilli (AFB), and when the prevalence of tuberculosis began to decrease as the social and economic status improved, a new concept about NTM infection began to arise. This was a result of keener bacteriologic and clinical correlation, as well as a better understanding of the relationship between weak skin test reactions to tuberculin and infection with organisms other than the tubercle bacillus(22).

Finally, in 1954, Timpe and Runyon correlated the known facts about the relationship between human pulmonary disease and NTM and provided the first working classification of the organisms(23).

3.1 EPIDEMIOLOGY

NTMwere considered as colonizers or ignored as environmental contaminants in the past. But the scenario has changed now and they are increasingly being recognized as important pathogens in both immunocompromised or immunosuppressed and even immunocompetent individuals. The rates of NTM infection have increased significantly in recent years, and rates vary widely depending on the demography and geographic location. Most epidemiological studies and case reports are from developed nations. However, in countries like India, NTM diseases often goes unrecognized and are misdiagnosed. The pulmonary disease due toNTM are often misidentified as tuberculosis.

3.1.1 EPIDEMIOLOGY BEFORE AIDS EPIDEMIC

Diseases caused by the NTM were sporadic in nature before the AIDS epidemic. Cases were chronic in nature resembling Pulmonary tuberculosis (TB),*Mycobacterium kansasii* and *Mycobacterium avium*complex(MAC) being the most common pathogens. Most cases were in middle-aged men in their sixth decade of life who had previous chronic lung diseases like pneumoconiosis, past tuberculosis, chronic bronchitis, chronic obstructive pulmonary disease, bronchiectasis or chronic aspiration from esophageal diseases. Less common presentations were cervical lymphadenitis, skin abscesses, or, in very rare cases, disseminated disease(24,25).

3.1.2 EPIDEMIOLOGY AFTER AIDS EPIDEMIC

The AIDS epidemic in the 1980s brought a dramatic change to the epidemiology of NTM infection. NTMinfections are primarily confined to respiratory system in immunocompetent individuals. But, in individuals with AIDS and other immunodeficiencies, it can lead to disseminated infections. Disseminated MAC infections were one of the opportunistic infections which increased dramatically following the AIDS epidemic. There were only 78 cases of disseminated NTM infections reported before the AIDS epidemic(24). But, according to the CDC, during June 1,1981 through August 31, 1987, there were 2,269 patients with disseminated NTM among the 41,349 patients with AIDS. Ninety six percent of these infections were due to MAC. The prevalence of MAC infections were reported to be as high as 50% among the AIDS patients in various studies during early 1990s(26).

3.1.3 BURDEN OF NTM DISEASES-GLOBAL

The exact rate or frequency of infections due to the different species of NTM is not known. In many countries including the United States, diseases due to NTM are not notifiable. This has led to a gap in our knowledge on the incidence and prevalence of the disease. Furthermore, most studies use the indicator called "isolation prevalence" as a surrogate marker for the diseaseprevalence, though the mere isolation of an NTM species does not necessarily indicate disease. If we look at available data, it is clear that the burden of NTM disease has been increasing in developed nations likeUnited States where the incidence of TB is low and there is excellent infrastructure to diagnose the

disease(27–29).The most common NTM species causing human disease in the United States are MAC and *Mycobacterium kansasii*. In a systematic review which included 22 studies that evaluated the trends in the rate of NTM disease across the United States, Canada, Europe, Australia, and Japan between 1946 and 2014, the incidence of NTM diseases increased by 75 percent in the above regions (30).

3.1.4 BURDEN OF NTM DISEASES-INDIA

In developing countries like India, the burden of NTM diseases are under reported due to misdiagnosis, lack of diagnostics and awareness of the disease. The overall rate of isolation of NTM has been reported to range from 0.5% to 8.6%. There is no data available on their geographical distribution(31). Prevalence of clinically significant NTM among specimens received in a study from north India during 2013-14 was 1.2% with *Mycobacterium intracellulare* being the most common species (32). A retrospective study from a tertiary care centre in south India during the time period 1999-2004 found that the prevalence of NTM was 3.9%.and the most commonly identified species were *Mycobacterium chelonae* and *Mycobacterium fortuitum*(33). In a study from central India in Madhya Pradesh during the period 2013-2015, 263 (29%) out of 906 mycobacterial culture isolates were identified as NTM. Among these, the predominant species were *Mycobacterium abscessus* followed by *Mycobacterium fortuitum*, *Mycobacterium intracellulare* and *Mycobacterium chelonae*(34).

3.2 ETIOLOGICAL AGENT

The term non-tuberculous mycobacteria (NTM) refers to those mycobacterial species other than the *Mycobacterium tuberculosis* complex (*M. bovis, M. africanum, M. microti, M. canetti, M. caprae, M. pinnipedii, M suricattae* and *M. mungi*) and those organisms causing leprosy (M. *leprae and M. lepromatosis*)(35).They are known by various synonyms like "atypical, "anonymous," "mycobacteria other than tuberculosis," "environmental," "environmental opportunistic", and most commonly, "nontuberculous, "or "NTM."(24)

3.2.1 TAXONOMY

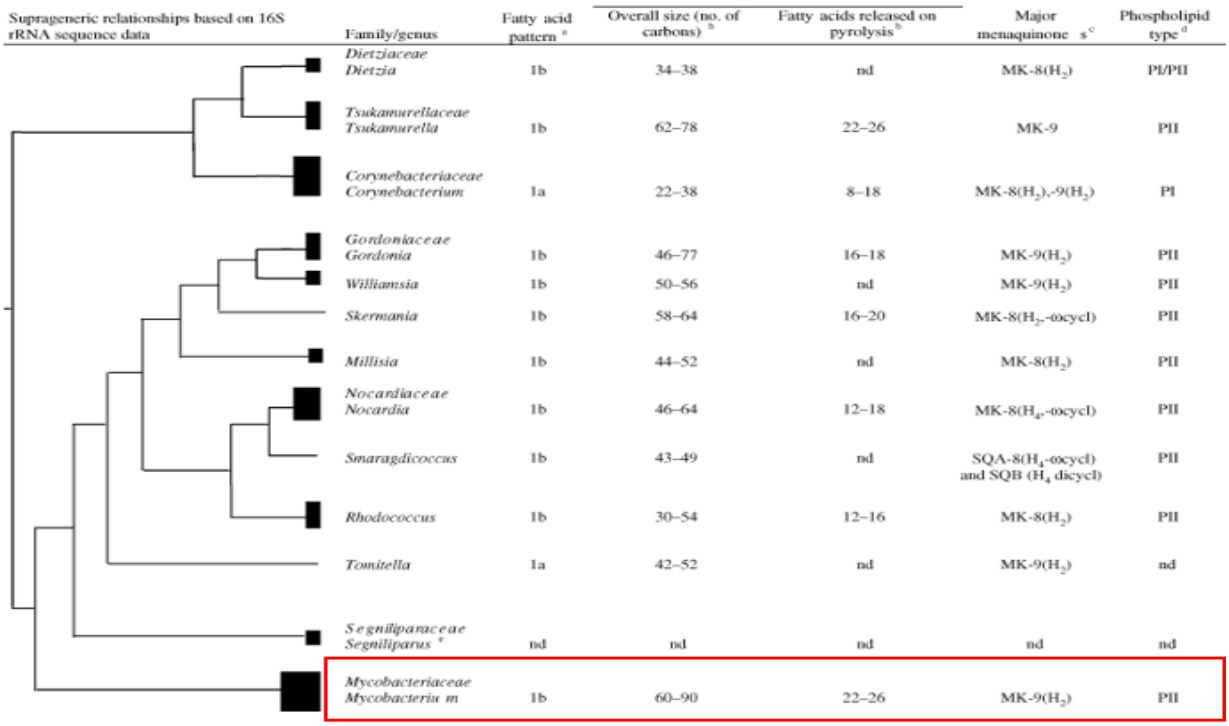
Classification of mycobacteria began in 1896 by Lehmann and Neumann. They proposed the genus Mycobacterium to include the tubercle and leprosy bacilli, organisms which had previously been classified as *Bacterium tuberculosis* and *Bacterium leprae.* The number of validly described species, including synonyms, of Mycobacterium currently stands at 197(2).

Table. 2 Taxonomy of Non-Tuberculous Mycobacteria–

Rank	**Reference**
Kingdom -Bacteria	(Cavalier-Smith, 2002)(36)
Subkingdom -Posibacteria	(Cavalier-Smith, 2002)
Phylum- Actinobacteria	(Cavalier-Smith, 2002)
Order Corynebacteriales	(Magee and Ward, 2015) (37)
Suborder- Corynebacterineae	(Stackebrandt E, 1997)(38)
Family- Mycobacteriaceae	(Chester, 1897)(39)
Genus- Mycobacterium	(Lehmann and Neumann, 1896)(40)

ORDER Corynebacteriales- Characteristics-

Order Corynebacteriales form a distinct monophyletic branch in the 16S rRNA actinobacterial gene tree. It encompasses the families Corynebacteriaceae; Gordoniaceae; Mycobacteriaceae; Nocardiaceae; Segniliparaceae; Tsukamurellaceae; and the genera *Hoyosella* and *Turicella*(41). They are aerobic or facultatively anaerobic, grampositive and catalasepositive actinomycetes which may form a branched substrate mycelium that fragments into coccoid-to rod-shaped element or presents as branched filaments, cocci, or pleomorphic forms. They are chemoorganotrophic. The cell wall peptidoglycan contains *meso*- diaminopimelic acid and is of the A1γ type. Arabinose and galactose are major cell wall sugars.The polar lipids of the plasma membrane are composed of hydrophilic head and fatty acid chains. Palmitic, octadecenoic and tuberculostearic are the major fatty acid constituents(42).Mycolic acids (2-alkyl, 3-hydroxy long-chain fatty acids) are the hallmark of the cell envelope. Members of families and genera classified in the order Corynebacteriales can be distinguished from one another and from corresponding taxa in the phylum Actinobacteria by taxon-specific 16S rRNA oligonucleotide sequences. This should be supported by chemical markers like fatty acid pattern, number of carbon atoms in mycolic acid, fatty acids released on pyrolysis, major menaquinone and phospholipid type.



Suprageneric relationships based on 16S rRNA sequence data	Family/genus	Fatty acid pattern [a]	Overall size (no. of carbons) [b]	Fatty acids released on pyrolysis [b]	Major menaquinone s [c]	Phospholipid type [d]
	Dietziaceae *Dietzia*	1b	34–38	nd	MK-8(H_2)	PI/PII
	Tsukamurellaceae *Tsukamurella*	1b	62–78	22–26	MK-9	PII
	Corynebacteriaceae *Corynebacterium*	1a	22–38	8–18	MK-8(H_2),-9(H_2)	PI
	Gordoniaceae *Gordonia*	1b	46–77	16–18	MK-9(H_2)	PII
	Williamsia	1b	50–56	nd	MK-9(H_2)	PII
	Skermania	1b	58–64	16–20	MK-8(H_2,-ωcycl)	PII
	Millisia	1b	44–52	nd	MK-8(H_2)	PII
	Nocardiaceae *Nocardia*	1b	46–64	12–18	MK-8(H_4,-ωcycl)	PII
	Smaragdicoccus	1b	43–49	nd	SQA-8(H_4-ωcycl) and SQB (H_4 dicycl)	PII
	Rhodococcus	1b	30–54	12–16	MK-8(H_2)	PII
	Tomitella	1a	42–52	nd	MK-9(H_2)	nd
	Segniliparaceae *Segniliparus* [e]	nd	nd	nd	nd	nd
	Mycobacteriaceae *Mycobacteriu m*	1b	60–90	22–26	MK-9(H_2)	PII

FIGURE 1. Order Corynebacteriales (Adapted from Bergey's Manual® of Systematic Bacteriology: Volume Five: the Actinobacteria(41))

a- Fatty acid classification according to Kroppenstedt. ()-Numbers refer to the type of fatty acid biosynthetic pathway and letters to the types of fatty acids (FA) synthesized.

b- Mycolic acid size (number of carbons) and fatty acids released on pyrolysis were detected by the use of GC (Gas Chromatography) and MS (Mass Spectrometry)

c- MK- 9(H2), notation for dihydrogenated menaquinone with nine isoprene units

d- Characteristic phospholipids: PI, nitrogenous phospholipids absent (with phosphatidylglycerol variable); PII, only phosphatidylethanolamine

e- The wall chemotype of Segniliparus has still to be established.(43)

FAMILY *Mycobacteriaceae*

The family *Mycobacteriaceae* contains the type genus *Mycobacterium*. Mycobacteria are aerobic to microaerophilic actinomycetes which usually form slightly curved or straight, nonmotile rods [(0.2-0.6) X (1.0-10) µm]. Cells are acid-fast at some stages of growth, and are considered gram positive although they are not readily stained by Gram's method. They do not form capsules, conidia, or endospores, rarely exhibit visible aerial hyphae, are catalase positive, produce acid from sugars oxidatively, and, with the exception of strains which do not grow in vitro, can be divided into rapid and slow growers. It is also characterized by the presence of mycolic acids with 60-90 number of carbon atoms and the predominance of dihydrogenated menaquinones with nine isoprene unit, MK- 9(H2)(37).

GENUS *Mycobacterium*

The genus encompasses saprophytes, as well as facultative and obligate pathogens. Four groups of human pathogens are recognized in the *Mycobacterium* genus: *Mycobacterium tuberculosis* complex, *Mycobacterium leprae*, slowly growing NTM and rapidly growing NTM(43,44).

Genus characteristics are as follows:

1) Acid –alcohol fastness (resists decolorization by an acidified alcohol after staining with a primary stain.

2) The presence of mycolic acids which can been cleaved into fatty acid methyl esters by pyrolysis.

3) 61-71 % G+C content of the DNA, *Mycobacterium leprae* being the only exception (51%).

3.2.2 CLASSIFICATION OF MYCOBACTERIA

One of the earliest classifications of NTM was by Krause in 1920. He reviewed the existing knowledge of the acid-fast bacilli that were widely distributed in nature during that period. He divided them into three groups- the free-living or saprophytic bacilli; those associated with healthy animals or their products (bacilli in dung, milk, and butter), and those associated with tuberculosis-like disease of cold-blooded animals like turtles, fish, and snakes. He documented existence of 40 different varieties of acid-fast bacilli exclusive of the tubercle bacillus (13).

The general view of mycobacteriologists by 1950 was that there were three varieties or species of *M. tuberculosis:* human, bovine and avian. In addition to these, there were several species of well-known "saprophytic" organisms like *M. phlei* and *M. fortuitum.* Several attempts to classify the mycobacteria were made by bacteriologists, but were not widely accepted by clinicians. As culturing of sputum became common practice during the post-WorldWar II era and more “atypical” mycobacteria were identified, the need for a clinically applicable method to classify mycobacteria was needed. By 1954, Timpe and Runyon developed a system of classification that suited the needs of the time(23,45). In this classification, mycobacteria, apart from those in the *M. tuberculosis* complex and members of non-cultivable taxa, were divided into four overtly artificial groups based on growth rates and ability to produce pigment.

Table 3. The Timpe-Runyon Classification

Group	Pigment	Rate of growth	Examples
I	Photochromogenic	14-21 days	*M.kansasii, M. marinum, M. simiae, M. asiaticum*
II	Scotochromogenic	10-14 days	*M.gordonae, M. scrofulaceum, M. flavescens*
III	Nonphotochromogenic	14-21 days	MAC, *M. xenopi, M. ulcerans, M. terrae, M. haemophilum*
IV	Variable	5-7 days	*M. abscessus, M. chelonae, M. fortuitum*

Ref: Runyon EH, 1959(45)

Types I, II, and III, slow growers, take 7 days or more to grow and are differentiated by the colour of colonies. Members within the two groups- slow and rapid growers- can be distinguished using a combination of phenotypic properties which will be described later. The taxonomic status of some of these species was, however, uncertain and it is largely due to the efforts of the International Working Group of Mycobacterial Taxonomy (IWGMT) that major progress has been made in this area(46). Another clinically oriented classification is the Woods and Washington Classification (47).

Table 4. Woods and Washington Classification scheme

1. **Species Potentially Pathogenic in Humans**	
Slow growers:	
M. avium complex	
M. kansasii	*M. malmoense*
M. scrofulaceum	*M. simiae*
M. xenopi	*M. genavense*
M. szulgai	*M. marinum*
M. haemophilum	*M. ulcerans*
M. celatum	
Rapid growers:	
M. fortuitum group, M. chelonae/abscessus group	
2. Saprophytic Mycobacteria Rarely Causing disease in Humans	
M. gordonae	*M. shimoidei*
M. asiaticum	*M. gastri*
M. terrae	*M. nonchromogenicum*
M. triviale	*M. paratuberculosis*
3. Species with an Intermediate Growth Rate	
M. flavescens	
4.Other Rapidly Growing Species	
M. thermoresistible	*M. mucogenicum* group
M. neoaurum	*M. mageritense*
M. smegmatis group	*M. wolinskyi*

The availability of molecular methods has made the Runyon classification obsolete for clinical purposes andresulted in a marked increase in the identification of new species.

Sequencing of 16S rRNA and phylogenetic analysis can be used to distinguish various species.

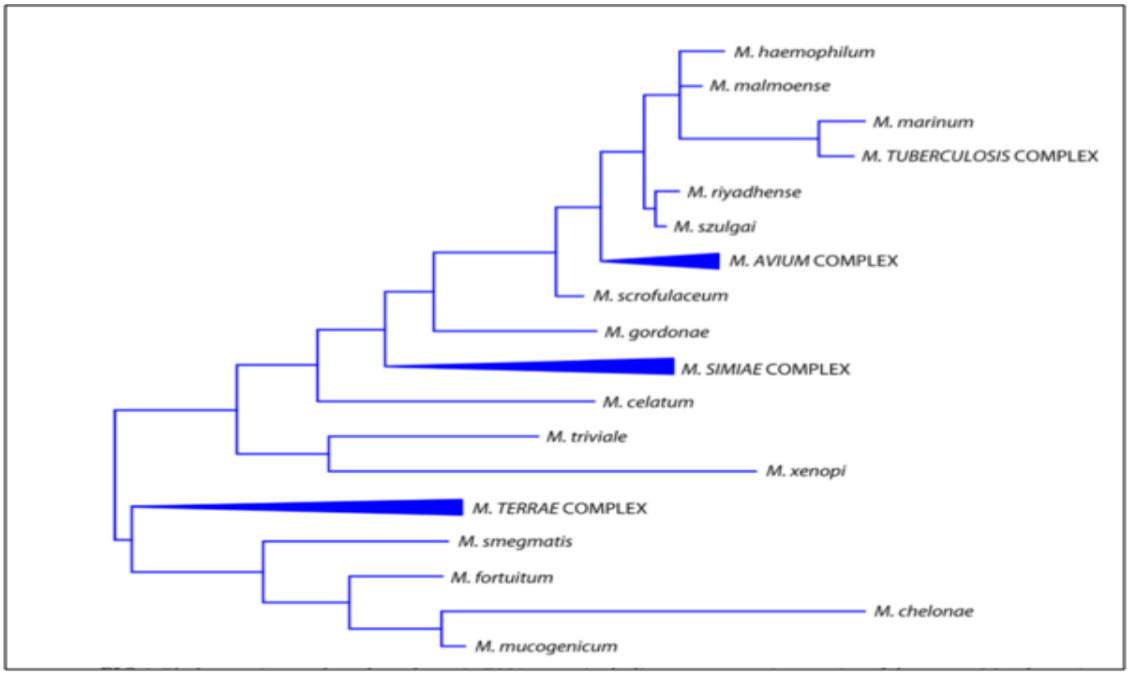


FIGURE 3. Phylogenetic tree of NTM, based on the 16S rRNA.(Adapted from Tortoli et al, 2014)

Table 5. Clinically important slowly growing NTM

***Mycobacterium avium* complex (MAC)**	*M.avium subsp. avium, M. avium subsp. paratuberculosis, M. avium subsp. silvaticum, M. avium subsp. hominisuis; M. intracellulare; M. chimaera, M. yongonense, M. timonense, M. marseillense, M. columbiense, M. arosiense, M. vulneris*
***M.simiae* complex**	*M.simiae, M.lentiflavum, M.sherrisii, M.heidelbergense, M.parmense, M.europaeum, M. parascrofulaceum, M.lentiflavum, M. triplex, M.florentinum*

***M. terrae* complex**	*M.terrae, M.nonchromogenicum, M.heraklionense, M.kumamotonense, M.longobardum, M.arupense, M.nonchromogenicum*
Others:*M.kansasii, M.scrofulaceum, M.marinum, M.malmoense, M.szulgai, M.celatum, M.interjectum, M.conspicuum, M.ulcerans, M.haemophilum, M.xenopi, M.malmoense*	

Table 6. Clinically important rapidly growing NTM

Six major groups of RGM [based on pigmentation and genetic relatedness]	**Species within group**
***Mycobacterium fortuitum* group**	*M. fortuitum, M. peregrinum, M. senegalense, M. setense, M. septicum, M. porcinum, M. houstonense, M. boenickei, M. brisbanense, M. neworleansense*
***Mycobacterium chelonae/ abscessus* group**	*M. chelonae, M. immunogenum, M. abscessus subsp. abscessus (formerly M. abscessus), M. abscessus subsp. bolletii (formerly M. massiliense and M. bolletii), M. salmoniphilum, "M. franklinii"*
***M. mucogenicum* group**	*M. mucogenicum, M. aubagnense, M. phocaicum*
***M. smegmatis* group**	*M. smegmatis (formerly M. smegmatis sensu stricto), M. goodii*
Early pigmented RGM	*M. neoaurum, M. canariasense, M. cosmeticum, M.*

	monacense, M. bacteremicum
***M. mageritense/M. wolinskyi* group**	*M. mageritense, M. wolinskyi*

3.3 STRUCTURE OF NON-TUBERCULOUS MYCOBACTERIA

The chemical nature of mycobacterial envelope is different from those of both Gram-positive and Gram-negative bacteria; for example, the lipid content of the cell envelope of Mycobacteria may represent up to 40% of the cell dry mass, compared to less than 5% in other Gram-positive bacteria and only 10% in Gram-negative bacteria(48).They give the property of acid fastness and the tendency of cells to clump together. Permeability to drugs and other metabolites is 10–100-fold lower than that of the notably impermeable bacillus like *Pseudomonas aeruginosa*.

Mycobacterial envelope consists of a plasma membrane, which is homologous to plasma membranes of other bacteria, surrounded by a complex wall of carbohydrates and lipids organized in an outer membrane bilayer, which is in turn surrounded by an outer layer,called ‘capsule’ in the case of pathogenic species(49). A compartment analogous to the periplasmic space in Gram-negative bacteria also exists in Mycobacteria. Cryo-electron microscopy,which maintained the integrity of the lipid structures, was performed for the detailed ultrastructural analysis of Mycobacteria (50).

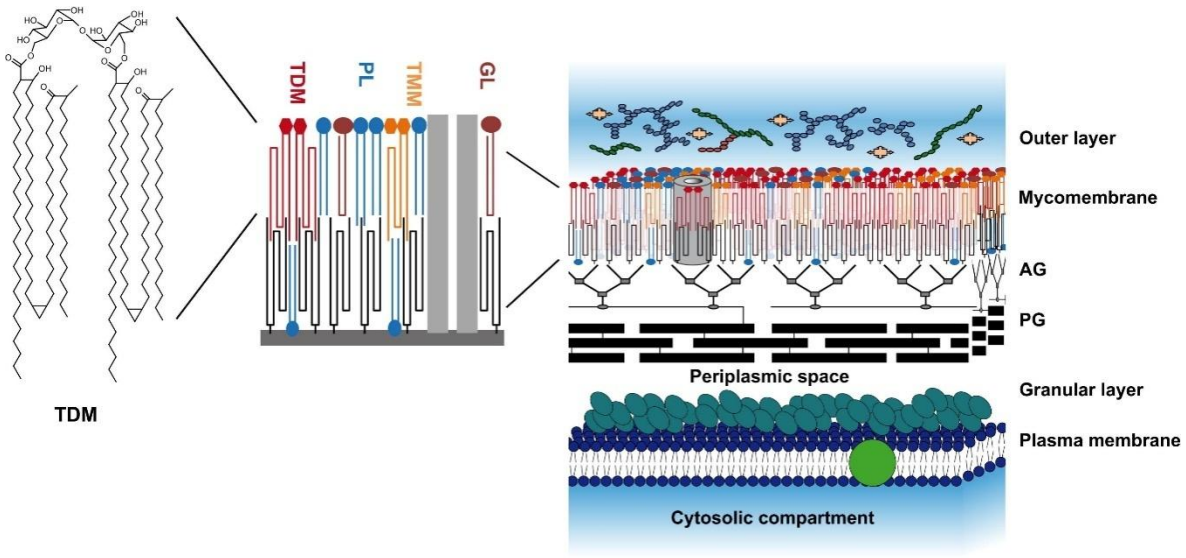


FIGURE 4. Cell wall structure of Mycobacteria.[AG, arabinogalactan; PG, peptidoglycan ;TDM- Trehalose dimycolate; PL- Phospholipid; TMM- Trehalose monomycolates; GL- Glycopeptolipid]-Adapted from Marrakchi *et al*, 2014 (51).

Plasma membrane: On visual inspection of ultrathin section, the plasma membrane of the Mycobacteria appears similar to other biological plasma membrane. Lipid composition of the plasma membranes between those of rapid- and slow-growing *Mycobacterium* species is the same. Polar lipids, mainly phospholipids, assemble into a lipid bilayer in association with the protein(52). Palmitic, octadecenoic and 10-methyloctadecanoic (or tuberculostearic) acid are the major fatty acid constituents. Main phospholipids (PL) are phosphatidylinositol mannosides, phosphatidylglycerol, cardiolipin and phosphatidylethanolamine.

Periplasmic space: It exist between the plasma membrane and cell wall. They are approximately 20-nm thick and they contain a granular layer.

Cell wall: The wall of mycobacteria is a tripartite complex which consists of a covalently linked 'cell wall skeleton', composed mainly of peptidoglycan (PG), arabinogalactan (AG) and Mycomembrane enclosed by a noncovalently linked capsule made up of polysaccharides and proteins. This is called the "mycolyl arabinogalactan peptidoglycan" (mAGP) complex(53). It gives shape to the cell.

Peptidoglycan: it is composed of repeating units of *N*-acetylglucosamine and *N*-acetyl/glycolylmuramic acid cross-linked by short peptides and has major amounts of meso-diaminopimelic acid, arabinose, and galactose.

Arabinogalactan: it is a complex, branched heteropolysaccharide. It contains a galactan chain composed of alternating 5- and 6-linked d-galactofuranosyl residues.

Mycolic acids of mycobacteria are very long-chain (60-90 carbon atoms) α-branched and β-hydroxylated fatty acids with an aliphatic side chain at position 2(49). On pyrolysis, mycolic acid methyl esters yield long-chain fatty acid methyl esters. The mycolic acid methyl esters of mycobacteria yield C22 to C26 fatty acid methyl esters on pyrolysis by gas chromatography. Non-mycobacterial mycolic acids have relatively simple structures and vary in chain length (22-74 carbon atoms) and in the number of cis double bonds (0-5) and release shorter fatty methyl acid esters.

The mycomembrane exhibits a non-conventional bilayer organization in which the inner leaflet is made of very long-chain Mycolic acid (MA) linked to Arabinogalactan (AG), which in turn is covalently attached to Peptidoglycan (PG). The outer leaflet of the mycomembrane is presumably composed of free - *i.e.* non-covalently bound to the cell – lipids – which include phospholipids (PL), trehalose monomycolates (TMM) and

trehalose dimycolates(TDM), glycopeptidolipids (GPL), and lipoglycans. The glycopeptidolipids (GPL) are produced by NTM but not *Mycobacterium tuberculosis*. The composition and concentration of GPL vary between NTM species and can impact colony morphology. *M. abscessus*-containing GPL show a smooth morphotype; those lackingGPL manifest as rough morphotypes. *M. abscessus* smooth variants survive in the environment, whereas the rough variant appears to arise only during the course of infection(54).To deter recognition by toll-like receptor 2 (TLR2) present on innate immune cells, GPL of smooth *M. abscessus* form an outer layer that covers the mycobacterial TLR2 ligand phosphatidyl-myo-inositol mannosides, effectively preventing the glycolipid from being recognized by TLR2 (55,56).

Outer layer (OL): it is also called as the capsule, in case of pathogenic species. The capsule of mycobacterial pathogens such as *Mycobacterium tuberculosis* is mainly composed of glucan and proteins, with only a tiny amount of lipids whereas the outer layer of non-pathogens is primarily constituted of proteins.

They can be stained with basic dyes such as carbol fuchsin and cannot be decolorized with acid-alcohol. This unique property is termed “acid-fastness” and is the basis of the Ziehl-Neelsen staining technique for the identification of mycobacteria. The acid-alcohol-fast property of these bacteria could be related to the lipid barrier of the wall mycolyl-arabinogalactan hindering the penetration of the acid. This indicates that differences in the degree of acid-alcohol fastness between different organisms might reflect variations in the chemical nature of their walls(57).

3.4 HABITAT OF NON-TUBERCULOUS MYCOBACTERIA

NTMs are ubiquitous in the natural and man-made environment. They are natural inhabitants of soils, rivers, lakes and streams(58). They enter the surface waters from the soil, especially acidic pine forest or coastal swamp soils. They have predilection for soil with an acidic pH and the presence of humic and fulvic acids, which stimulate their growth. Due to the presence of lipid-rich outer membrane, they are resistant to chlorine, chloramine and ozone. In fact, NTMs are 40-fold more resistant to chlorine than *Pseudomonas aeruginosa* and 500-fold more resistant to chlorine than *Escherichia coli*(59). Coupled with the ability to survive in low nutrient settings, it can multiply and reach end users of the water supply. NTM can also survive in the drinking water pipeline system through biofilm formation leading to its slow and persistent release. They can easily be aerosolized due to the hydrophobic nature and get released in showers, water taps, hot tubs or spas, and humidifiers of air conditioning system(60).

3.4.1 TRANSMISSION OF NON-TUBERCULOUS MYCOBACTERIA

Unlike *Mycobacterium tuberculosis*, there are no convincing data demonstrating human-to-human transmission of infection. Thus, the concept prevails that these organisms are acquired from the environment*via* inhalation (most common), ingestion, and skin-to-skin contact(61). Water is the primary source of MAC infection in humans(62). DNA-based fingerprints of *Mycobacterium avium* isolates from AIDS patients in a study were identical to those of isolates recovered from their drinking water(63). There are several theories regarding the increasing incidence of infection. One relates to showering rather

than bathing in a tub. Showering in a closed stall exposes the user to a higher aerosol concentration of NTM(64). In addition, to save energy, water heaters have lower temperatures now, which could allow more NTM growth in the water. Water filters may serve as a breeding ground for organisms and allow for higher concentrations of mycobacteria compared to unfiltered water(65). Furthermore, environmental exposure to these organisms must be common because data from several countries shows that a substantial fraction of children have delayed cutaneous hypersensitivity responses to MAC antigens, a fraction that increases with age. Finally, there are data to indicate that aspiration of water, through swallowing or gastroesophageal reflux, is a way that mycobacteria gain access to the lungs to cause disease(66).

Skin and soft tissue infections usually occur due to contact with water and direct inoculation with contaminated intravenous infusion fluids and medicines or unsterile injection needles or during trauma (67,68).

3.4.2 RISK FACTORS ASSOCIATED WITH NTM DISEASE

Although it is not clear why NTM diseases have been increasing, there are several contributing factors, such as, an increase of mycobacterial infection sources in the environment, an increase in the number of susceptible individuals, improvements of laboratory detection techniques, and increased awareness of NTM diseases(69). Although they are found easily in water and soil, infections are not common in the human population. Most NTM are significantly less pathogenic than *Mycobacterium tuberculosis* and require some degree of host impairment to result in disease. The host

impairment can either be chronic lung disease or defects in the immune system of which the former is more common. NTM disease has been described in association with prior tuberculosis, bronchiectasis, cavitary lung disease, chronic obstructive pulmonary disease including 1-antitrypsin deficiency, cystic fibrosis, pneumoconiosis, pulmonary alveolar proteinosis and chronic lung injury due to aspiration from gastro-esophageal disorders(29,70,71). A genetic link has been hypothesized as many females who develop bronchiectasis and NTM infection share a similar body type characterized by tall slender stature with a higher frequency of pectus excavatum, kyphoscoliosis and mitral valve prolapse than females who do not have NTM infection. This condition was first described in 1989 and later referred to as the ‘‘Lady Windermere Syndrome’. referring to the chronic suppression of normal cough reflex as portrayed by the character(72,73). A number of inherited and acquired defects in the host immune response, particularly those that affect the Th1 cell and macrophage pathway, have been associated with increased susceptibility to NTM infections. Many of these host immune defects are associated especially with disseminated infection.

Table 7. Host immune defects associated with disseminated NTM infection

1. Mutations in interferon (IFN)- γ receptor 1, IFN-γ receptor 2, interleukin (IL)-12 p40 and the IL-12 receptor
2. Mendelian susceptibility to mycobacterial diseases (MSMD), is caused by genetic defects in the mononuclear phagocyte/T helper cell type 1 (Th1) pathway
3. Auto-antibodies to interferon gamma

4. CD4 lymphopenia due to HIV or other causes
5. Use of TNF-alpha inhibitors, particularly infliximab and adalimumab
6. Signal transducer and activator of transcription 1 (STAT1) deficiency

Protective factors-Epidemiologic studies from Europe have suggested that childhood immunization with Bacillus Calmette-Guerin (BCG) vaccine is associated with a reduced risk of childhood cervical lymphadenitis due to NTM(74).

3.5 CLINICAL PRESENTATION

In broad terms, NTM cause four distinct clinical syndromes:

●Progressive pulmonary disease in older persons caused primarily by *Mycobacterium avium* complex (MAC) and *Mycobacterium kansasii*

●Superficial lymphadenitis, especially cervical lymphadenitis, caused mostly by MAC and*Mycobacterium scrofulaceum*.

●Skin and soft tissue infection usually as a consequence of direct inoculation.

●Disseminated disease in severely immunocompromised patients.

Table 8.Diseases caused by NTM and the etiologic species:

Clinical disease	**Etiologic species**		
	Common	**Unusual**	
Chronic bronchopulmonary disease	MAC *M.kansasii* *M.abscessus* *M.malmoense* *M.xenopi*	*M.simiae* *M.szulgai* *M.fortuitum* *M.celatum* *M.gordonae*	*M.asiaticum* *M.shimodii* *M.smegmatis* *M.haemophilum*

Lymphadenitis	MAC *M.scrofulaceum* *M.malmoense*	*M.fortuitum* *M.chelonae* *M.kansasii* *M.abscessus* *M.haemophilum*	*M.lentiflavum* *M.interjectum* *M.heidelbergense* *M.bohemicum*
Skin and soft tissue disease	*M.ulcerans* *M.marinum* *M.chelonae* *M.abscessus* *M.fortuitum*	*M.kansasii* *M.haemophilum* *M.malmoense* *M.smegmatis*	
Otitis media	*M.abscessus* *M.chelonae*		
Tenosynovitis	MAC *M.marinum*	*M.fortuitum* *M.abscessus* *M.chelonae*	*M.kansasii* *M.haemophilum* *M.scofulaceum*
Osteomyelitis	*M.fortuitum* *M.abscessus*	*M.xenopi* *M.marinum* *M.smegmatis* *M.nonchromogenicum*	*M.malmoense* *M.xenopi* *M.szulgai* *M.kansasii*
Catheter related infections	*M.fortuitum* *M.abscessus* *M.chelonae*	*M.mucogenicum* *M.neoaurum* *M.aurum*	
Prosthetic valve infections	*M.fortuitum* *M.chelonae*	*M.gordonae*	
Surgical site infections	*M.fortuitum*	*M.simiae*	

	M.abscessus
	M.chelonae

Reference: Wagner et al,2004(75)

3.5.1 PULMONARY DISEASE

NTM can cause three distinct types of pulmonary disease: fibrocavitary disease, nodular-bronchiectatic disease and hypersensitivity pneumonitis. When NTM lung disease initially gained interest in the late 1950s, it was mainly a disease of men who were miners and smokers; their upper lobe cavitary disease was difficult to distinguish from pulmonary tuberculosis. They were older than the usual population at the sanatoria with limited response to treatment. During the 1980s, the nodular bronchiectatic form was first noticed in mainly female patients without a history of chronic lung disease, defined by nodular and bronchiectatic lesions radiologically and predominantly affecting the middle lobe and lingula. More than a decade later, hypersensitivity pneumonitis was reported, often related to exposure to aerosols generated by indoor hot tubs or metalworking fluid. Most common agents associated with pulmonary disease are *Mycobacterium avium, Mycobacterium intracellulare, Mycobacterium. kansasii, Mycobacterium malmoense* (Europe), *Mycobacterium xenopi* (outbreaks linked to hot water systems in buildings), and *Mycobacterium simiae* (linked to groundwater sources).

In the more familiar fibrocavitary form, upper lobe disease occurs most commonly in older male smokers with chronic pulmonary symptoms due to underlying lung disease.

Symptoms and radiographic changes may be difficult to differentiate from the underlying disease. It usually progresses faster than the nodular type. These patients are almost universally diagnosed as a consequence of suspected tuberculosis.

The nodular-bronchiectatic form of MAC lung disease appears most frequently in nonsmoking women over the age of 50 who do not have a history of underlying lung disease, but invariably have bronchiectasis at the time of NTM lung disease diagnosis. Body morphotype is a possible predisposition to this second form of disease. Some patients with fibronodular NTM lung disease appear to have similar clinical characteristics and body types, including scoliosis, pectus excavatum, mitral valve prolapse, and joint hypermobility(76,77).

Hypersensitivity pneumonitis is an occupational disease very frequent among people using metal-working fluids.Cases have also been described in immunocompetent individuals with history of exposure to hot water aerosols from hot tubs/spa pools (known as “hot tub lung”), showers, and swimming pools. It has a subacute presentation with dyspnea and fever. Rapid growers,in particular *Mycobacterium immunogenum*, are most frequently involved(78).

Patients afflicted with cystic fibrosis, a genetic disease, are highly susceptible to bacterial infections of the chest. Infections as a result of NTM have increasingly been reported with several species, MAC being the most common, followed by *Mycobacterium abscessus*in patients with*CFTR* gene (79).

In lipoid pneumonia, which is a specific form of lung inflammation that develops when lipids enter the bronchial tree, a strong association with the isolation of mycobacteria, predominantly comprising rapid growers, has been reported, although a causal correlation has not been demonstrated(80).

3.5.2 LYMPHADENITIS

Lymphadenitis caused by NTM usually affects lymph nodes at a single site. The submandibular and cervical lymph nodes are most frequently affected, although axillary and inguinal lymphadenitis have been observed. Patients are typically, though not exclusively, children, under the age of 8 years. This disease is relatively benign and most patients present with an enlarged lymph node without constitutional symptoms; in more advanced disease, fluctuating masses with violaceous overlying skin are seen. Even if multiple nodes are involved, disease is usually unilateral(81). Until mid-1980s, the majority of cases were caused by *Mycobacterium scrofulaceum*, but now *Mycobacterium avium* is the most common pathogen throughout the developed world(25).

3.5.3 SKIN AND SOFT TISSUE INFECTIONS

Localized skin and soft tissue infections due to NTM can be divided into three main disease phenotypes; two of them are named diseases.

Localized NTM skin disease such as the wound or injection site infections are the first type. These usually follow medical procedures (contaminated fluids, anesthetic, prosthesis like mesh), tattooing or cosmetic therapies that involve incisions in or

injections into skin or subcutaneous fat and inoculation of contaminated products. Outbreaks have also been noted in beauty salons where foot baths were heavily contaminated with NTM. Rapid growers like*Mycobacterium fortuitum*, *Mycobacterium chelonae* and*Mycobacterium abscessus*are the most frequent causative agents of these diseases, but cases caused by recently described new species, such as *Mycobacterium goodii* and *Mycobacterium massiliense*are increasingly been reported(80,81).

The second is **Buruli ulcer**, a severe skin disease caused by *Mycobacterium ulcerans* that progresses from nodular skin lesions into large ulcers. This disease is endemic to parts of West Africa and Australia (its former name was Bairnsdale ulcer), but is also seen in specific foci in Latin America and China.

The third is **fish tank granuloma** (previously also called **swimming pool granuloma**) caused by infection of existing skin abrasions by *Mycobacterium marinum*, acquired during fish- or water-related activities. This usually leads to a single 1-3 cm diameter, papulo-nodular, verrucous, or ulcerated granulomatous lesion, mostly on the hand or lower arm. The skin disease caused by *Mycobacterium marinum* may progress to form multiple lesions in a typical sporotrichoid pattern. In immunocompromised patients, the infection can spread to underlying bony structures, joints and regional lymph nodes.

3.5.4 DISSEMINATED INFECTIONS

Disseminated mycobacterial infections develop almost always in severely immunocompromised people; the best known are those that affect HIV-infected patients.

The respiratory apparatus and the gastrointestinal tract are the two major routes of infection. The main symptoms and signs include high fever, diarrhoea, weight loss, abdominal pain, sweating, hiccups, anemia, hepatomegaly and splenomegaly. The progression of disease may be very rapid and even fatal.

Disseminated MAC was one of the first opportunistic infections detected in AIDS patients; it has been estimated to affect approximately 20-40% of subjects with CD4 lymphocyte counts less than 50/µL in the absence of effective anti-retroviral therapy(82). In such patients, infections caused by *M. avium* are four-fold more frequent than those due to M. *intracellulare*. Various NTM species are responsible for disseminated infections in AIDS patients; among them, *M. genavense,* a species which remains frequently undiagnosed because of its inability to grow on conventional solid media, has been estimated to be involved in as high as 10% of the cases attributable to MAC(83).

Currently, disseminated NTM disease is quite rare in HIV-infected patients due to the treatment withHighly Active Antiretroviral Therapy (HAART). An important predisposing condition to disseminated mycobacterial infections is the immunosuppression established to prevent the rejection of transplanted organs. Disseminated mycobacterial infectionsmay develop concomitantly with anticancer therapy. They originate, in most cases, from infected catheters and should be suspected when a patient remains febrile despite negative blood cultures.

Mycobacterium chimaera is an emerging MAC species that has been generally been thought to have relatively low virulence, at least as a pulmonary pathogen. Two outbreaks of prosthetic valve infections and associated disseminated infection after cardiac surgery were associated with *Mycobacterium chimaera*, in which the source was determined through epidemiologic and molecular analysis to be the heater-cooler unit used for cardiac bypass procedures, were first described in 2015 in Europe and the United States and subsequent clusters have been reported elsewhere(84).

Catheter-related sepsis, mainly as a result of rapidly growing mycobacteria, has also been reported in hemodialysis patients and in surgical patients, primarily following cardiac operations.

In recent years, the administration of antibodies against interferon (IFN)-γ has become a frequent practice for the treatment of many immune mediated inflammatory diseases. A side effect of the impairment of macrophage activation, consequent to the biological therapy, can predispose to mycobacterial infections. A number of cases, mostly disseminated, have been reported, some of which do not respond to treatment(80).

3.6 LABORATORY DIAGNOSIS

Since NTM are ubiquitous in environment, itsisolation from non-sterile body sites especially the respiratory tract does not imply true infection or disease, per se. Repetitive isolation, signs of clinical disease, radiological abnormalities, the exact species isolated and predisposing conditions of the patient involved are all helpful in determining whether

the isolated mycobacteria are to be considered as the causative agent of the patient's disease. The American Thoracic Society has published a set of diagnostic criteria to aid in the diagnosis of NTM. A combination of clinical, radiologic, and bacteriologic features has been suggested for diagnosis of true pulmonary NTM disease. Evaluation should include, beside appropriate history and physical examination, chest imaging using high- resolution computed tomography (HRCT), unless there is obvious cavitation by chest X ray. HRCT views may show scattered areas of bronchiectasis and often mixed nodular and cavitary infiltrates, with an occasional "tree in bud" pattern. At least three sputum samples need to be collected for AFB smear and culture. These criteria are established the best for disease caused by MAC, *M. kansasii*, and *M. abscessus*. If possible, NTM should be identified at the species level.

Table 9.Clinical and microbiological criteria for diagnosing nontuberculous mycobacterial lung disease

Clinical (both required)

1. Pulmonary symptoms, nodular or cavitary opacities on chest radiograph, or a high-resolution computed tomography scan(HRCT) that shows multifocal bronchiectasis with multiple small nodules AND
2. Appropriate exclusion of other diagnoses.

Microbiological

1. Positive culture results from at least two separate expectorated sputum samples; if the results from one are nondiagnostic, repeating sputum smears

and cultures should be considered. OR

2. Positive culture result from at least one bronchial wash or lavage. OR
3. Transbronchial or other lung biopsy with mycobacterial histopathological features (granulomatous inflammation or acid-fast bacilli) and positive culture for NTM or biopsy showing mycobacterial histopathological features (granulomatous inflammation or acid-fast bacilli) and one or more sputum or bronchial washings that are culture positive for NTM.

Additional considerations

4. Expert consultation should be obtained when NTM are recovered that are either infrequently encountered or that usually represent environmental contamination.
5. Patients who are suspected of having NTM lung disease but do not meet the diagnostic criteria should be followed up until the diagnosis is firmly established or excluded.
6. Making the diagnosis of NTM lung disease does not, *per se,* necessitate the institution of therapy, which is a decision based on the potential risks and benefits of therapy for individual patients.

Ref: Griffith et al**.,** An Official ATS/IDSA Statement: diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases,2007(44)

3.6.1 IMAGING

The radiographic pattern of disease can usually be separated into predominantly cavitary versus nodular/bronchiectatic. Some generalizations can be made: Cavitary disease in the upper lung zones, similar to pulmonary tuberculosis, is seen in approximately 90 percent of patients with *M. kansasii* infection and perhaps 50 percent of those with MACinfection. The cavities caused by these organisms tend to have thinner walls and less parenchymal opacity surrounding it than those caused by *M. tuberculosis*(85,86).

At least 50 percent of patients with MAC lung disease have radiographic abnormalities characterized by nodules associated with bronchiectasis or nodular or bronchiectatic disease. The nodules and bronchiectasis are usually present within the same lobe and occur most frequently in the right middle lobe and lingula(87).

Solitary nodules and dense consolidation have also been described. Pleural effusions are uncommon, but reactive pleural thickening is frequently seen.

3.6.2 SMEAR MICROSCOPY

Acid-fast microscopy is the fastest, easiest, and least-expensive tool for the rapid identification of patients with mycobacterial infections, and semiquantitative results of smear examinations may be an important aid in determining the clinical significance of specimens with NTM isolates. Bacterial cells are generally pleomorphic, ranging from long, filamentous forms to short, thick rods. Branching typically is absent or rudimentary. However, microscopy is unable to distinguish withinthe Mycobacterium genus and

between viable and nonviable mycobacteria. Thesensitivity of microscopy is influenced by numerous factors, such as type of specimen, quality of specimen collection, number of mycobacteria present in the specimen, method of processing (direct or concentrated), method of centrifugation, staining technique and quality of examination.

Ziehl-Neelsen (ZN) staining has a low sensitivity of 22–43% for a single smear. Maximum sensitivity is up to 60% when compared with that of cultures. The threshold of detection of AFB in sputum is 10^4 to 10^5 bacilli per ml. To overcome the above-mentioned disadvantages of conventional method of staining, Light-Emitting Diode (LED) fluorescence microscope was introduced. It uses an auramine-rhodamine or auramine O dye (acid-fast fluorochrome), weaker acid as a decolorizer, and a counter stain with a quencher such as potassium permanganate. Fluorescent microscopy has 80-90% sensitivity compared to culture(88). LEDfluorescent microscopy has been recommended by World Health Organisation(WHO) in 2010 as the preferred microscopic technique in high burden labs (44,89).

3.6.3 CULTURE

Sputum, induced sputum, bronchial washings, bronchoalveolar lavage or transbronchial biopsy samples can be used to evaluate individuals suspected to have NTM-pulmonary disease. Whenever possible, less invasive sampling should be attempted first to minimize procedural risks. Respiratory samples should be processed within 24 hours of collection (or refrigerated at 4°C if delays are anticipated) (44).Other samples to be collected are lymph node biopsy, blood, exudate, tissue biopsy etc. depending on clinical scenario.

Both liquid and solid media are used for mycobacterial culture. Cultures grown on solid media allow for the observation of colony morphology, growth rates, species categorization based on pigmentation, and quantitation of the infecting organism. Liquid systems are more sensitive and reduce the delay in the detection of NTM, but they are prone to contamination by other microorganisms and bacterial overgrowth(44).

Positive sputum cultures for NTM must be interpreted cautiously since these organisms have variable virulence and can be recovered from the respiratory tract without causing progressive infection (transient infection or colonization). In addition, NTM are common in the natural environment and may contaminate laboratory specimens. Tap water especially (liquid or frozen) may contain NTM and contaminate clinical and laboratory specimens.

NTM frequently occurs in the context of underlying lung disease as in concurrent tuberculosis, which can also cause symptoms. As a result, the distinction between transient infection or contamination and true infection is sometimes difficult. Isolation of *M. gordonae, M. mucogenicum, M. nonchromogenicum, M. haemophilum, M. flavescens, M. gastri, M. terrae, or M. triviale* indicates probable colonization or contamination. These organisms are generally not pathogenic for humans except in the context of severe cellular immunodeficiency, such as AIDS. Expert consultation should be obtained when NTM are recovered that are either infrequently encountered or that usually represent environmental contamination(44). Fortunately, lung disease caused by NTM is often indolent, so careful assessment over time is possible.

Lowenstein-Jensen (LJ) is a conventional medium which is excellent for the growth of both *Mycobacterium tuberculosis* and NTM(44). Guidelines and Recommendation for culture of NTM as per ATS (American Thoracic Society) include two types of medium, one solid medium (LJ or Middlebrook medium) and one liquid medium culture system BACTEC (Becton-Dickenson Diagnostics), MB Redox (Heipha Diagnostika), BacT/ALERT® MP (bioMerieux, France), MGIT (BD Diagnostics) or Septi-check (BD Diagnostics).Liquid media require additives such as OADC in order to provide enrichment (mixture of bovine albumin, dextrose, catalase, and oleic acid) and PANTA antibiotic (mixture of polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin) to inhibit the growth of contaminants.The conventional method for the growth of mycobacteria is time-consuming (6–8 weeks), but they are considered "gold standard." Liquid-based culture has high sensitivity because the growth of mycobacteria can be detected within 1–2 weeks. So, they are always used in combination with the conventional LJ method. The final report can be sent after 6 weeks if no growth is detected in the liquid-based culture and 8 weeks of incubation in case of the LJ slants(88).

Slowly growing mycobacteria need at least 7 days for the growth to appear on solid media. Chromogenic NTM species produce pigment that can range from light yellow and yellow to orange or even rose-colored. Within the pigmented mycobacteria, the photochromogens are nonpigmented in the dark and produce pigmentation only after exposure to light. The scotochromogens produce pigment when grown in the dark. The

color may intensify with the increasing age of the culture. The nonchromogenic species do notproduce pigmentation when grown either in the dark or after exposure to light(57).

Mycobacteria are categorized as rapid growers, by definition, if they grow on a solid medium within 7 days after subculture. The young colonies appear smooth and hemispherical, usually with a butyrous or waxy consistency. Colonies of *M. fortuitum* group and *M. chelonae/M. abscessus* group are typically nonpigmented but may appear off-white or faintly cream-colored. Some strains produce rough colonies with short aerial hyphae, best observed under a stereomicroscope. The above mycobacteria often have the capability of growing on MacConkey agar without crystal violet. Additionally, they may grow on routine 5% sheep blood agar, initially appearing as tiny pinpoint colonies. Some strains of *M. fortuitum* may grow within 48 hours. Microbiologists must perform acid-fast staining in addition to Gram's stain if the correct identification is to be made. The bacterial cells appear as short, slender, irregularly staining gram-positive bacilli(90).

Mycobacterium species differ in the ability to grow at certain temperatures. For slowly growing species, the minimum set of temperatures for incubation comprise 30 ± 1°C and 36 ± 1°C. Most slowly growing mycobacterial species grow well at 35 to 37°C, but others, including *M. marinum*, grow better at a lower temperature (30°C), especially on primary isolation. Therefore, samples from skin or lymph nodes and other tissue specimens from the body periphery should routinely be incubated both at 30 ± 1°C and at 36 ± 1°C. Additional media incubated at 22 to 25°C and 42°C may be necessary for

optimal growth of some species, such as *M. haemophilum* (28 to 32°C), *M. xenopi* (42°C) and *M. conspicuum* (22 to 31°C)(57).

3.6.4 IMMUNOCHROMATOGRAPHIC TEST

MPT 64 TB Ag Kit is a simple and rapid immunochromatographic assay developed by SD Bioline, which facilitates detection and differentiation of *M. tuberculosis* complex isolates from NTM.Studies reported a sensitivity of up to 97-100% and specificity of up to 100%. However, it cannot be used for speciation of NTM(91,92).

3.6.5 SPECIES IDENTIFICATION

Because treatments and outcomes differ depending on the NTM species, the accurate identification of NTM species is very important(93,94) . Traditional biochemical tests and high-performance liquid chromatography for NTM identification have been replaced by molecular methods such as line probe assay, PCR-RFLP (restriction fragment length polymorphism), real-time PCR, DNA sequencing, and matrix-assisted laser desorption ionization–time of flight (MALDI-TOF)mass spectrometry.

3.6.6 BIOCHEMICAL IDENTIFICATION

The rate of growth, pigmentation of colonies, and various biochemical reactions are used for phenotypic identification of NTM species.

Table 10. Key biochemical tests for NTM species identification

1. Nitrate reductase activity
2. Niacin accumulation test
3. Tween 80 hydrolysis
4. Growth on MacConkey agar
5. Tellurite reduction test
6. Urease activity
7. Catalase activity at 68°C
8. Arylsulfatase production
9. Tolerance to 5% NaCl
10. Pyrazinamidase activity
11. ß-glucosidase activity

Reference- Koneman's Colour Atlas, 7thedition(90).

The utility of the tests is decreasing currently, especially for the slow growers, as most of the novel species cannot be identified using the biochemical tests.

3.6.7 MOLECULAR METHODS:

Molecular methods are needed for identification of newer species of NTM that are implicated in human infection. However, phenotypic characteristics of NTM such as colony morphology, pigmentation, and growth rate must be correlated with molecular identification. Molecular testing is based on the principle of nucleic acid amplification which allows faster and precise identification of the mycobacterium species.

3.6.8 LINE PROBE ASSAY

Line probe assays are based on nucleic acid amplification and reverse hybridization to detect various species of NTM from clinical samples and culture isolates. The commercial assays available are GenoType®Mycobacterium common mycobacteria/additional species (CM/AS) assay (Hain Lifescience) and INNOLiPA Mycobacteria assay (Innogenetics). This method targets the 23S ribosomal RNA gene region, followed by reverse hybridization on nitrocellulose membrane strips.GenoType®Mycobacterium CM recognizes 15 common *Mycobacterium* species, including *M. tuberculosis* complex whereas the GenoType®MycobacteriumAS is able to identify 16 additional less common NTM species. In this technology, the target sequences are first amplified by PCR using biotinylated primers.INNO-LiPA MYCOBACTERIA V2 (Innogenetics, Gent, Belgium) is able to determine the presence of mycobacteria at the genus level for 16 different species using a broad-range PCR directed against the 16S to 23S internal transcribed spacer region. The multiple probes are immobilized on a nitrocellulose strip, and the amplified product is applied to the strip, which is the reverse of a Southern blot. Lines form at the site of amplicon-probe hybridization. Evaluation of GenoType®Mycobacterium CM/AS assay showed that sensitivity and specificity were 97.9% and 92.4% for the CM kit and 99.3% and 99.4% for the AS kit(95). INNO-LiPA was evaluated and found to be 100% sensitive and 94% specific(96).

Disadvantages: Laborious and needs both a good molecular biology set up and trained laboratory personnel.Members of the *M. abscessus* complex cannot be differentiated using this assay. LPA has been found to perform better on culture isolates.

3.6.9 MALDI-TOF

Matrix-assisted laser desorption ionization–time of flight(MALDI-TOF) massspectrometry is revolutionizing the identification of microorganisms and has been recently applied to the identification of mycobacteria. MALDI-TOF mass spectrometry analysis of mycobacteria involves several steps including inactivation, extraction, and analysis. Several different methods have been described for the inactivation and extraction of mycobacterial cells. Inactivation can be done by ethanol or heat or mechanical lysis using either a micropestle or by bead beating techniques. Extraction is performed by adding formic acid and acetonitrile to the inactivated mycobacterial cells. All inactivation and extraction steps should be performed within a BSL- 3 facility using the appropriate personal protective equipment (PPE). Following inactivation and extraction, an aliquot is spotted onto a solid target support plate and overlaid with a chemical matrix (for example, alpha-cyano-4-hydroxy-cinnamic acid). The sample plate is loaded into the instrument, and mycobacterial proteins are ionized using a laser. The ionized proteins then move through the time of flight chamber and are separated based on the mass-to-charge ratio of the ions, such that the lighter proteins hit the detector first, followed by the heavier proteins. A mass spectrum is produced as the ions collide with the ion detector and serves as a “fingerprint” for identification of the mycobacterial

species. Currently, there are two commercial MALDI-TOF platforms available for microbial identification, the Vitek®MS (bioMerieux,NC) and the Biotyper system (Bruker Daltonics,MA). Databases supplied with both instruments contain entries for many NTMs, including those most commonly encountered in the clinical laboratory. Recent studies have demonstrated the ability of MALDI-TOF mass spectrometry to serve as a rapid and accurate means for the identification of mycobacteria from both solid and liquid media(97–99).

Advantages: According to a study using the latest version of the database, 94% of the mycobacterial isolates tested can be identified up to the species or complex level(100). Although the cost of MALDI-TOF mass spectrometry instrument is not insignificant, studies have shown that this technique has minimal associated reagent costs, requires minimal technologist time, can be utilized for a wide variety of microorganisms, and provides a reduction in turnaround time for organism identification, leading to an overall reduction in costs.

Disadvantages:A potential limitation in comparison to molecular sequencing methods is the requirement for a moderate amount of growth present on solid media rather than the scant growth required for sequencing. This problem is most evident when identifying the slowly growing mycobacteria. (57)

3.6.10 DNA SEQUENCING

Gene sequencing is the reference method for the identification of NTM species and may be performed for rare species or for precise identification at the subspecies level. Sequencing of the 16SrRNAgene allows discrimination at the species level or to the

complex level, such as for MAC. However, single-target sequencing cannot be used to accurately differentiate species, and for a higher level of discrimination, up to the subspecies level, sequencing of several key targets, such as *hsp65*, *rpoB*, and the 16S-23S internal transcribed spacer, is required(101).

16S rRNA sequencing:

The 16SrRNA gene is composed of approximately 1,500 base pairs. It is a highly conserved gene composed of two major sequences known as regions A and B. For mycobacterial species identification, partial sequencing of region A, which contains most of the species-specific sequence variations ("signature sequences"), is often adequate, while region B maybe confirmatory. This has been used to achieve rapid and accurate identification of the most common clinically relevant mycobacteria. After DNA extraction from culture, a PCR is performed using the specific primers. The sequence is then determined through capillary-based sequencing, and the product is submitted as a query to a genetic database that is maintained and updated by the manufacturer. All of the RGM (except for *M. chelonae* and the *M.abscessus* group, which each contain one copy) contain two copies of the 16S rRNA gene(101). Generally, members of the genus Mycobacterium are closely related to each other and may differ by only a few base pairs or none. However, differentiation of *M. chelonae* and *M. abscessus* and some species within the *M. fortuitum* group requires sequencing of 16S rRNA gene sites outside regions A and B. *M. chelonae* and *M. abscessus* differ by only 4 bp in the 16S gene, so complete 16S rRNA sequence analysis is required for species identification unless other gene targets are sequenced(90,102).

ITS 1 Region sequencing:

Another target is the internal transcribed spacer sequence, which separates the 16S and 23S rRNA genes and is denominated ITS 1. The sequence of this fragment comprises only 200 to 330 bp and thus can easily be analyzed. Several sets of primers that enable the amplification and sequencing of the complete fragment have been published(103). For the ITS 1 sequence, a high variability that is used for species identification has been shown. Two or more sequence variants have been observedfor some species, mainly for rapidly growing species and occasional slowly growing species *(M. simiae* and *M. xenopi*). As with the 16S rRNA gene sequence, *M. marinum* and *M. ulcerans* have identical ITS sequences and thus cannot be differentiated by this analysis(57,104).

***hsp65* genesequencing:**

The 65-kDa heat shock protein gene (*hsp65*) also has hypervariable regions, the sequences of which may be used for identification to the species level. Like the *hsp65* PRA(PCR Restriction fragment Analysis), sequence analysis of this gene involves the same 441-bp segment (105). *hsp65* is not as well-conserved as the 16S rRNA gene sequence. But it has proven useful for species-level identification of closely related RGM isolates, such as *M. abscessus* and *M. chelonae*. These two species differ by only 3 of approximately 1,500 base pairs of the 16S rRNA gene, while they differ by almost 30 base pairs in the 441-bp *hsp65* gene. In addition, species such *as M. fortuitum, M. septicum, M. peregrinum, M. houstonense*, and *M. senegalense* are more readily discriminated by hsp65 gene analysis than by 16S rRNA gene analysis (92). As with all

gene sequencing methods, the integrity and updating of the database for *hsp65* gene sequences remains the major limitation for this method(42).

***rpoB* gene sequencing:**

The *rpoB* gene is a single-copy gene that encodes the β subunit of RNA polymerase and has been used recently in the identification of RGM, including the identification of several new species (106,107).Sequencing of the *rpoB* gene has an advantage over16S rRNA sequencing in that a single site without a deletion or insertion is usually small enough to be sequenced directly in both directions at one time and contains enough information to identify many of the RGM to the species level. Recent studies have shown that the complete *rpoB* sequences vary from 84.3% to 96.6% for most species, compared to a 95.7% to 99.7% variation by sequencing of the 16S rRNA gene(108).The most commonly used sequence for RGM is a 723-bp fragment in region V(106). Just as for other sequence databases, a sufficient, updated, and quality-controlled database is necessary for accurate identification of RGM to the species level.

3.6.11 OTHER MOLECULAR TESTS FOR NTM

Sequence analysis of target genes for the identification of mycobacteria may not be practical for routine clinical laboratories. Commercially available assays that are based on liquid- or solid-phase hybridization have been shown to be easily implemented into a routine workflow. They are intended for the detection of some of the most common*Mycobacterium* species and can be performed from both solid and liquid media.

Identification using AccuProbe- The AccuProbe test was the first commercial molecular assay for the identification of selected mycobacterial species from positive culture media. Probes are available for the identification of *Mycobacterium tuberculosis* complex, *M. avium, M. intracellulare*, MAC, *M. gordonae*, and *M. kansasii*. All probes are FDA cleared and commercially available. Species identification is based on the hybridization of specific DNA probes to rRNA of the bacteria. Briefly, by heat treatment and sonication, nucleic acids, including the target 16S rRNA, are released from the mycobacteria. A specific DNA probe hybridizes with the target rRNA. Finally, the DNA-rRNA hybrid molecule can be detected by chemiluminescence. The results are obtained within 2 hours. The utility of these tests has been proven in many studies and by usage in many laboratories worldwide. Specificity of the test varies from 90-100% and sensitivity is nearly 100 % (109,110).

Disadvantages: The probes are limited to a few, albeit important, mycobacterial species, necessitating the performance of additional tests for identification of species for which there are no probes. The need to perform individual tests for each target species renders the tests expensive and, if not performed in parallel, also time-consuming. Studies have also shown cross-reactions between the *M. intracellulare* probe andseveral other slowly growing mycobacterial species(57).

3.7 DRUG SUSCEPTIBILITY TESTING

Drug susceptibility testing (DST) for NTM is difficult and controversial because of discrepancies between in vitro susceptibility and in vivo clinical outcomes. The gold

standard method for DST of NTM is broth microdilutionperformed according to a standardized protocol from the Clinical and Laboratory Standards Institute. The Minimum Inhibitory Concentration (MIC), expressed in µg/ml,have been determined using concentrations derived from serial twofold dilutions indexed to base 1 (e.g., 1, 2, 4, 8, 16, and 32 µg/ml). It represents the "true value" which is the closest agreement between the average value obtained from a large series of test results and an accepted reference value. The "true value" is somewhere between the lowest test concentration that inhibits the growth of organism (i.e. MIC reading) and the next lower test concentration(111).

For **slowly growing NTM** such as MAC, macrolide susceptibility testing, specifically clarithromycin is recommended as the class agent for DST. Macrolide resistance in MAC is caused by mutations in the macrolide binding site of 23S rRNA. Rifampin and clarithromycin are the currently recommended drugs for primary susceptibility testing for *M. kansasii*. DST reading is taken at 10- 14 days, if growth controls are satisfactory.

For **rapidly growing mycobacteria,** the agents that should be tested are amikacin, cefoxitin, ciprofloxacin, clarithromycin, doxycycline (or minocycline), imipenem, linezolid, moxifloxacin, trimethoprim-sulfamethoxazole, and tobramycin. Readings of DST is taken at 3-5 days, provided the growth controls are satisfactory.

Inducible macrolide resistance to clarithromycin is observed during in vitro DST after prolonged incubation (susceptible at day 3 but resistant at day 14) or after preincubation in macrolide-containing media. Therefore,it is recommended that the final reading for

clarithromycin be performed after at least 14 days. This inducible resistance to clarithromycin is due to a functioning erythromycin ribosomal methylase *erm*(41) gene, which is present in most strains of *M. abscessussubsp. abscessus* but not in *M.abscessus subsp. massiliense*(111,112).

3.8 MANAGEMENT OF NTM DISEASES

A diagnosis of NTM lung disease does not necessitate the initiation of antibiotic therapy against NTM species (1). Instead, this decision should be made based on the potential risks and benefits for individual patients of a prolonged course of treatment with multiple antibiotics. Initiation of NTM treatment should be individualized based on disease type, comorbid conditions, and age Once the decision has been made to initiate treatment for NTM lung disease, the treatment regimen should be formulated according to established guidelines, understanding that a substantial proportion of the current guidelines rely upon expert opinion rather than randomized clinical trials. However, adherence to the current guidelines for treating NTM lung disease is poor (44,111).

Table 11. Management of NTM diseases

Clinical presentation	Species	Treatment
Pulmonary disease	MAC	• Macrolide, rifamycin, ethambutol daily or three times a week (t.i.w) ± streptomycin/amikacin t. i. w
	M. kansasii	• Rifampin, ethambutol, isoniazid daily or rifampin, macrolide, ethambutol daily or t. i. w
	M.simiae	• No regimen with good clinical data on efficacy
	*M.abscessus (*subsp. a*bscessus*and *bolletii)*	• Three or four of the following: amikacin, cefoxitin, imipenem, tigecycline, linezolid, macrolide (macrolides may be inactive if *erm* gene is functional)
	M. abscessus subsp. *massiliense*	• Macrolide plus two of the following: amikacin, cefoxitin, imipenem, linezolid
	M. szulgai, M. malmoense,M. xenopi	• Same as MAC regimen

Adapted from British Thoracic Society guidelines for the diagnosis and management of NTM-PD, 2017(35,81)

For patients with pulmonary disease, treatment is continued for at least 12 months after sputum cultures become negative(44).

Table 11. Management of extrapulmonary and disseminated NTM infections

Clinical presentation	Species	Treatment
Cervicofacial lymphadenitis	MAC	• Surgical excision was more effective than antibiotic therapy (cure rate of 96% vs 66% for a 3-month regimen of rifabutin- clarithromycin)
Disseminated- at least for 12 months of therapy	MAC (if macrolide susceptible)	• Macrolide, rifamycin, ethambutol daily or t.i.w • i. v aminoglycosideduring the initial 8 to 12 weeks of therapy
	MAC (if macrolide resistant)	• Ethambutol plus rifabutin plus an aminoglycoside (parenteral) • Surgical intervention (e g, valve replacement, joint replacement, or debridement of infected bone) if required
	M. chimaera	• Macrolide, rifamycin, ethambutol daily or t.i.w
Disseminated RGM infections		• No evidence-based treatment available • Surgical debridement wherever possible plus 4 months of two-drug antibiotic treatment based on susceptibility test results
	M. fortuitum complex	• Combination of quinolones, co-trimoxazole, tobramycin, doxycycline and imipenem
	M. abscessus	• Amikacin, imipenem, cefoxitin, linezolid and macrolides(if no functional *erm* gene)

References: (113–115)

4.MATERIAL AND METHODS

4.1 STUDY DESIGN

This is a prospective diagnostic studyconducted for a period of 21 months.

4.2 ETHICAL APPROVAL

The approval for conducting the study was obtained from the Institutional Review Board, Christian Medical College, Vellore

4.3 STUDY DURATION

The study was conducted for a period of 21 monthsfrom January 2017 to September 2018.

4.4 STUDY SAMPLES

NTM isolates cultured from clinical samples received at the Department of Clinical Microbiology for mycobacterial culture/ culture and drug susceptibility testing.

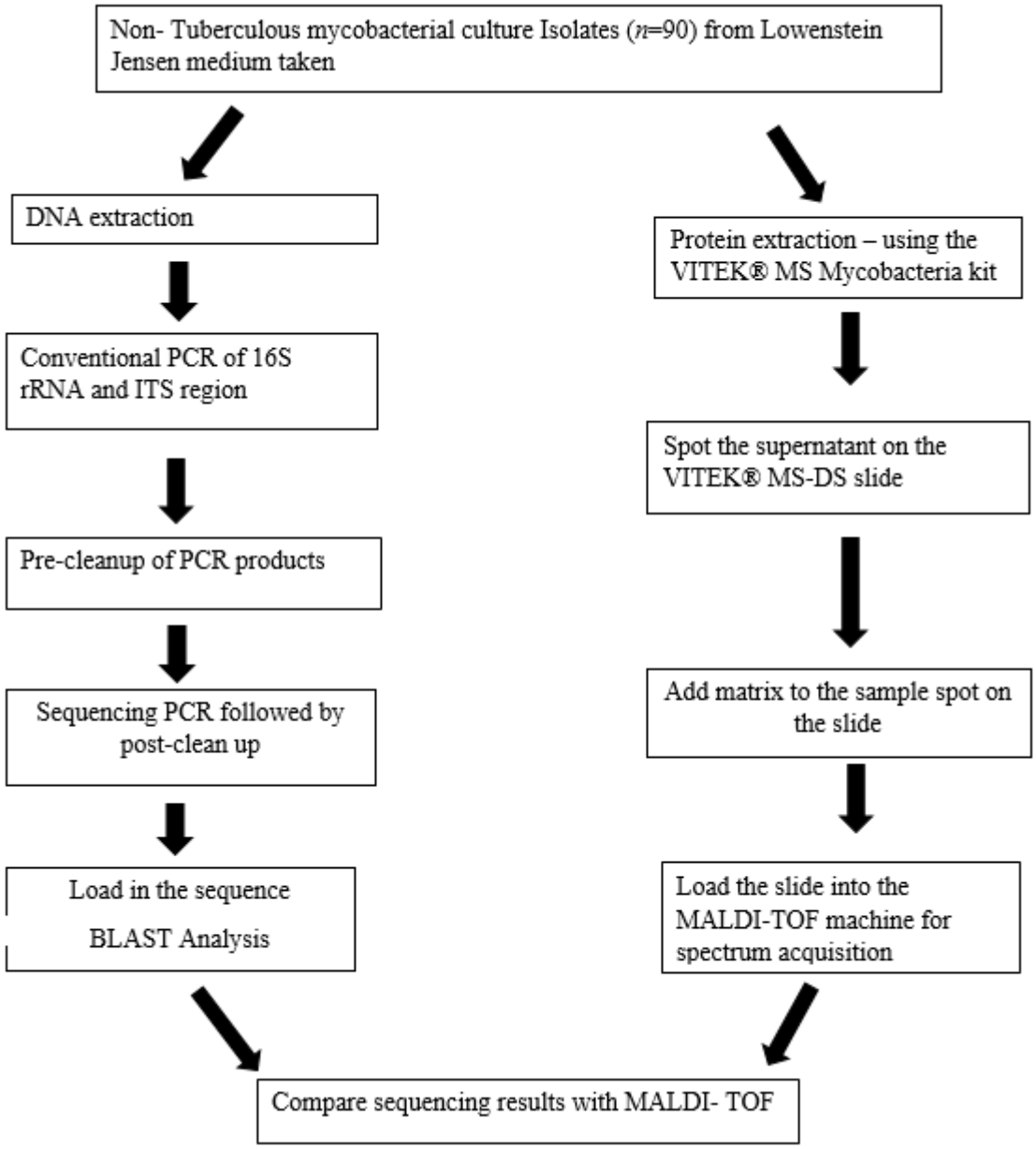
4.5 SAMPLE SIZE CALCULATION

Sample size was taken as 90 as this is a pilot study since the exact prevalence of NTM diseases in our setting is not known.

4.6 DATA SOURCE

Relevant clinical data regarding the clinical isolate was obtained from electronic patient records (Clinical work station) of the institution.

4.7STUDYALGORITHM



4.8CONVENTIONAL TESTS

Samples are cultured on Lowenstein Jensen media (solid) or Middlebrook 7H9 broth in BACTEC Mycobacterial Growth Indicator Tube (MGIT) 960 automated system as per the treating clinician's request. The optimal temperature for growth of most NTMspecies is between 28°C and 37°C. Positive MGIT cultures are sub cultured on LJ media.

The culture growth on LJ should be examined after 3 and 7 days and weekly thereafter for 6 to 9 weeks. Cultures that show visible colonies on LJ media are taken for further identification.

Figure 5. Phenotypic features for identification of NTM from LJ media:

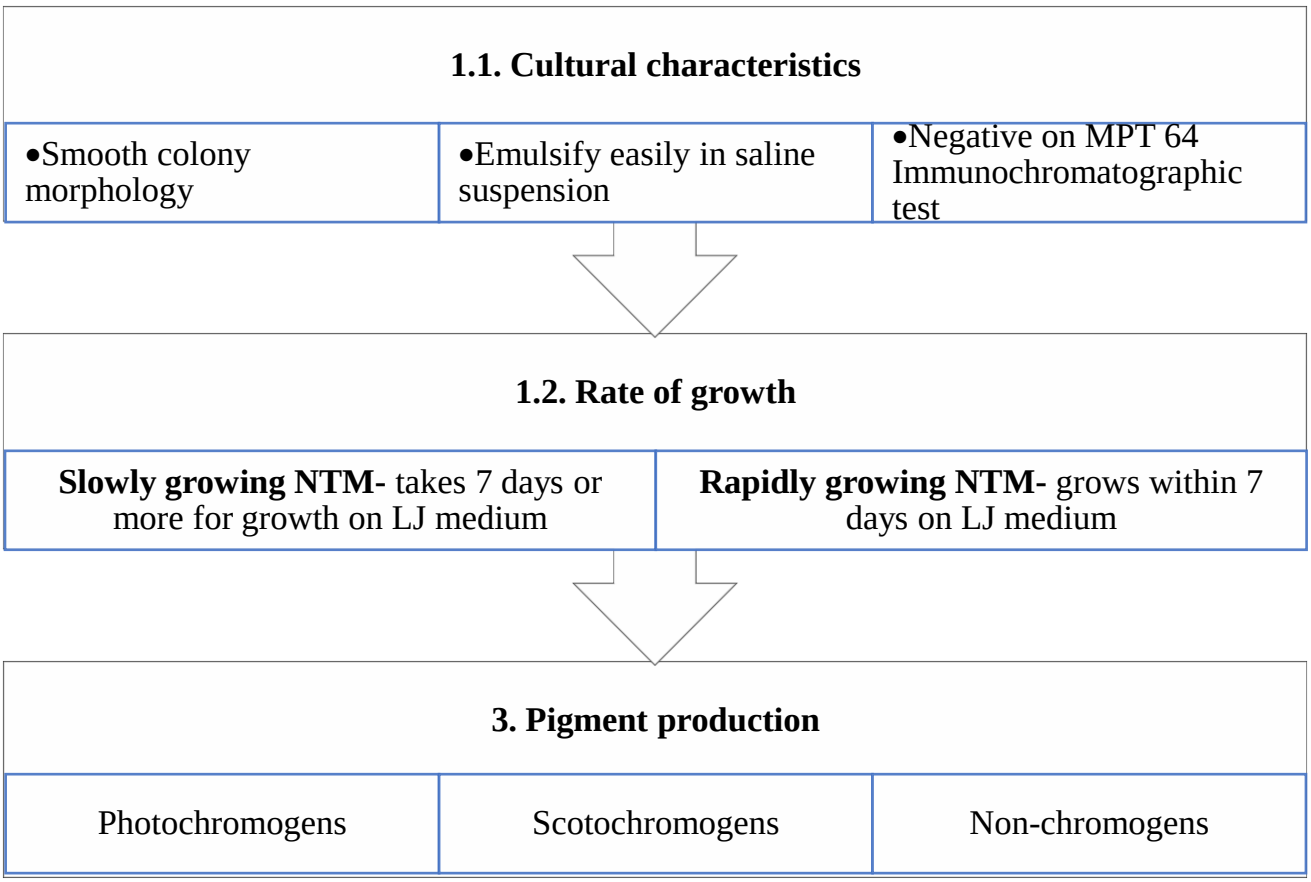
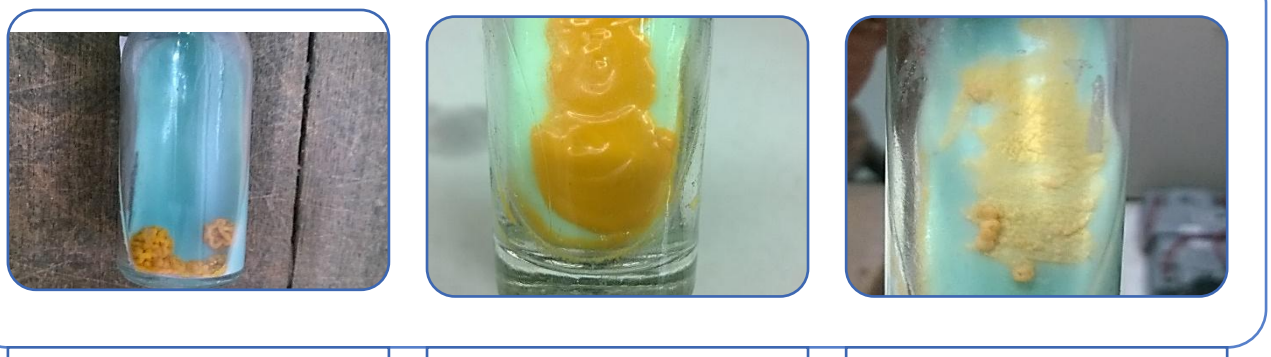


Figure 6. Classification based on pigment production



i.Photochromogens: produce a pigment only on the slopes exposed to light. Eg: *M. kansasii*

Scotochromogens: produces pigment both in the presence and absence of light.

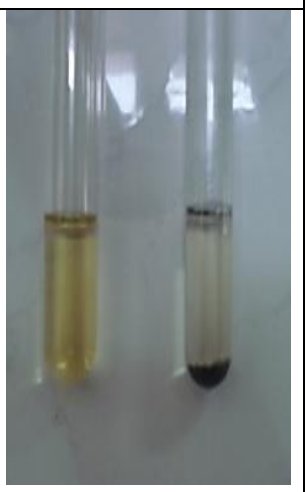
Eg: *M. gordonae*

Non-chromogens: does not produce pigment in the presence or absence of light. Eg: *M. avium, M. fortuitum*

3. Biochemical tests

Table 11. Common biochemical tests

Test	Principle	Result	Observation
Nitrate reduction test	Nitratereductase enzyme reduces nitrate (NO_3^-) to nitrite (NO_2^-) or free nitrogen gas	**Positive:** medium turns red after the addition of nitrate reagents **Negative:** medium turns red after the addition of the zinc	

Arylsulfatase test	Arylsulfatase hydrolyzes the bond between the sulfate and the aromatic rings of tripotassium phenolphthalein	**Positive:** pink colour when alkali is added to the medium **Negative:** colourless	
Test for urease production	Ureaseenzymesplit urea in the urea broth into ammonia resulting in alkalinity	**Positive:** media turns pink colour **Negative:** media remains yellow in colour	
Test for tellurite reduction	To determine the ability of *Mycobacterium spp.* to reduce potassium tellurite to metallic tellurium	**Positive:**Black precipitate around the colonies or the solution **Negative:** no black precipitate	

Slow growers are differentiated in the lab using tellurite reduction, nitrate reduction, tolerance to LJ with 5% NaCl and urease production tests.

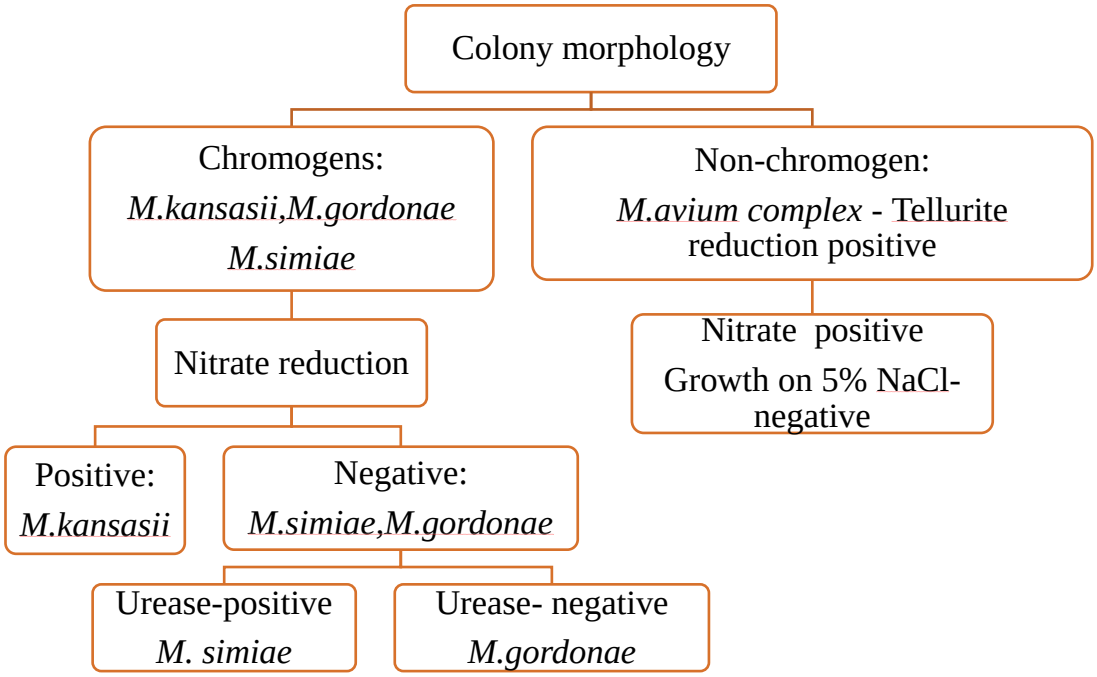


Figure7. Algorithm for biochemical identification of common slow growers.

Rapid growers are identified in the lab by arylsulfatase activity (at 3 days), nitrate reduction, tolerance to LJ with 5% NaCl.

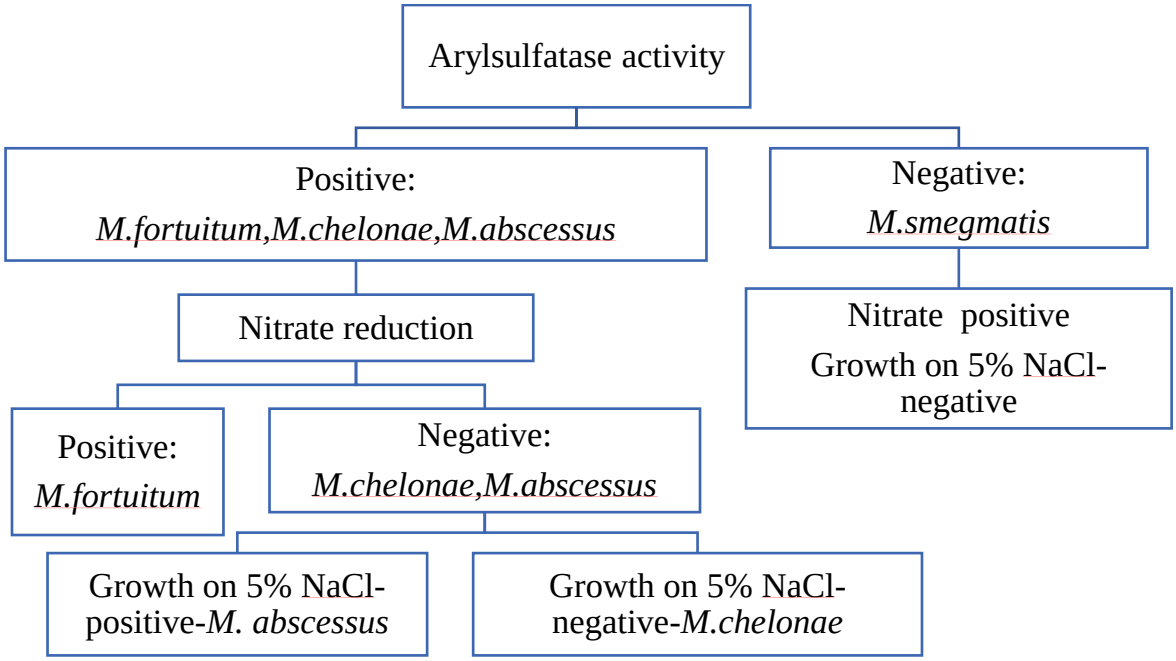


Figure 7. Algorithm for biochemical identification of common rapid growers.

Table 12.Conventional method of NTM species identification using biochemical tests:

Slow growers:	**MPT 64**	**Tellurite reduction**	**Nitrate reduction**	**Tolerance to 5% NaCl**	**Urease**	**Aryl Sulfatase activity**
M. kansasii	-	+	+	-	+	-
M. marinum	-	+	-	-	+	V
M. simiae	-	+	-	-	+	-
M.scrofulaceum	-	±	-	-	+	-
M. szulgai	-	±	+	-	+	-
M. gordonae	-	-	-	-	-	-
M. avium intracellulare complex	-	+	-	-	-	-
M. malmoense	-	+	-	-	-	-
M. xenopi	-	+/-	-	-	-	+
Rapid Growers:						
M.smegmatis	-	+	+	+	Not done	-
M. fortuitum	-	+	+	+	-do-	+

M.chelonae	-	-	-	-	-do-	+
M.abscessus	-	-	-	+	-do-	+

4.10 Line probe assay:

The line probe assay used for identification of NTM species in this study is Genotype Mycobacterium CM (VER 2.0) (Hain Lifescience).

Principle: LPA is based on solid phase reverse hybridization of biotinylated PCR amplicons of the target region to oligonucleotide probes arranged on a membrane strip.

Methodology:

The whole procedure is divided into three steps: DNA extraction (from positive cultures); a multiplex PCR amplification with biotinylated primers and reverse hybridization.

DNA extraction: Mycobacteria grown on the solid culture media is used as the starting material. DNA is extracted using the GenoLyse® kit.

Amplification:Following DNA extraction, nucleic acids are selectively replicated in an amplification reaction using the amplification mix-A and B in the thermal cycler.

Reverse hybridization:

1. The hybridization buffer was prewarmed to 50^{o}c in a water bath.

2. Transfer 20 µl of denaturing solution to the wells and add 20 µl amplified product. Mix well and incubate for 5 min.

3. Add 1ml of hybridization buffer to each lane and this was mixed to make homogeneous colour.Place the strips in respective wells.

4. The program P3S1(GT FAST) is used on the Twincubator. The machine is set at Step 1(20 minutes, shaking at 50^{o}C), while the stringent solution is warmed in a water bath to a temperature of 50^{o}C.

5. The hybridization buffer was aspirated completely with pipette and tapped onto tissue paper.

6. Add 1 ml of stringent solution to each lane. The Twincubator was set to Step 2 (10 minutes, shaking at 50^{o}C).

7. Aspirate the stringent solution completely with a pipette and taponto tissue paper and add 1 ml of conjugate solution.

8. Step 3 (20 minutes, shaking) is set up in Twincubator.

9. Remove the conjugate completely by tapping off onto tissue paper

10. One ml of Rinse solution is added and the step is selected in the Twincubator (1 minute, shaking).

11. One ml distilled water is added and the washing step is selected in the Twincubator (1 minute, shaking). Remove the water completely by tapping off onto tissue paper.

12. One ml of substrate solution is added to each lane and placed in the Twincubator (5-10minutes, stationary). Cover the Twincubator with aluminium foil till the substrate incubation is over.

13. The substrate is removed completely and tapped off onto tissue paper. 1 ml distilled water is added. The water is removed completely and tappedoff onto tissue paper.

14. The strips were stuck on the evaluation sheet while wet and results interpreted as per Genotype® Mycobacterium CM interpretative chart.

4.10.3 MALDI-TOF

Principle: Mass spectrometry is an analytical technique in which samples are ionized into charged molecules and ratio of their mass-to-charge (m/z) can be measured. It is the ionization that involves a laser striking a matrix of small molecules to make the analyte molecules into the gas phase without fragmenting or decomposing them. The basic principle of time-of-flight is that ions of different m/z are dispersed in time during their flight along a field-free path of known length. Provided that all the ions start their journey at the same time, the lighter ones will arrive earlier at the detector than the heavier ones.

Procedure: Performed in BSL III laboratory till the inactivation step.

<u>Protein extraction-</u>

1. For each organism to be tested, transfer 0.5mL of ethanol to a tube with glass beads.

2. Use a 1 µL loop to pick up and transfer two loopful of the test organism to the tube.

3. Homogenize the organism in a bead beater for 5minutes at 6.5 meter/second.

4. Remove the screw capped tube from bead beater and incubate it at room temperature for 10 minutes.

5. Vortex for 5 to 10 seconds and immediately transfer suspension into an empty 2 mL round-bottomed tube using a pipette and avoiding transfer of any glass beads.

6. Centrifuge sample for 2 minutes at 14,000 G to create a pellet.

7. Discard all the supernatant ethanol using a pipette.

8. Add 10 µL of formic acid to the pellet. Vortex it for 5 seconds.

9. Add 10 µL of acetonitrile and mix using a vortex for 5 seconds.

10. Centrifuge for 2 minutes at 14,000 G to create a pellet.

11. Transfer 1.5 µL of the supernatant onto the target slide spots. Allow each spot to dry completely.

12. Add 1 µL of α-cyano-4-hydroxycinnamic acid(matrix) to each target slide spot. Allow matrix to dry.

Target slides were kept at room temperature and analyzed using Vitek®MS v3.0, which includes amass spectrometer linked to a reference database. Laser shots are targeted at different positions within the sample well which will produce up to 100 mass profiles of protein that are summed into a single, raw mass spectrum.

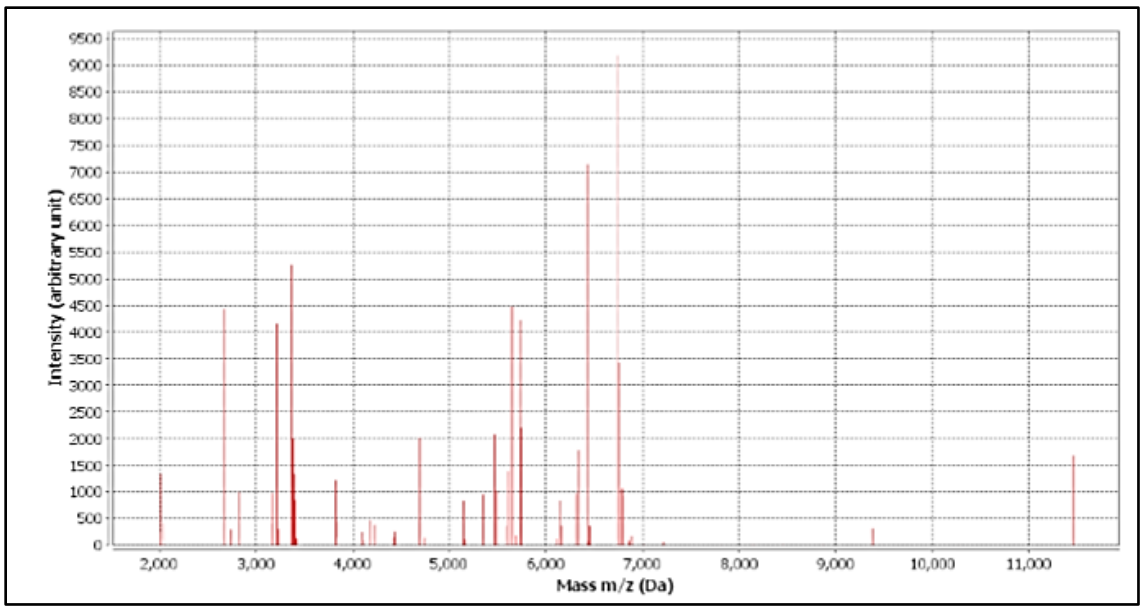


Figure 8. Mass spectra of an NTM isolate

The spectrum is then processed by baseline correction, de-noising, and peak detection to identify the well-defined mass/charge peaks.Spectra are then acquired and analyzed using Myla 4.0 software with database version 3.0.

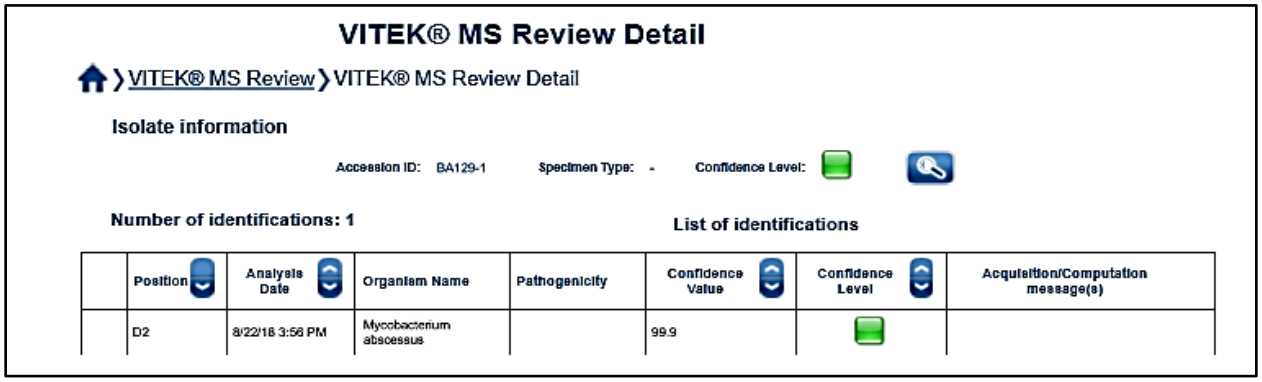


Figure 9. Result of NTM species identification by MALDI-TOF

4.11 INDEX TEST- DNA SEQUENCING

DNA sequencing is the term which refers to methods for determining the order of the nucleotide bases adenine,guanine,cytosine and thymine in a molecule of DNA.

Sanger sequencing:

The key principle of the method is the use of di-deoxynucleotide triphosphates (ddNTPs) as DNA chain terminators.

STEP 1: DNA EXTRACTION FOR SEQUENCING:

1. Pure culture of the NTM from Lowenstein Jensen media (solid media) is selected.
2. Emulsify one or two loopful of the culture in 200 microliters of sterile normal saline taken in an Eppendorf tube.Pulse vortex for 15 seconds.
3. Add 100 microliter of Lysozyme. Pulse vortex for 15 seconds.Incubate at 37^{o}c for 1 hour.
4. Add 20 microliter of Proteinase K. Pulse vortex for 15 seconds. Incubate at 56^{o}c for 30 minutes.
5. Add 200 microliter of Alkaline Lysis(AL) buffer. Pulse vortex for 15 seconds. Incubate at room temperature for 10 minutes
6. Add 250 microliter of 99.9% Ethanol. Pulse vortex for 15 seconds.
7. Transfer the whole content to a DNA spin column without touching the rim. Avoid air bubbles.Centrifuge at 8000 rpm * 1 minutes
8. Discard the collection tube with the flow through.Take a new collection tube.

9. Add 500 microliter of Wash buffer 1(AW1). Centrifuge at 8000 rpm x 1 minutes. Discard the flow through.
10. Add 500 microliter of Wash buffer 2(AW2). Centrifuge at 14000 rpm x 3 minutes. Discard the flow through.
11. Centrifuge at 10000 rpm x 1 min (Dry spin). Place a new collection tube
12. Add 100 microliter of Elution Buffer. Keep it at Room temperature for 5 minutes.
13. Centrifuge at 8000 rpm x 2 minutes.
14. Transfer the eluate into another Eppendorf tube. Label it properly. Store the DNA at -20^{o}c.

Step 2: DNA Quantification:

For quantitating DNA or RNA, readings should be taken at wavelengths of 260 nm and 280 nm. The reading at 260 nm allows calculation of the concentrate on of nucleic acid in the sample. The reading at 280 nm gives the amount of protein in the sample.

- DNA quantification [in nanogram/µl] is given by the following formula

=Absorbance at 260 nanometer (OD260) x 50 nanogram/ µl x 10 (dilution factor)

For Sanger sequencing the DNA concentration of 20-25 nanogram/ µl is ideal.DNA purity can be identified dividing the readings at 260 by reading at 280 (nm). Pure preparations of DNA should have OD260 /OD280 values of 1.8. If there is contamination with protein or phenol, this ratio will be significantly less than the values given above, and accurate quantitation of the amount of nucleic acid will not

be possible. Once the DNA quantification is satisfactory, the extraction product can be used for sequencing.

Step 3: Conventional PCR (Polymerase Chain Reaction) for amplifying the targetDNA:

In this study, two targets are used for sequencing- 16S rRNA and Internal TranscribedSpacer (ITS) region

16S rRNA primer sequence:Universal 16S rRNA primers first described by Turner *etal* was used for the sequencing(116)

16S rRNA Forward: 5'-AGAGTTTGATCCTGGCTCAG-3'

16S rRNA Reverse: 5'-ACGGTTACCTTGTTACGACTT-3'

ITS primer sequence:Reference: Yu et al, PloS,2014(117)

ITS-Forward (16SrRNA)- 5'-GTGGGATCGGCGATTGGGAC-3'

- positions 1280 to 1299 of the 16S rRNA gene

ITS-Reverse (23SrRNA)- 5'- CCACCATGCGCCCTTAGACAC -3'

- positions 7 to 27 of the 23S rRNA gene

Table 13. Cycling conditions of 16S rRNA PCR

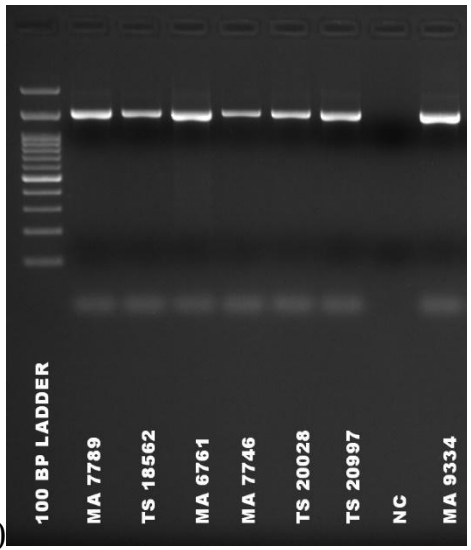
Initial denaturation	95 °C for 15 minutes	1 cycle
Denaturation	95 °C for 1 minute	30 cycles
Annealing	52 °C for 30 seconds	
Extension	72 °C for 1.5minutes	
Final Extension	72 °C for 10 minutes	1 cycle
Hold	4 °C	∞

Table 14. Cycling conditions of ITS PCR

Initial denaturation	94 °C for 5 minutes	1 cycle
Denaturation	94 °C for 30 seconds	38 cycles
Annealing	55 °C for 30 seconds	
Extension	72 °C for 1 minute	
Final Extension	72 °C for 10 minutes	1 cycle
Hold	4 °C	∞

Gel documentation after conventional PCR:

(a)



(b)

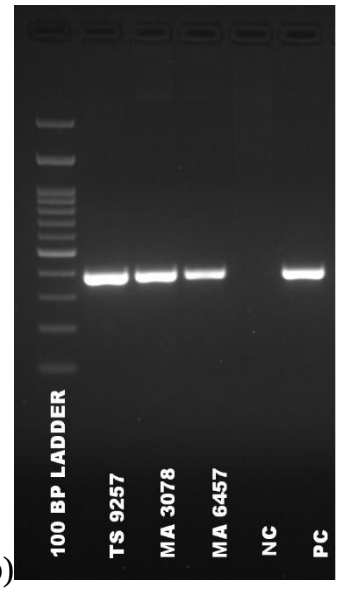


Figure10. a. Gel picture of 16S rRNA PCR- Product size approximately 1500 bp.

b. Gel picture of ITS region PCR- Product size approximately 350 bp

Pre-sequencing clean up

1. Add 5 volumes of PB(Binding) buffer to 1 volume of the PCR reaction and transfer to QIAquick™ column.Centrifuge at 13000 rpm for 1 minute and discard the flow through.

2. Add 750 µl of PE(Ethanol) buffer, Centrifuge at 13000 rpm for 1 minute and discard the flow through.

3. Centrifuge at 13000 rpm for 1 minute to remove residual wash buffer.

4. Place a new 1.5ml micro centrifuge tube then add 50µl of elution buffer in the center of the QIAquick™ membrane and centrifuge 13000rpm for 1minute.

5. The purified DNA is to be analyzed on a gel and add 1 volume of loading dye to 5 µl purified DNA.Check the quantification of DNA by using spectrophotometry.

SEQUENCING PCR REACTION: The reagents required for one PCR reaction include ready reaction (RR) mixture 0.5 µl, sequencing buffer 1.75 µl, primer 0.5 µl and milli Q water 6.25 µl.Add 1 µl of DNA template to the above to make the total volume of 10 µl and load into thermal cycler.

Table 15. Cycling conditions of sequencing PCR

Initial denaturation	96 °C for 1 minutes	1 cycle
Denaturation	96 °C for 10 seconds	25 cycles
Annealing	50 °C for 5 seconds	
Extension	60 °C for 5 minutes[81]	
Hold	4 °C	1 cycles

Post clean up

Procedure

1. Transfer the reaction product into a 1.5 ml micro centrifuge tube.

2. Make a master mix of 1 of 10 µl Milli-Q and 2 µl of 125 mM EDTA per reaction.

3. Add 12 µl of master mix I to each reaction containing 10 µl of reaction. Make sure the contents are mixed.

4. Make master mix II of 2 µl of 3M Sodium acetate (NaOAc) pH 4.6 and 50 µl of ethanol per reaction. Add 52 µl master mixes II to each reaction.

5. Mix the content well and incubate at room temperature for 15 minutes.

6. Spin at speed of above 12000rpm for 20 minutes at room temperature and discard the supernatant.

7. Add 250 µl of 70 % of ethanol and spin at above 12000 rpm for 10 minutes at room temperature and discard the supernatant.

8. Add 12-15 µl of Hi-Di formamide, transfer to sample tube cover with septa, denature snap chill and proceed for electrophoresis.

The DNA fragments on each strand will be sequenced with the primers used in the initial PCR amplification by using the ABI PRISM BigDye Terminator Cycle Sequencing kit V3.1 (Applied Biosystems, USA) and the sequencing was carried out on the ABI 3500 Genetic Analyzer (Applied Biosystems, USA).

Sequence analysis

First, merge the raw sequences of forward and reverse primers in EMBOSS merger to get the double stranded DNA information. The resulting sequences were BLAST matched against the reference sequences in the NCBI database.

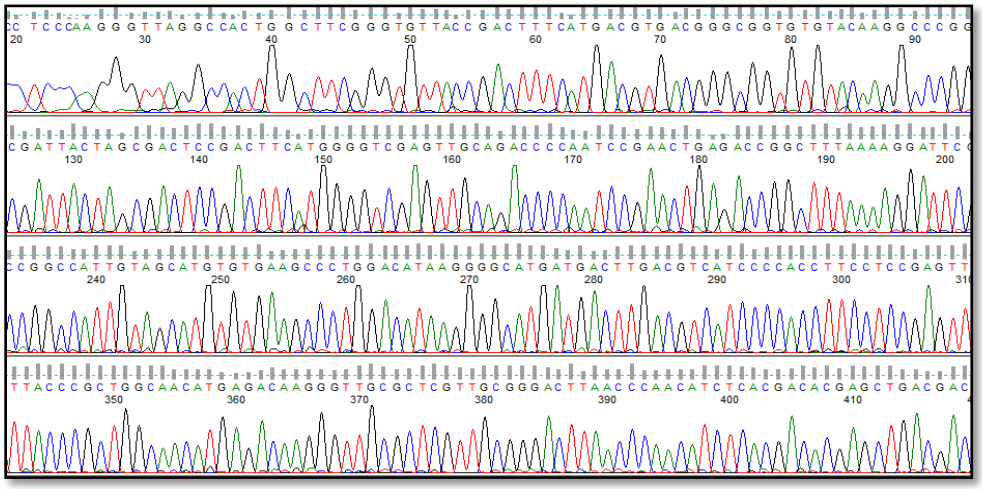


Figure 12. Chromatogram of the DNA sequence

Description	Max score	Total score	Query cover	E value	Ident	Accession
Mycobacterium abscessus strain FLAC045, complete genome	2407	2407	100%	0.0	100%	CP014958.1
Mycobacterium abscessus strain FLAC008, complete genome	2407	2407	100%	0.0	100%	CP014954.1
Mycobacterium abscessus strain FLAC007, complete genome	2407	2407	100%	0.0	100%	CP014953.1
Mycobacterium abscessus strain FLAC004, complete genome	2407	2407	100%	0.0	100%	CP014951.1
Mycobacterium abscessus subsp. bolletii strain zj-1016 16S ribosomal RNA gene, partial sequence	2407	2407	100%	0.0	100%	KP736078.1
Mycobacterium abscessus strain zj-972 16S ribosomal RNA gene, partial sequence	2407	2407	100%	0.0	100%	KP736043.1
Mycobacterium abscessus strain 4529, complete genome	2407	2407	100%	0.0	100%	CP009616.1
Mycobacterium abscessus strain DJO-44274, complete genome	2407	2407	100%	0.0	100%	CP009615.1
Mycobacterium abscessus subsp. massiliense str. GO 06, complete genome	2407	2407	100%	0.0	100%	CP003699.2
Mycobacterium abscessus subsp. bolletii gene for 16S ribosomal RNA, partial sequence, strain: A1	2407	2407	100%	0.0	100%	AB548592.1
Mycobacterium massiliense strain INCQS 594 16S ribosomal RNA gene, partial sequence	2407	2407	100%	0.0	100%	GU143891.1
Mycobacterium abscessus partial 16S rRNA gene, strain ITG 98-1292	2407	2407	100%	0.0	100%	AJ419970.1

Figure 13. BLAST(Basic Local Alignment Search Tool) analysis of the sequence using NCBI Genbank® database (https://blast.ncbi.nlm.nih.gov/)

5. RESULTS

A total of 90 consecutive clinically significant NTM isolates were included in the study. These were isolated from samples that were received in the laboratory for routine mycobacterial diagnosis between January 2017 and September 2018.

5.1 Demographic data:

5.1.1 Age: The samples belonged to patients aged between 0 to 74 years. Highest number of samples were received from patients in the 41 to 50 years age group. The mean age was 42 years.

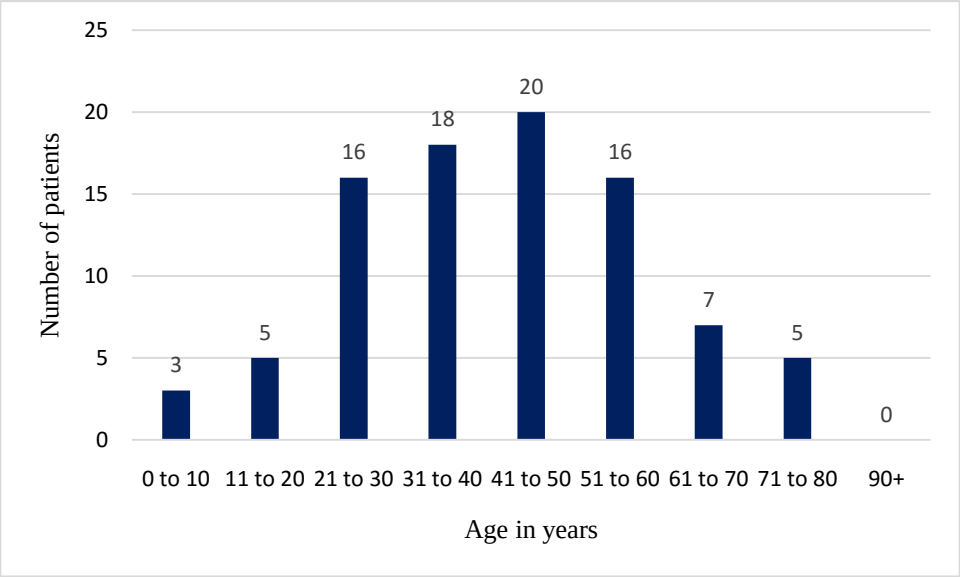


Figure 14. Age-group wise distribution

5.1.2 Gender: Of the 90 patients, 50 (56%) were male and 40 (44%) were female.

5.1.3 Geography: The patients in this study came from different parts of India and also from neighboring countries Bangladesh and Nepal. Majority of them came from Tamil

Nadu (25, 28%), West Bengal (23, 26%), Jharkhand (12, 13%) and Andhra Pradesh (11%).

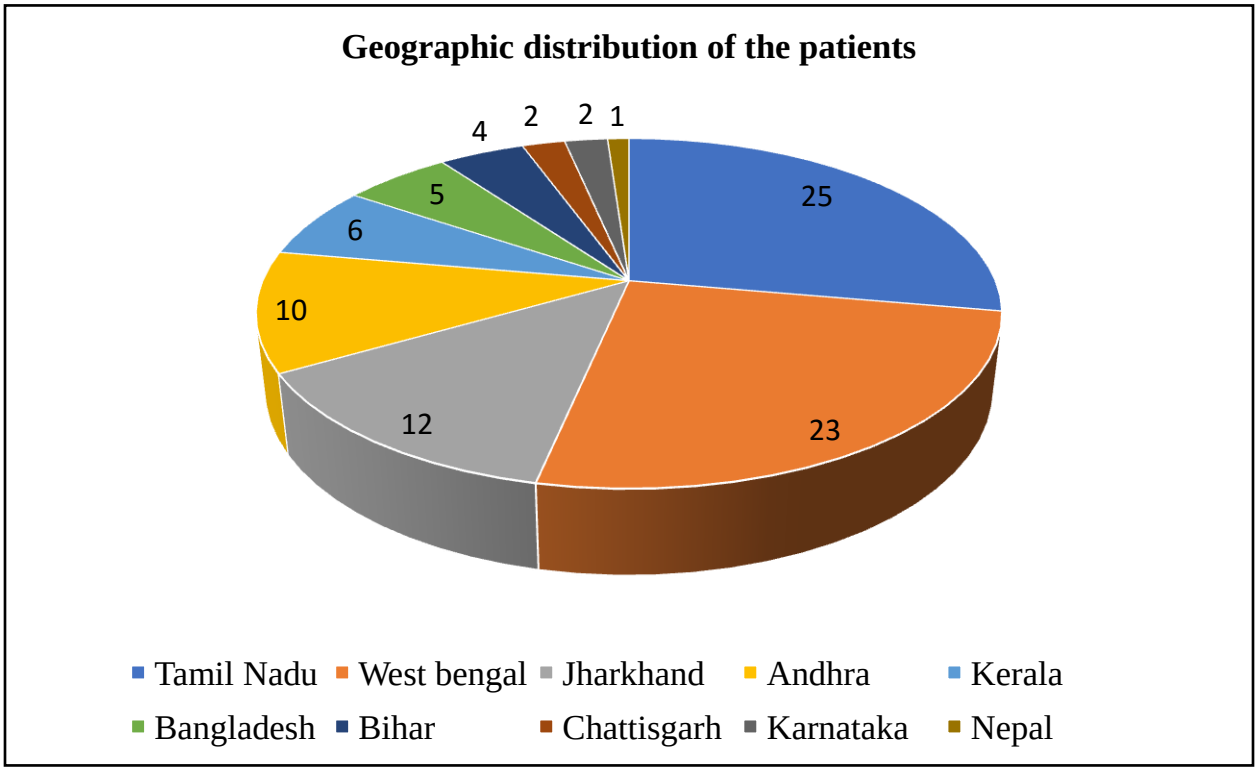


Figure 15. Geographic distribution of patients

5.2 Clinical specimen

Of the 90 isolates, 50 were isolated from the respiratory tract- sputum (39 samples), bronchoalveolar lavage (8 samples), lung biopsy (2 samples) and endotracheal aspirate (1 sample).

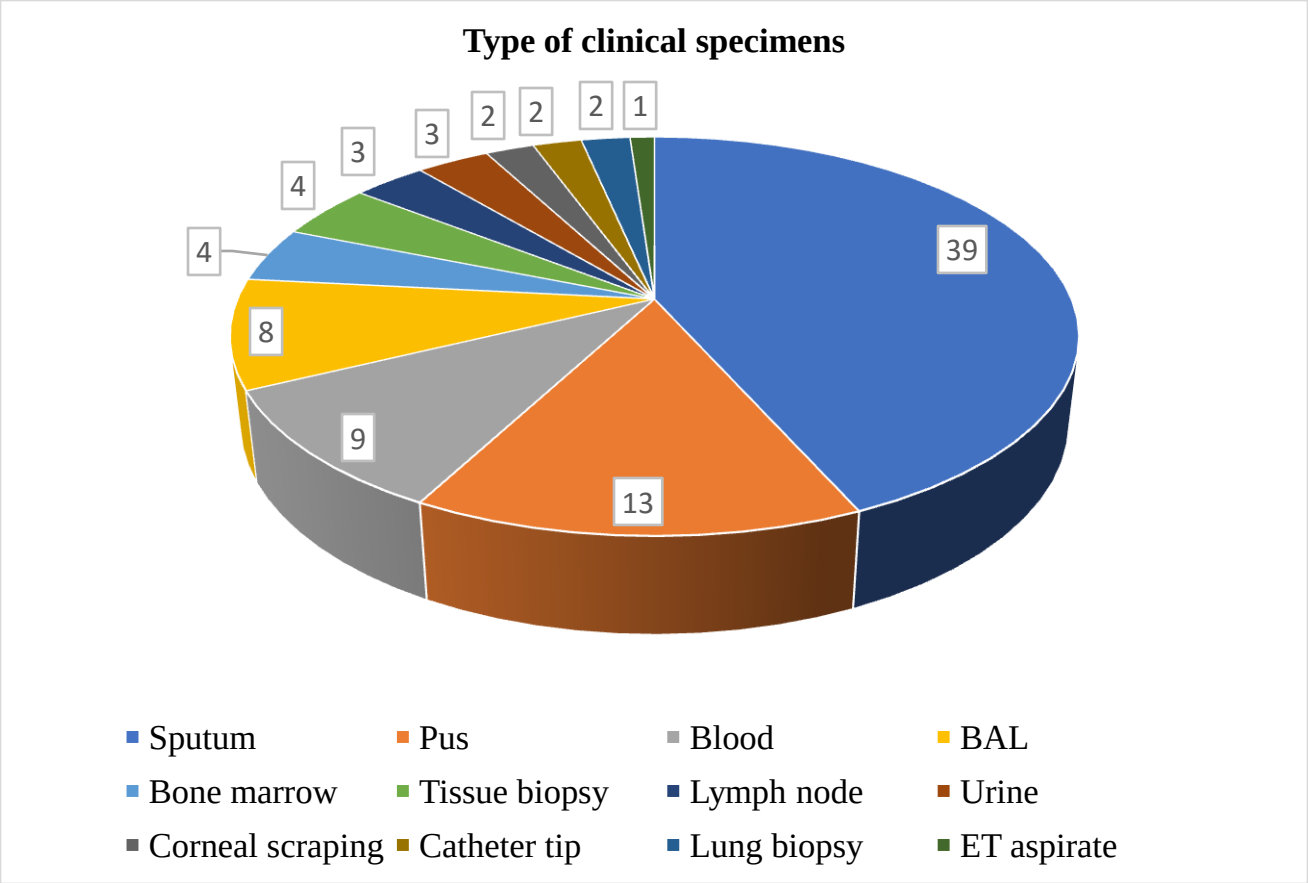


Figure 16. Sample distribution

5.3 Non-Tuberculous Mycobacterial species identification

In this study, out of the 90 isolates, 49 (54%) were slow growers and 41 (46%) were rapid growers.

Based on 16S rRNA sequencing, which is the gold standard, they were identified up to the species level.

5.3.1 Slowly growing NTM species

The most common slow growingNTMs isolated in this study were *M. intracellulare* (19/90, 21%) followed by *M. simiae* (8/90, 8.8%)and *M. avium* (6/90, 6.6%). Species with intermediate growth rate such as *M. flavescens* and *M. novocastrense*have been included along with slow growers in this study. As shown in the figure below the other slow growing NTM species identified were M. avium complex, *M. kansasii*, *M. szulgai*, *M. yongonense*, *M. timonense*, *M. scrofulaceum*, *M. parascrofulaceum* and *M. europaeum.*

One isolate which could only be speciated upto genus level by 16S rRNA sequencing was identified as *M. europaeum* by ITS sequencing.

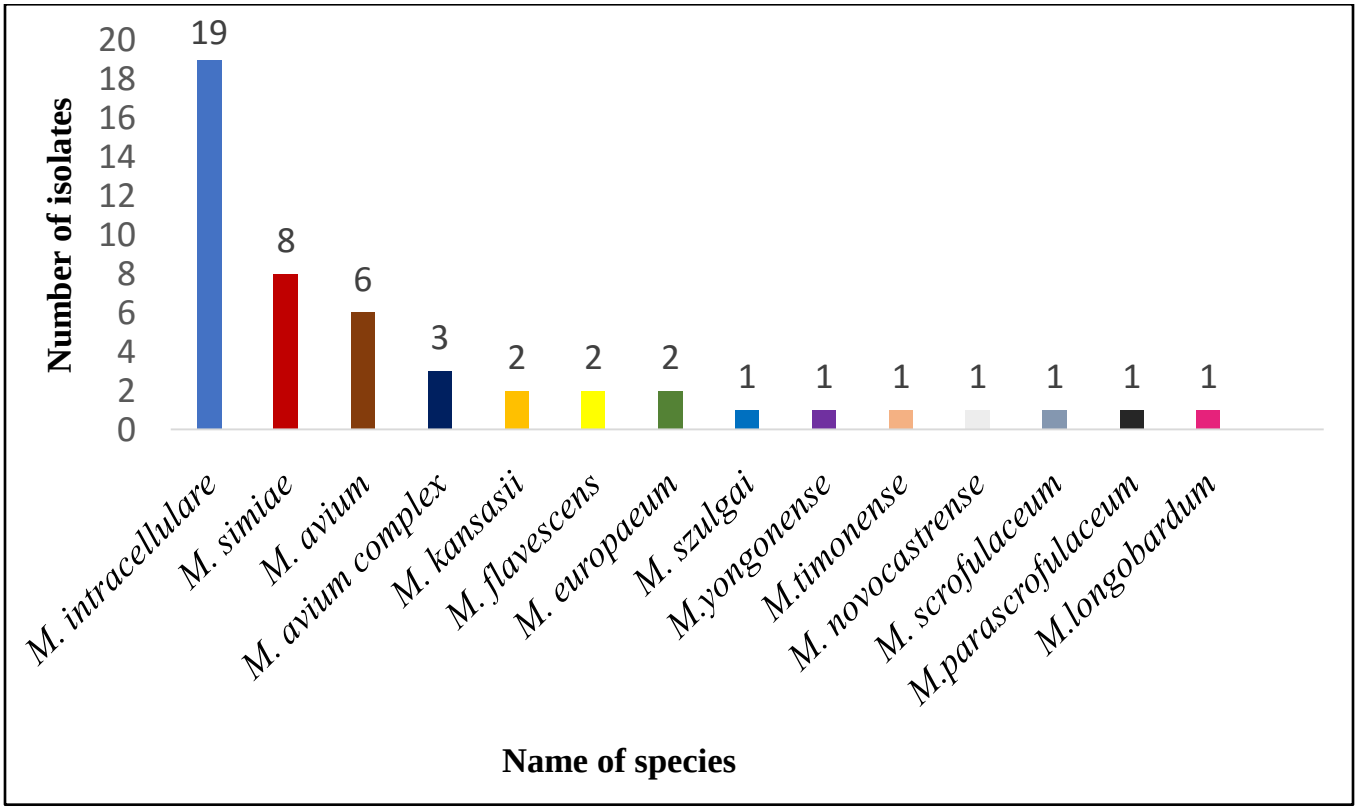


Fig 17. Slowly growing NTM species

5.3.2 Rapidly growing NTM species

The most common rapidly growing NTM isolated in this study were *M. abscessus (n*=26, 63%) followed by *M. fortuitum* (*n*=10, 24%). Other species isolated were *M. farcinogenes, M. bacteremicum, M. phocaicum* and *M. goodii*.

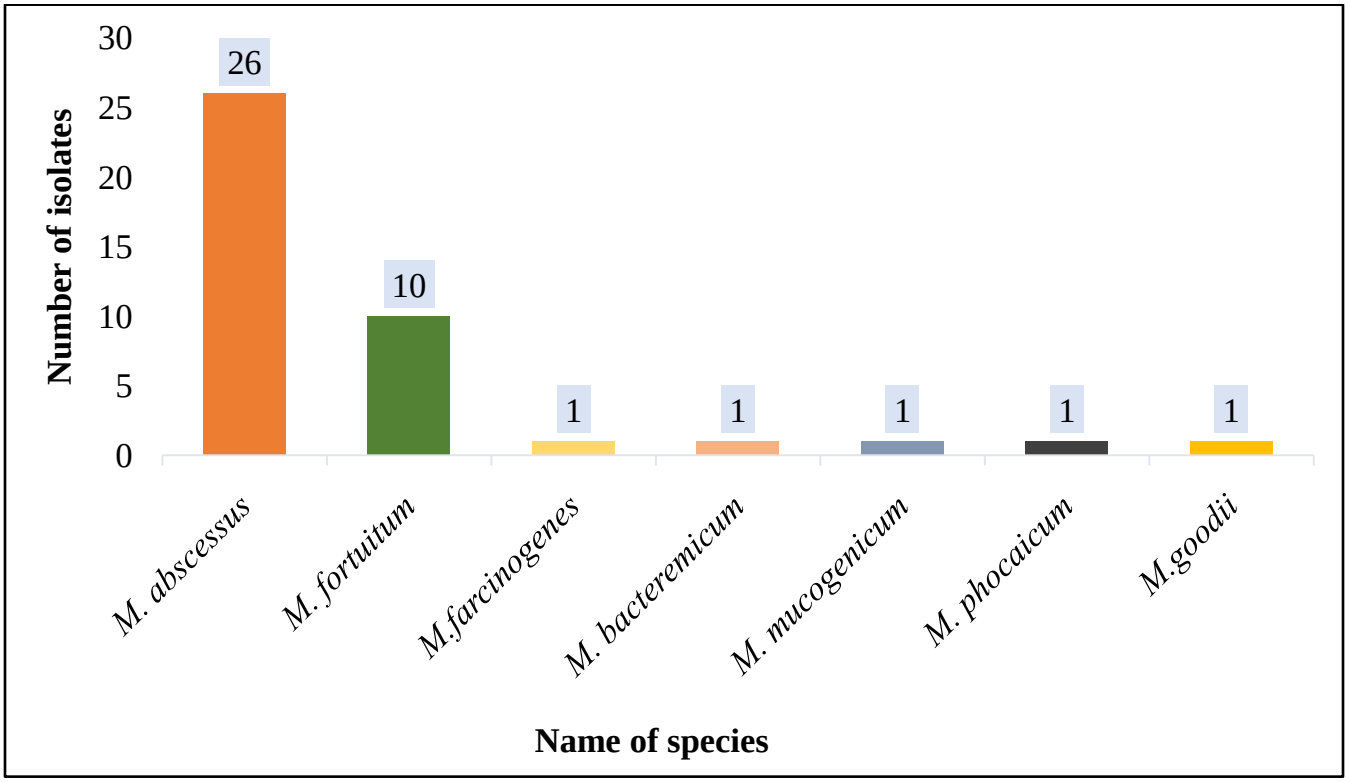


Figure 18. Rapidly growing NTM species

5.4 Clinical presentationof slowly growing NTM:

Pulmonary disease: The most common clinical form of disease caused by slowly growing NTM isolated in this study was pulmonary disease. Of the 49 patients who presented with NTM pulmonary disease, 40 (81.6%) were due to slow growers. Among

these isolates, 17 were *M. intracellulare*, 7 were *M. simiae*, 3 were MAC (which were identified as *M. intracellulare* by ITS sequencing), *M. avium*, *M. kansasii* and *M. europaeum* were 2 each by 16S rRNA sequencing. Other rare species like *M. timonense, M. yongonense, M. szulgai, M. parascrofulaceum* and *M. scrofulaceum* were isolated from 1 patient each. Two NTMs isolated from sputum were not clinically significant and were possible colonizers and it was identified as *M. flavescens* and *M. longobardum*. Twenty-five patients (25/50= 50%) had past history of tuberculosis and twenty (20/50= 40%) of them had associated bronchiectasis.

Disseminated infection: This was the second most common presentation of slow growers. Seven patients had disseminated infections with *M. avium* (isolated from 4 patients), *M. intracellulare* (2 patients) and *M. simiae* (1 patient), isolated from bone marrow and lymph node specimens. Six (86%) of these patients had acquired immune deficiency syndrome (AIDS) with CD_4 cell count less than 150 cells/µl at the time of diagnosis. Median CD_4 count of these patients was 124 cells/µl.

One patient was 3 years old with Severe Combined Immunodeficiency Disorder (SCID) from whom *M. avium* was isolated from the middle ear tissue biopsy.

Skin and soft tissue infections: There were no patients who presented with skin and soft tissue infections associated with slow growers in this study.

Contaminants:*M. novocastrense* and *M. flavescens* were isolated from bone marrow biopsy and urine sample respectively, which were considered to be contaminants as there was no clinical signs or symptoms consistent with NTM disease.

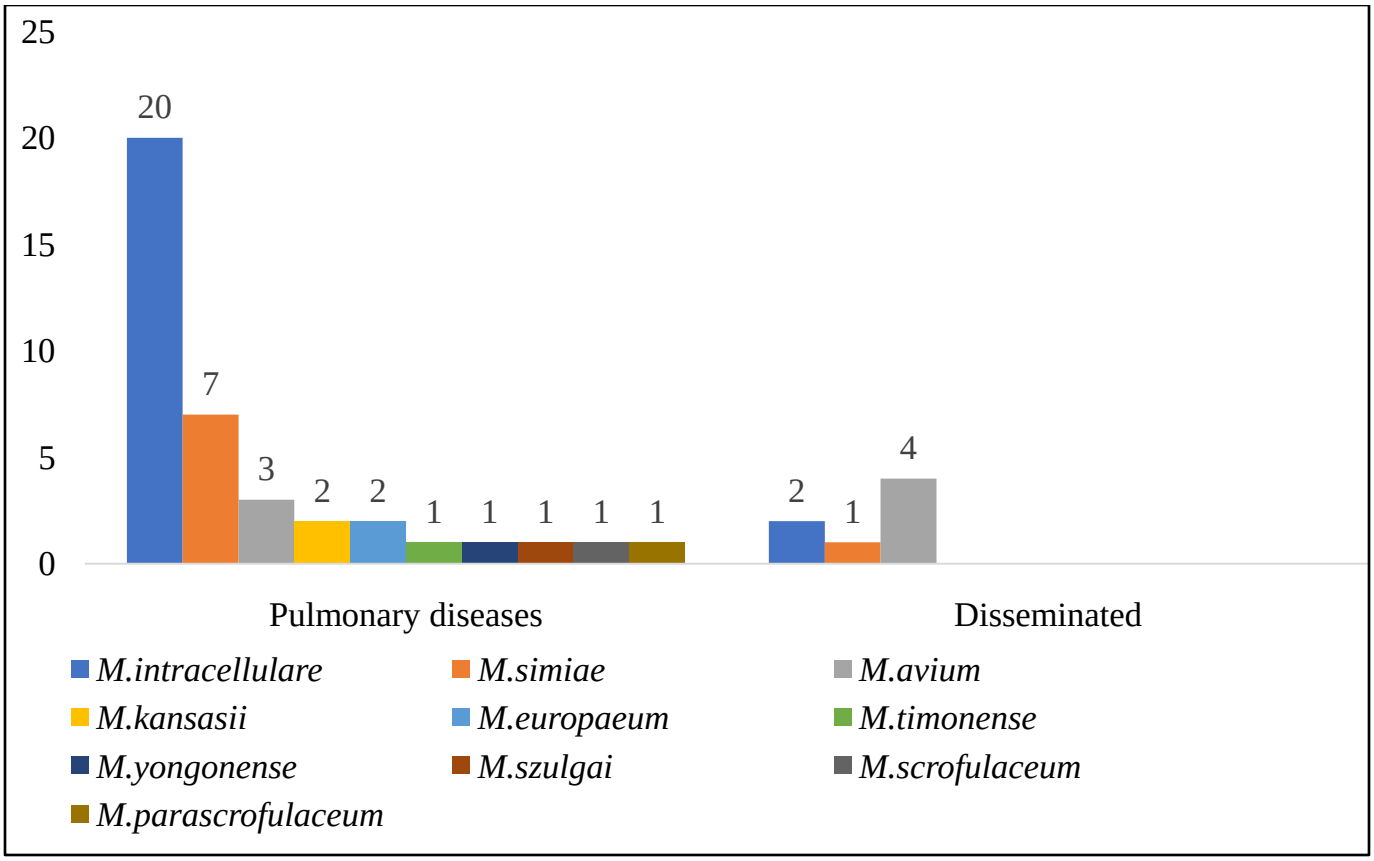


Figure 19. Clinical spectra of slowly growing NTMs

5.5 Clinical presentationof rapidly growing NTM:

Skin and soft tissue infections: The predominant clinical manifestation due to rapidly growing NTMs were skin and soft tissue infections (16/41 =39%). Most of them were due to post- operative wound infections (9/16, 56%), with inguinal hernia repair with mesh placement being the predominant type of surgery associated with the disease (3 cases). Other surgeries implicated were laparotomy, laparoscopic surgeries of abdomen and caesarean section. Two patients presented with injection site abscess. Other clinical presentations were breast abscess, skull base osteomyelitis, abscess at chemotherapy port site, traumatic injury to the flexor zone and discharging sinus (in one patient each).

Twelve out of the 16 cases were due to *M. abscessus*, 3 were caused by *M. fortuitum* and 1 by *M. farcinogenes*.

Blood stream infection/ line associated infections: *M. abscessus*was associated with 7 cases of blood stream infection. The patients who were admitted in the hospital for more than 2 weeks, who had various risk factors like presence of central venous line, invasive procedures done on the cardiovascular system (stent, valve repair) and therapy with immunosuppressants for haematological malignancies or treatment with immunomodulators for disorders of immune system were the ones that presented with NTM bacteremia. *M. fortuitum* and *M. phocaicum* was identified from one case of blood stream infection each.

Pulmonary infections: Rapid growers were isolated from respiratory samples of 9 patients. *M. fortuitum* was isolated in 5 patients, out of which only 3 were clinically significant. *M.abscessus* was isolated from the samples of 4 patients from multiple samples from the same patient. One of them was an infant with lipoid pneumonia who succumbed to the infection.

Ocular infections: Two patients who had the history of corrective eye surgery for strabismus and cataract surgery presented with ocular infections with *M. abscessus*.

Infections of the urinary tract: *M. abscessus* was isolated from 2 patients. One of them had past history of genitourinary tuberculosis and the other had previous surgery with ureteric stent placement.

Rare presentations:*M. mucogenicum* was isolated from the bone marrow biopsy of a patient with history of pyrexia of unknown origin of 4 weeks duration.

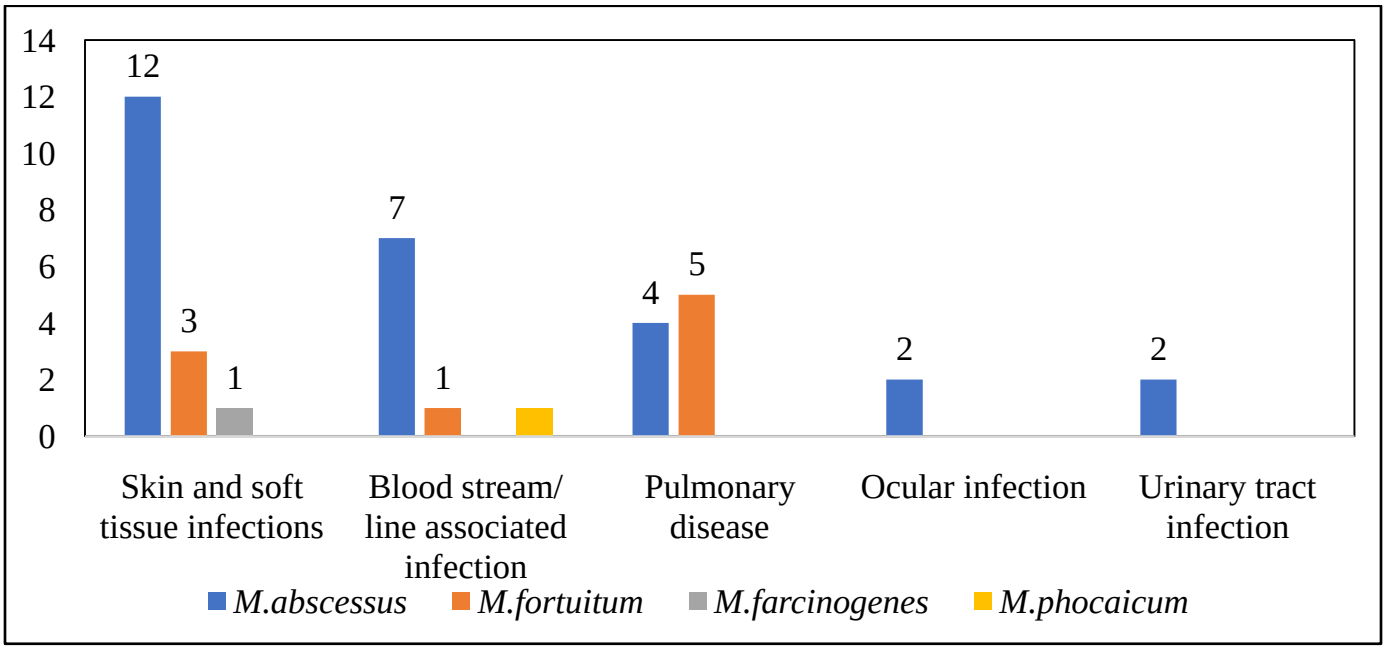


Figure 20. Clinical spectra of rapidly growing NTMs

5.6 Laboratory data

5.6.1 MALDI TOF identification

MALDI-TOF mass spectrometry was performed on all the 90 isolates. It could identify 75 of the 90 isolates up to species or complex/group level. It could not identify 13(13/90= 14%) isolates, out of which 11 were slow growers. Two isolates were wrongly identified as non-mycobacterial species. As mentioned in table 13, the concordance of MALDI-TOF with the gold standard 16S rRNA sequencing was 83.33%.

5.6.2 Internal transcribed spacer (ITS) sequencing

ITS sequencing was performed on all the slow growers and *M. avium* was identified upto subspecies level. Out of the 6 *Mycobacterium avium* isolates, all were identified as *M. avium* subsp. *hominisuis*. The results were compared with 16S rRNAsequencing and MALDI-TOF as described in the table 15 below.

5.6.3 Concordance

Table 15. Concordance between the results of 16S rRNA sequencing, ITS sequencing and MALDI-TOF

Tests compared (Total tests, n=90)	Concordance	95% confidence interval
16S rRNA sequencing vs MALDI TOF	83% (cumulative)	74.00- 90.4%
• **For rapid growers**	94.5%	81.3- 99.4%
• **For slow growers**	77.5%	63.9- 81.4%
16S rRNA sequencing vs ITS sequencing	95.5%	82.4- 99.7%
ITS sequencing vs MALDI-TOF	76%	63.9- 81.4%

5.7 Cost analysis

5.7.1 Particulars for costing of Sanger sequencing

Table 16. Sanger's sequencing cost per DNA sample

a. Material cost (chemicals and reagents)-	INR
DNA extraction per sample	250
Reagent kit with RR mix for testing two DNA targets- 16S rRNA	3000.00

and ITS (Applied Biosystems) (@Rs.1500/- per target)	
b. Consumables	
1. Gloves 5 sets (@ Rs. 20/- per set)	100.00
2. Pipette tips 50 Nos. (@Rs.2.90 each)	145.00
3. Pipettes (1000 µl, 100 µl, 10 µl)	15.00
4. Discard Lotion (@Rs. 50/-)	50.00
5. Eppendorf tubes (@Rs. 1.5/- per tube)	11.50
6. PCR tubes (@Rs. 0.5/- per tube)	2.00
	323.50
Total for one gene	**3573.50**

5.7.2 Particulars for costing of MALDI-TOF mass spectrometry

Table 17. MALDI-TOF cost per sample	
a. Material cost (chemicals and reagents)-	**INR**
1.Protein extraction per sample (Rs 46000 / 100 tests)	460.00
2. VITEK®MS-DS slide-1 spot	100.00
3. VITEK®MS CHCA matrix- 1µl	10.00
b. Consumables	
1. Gloves 2 sets (@ Rs. 20/- per set)	40.00
2. Pipette tips 10 Nos. (@Rs.2.90 each)	29.00
3. 1 µl disposable loop-2 no. s	7.00
Total for one sample (exclusive of tax)	**646.00**

6 DISCUSSION

Non-tuberculous mycobacteria (NTM) are a group of more than 190 species of mycobacteria apart from *Mycobacterium tuberculosis* complex and *Mycobacterium leprae*. They are an important public health concern worldwide as the cause of opportunistic infection, particularly in patients having acquired immunodeficiency syndrome and those on iatrogenic immunosuppression(118).

According to the American Thoracic Society (ATS)/Infectious Diseases Society of America (IDSA) statement of 2007, when NTM are suspected as the etiology of disease, definitive diagnosis should always be supported by repeated isolation of NTM from two or more specimens of the patient or a single specimen if it is collected aseptically from a sterile body site(44). The British Thoracic Society guideline of 2017 also recommends that NTM should be identified to the species level using validated molecular or mass spectrometry techniques as the treatment is species-specific and pathogens must be distinguished from environmental contaminants(35). Thus, timely and accurate identification of NTM is required to guide therapy (patient outcome) and for epidemiological reason.

For many years, the classification developed by Runyon was utilized in clinical laboratories to provide a convenient way to differentiate among NTM based on the rate of growth of the and pigment production(23). However, it could not identify NTM upto the species level. Speciation of clinically isolated NTMin the laboratory was initially performed using an array of biochemicals and later with High Performance liquid

chromatography (HPLC) and then in recent years with Line probe assays. All three had limited utility as they were restricted to identification of the most frequently isolated NTM species(42). Due to this, molecular sequencing of 16Sr RNA and Internal Transcribed Spacer (ITS) regions were developed, which will not only give an accurate identification but also be able to identify novel species. However, the high cost of the technique limits its use for routine diagnostics. Therefore, a novel technology called Matrix Assisted Laser Desorption/Ionization -time of flight (MALDI-TOF)was introduced, which is a simple, rapid, less expensive (after the initial purchase of the mass spectrometry instrument) and accurate method of identifying the NTM species using mass spectrometry of proteins. This method identifies organisms based on the creation of a unique species-specific spectral profile (fingerprint) produced by extracted ribosomal proteins with specific mass to charge ratios. Hence, we have compared the 16S rRNA (gold standard) and ITS sequencing with MALDI-TOF (index test) for the accurate identification of NTM species(100).

6.1 Demographic data

6.1.1 Age and gender:

Unlike *M. tuberculosis*, NTM associated with human disease is not reportable in most countries, including India. Hence, the exact age and gender distribution is not known. In our centre, as depicted in figure no.14, highest number of patients with NTM disease were in the 41 to 50 years age group (22%). A study by Umrao *et.al* from north India found that patients of age more than 55 years were predominant (36.2%) in their setting.

Out of the 90 patients included in this study, 50 (56%) were male and 40 (44%) were female. This data correlates with a study conducted by Jesudason *et.al* at a tertiary-care centre from Vellore, south India(33). However, a multi-centric study from United States by Prevots *et.al* showed a higher prevalence in women when compared with men (1.1 to 1.9-fold higher)(119).

6.1.2 Geographic distribution

Since the study was done at a tertiary-care hospital in South India, majority of the patientswere from Tamil Nadu (25/90= 28%) and Andhra Pradesh (10/90=11%). There were a considerable number of patients from north Indian states such as West Bengal (23/90= 26%), Jharkhand (12/90= 13%), Bihar (4/90= 4.4%) and Chattisgarh (2/90= 2%); neighbouring countries like Bangladesh (6/90= 6%) and Nepal (1/90= 1.1%).

6.2 Clinical specimen

In this study, NTM were isolated from 50 (55%) pulmonary samples, sputum being the most common sample and 40 (45%) extrapulmonary samples as depicted in Figure 16. However, according to the population based laboratory surveillance in Oregon, United States, there was a significantly higher number (80–85%) of NTM pulmonary disease(120). In a study from north India by Umrao et.al, total of 79.4% of the NTM were recovered from pulmonary and only 18.2% from extrapulmonary samples(34).

6.3 Species identification

Distribution of NTM species that are isolated from clinical samples differs strongly by region.In this study, out of the 90 isolates, 49 (54%) were slow growers and 41 (46%) were rapid growers. We used 16S rRNA sequencing as the reference standard for species identification.

6.3.1 Slow growers

Thirteen species of slow growers were identified from the samples included in this study. *M. intracellulare* (19/90= 21%) was the predominant species followed by *M. simiae* (8/90= 8.8%)and *M. avium* (6/90= 6.6%). As depicted in figure 17, the other slow growing NTM species identified were *M. kansasii, M. szulgai, M. yongonense, M. timonense, M. scrofulaceum, M. parascrofulaceum, M. europaeum, M. flavescens* and *M. novocastrense.* This is similar to the study conducted in north India by Umrao et.al, in which *M.intracellulare* and *M.avium*were the most common slow growers isolated(34).

6.3.2 Rapid growers

Seven species of rapid growers were isolated in this study as shown in figure 18, with *M. abscessus* being the most common (26/90= 28.8%). Other species were *M.fortuitum* (10/90= 11%), *M. farcinogenes, M. phocaicum, M. mucogenicum, M. bacteremicum* and *M.goodii*. Data from India, USA and multi-national studies by NTM-NET also reported *M. abscessus* to be the most pathogenic rapidly growing NTM to humans(34,121,122).

6.4 Clinical presentation

6.4.1 Clinical presentation of slow growers

The predominant clinical presentation of slow growers in our setting during the study period was pulmonary disease. Among the 49 pulmonary isolates in this study, 40 (81.6%) were slow growers. Most common slowly growing NTM species isolated were *M. intracellulare* (17/49= 34.6%) followed by *M. simiae* (7/49=14%), MAC (3/49=6%), *M. avium* (2/49=4%), *M. kansasii* (2/49=2%), *M. europaeum* (2/49=4%), *M. timonense* (1/49=2%), *M. yongonense* (1/49=2%), *M. szulgai* (1/49=2%), *M. parascrofulaceum* (1/49=2%),*M. scrofulaceum* (1/49=2%*), M.flavescens*(1/49=2%*) and M. longobardum*(1/49=2%*).*

NTM-Network European Trials Group (NET) network conducted a multi-centric study in 2008 to find the relative distribution of the different NTM from pulmonary samples. 91 different NTM species were identified using line probe assay. *Mycobacterium avium* complex (MAC) predominated in most countries (47%), followed by *M. gordonae* (11%). *M. gordonae* and *M. xenopi* had a high rate of isolation in Europe. Isolates from Asia (Japan, South Korea and Taiwan) were predominantly MAC (54%) followed by a significant proportion of rapid growers (31%)(121).In a study from north India by Umrao *et.al*, the slow growers were isolated less frequently (41%) compared to rapid growers (59% of isolates) (34).

The second commonest clinical presentation of slow growers was disseminated illness. Slow growers such as*M. avium* (4/7=57%),*M. intracellulare* (2/7= 58.5%) and *M.*

simiae(1/7=14%) were isolated from bone marrow and lymph node specimens of 7 patients in this study. Out of the 7 patients, 6 were diagnosed with Acquired Immunodeficiency Syndrome. Median CD_4 count of the patients in this study was 124 cells/cu.mm. Our findings are consistent with other studies on immunocompromised individuals by Varley *et.al* and Dhar *et.al* with disseminated infection in which *M.avium* is the most common species implicated (123,124).However, Varley et al found very low median CD4 count of 10cells / mm3 in their patients with disseminated NTM disease(13).

6.4.2 Clinical presentation of rapid growers

Most rapid growers were associated with extrapulmonary infections (post-operative wound infection, bacteremia, ocular infection, urinary tract infection) in our setting with *M. abscessus* (23 out of 32 cases =71.8%) being the most common. *M. fortuitum*(4/32=12.5%), *M. farcinogenes* (1/32= 3%), *M. phocaicum* (1/32= 3%), *M. mucogenicum* (1/32=3%), *M. bacteremicum* (1/32=3%) and *M.goodii* (1/32=3%) are the other species identified from these samples. This is in agreement with the results from east Asian countries and north India(34,121,125). However, in a study done in 116 patients by Helou *et.al* at USA, most commonly encountered mycobacterial species was *M. mucogenicum*,(39%) (126).

Among the 9 rapid growers (18.4%) causing pulmonary disease, 5 were *M. fortuitum* (5/49= 10%) and 4 were *M. abscessus* (4/49=8%). Pulmonary disease is an uncommon presentation of rapid growers in our setting while comparing with NTM-NET study

.Umrao *et.al*found a high prevalence of *M.abscessus* (31%) followed by *M.fortuitum* (20%) in North India in patients with pulmonary disease(34).

6.4 Risk factors of NTM

Pulmonary NTM infections often occur in the context of preexisting lung disease, especially bronchiectasis, pneumoconiosis, chronic obstructive pulmonary disease (COPD)and cystic fibrosis. In this study, twenty-five patients (25/50= 50%)had past history of tuberculosis and twenty (20/50= 40%) of them had associated bronchiectasis.

Lung damage due to previous tuberculosis is a risk factor for NTM pulmonary disease according to various studies (127,128). Studies done by Xu *et al* from China, Mirsaeidi *et al* from USA and Fowler *et al* from UK have analyzed the rate of NTM disease in patients with bronchiectasis and it was found to be variable from 5 to 30% (129–131).

Most common extrapulmonary manifestation in this study was skin and soft tissue infections (16/90 =17%) and predominant type was post- operative wound infections.This is consistent with the rise in prevalence of SSTI due to NTM in various parts of the world. A study from Oregon, USA, found the incidence of SSTI to be 0.9 cases per 100,000 patients and data from Taiwan suggests an increasing incidence of SSTI of 1.67 to 6.7 per 100,000 population over 8 years(132,133).

NTM bacteremia was seen in 7 patients on prolonged hospitalization of more than 2 weeks with central venous line in-situ,who had undergone invasive procedures of the cardiovascular system (stent, valve repair) and therapy with immunosuppressants for

haematological malignancies orimmunomodulators for immune system disorders. This correlates with studies from other centers(134).

6.5 Species identification of NTM

6.5.1 MALDI-TOF

MALDI-TOF-MS is one of the newest techniques introduced for the identification of NTM. The accuracy of the MALDI- TOF- MS is dependent upon obtaining good quality spectra which can be challenging due to the complex cell walls of mycobacteria.

In this study, isolates were tested using the VITEK MS system (bioMérieux, France), and the results were analyzed using knowledge base Version 3.0, which includes 49 Mycobacterium species [45 NTM (including 23 slow growers and 22 rapid growers) and 4 MTBC]. Seventy five of the 90(83.3%) isolates tested were identified up to species or complex/group level. 13/90(14%) were not identified and 2/90(2%) were misidentified as non-mycobacterial species. Of the 13 species which were not identified, 4 were not in the database, namely *M. europaeum*(2 isolates), *M. yongonense* and *M. longobardum*. However, *M. intracellulare (*6/90= 6.6%), *M. abscessus*(2/90= 2%),and *M. avium*(1/90= 1.1%) were available in the database, but were not identified. Other species which were misidentified- *M. timonense* was identified as *M. intracellulare*, *M. parascrofulaceum* was identified as *M. scrofulaceum*, *M. bacteremicum* was identified as *M. neoaurum* and *M. farcinogenes* was identified as *M. fortuitum* group due to the close phylogenetic relationship between these species.

In a similar study done by Luo *et.al* on evaluation of VITEK MS version 3.0, 425/507(83.8%) isolates were initially identified, and 51/507(10.1%) additional isolates were identified on repeat, with 23/507 (4.5%) isolates remaining unidentified and 8/507(1.6%) isolates misidentified at species level. Among slow growers, only 2/56 (3.6%) *M. avium* and 3/153 (2%) *M. intracellulare* were unidentified, which was marginally better than our findings(135).

Limitations of the MALDI-TOF-MS with NTM include lack of discrimination with some clinically important closely related species and subspecies including*;M. chelonae* and *M. abscessus*; *M. intracellulare* and *M. chimaera*; *M. mucogenicum* and *M. phocaicum;* subspecies of *M. avium* and *M. abscessus;* and species withinthe *M. fortuitum group*. If no identification is achieved with a good quality spectrum, this may mean that the organism is not represented in the database being applied.These databases are being developed and updated, and there is no public database to use as an additional reference to the commercial companies that market MALDI instrumentation. In contrast, if the spectral pattern is poor, there may be mixed culture present, and colony purity should be confirmed.

6.5.2 16S rRNA sequencing

Sequencing of the16S rRNA gene (approximately 1500-bp) is the most universally used sequencing method for identification of bacteria and also is considered a gold standard for identification of NTM species. Region A at the 5′ end contains most of the species-specific or “signature sequences” in mycobacteria. In this study, partial sequencing of

16S rRNA gene of 90 NTM isolates was performed, the details of which are discussed above. Three isolates which were identified as *M. avium* complex by 16S rRNA sequencing, were found to be *M. intracellulare* on ITS sequencing(42).

6.5.3 Internal transcribed spacer (ITS) sequencing

The sequence of the ITS 1 region is a 200–330-bp target region that separates the 16S and 23S rRNA genes. Several primer sites for amplification and sequencing of this segment have been proposed. The ITS 1 sequence has a high variability that can be used for species discrimination especially with slowly growing species including MAC.

In this study, ITS sequencing was performed on the slow growers. All the MAC isolates were identified upto the species level. *M. avium* was identified upto subspecies level. Out of the 6 *Mycobacterium avium* isolates, all were identified as *M. avium* subsp. *hominisuis*, which is the most pathogenic subspecies to humans according to various studies(81,136). Therefore, ITS sequencing can be used as an additional method over 16s rRNA sequencing for identification of slow growing as well as novel / rare NTM species.

6.5.4 MALDI-TOF Vs sequencing

Total concordanceof MALDI-TOF with the gold standard 16S rRNA sequencing was 83.33%(95% CI:74.00- 90.4%). Concordance of MALDI-TOF for the rapid growers with 16S rRNA sequencing was 94.5% (95% CI:81.3- 99.4) and for slow growers it was 77.5% (95% CI: 63.9- 81.4).

The concordance of MALDI-TOF with ITS sequencing (for slow growers) was found to be 76% (95% CI: 63.9- 81.4).

In a study done by Barbara Body *et.al*, 92% percent of the slowly growing NTM and 98% of the rapidly growing mycobacteria (RGM) were correctly identified to the species, complex, or group level by MALDI-TOF (using VITEK MS ver 3.0) compared to sequencing of partial 16S rRNA genes and several additional housekeeping genes (*rpoB, hsp65, secA*,ITS, *gyrB*, and *erm* genes)(100).However, in our study the concordance between MALDI-TOF and sequencing for identification of slow growers was lower. There were no similar studies from India.

6.5.5 Cost analysis: MALDI-TOF Vs sequencing

MALDI-TOF is less expensive per test, after the initial purchase of the mass spectrometry instrument, which makes it a suitable assay for routine diagnostics. Sequencing is 5 to 6 times more expensive than MALDI-TOF and therefore should be used as a confirmatory test.

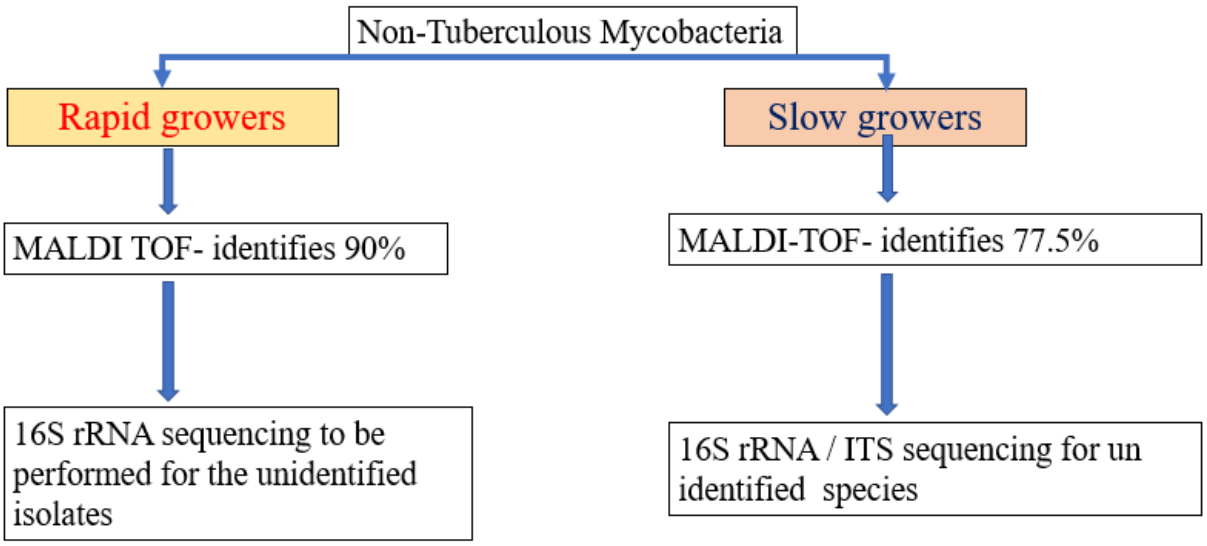
6.5.6 Technology

MALDI-TOF requires only protein extraction, which is a simple technique which can be performed by any laboratory technologist in a routine clinical microbiology laboratory setting. However, sequencing needs trained personnel for the procedure in a molecular laboratory setting.

6.6 Turnaround time

MALDI-TOF is a rapid method which will take only 1.5 to 2 hours for the whole procedure, whereas sanger sequencing will take on average 12- 24 hours of turnaround time(42).

Proposed diagnostic algorithm for routine speciation of NTMs in a diagnostic laboratory:



7. LIMITATIONS

a) ITS sequencing of rapid growers

ITS sequencing of rapid growers could not be done as the primers we selected amplified only the ITS region of slow growers.

b) 16S rRNA sequencing

Three isolates could not be included in this study as the Lowenstein Jensen medium was contaminated and the sequencing identified it as non-mycobacterial species or uncultured bacterium clone.

8. SUMMARY

- 90 Mycobacteria isolates was included in this study to evaluate the MALDI TOF against Molecular sequencing methods for the rapid and accurate speciation of Non-tuberculous mycobacteria
    - Slow growing NTM- 49
    - Rapidly growing Mycobacteria- 41
- Most common Slow growing NTM was *M.intracellulare*(21%) followed by *M. simiae (8*%)
    - Most common clinical presentation was pulmonary infection (55%)
- Most common rapidly growing NTM was *M. abscessus*(28.8%) followed by *M.fortuitum* (11 %)
    - The most common clinical presentation was skin and soft tissue infection (56%)
- 83 % (70/90) NTMs were identifies correctly upto species or complex/ group level using MALDI TOF
- The concordance of various tests was as follows
    - MALDI TOF vs 16s rRNA sequencing- Slow growers
        - 77.5% (95% CI: 63.9- 81.4)
    - MALDI TOF vs 16s rRNA sequencing – Rapid growers
        - 94.5% (95% CI:81.3- 99.4)

CONCLUSION

MALDI-TOF assay can be used as a rapid and cost-effective method for identification of NTM in a routine diagnostic laboratory especially for the rapidly growing NTMs. However, for the slow growing and novel or rare NTMs additional sequencing of 16s rRNA and ITS region need to be done for accurate identification.

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OFFICE OF RESEARCH
INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE, VELLORE, INDIA

Dr. B.J. Prashantham, M.A., M.A., Dr. Min (Clinical)
Director, Christian Counseling Center,
Chairperson, Ethics Committee.

Dr. Anna Benjamin Pulimood, M.B.B.S., MD., Ph.D.,
Chairperson, Research Committee & Principal

Dr. Biju George, M.B.B.S., MD., DM.,
Deputy Chairperson,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

May 14, 2018

Dr. Ann Susan Sam,
PG registrar,
Department of Microbiology,
Christian Medical College,
Vellore – 632 002.

Sub: **Fluid Research Grant: New Proposal:**
Sequencing and MALDI-TOF- tools for accurate identification of Non-tuberculous Mycobacteria-a Pilot study.
Dr. Ann Susan Sam , Employment Number: 21309, PG Registrar, Clinical Microbiology, Dr. Joy Sarojini Michael, Emp. No : 50199 Microbiology, Ms. R Ranjani, Microbiology, Dr. Marilyn Ninan, Microbiology, Dr. Naveen Kumar, Microbiology.

Ref: IRB Min. No. 11224 [DIAGNO] dated 05.03.2018

Dear Dr. Ann Susan Sam,

I enclose the following documents:-

1. Institutional Review Board approval 2. Agreement

Could you please sign the agreement and send it to Dr. Biju George, Addl. Vice Principal (Research), so that the grant money can be released.

With best wishes,

Dr. Biju George
Secretary (Ethics Committee)
Institutional Review Board

Dr. BIJU GEORGE
MBBS., MD., DM.
SECRETARY - (ETHICS COMMITTEE)
Institutional Review Board,
Christian Medical College, Vellore - 632 002.

Cc: Dr. Joy Sarojini Michael, Dept. of Microbiology, CMC, Vellore

Ethics Committee Blue, Office of Research, 1st Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002
Tel: 0416 – 2284294, 2284202 Fax: 0416 – 2262788, 2284481 E-mail: research@cmcvellore.ac.in

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Ref: IRB Min. No. 11224 [DIAGNO] dated 05.03.2018

Dear Dr. Ann Susan Sam,

The Institutional Review Board (**Blue**, Research and Ethics Committee) of the Christian Medical College, Vellore, reviewed and discussed your project titled "Sequencing and MALDI-TOF-tools for accurate identification of Non-tuberculous Mycobacteria-a Pilot study" on March 05th 2018.

The Committee reviewed the following documents:

1. IRB Application format
2. Signature Page
3. Cvs of Drs. Ann, Merlin N, Naveen Kumar, Joy Sarojini, Ms. Ranjani.
4. No. of documents 1- 3.

The following Institutional Review Board (Blue, Research & Ethics Committee) members were present at the meeting held on March 05th 2018 in the Jacob Chandy Hall, Paul Brand Building, Christian Medical College, Vellore 632 004.

Ethics Committee Blue, Office of Research, 1st Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002
Tel: 0416 – 2284294, 2284202 Fax: 0416 – 2262788, 2284481 E-mail: research@cmcvellore.ac.in

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Director, Christian Counseling Center,
Chairperson, Ethics Committee.

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Chairperson, Research Committee & Principal

Dr. Biju George, M.B.B.S., MD., DM.,
Deputy Chairperson,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

Name	Qualification	Designation	Affiliation
Dr. Biju George	MBBS, MD, DM	Professor, Haematology, Research), Additional Vice Principal , Deputy Chairperson (Research Committee), Member Secretary (Ethics Committee), IRB, CMC, Vellore	Internal, Clinician
Dr. Mathew Joseph	MBBS, MCH	Professor, Neurosurgery, CMC, Vellore	Internal, Clinician
Dr. B. J. Prashantham	MA(Counseling Psychology), MA(Theology), Dr. Min(Clinical Counselling)	Chairperson, Ethics Committee, IRB. Director, Christian Counseling Centre, Vellore	External, Social Scientist
Dr. Sowmya Sathyendra	MBBS, MD (Gen. Medicine)	Professor, Medicine III, CMC, Vellore	Internal, Clinician
Dr. Thomas V Paul	MBBS, MD, DNB, PhD	Professor,Endocrinology, CMC, Vellore	Internal, Clinician
Mr. C. Sampath	BSc, BL	Advocate, Vellore	External, Legal Expert
Dr. Jayaprakash Muliyil	BSc, MBBS, MD, MPH, Dr PH (Epid), DMHC	Retired Professor, CMC, Vellore	External, Scientist &Epidemiologist
Ms. Grace Rebekha	M.Sc., (Biostatistics)	Lecturer, Biostatistics, CMC, Vellore	Internal, Statistician
Dr Sneha Varkki	MBBS, DCH, DNB	Professor, Paediatrics, CMC, Vellore	Internal, Clinician
Dr. Shyam Kumar NK	MBBS, DMRD, DNB, FRCR, FRANZCR	Professor, Radiology, CMC, Vellore	Internal, Clinician
Mrs. Pattabiraman	BSc, DSSA	Social Worker, Vellore	External, Lay Person
Mrs. Nirmala Margaret	MSc Nursing	Professor, Medical Surgical Nursing, CMC, Vellore	Internal, Nurse

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Dr. Biju George, M.B.B.S., MD., DM.,
Deputy Chairperson,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

Dr. John Antony Jude Prakash	MBBS, MD	Professor, Clinical Microbiology, CMC, Vellore.	Internal, Clinician.
Dr. Ajith Sivadasan	MD, DM	Professor, Neurological Sciences, CMC, Vellore	Internal, Clinician

We approve the project to be conducted as presented.

Kindly provide the total number of patients enrolled in your study and the total number of Withdrawals for the study entitled: "Sequencing and MALDI-TOF- tools for accurate identification of Non-tuberculous Mycobacteria-a Pilot study" on a monthly basis. Please send copies of this to the Research Office (research@cmcvellore.ac.in).

Fluid Grant Allocation:

A sum of 1,00,000/- INR (Rupees One Lakh Only) will be granted for 2 years. 50,000/- INR (Rupees Fifty Thousand only) will be granted for 12 months as an Ist Installment. The rest of the 50,000/- INR (Rupees Fifty thousand only) each will be released at the end of the first year as 2 nd Installment.

Yours sincerely,

Dr. Biju George
Secretary (Ethics Committee)
Institutional Review Board

CHRISTIAN MEDICAL COLLEGE VELLORE INDIA
Dr. BIJU GEORGE M.B.B.S., M.D., D.M.
SECRETARY - (ETHICS COMMITTEE)
Institutional Review Board,
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IRB Min. No. 11224 [DIAGNO] dated 05.03.2018

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id	sid	age	add	samp	growth	col	biochem	n16s	its	maldi	lpa	hiv	cd4	tb	ctb	tbsite	brect	copd	malig	trans	immsu	line	line	pop	pops	dm	prosthetic	device	sx	surg	CD
1	TS 9257	31	Jharkhand	sputum	slow	smooth cre	NO ID	M.intracellulare	M.intracellulare	M.intracell	M.intracell	no		yes	no	PULMONARY	yes	no	no	no	no	no		no		no	no		no		
2	MA 3078	28	West bengal	lymph nod	slow	smooth cre	M.avium	M.avium	M.avium subsp.hominisuis	M.avium	M.avium	yes	124	yes	no	PULMONARY	no	no	no	no	no	no		no		no	no		no		PLHIV
3	MA 6141	55	Chattisgarh	TBLB	rapid	smooth cre	M.chelonae	M.abscessus	pending	M.abscess	M.abscess	no		yes	no	PULMONARY	no	no	no	no	no	no		no		no	no		no		
4	TS 16379	49	Bihar	sputum	slow	smooth cre	NO ID	M.intracellulare	M.intracellulare	No ID	not done	no		yes	no	PULMONARY	no	no	no	no	no	no		no		no	no		no		
5	MA 6457	46	West bengal	sputum	slow	yellow	M.intracellula	M.intracellulare	M.intracellulare	NO ID	not done	no		yes	no	PULMONARY	no	no	no	no	no	no		no		no	no		no		
6	MA 7789	23	Andhra	sputum BN	slow	yellow	M.intracellula	M.intracellulare	M.intracellulare	M.intracell	M.intracell	yes	6	no	no		no	no	no	no	no	no		no		no	no		no		DISSEMINATED
7	MA 6761	27	West bengal	tissue biop	rapid	smooth cre	M.abscessus	M.abscessus	pending	M.abscess	not done	no		no	no		no	no	no	no	no	no	n/a	yes	upper	no	no	n/a	yes	CHOLECYSTECTOMY	
8	MA 7746	74	Tamil Nadu	sputum	slow	yellow	NO ID	M.simiae	M.simiae	M.simiae	no ID	no		no	no		yes	no	no	no	no	no		no		no	no		no		
9	MA 9780	43	West bengal	BAL	slow	fine colonie	NO ID	M.europaeum	M.europaeum	M.gordona	not done	no		no	no		yes	no	no	no	no	no		no		no	no		no		
10	TS 20977	59	Tamil Nadu	sputum	slow	smooth cre	NO ID	M.simiae	M.simiae	M.simiae	No ID	no		no	no		yes	no	no	no	no	no		no		no	no		no		
11	TS 19444	48	Andhra	sputum	slow	smooth cre	NO ID	M.simiae	M.simiae	M.simiae	not done	no		yes	no	PULMONARY	no	no	no	no	no	no		no		yes	no		no		
12	MA 10633	57	Kerala	sputum	slow	yellow	NO ID	M.simiae	M.simiae	M.simiae	not done	no		no	no		no	no	no	no	no	no		no		no	no		no		
13	BA 39160	72	Bangladesh	blood	rapid	smooth cre	M.abscessus	M.abscessus	pending	M.abscess	not done	no		no	no		no	no	yes	no	yes	no		no		yes	no		no		INTRINSIC SECONDARY HYPOPITUITARISM ON STEROIDS
14	MA 9178	33	West bengal	BAL	slow	smooth cre	NOT DONE	M.intracellulare	M.intracellulare	M.intracell	not done	no		no	no		yes	no	no	no	no	no		no		no	no		no		
15	TB 1424	25	Jharkhand	tissue biop	rapid	smooth cre	M.abscessus	M.abscessus	pending	M.abscess	not done	no		no	no		no	no	no	no	no	no	n/a	yes	ANT A	no	no	n/a	yes	LAPAROTOMY	
16	MA 9742	71	West bengal	sputum	slow	smooth cre	NO ID	M.intracellulare	M.intracellulare	M.intracell	not done	no		no	no		yes	no	no	no	no	no		no		no	no		no		
17	MA 8645	45	West bengal	BAL	slow	smooth cre	NO ID	M.intracellulare	M.intracellulare	NO ID	M.intracell	no		yes	no	PULMONARY	yes	no	no	no	no	no		no		no	no		no		
18	MA 8054	67	Tamil Nadu	sputum	rapid	smooth cre	M.abscessus	M.fortuitum	M.fortuitum	M.fortuitu	not done	no		no	no		no	no	no	no	no	no		no		yes	no		no		
19	MA 5676	32	Jharkhand	pus	rapid	smooth cre	M.abscessus	M.abscessus	pending	M.abscess	not done	no		yes	no	NA	no	no	no	no	no	no	n/a	no	n/a	no	no	n/a	no	n/a	SKULLBASE OSTEOMYELITIS WITH PARAPHARYNGEAL ABSCESS	
20	MA 4241	59	Tamil Nadu	sputum	slow	yellow	M.kansasii	M.kansasii	M.kansasii	M.kansasii	not done	no		yes	no	PULMONARY	no	no	no	no	no	no		no		yes	no		no		
21	MA 8410	40	West bengal	urine	rapid	smooth cre	M.abscessus	M.abscessus	PENDING	M.abscess	not done	no		yes	no	GENITOURIN	no	no	no	no	no	no		no		no	no		no		
22	MA 5812	18	Jharkhand	Lung biops	rapid	smooth cre	M.fortuitum	M.fortuitum	M.fortuitum	M.fortuitu	not done	no		yes	no	PULMONARY	yes	no	no	no	no	no		no		no	no		no		CYSTIC BRONCHIECTASIS
23	MA 1101	58	Kerala	urine	rapid	smooth cre	M.abscessus	M.abscessus	Pending	M.abscess	not done	no		no	no		no	no	no	no	no	no		no		yes	yes	DJ STENT	no		
24	BA 1983	50	Bangladesh	blood	rapid	smooth cre	M.abscessus	M.abscessus	PENDING	M.abscess	not done	no		no	no		no	no	no	no	no	no		no		yes	no		yes	PCI	INFECTIVE ENDOCARDITIS- POST PTCA TO LAD
25	PT 4522	43	West bengal	tissue biop	rapid	smooth cre	M.abscessus	M.abscessus	PENDING	M.abscess	not done	no		no	no		no	no	no	no	no	no	n/a	yes	LEFT I	no	no	n/a	no	n/a	OLD INFECTED FLEXOR ZONE V INJURY	
26	MA11199	26	Kerala	BAL	slow	smooth cre	NO ID	M.avium	M.avium subsp.hominisuis	M.avium	not done	no		no	no		no	no	no	no	no	no		no		no	no		no		
27	MA 11294	3	Tamil Nadu	tissue biop	slow	smooth cre	NO ID	M.avium	M.avium subsp.hominisuis	NO ID	not done	no		no	no		no	no	no	no	no	no	n/a	no	n/a	no	no	n/a	no	n/a	MIDDLE EAR BX- SCID T-B- NK+	
28	BA 1988	66	Tamil Nadu	blood	rapid	smooth cre	M.abscessus	M.abscessus	PENDING	M.abscess	not done	no		no	no		no	no	no	no	no	no		no		no	yes	TRACHEOSTOM	no		C/C INFLAMMATORY DEMYELINATING POLYNEUROPATHY- PROB TB ON ATT
29	MA 9975	52	West bengal	sputum	slow	fine colonie	NO ID	M.intracellulare	M.intracellulare	NO ID	not done	no		no	no		no	yes	no	no	no	no		no		no	no		no		
30	TS 622	37	Jharkhand	sputum	rapid	smooth cre	M.abscessus	M.abscessus	PENDING	NO ID	not done	no		yes	no	PULMONARY	yes	no	no	no	no	no		no		no	no		no		
31	MA 9914	60	Kerala	sputum	slow	yellow	NO ID	M.intracellulare	M.intracellulare	M.intracell	not done	no		yes	no	DISSEMINATI	no	no	no	no	yes	no		no		no	no		no		
32	MA 1409	48	Andhra	bone marr	slow	smooth cre	NO ID	M.avium	M.avium subsp.hominisuis	M.avium	not done	yes	127	no	no		no	no	no	no	no	no		no		no	no		no		PROBABLE DISS MAC
33	MA 533	41	Andhra	sputum	rapid	smooth cre	M.fortuitum	M.fortuitum	PENDING	M.fortuitu	not done	no		yes	no	PULMONARY	no	no	no	no	no	no		no		no	no		no		
34	MA 1280	23	West bengal	pus	rapid	smooth cre	M.abscessus	M.abscessus	PENDING	M.abscess	not done	no		no	no		no	no	no	no	no	no	n/a	no	n/a	no	no	n/a	no	n/a	RT BREAST ABSCESS	
35	EY 276	18	Tamil Nadu	corneal scr	rapid	smooth cre	M.abscessus	M.abscessus	PENDING	M.abscess	not done	no		no	no		no	no	no	no	no	no		no		no	yes	AADI IMPLANT	yes		SQUINT SX LEFT EYE
36	MA 6390	0	Andhra	ETA	rapid	smooth cre	M.abscessus	M.abscessus	PENDING	M.abscess	M.abscess	no		no	no		no	no	no	no	no	no		no		no	no		no		
37	MA 228	55	West bengal	sputum	slow	smooth cre	NO ID	M.intracellulare	M.intracellulare	M.intracell	not done	no		yes	no	PULMONARY	yes	no	no	no	no	no		no		no	no		no		
38	TS 2315	48	Tamil Nadu	sputum	slow	smooth cre	NO ID	M.longobardum	M.celatum	NO ID	not done	no		no	no		no	yes	no	no	no	no		no		yes	no		no		SIGNI DOUBTFUL
39	BA 35338	26	Andhra	blood	rapid	orange	NO ID	M.bacteremicum	M.neoaurum	M.neoauru	Mycobacte	no		no	no		no	no	no	no	yes	no		no		no	no		no		NEUROMYELITIS OPTICA- ON STEROIDS, RITUXIMAB
40	BP 15000	2	Tamil Nadu	blood	rapid	smooth cre	M.abscessus	M.abscessus	PENDING	M.abscess	not done	no		no	no		no	no	yes	no	yes	yes	cent	no		no	no		no		T CELL ALL ON RELAPSE PROTOCOL CHEMO
41	BA 33801	19	West bengal	blood	rapid	smooth cre	M.abscessus	M.phocaicum	Mycobacterium sp.	P.stutzeri	not done	no		no	no		no	no	no	no	yes	yes	cent	no		no	no		no		MYASTHENIA GRAVIS ON IMMUNOMODULATOR-RITUXIMAB
42	EY 323	67	Tamil Nadu	corneal scr	rapid	smooth cre	M.abscessus	M.abscessus	PENDING	M.abscess	not done	no		no	no		no	no	no	no	no	no		yes	RIGHT	no	yes	INTRAOCULAR	yes		RT EYE CATARACT SX
43	MA 1362	20	Andhra	lymph nod	slow	smooth cre	NO ID	M.avium	M.avium subsp.hominisuis	M.avium	not done	yes	107	yes	no	DISSEMIINAT	no	no	no	no	no	no		no		no	no		no		PLHIV
44	TS 3958	31	Andhra	sputum	slow	smooth cre	NO ID	M.yongonense	M.intracellulare	NO ID	not done	no		no	no		no	no	no	no	no	no		no		no	no		no		
45	MA 365	42	Jharkhand	sputum	slow	yellow	NO ID	M.szulgai	M.szulgai	M.szulgai	not done	no		yes	no	PULMONARY	no	no	no	no	no	no		no		no	no		no		
46	MA 136	69	Andhra	sputum	slow	smooth cre	NO ID	M.intracellulare	M.intracellulare	M.intracell	not done	no		no	no		yes	no	no	no	no	no		no		no	no		no		
47	MA 10996	33	Jharkhand	bone marr	slow	smooth cre	NO ID	M.intracellulare	M.intracellulare	M.intracell	not done	yes	5	no	no		no	no	no	no	no	no		no		no	no		no		DISSEMINATED
48	P 813	25	Tamil Nadu	pus	rapid	smooth cre	M.abscessus	M.abscessus	PENDING	M.abscess	not done	no		no	no		no	no	no	no	no	no	n/a	no	n/a	yes	no	n/a	no	n/a	TYPE I DM INJECTION ABSCESS	
49	TS 23812	48	West bengal	BAL	slow	yellow	NO ID	M.intracellulare	M.intracellulare	M.intracell	not done	no		no	no		no	yes	no	no	no	no		no		no	no		no		
50	TS 20224	63	West bengal	sputum	slow	smooth cre	NO ID	M.timonense	M.timonense	M.intracell	not done	no		no	no		no	yes	yes	no	no	no		no		no	no		no		SIGNI TO CORRELATE CLINICALLY
51	MA 10818	50	West bengal	BAL	slow	smooth cre	NO ID	M.intracellulare	M.intracellulare	M.intracell	not done	no		no	no		yes	no	no	no	no	no		no		no	no		no		
52	MA 10489	57	Jharkhand	sputum	slow	smooth cre	NO ID	M.avium	M.avium subsp.hominisuis	M.avium	not done	no		yes	no	PULMONARY	no	no	no	no	no	no		no		no	no		no		
53	MA 7165	45	Bangladesh	pus	rapid	smooth cre	M.abscessus	M.abscessus	PENDING	M.abscess	not done	no		no	no		no	no	no	no	no	no	n/a	yes	INCISI	no	yes	MESH PLACEM	no	n/a	n/a	
54	BA 21926	38	Tamil Nadu	blood	rapid	orange	NO ID	Mycobacterium spp	Pending	S.equorum	Mycobacte	no		no	no		no	no	no	no	no	no		no		no	no		no		CONTAMINANTS INCL RAPIDLY GROWING NTM
55	TS 6440	40	Bihar	sputum	slow	smooth cre	NO ID	M.intracellulare	M.intracellulare	NO ID	not done	no		no	no		yes	yes	no	no	no	no		no		yes	no		no		
56	TS 7361	36	Bihar	sputum	slow	smooth cre	M.intracellula	M.intracellulare	M.intracellulare	M.intracell	not done	no		yes	no	PULMONARY	yes	no	no	no	no	no		no		no	no		no		
57	PP 1669	54	Tamil Nadu	pus	rapid	smooth cre	M.fortuitum	M.fortuitum	PENDING	M.fortuitu	not done	no		no	no		no	no	no	no	no	no	n/a	yes	RT IN(	no	yes	MESH PLACEM	yes	n/a	RT INGUINAL NONHEALING SURGICAL WOUND	
58	TS 17410	54	Tamil Nadu	sputum	slow	smooth cre	NO ID	M.avium complex	M.intracellulare	M.intracell	not done	no		yes	no	PULMONARY	yes	no	no	no	no	yes		no		yes	no		no		
59	MA 1251	34	Tamil Nadu	pus	rapid	smooth cre	M.abscessus	M.abscessus	PENDING	M.abscess	not done	no		no	no		no	no	no	no	no	no	n/a	yes	ABDO	no	no	n/a	no	n/a	n/a	
60	MA 833	48	Tamil Nadu	sputum	slow	smooth cre	NO ID	M.simiae	M.simiae	M.simiae	not done	no		yes	no	PULMONARY	yes	no	no	no	no	no		no		no	no		no		
61	OH18554	41	Tamil Nadu	sputum	slow	smooth cre	NO ID	M.avium complex	M.intracellulare	M.intracell	M.intracell	no		yes	no	PULMONARY	no	no	no	no	no	no		no		no	no		no		
62	OH	59	Kerala	pus	rapid	smooth cre	M.chelonae	M.abscessus	Pending	NO ID	not done	no		no	no		no	no	no	no	no	no	n/a	yes	INGU	no	yes	HERNIAL MESH	yes		INGUINAL HERNIOPLASTY
63	MA 7188	51	Tamil Nadu	sputum	slow	dry cream	NO ID	M.intracellulare	M.intracellulare	M.intracell	M.intracell	no		no	no		yes	no	no	no	no	no		no		no	no		no		
64	MA 6577	50	Tamil Nadu	sputum	slow	smooth cre	NO ID	M.avium complex	M.intracellulare	NO ID	M.intracell	no		yes	no	PULMONARY	no	no	no	no	no	no		no		no	no		no		
65	TS 4243	30	Jharkhand	sputum	rapid	smooth cre	M.fortuitum	M.fortuitum	M.fortuitum	M.fortuitu	not done	no		no	yes	PULMONARY	no	no	no	no	no	no		no		no	no		no		NR
66	TS 6321	40	Bihar	sputum	rapid	smooth cre	M.fortuitum	M.fortuitum	M.fortuitum	M.fortuitu	not done	no		yes	no	MDR PULMO	no	no	no	no	no	no		no		yes	no		no		NR
67	MA 2470	72	Jharkhand	bone marr	intermediate	orange	NO ID	M.novocastrense	PENDING	M.flavesce	not done	yes	132	yes	no	PULMONARY	no	no	no	no	no	no		no		no	no		no		NR
68	TU 183	33	Tamil Nadu	urine	intermediate	orange	NO ID	M.flavescens	PENDING	M.flavesce	not done	no		yes	no	TB MENINGI	no	no	no	no	no	no		no		no	yes	URINARY CATH	no		NR
69	ID	41	Tamil Nadu	sputum	slow	yellow	M.kansasii	M.kansasii	M.kansasii	M.kansasii	not done	no		yes	no	PULMONARY	no	no	no	no	no	yes		no		yes	no		no		
70	TS 11537	46	Tamil Nadu	BAL	rapid	smooth cre	M.abscessus	M.abscessus	PENDING	M.abscess	not done	no		yes	no	PULMONARY	no	no	no	no	no	no		no		no	no		no		
71	TS 6333	38	Bangladesh	sputum	intermediate	orange	NOT DONE	M.flavescens	PENDING	M.flavesce	not done	no		no	no		no	no	yes	no	no	no		no		no	no		no		NR
72	MA 5496	47	West bengal	pus	rapid	dry cream	M.fortuitum	M.fortuitum	PENDING	M.fortuitu	not done	no		no	no		no	no	no	no	no	no	n/a	yes	ABDO	no	no	n/a	yes	LAPAROSCOPIC APPENDICECTOMY	
73	MA 4461	40	Nepal	lymph nod	slow	yellow	NOT DONE	M.simiae	M.simiae	M.simiae	NO ID	yes	81	yes	no	TB lymphade	no	no	no	no	no	no		no		no	no		no		AML PLHIV DISSEMINATED NTM
74	MA 5379	35	West bengal	sputum	slow	yellow	not done	M.parascrofulaceum	M.parascrofulaceum	M.scrofula	not done	no		yes	no	PULMONARY	yes	no	no	no	no	no		no		no	no		no		
75	PP 3666	24	West bengal	pus	rapid	smooth cre	M.fortuitum	M.fortuitum	PENDING	M.fortuitu	not done	no		no	no		no	no	yes	no	yes	yes	CEN	no	n/a	no	no	n/a	no	n/a	LINE ABSCESS	
76	BV 428	53	West bengal	catheter ti	rapid	smooth cre	M.abscessus	M.abscessus	PENDING	M.abscess	not done	no		no	no		no	no	no	no	no	no		no		yes	yes	LAD STENT	yes		PTCA -2017 DEC
77	BV 433	15	West bengal	catheter ti	rapid	smooth cre	M.abscessus	M.abscessus	PENDING	M.abscess	not done	no		no	no		no	no	yes	no	yes	no		no		no	no		no		APML
78	PP 3761	27	Tamil Nadu	pus	rapid	dry cream	M.fortuitum	M.farcinogenes	PENDING	M.fortuitu	not done	no		no	no		no	no	no	no	no	no	n/a	no	n/a	no	no	n/a	no	n/a	RT GLUTEAL ABSCESS	
79	MA 3970	32	West bengal	sputum	slow	yellow	NO ID	Mycobacterium spp.	M.europaeum	NO ID	not done	no		yes	no	PULMONARY	yes	no	no	no	no	no		no		no	no		no		NR
80	MA 6202	60	Chattisgarh	pus	rapid	dry cream	M.fortuitum	M.fortuitum	pending	M.fortuitu	Not done	no		no	no		no	no	no	no	no	no	n/a	no	n/a	yes	no	n/a	no	n/a	ABDOMINAL WALL INFN	
81	MA 5388	47	Jharkhand	sputum	slow	smooth cre	not done	M.intracellulare	M.intracellulare	M.intracell	M.intracell	no		yes	no	PULMONARY	no	yes	no	no	no	no		no		no	no		no		
82	MA 7428	29	West bengal	bone marr	rapid	smooth cre	M.abscessus	M.mucogenicum	pending	M.mucoge	not done	no		no	no		no	no	no	no	no	no		no		no	no		no		PUO X 4MO WITH SPONDYLODISCITIS AND MONOARTHRITIS RT KNEE
83	MA 5415	48	Bangladesh	BAL	slow	yellow	NOT DONE	M.scrofulaceum	M.europaeum	NO ID	NO ID	no		yes	no	PULMONARY	yes	no	no	no	no	no		no		no	no		no		

84	TS 12900	37	Jharkhand	sputum	slow	smooth cre	NOT DONE	M.intracellulare	M.intracellulare	M.intracell	not done	no		yes	no	PULMONARY	yes	no	no	no	no	no		no		no	no		no						
85	TP 170	50	Tamil Nadu	pus	rapid	smooth cre	M.abscessus	M.abscessus	PENDING	M.abscess	not done	no		no	no		no	no	no	no	no	no	n/a	no	n/a	no	no	n/a	no	n/a	SINUS DISCHARGE				
86	OH-SRICHI	27	Kerala	blood	rapid	smooth cre	M.abscessus	M.abscessus	PENDING	M.abscess	not done	no		no	no		no	no	no	no	no	no		no		no	no		no		AORTIC ROOT ABSCESS				
87	MA 6979	24	Tamil Nadu	sputum	slow	yellow	not done	M.simiae	M.simiae	M.simiae	not done	yes	251	no	no		no	no	no	no	no	no		no		no	no		no						
88	AFB 436	24	Karnataka	pus	rapid	smooth cre	M.abscessus	M.abscessus	Pending	M.abscess	M.abscess	no		no	no		no	no	no	no	no	no	n/a	yes	LSCS \	no	no	n/a	no	n/a	n/a				
89	BT 7770	22	Karnataka	blood	rapid	dry cream	M.fortuitum	M.fortuitum	Pending	M.fortuitu	M.fortuitum	group		no	no		no	no	no	no	no	no		no		no	no		no		DAMA				
90	MA 4527	74	Andhra	sputum	slow	smooth cre	not done	M.simiae	M.simiae	M.simiae	M.intracell	no		no	no		no	yes	no	no	no	no		no		no	no		no						