Isolation and characterization of Non tuberculous Mycobacteria from clinical specimens

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CERTIFICATE

This is to certify that the dissertation work entitled **"Isolation and characterization of Non tuberculous Mycobacteria from clinical specimens"** submitted by Dr.Kanimozhi A, is the work done by her during the period of study in this department from January 2013 to July 2014. This work was done under direct guidance of Dr.S.Parvathi, Professor and Head, Department of Microbiology, PSGIMS& R.

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CONTENTS

Abstract

Introduction: Non Tuberculous Mycobacteria commonly known as Atypical Mycobacteria are seen in the environment and have been isolated worldwide. The prevalence of NTM infections are increasing and the diagnosis is often difficult or unconvincing . Identification of isolates to the species level is important, as infections with different Mycobacterium species often require different management and pathogens must be distinguished from environmental NTM contaminants

Aim To isolate and characterize different species of Non tuberculous Mycobacteria from various clinical specimens

Materials and methods: The study was conducted in patients of PSG hospitals with clinical suspicion of tuberculosis during the period of January 2013 –July 2014.Sputum,tracheal aspirate ,BAL, pus and tissue were obtained as specimens. NTMs were isolated and speciated by using conventional biochemical tests.Immunochromatography (TBcID test) test was used to differentiate NTM from *M.tuberculosis*. Comparative evaluation of a commercial niacin strip test

kit(BBLTM TaxoTM TB NiacinTest Strips) with conventional niacin test was done.

Results: A total of 3050 samples were received and processed in Mycobacteriology laboratory during the study period. Incidence of NTM during the study period was found to be 1.3%. Out of 363 culture positive acid fast bacilli, 40(11.01%) were found to be Non tuberculous Mycobacteria. Among them 67.5% and 32.5% were rapid growers and slow growers respectively. The most common NTM isolated was *Mycobacterium fortuitum*(27 %).

Conclusion: The incidence of Non Tuberculous Mycobacteria was found to be 1.3%. The commercial niacin test kit and TBc ID test were found to be 100 % sensitive and specific and can be used as an alternative for rapid diagnosis of NTM.

Key words: NTM, TBcID test Commercial niacin strip test

INTRODUCTION

Mycobacterium belongs to the genus Actinobacteria and family Mycobactericeae. Mycobacteria can be classified into major groups :*M.tuberculosis* complex, *M.leprae* and Non Tuberculous Mycobacteria(NTM)

Non Tuberculous Mycobacteria commonly known as Atypical Mycobacteria are seen in the environment and have been isolated worldwide .Other synonyms are MOTT (Mycobacterium other than tubercle bacilli), anonymous (or) unclassified mycobacteria, tuberculoid or paratubercle bacilli .The better suited names are environmental or opportunistic mycobacteria as their natural habitat appears to be the soil or water and they cause opportunistic infections in human beings[1].

Non tuberculous mycobacteria have been classified into four groups by Runyon based on pigment production and rate of growth[1].Group I ,II ,III and IV includes Photochromogens, scotochromogens, nonphotochromogens and rapid growers respectively. Nearly 125 species have been identified till date. Few medically important species among them are *Mycobacterium avium complex, Mycobacterium ulcerans ,Mycobacterium kansasi, Mycobacterium fortuitum, Mycobacterium chelonae and Mycobacterium marinum.* Most common infection caused by atypical mycobacterium is pulmonary disease .*Mycobacterium avium complex* causes lymphadenopathy, pulmonary lesions or disseminated disease particularly in AIDS patients. *Mycobacterium fortuitum and Mycobacterium*

chelonae causes chronic abscesses in human beings. Both *Mycobacterium fortuitum and Mycobacterium kansasi* produces lung infections resembling that of Mycobacterium tuberculosis. Human - human transmission has no evidence or documentation but the environmental exposure is suspected to produce human disease.

Since few species produces lung disease exactly like that caused by *Mycobacterium tuberculosis*, some patients with NTM lung disease are falsely treated for tuberculosis who will never respond to anti tubercular treatment (ATT). Moreover just the isolation of NTM from a sputum sample may not indicate NTM lung disease, as it is an organism from the environment. In order to distinguish between contamination and true NTM disease the American Thoracic Society has established a specific diagnostic criteria .Hence when a patient is diagnosed to have NTM infection it is very important to correlate with clinical, radiographic and histological findings, and the clinician should notify the laboratory when results are inconsistent. Such an established and ongoing professional relationship between clinicians and the laboratory enables the recognition of inaccurate results earlier, and therefore may minimize any potential harm to the patient.

Non tuberculous mycobacteria also produces other infections like lymphadenitis, skin and soft tissue infections, bone infections , keratitis and disseminated diseases. The current extraordinary level of interest in NTM disease

is the result of two different reasons, they are its and more number of people with NTM fall in the category without diagnosis of AIDS. Another major factor contributing is interest and involvement in new techniques for identification of NTM from patient specimens[2]

Several biochemical and cell level identification are been in research for quick identification of these organisms. The advances in molecular techniques like 16s rRNA gene sequencing (which is accepted as standard for defining new species) has led to the discovery of newer species.

The prevalence of NTM infections are increasing and the diagnosis is often difficult or unconvincing. Treating modalities is also tricky and notorious with long term medications and less tolerance which proves it for less eradication[2].In India, Non tuberculous mycobacterial infections are under diagnosed and undertreated when compared to developed countries. Research on the Non tuberculous mycobacteria is also very minimal in South India. Recognition of each species and its sub species is important as the treatment is different for each species and they have to be differentiated from the environmental NTM contaminants[3.] Thus, the appropriate diagnosis and the timely treatment pattern of these mycobacteria for epidemiological purposes.

Hence, this study was undertaken to find out the prevalence of Atypical Mycobacterial infections and the species causing disease in Coimbatore.

REVIEW OF LITERATURE

The most acknowleged mycobacteria over centuries include,*Mycobacterium tuberculosis* and *Mycobacterium leprae*. The other known mycobacterial species are seen around us as saprophytes. Their tendency to cause the disease is identified as early as last century[3]. These were all identified as atypical mycobacteria, the term first coined by Pinner[4].They are also referred to as non tuberculous, anonymous, opportunistic and Mycobacteria other than tubercle bacilli (MOTT).Although they are referred with various names there is no universal term to name them, and Non tuberculous mycobacteria (NTM) seems to be more acceptable and has been approved by American Thoracic Society (ATS) in their statement[5]

History of NTM:

In the late 19th century is the time "tuberculosis" was described in chickens in 1868 where the history of Non tuberculous mycobacteria (NTM) started. But this organism was identified as one distinct or different from tuberculosis bacilli and it was later identified to be *Mycobacterium avium*. It was also found these organisms were not found to cause any the disease after injected into guinea pigs and therefore it was recognized varying from *Mycobacterium tuberculosis* and were considered it cannot cause any human disease. It was only in 1930s NTM

was found to be causing disease in humans. In 1943 was the first time it was identified in lung disease due to *M. avium complex* (MAC) in a person who had silicosis[8]

Thirty-five years ago, the Mycobacterium genus comprised only some 30 species. By 2004, it encompassed close to nearly 95 species.[9]

General characteristics:

Atypical mycobacteria are classified into four main groups. They are Photochromogens, Non photochromogens, Scotochromogens and Rapid growers .Each group contains many species which are identified as separate species by using many recent and improved techniques like polymerase chain reaction-restriction length polymorphism analysis (PRA), high-performance liquid chromatography (HPLC), 16S ribosomal DNA sequencing. Nearly 125 species have been identified till date.

Scientific classification:

Kingdom	**Bacteria**
Phylum	Actinobacteria
Order	Actinomycetales
Suborder	Corynebacterineae
Family	Mycobacteriaceae
Genus	Mycobacterium

Habitat

Since there were no clues of person to person transmission, the source of infection is identified as environment[8].They were also important environmental opportunistic pathogens of humans, animals, poultry, and fish[9, 10]. The past 3 decades evidence has predictable that the NTM were normal population of a variety of ecological habitat common with humans and animals, including natural

occurring waters, drinking water allocation systems, and soil[11].Various studies in India also proved its presence in environment. Study conducted from JALMA in 2004 has established among many mycobacteria, *M. avium*, *M. terrae* and *M. chelonae* in soil and *M. avium, M. kansasii, M. terrae, M. fortuitum* and *M. chelonae* in water [12].

Mode of Transmission:

Since the infection is said to be acquired from the environment and there is no person to person transmission[9], there arises a question that how it gets aerosolized from the environment. The mode of transmission was once reported to be aerosolized from sea water bubbles and hence higher incidence of atypical mycobacterial disease has been reported in wide spread coastal region in India[10].Even an epidemiological study at Chennai in 1979, revealed that one of the cause for BCG protection against tuberculosis in coastal region of India could be the sensitization of these individual with atypical mycobacteria[13]. It was found that in *M. abscessus, M. fortuitum, M. chelonae, M. marinum* and *M. ulcerans.* Cutaneous disease may occur by entry through the skin during any abrasion or surgery produced wound. It was well established even before 2000that NTM can multiply in the lungs even if the patient is immunocompetent. Though many levels are identification are there for these species their pathogenic role is not clear.

Infections caused by NTM:

In 1961, after the advent of antibiotics, diseases due to NTM are frequently reported in world literature. Some of the predisposing conditions which are important in pathogenesis of pulmonary disease due to NTM are pneumoconiosis, chronic obstructive pulmonary disease and healed tuberculosis[10] .Initially only 2 species or complexes have emerged as the predominant agents of disease, *Mycobacterium kansasi*, *Mycobacteriumaviumintracellulare and M.scrofulaceum* complex[12].Since then various spectrum of infections caused by different mycobacterial species has been proposed in various studies.

Important Atypical mycobacteria associated with infections[2]

INFECTIONS	COMMON NTM ASSOCIATED	UNCOMMON NTM
PULMONARY DISEASE	***M.aviumcomplex***,***M.abscesses*** ***M.kansasi***,***M.malmoense***	***M.chelonae***,***M.fortuitum*** ***M.scrofulaceum***,***M.celatum***
LYMPHADENITIS	***M.avium complex*** ***M.Malmoense***,***M.scrofulaceum***	***M.genavense*** ***M.hemophilium***
SKIN , SOFT TISSUE & BONE DISEASES	***M.chelonae***,***M.fortuitum*** ***M.ulcerans***,***M.abscesses***	***M.avium complex*** ***M.hemophilium***
DISSEMINATED DISEASE	***M.avium complex***, ***M.chelonae***,***M.hemophilium***, ***M.kansasi***	***M.malmoense*** ***M.conspicuum***, ***M.fortuitum***

Pathogenesis:

There are three important observations to describe the pathogenesis behind NTM infections[2] Firstly in patients with HIV, NTM infection disseminated typically only when the CD4 T-lymphocyte count had fallen below 50/l, showing that specific T-cell response were required for mycobacterial resistance .Secondly, in Non- HIV patient group, specific mutations in interferon (IFN)- γ and interleukin (IL)-12 synthesis and response pathways had association with genetic syndromes of disseminated NTM infections. Thirdly, there is also an association between bronchiectasis, nodular pulmonary NTM infections and a particular body habitus, predominantly in postmenopausal women (e.g., pectus excavatum, scoliosis, mitral valve prolapse)

Importance of different species of Non Tuberculous Mycobacteria:

Mycobacterium avium complex, Mycobacterium fortuitum and Mycobacterium kansasi were the most frequently reported potentially pathogenic species from various studies[13.]

Mycobacterium avium complex:

Two mycobacterial species namely *Mycobacterium avium* is responsible for disseminated disease and *Mycobacterium intracellulare* is commonly a respiratory pathogen together constitute *Mycobacterium avium intracellulare complex* [2].It has been commonly found to multiply in all sources of water, hot tubs and indoor

water systems. They have been reported to be the cause for various pulmonary infections ,lymphadenitis ,bone and joint infections and disseminated infections in Immunocompromised patients[15].MAC lung disease presents as 2 forms :apical fibrocavitory lung disease in males belonging to 40-50years age group and who have smoking and excessive alcohol use[16].The other form is the one with nodular and interstitial infiltrates seen in nonsmoking postmenopausal females and this form is labeled as "Lady Windermere syndrome"[17]

Mycobacterium kansasi:

For *M.kansasi* causing human disease tap water is the major reservoir [18].Various DNA based studies suggests that *M.kansasi* has many subtypes and that subtype I is the predominant one responsible for human infection[19].Risk factors for *M.kansasi* infection includes COPD ,pneumoconiosis, previous mycobacterial disease and alcoholism[20].They are known to cause pulmonary infections, bone and joint infection and disseminated infection in HIV patients[7]. *M.kansasi* is the next commonest organism to produce the disease in immunocompromised.[21].They have the similar symptoms as seen with tuberculosis and the symptoms are also identical to pulmonary tuberculosis in case of *M.kansasi*infection[2] Moreover they show more resistance to antitubercular drugs than *Mycobacterium tuberculosis*[7].

Mycobacterium fortuitum:

M.fortuitum –M.chelonae complex are rapidly growing organisms that most commonly involved in the infections involving soft tissues abscesses and injection site reactions and isolation from soil in different countries including India is frequent[7,15].DaCosta Cruz characterized the first strain of *M. fortuitum* following its recovery from the site of vitamin injection[22]. Literature reports of injection abscess due to NTM such as *M. fortuitum, M. chelonae*, and *M. abscessus* following the injection of vitamin preparation, DPT vaccine, iron dextran and penicillin have been documented[23]. An Indian case report from Bangalore has mentioned about injection abscess due to *M.fortuitum*in a 3 year old child[24].They are also known to cause joints , bursae and pulmonary infections and disseminated disease[7]. The most frequently reported etiological agent in NTM infection in patients wearing contact lenses or following a eye surgery like LASIK is *M.chelonae*[25].

M.fortuitum complex includes *M. fortuitum*, *M. peregrinum, M. houstonense,M. boenickei, M. mageritense* and*M. Senegalense* and separation of these species could be done only by molecular methods.These were also identified as sources of furunculosis as seen with *M.fortuitum* and rapid growers[26].

Mycobacterium abscessus:

Mycobacterium abscessus is seen to multiply in the respiratory tract and produce certain pulmonary conditions in high risk groups like prior pulmonary mycobacterial infection, particularly tuberculosis and in white people of female sex with age group more than sixty years of age and those with diagnosis of cystic fibrosis [27,28].**Mycobacterium abscessus** can produce biofilms in the drinking distilled water.This also produces skin related infections which might resolve spontaneously or only after either medical or surgical treatment[27,28]. A study in United states found that major endemic area for *M.abscesses* disease were the southeastern part from Florida to Texas[13]. It causes cutaneous disease usually followed by accidental trauma or surgery in a various clinical situation [29].In India, a case of bacteremia secondary to a nonhealing sternal wound due to *M. abscessus* following a CABG bypass graft procedure was reported in Haryana[30].A fatal case of native valve endocarditis in a hemodialysis patientdue to *Mycobacterium abscessus* was reported in Kuwait[31]

M.marinum:

It is pigmented slow growing organism causes swimming pool granuloma or fish tank granuloma. This mycobacteria is present commonly in all water related sources both fresh and salt water and mostly in the stagnated wated area like fish tanks, swimming pools which are not chlorinated[32].A case report revealed growth

of *M.marinum* seen in multiple non-inflammatory nodules with necrotic centers and erythema on the forearm of a 29 year old immunocompetent female with history of rose thorn prick from Thrissur,India[33].

Mycobacterium hemophilum:

It is a fastidious organism and it is slow growing in a enriched medium in the presence of haemin,ferric ammonium citrate or chololate agar for it to grow. Growth is seen good in the temperatures below thirty degrees celcius[7].In AIDS and patients after bone marrow transplantation it is found to produce life threatening infections[34]. The second risk group category consists of immunocompetent children in whom disease are caused which is clinically similar to that induced by infection with *M.avium* complex,*M.tuberculosis*,and ***M.scrofulaceum*** in whom *M. haemophilum* infection induces cervical and perihilar lymphadenitis[35]

Mycobacterium smegmatis :

It rarely cause significant infection, has been associated with infections of the bone like osteomyelitis, infections of the lymphnodes, infection of the surgical site in the sternum after cardiac surgery, infection of the catheter site, and infections of the breast like abscess formation or following mammoplasty[36].

Mycobacterium scrofulaceum:

It is commonly present in atmosphere in soil, and water, household dust and also seen as opportunistic infection with disseminated infections, childhood lymphadenitis, lung disorders, and cutaneous infections[38]. It was known to cause "scrofula" (cervical lymphadenitis) in children but during early 1980s and it was replaced by *Mycobacterium avium* complex[2].some studies shows that they account to nearly two percent of all the mycobacterial infections seen in AIDS individuals[38].

Mycobacterium terrae complex:

It comprises of multiple species including *M. triviale*, *M. terrae*, *M. nonchromogenicum*, and *M. hibernia*[39].Most of the isolates were previously presumed to be non pathogenic[40] but now they are proved to cause tenosynovitis of hand and cavitory pulmonary disease[41].*M. terrae* complex was also reported in individuals with recurrent urinary infections[42].

Mycobacterium ulcerans:

It is considered as third common mycobacterium following tuberculosis and Lepra, in immunocompetant individuals[43]. The skin lesions caused by them are indolent, progressive necrotic and with scalloped edges known as "buruli ulcers"[37,44]. Infections are acquired through abrasion of the skin or compromised

skin which comes in contact with the water sources or contaminated soil . They are seen mostly in young kids and adults resulting with scars and deformities in the extremities[37].

Mycobacterium xenopi:

This organism requires thermophile condition with temperature of minimum 45 degrees celcius .They capable of colonising hot water tanks of disinfecting machine resulting in pseudoepidemic infection by them due to contamination of surgical instruments[45].

Diagnosis of NTM

Speciation of NTM is more significant as a result of the variations in antibiotic susceptibility that determine treatment options[46].All NTM isolates not necessarily got to be extensively identified as they might be a contaminant too. Therefore, communication needs to be present between clinician and laboratorian that becomes important in diagnosis of NTM. The clinical significance of NTM isolates is influenced by many factors like, the recovery from multiple specimens, recovery of the organism in large quantities, or recovery of an NTM isolate from a normally sterile site likeblood[2].

Specimen collection

The statement issued by American Thoracic Society (ATS) and Infectious Disease Society of America (IDSA) on diagnosis and treatment of infections caused by NTM emphasizes that evaluation of patients suspected of having pulmonary NTM infection should include the following:"*Chest radiograph; chest high resolution computed tomography (HRCT) scan if cavitation is absent on radiography; three or more sputum specimens for acid fast bacilli (AFB) analysis and exclusion of other disorders such as tuberculosis*"[2]. Specimen collection should be done by avoiding potential sources of contamination, e.g. tap water in which environmental mycobacteria are most commonly present. Specimens must be collected in a container which is sterile, leak proof, appropriately labeled .Usually transport media and preservatives are not recommended. Refrigeration of specimens at 4 °C could be done just in case transportation is delayed by more than an hour.

Body fluid or abscess pus should ideally be collected by needle aspiration or surgical procedures. Sample collection should not be done using swabs as they often have very limited culture material and can also get dried leading decreased chances of isolating the NTM. The most sensitive ways of diagnosing infections due to NTM were found to be culture and histopathological examination of tissue biopsies. Care should be taken that tissue biopsy specimens are not wrapped in

gauze or diluted in liquid material. Tissue must be immersed in a small amount of sterile normal saline (not formalin) to avoid excessive drying in case if scanty tissue was available. Antibiotics like macrolides and quinolones ought to be restricted when RGM diseases are suspected as these can decrease the yield of RGM[2].

Digestion and decontamination

When specimens are collected from non sterile areas, it is mandatory to perform digestion and decontamination procedures. The most commonly used method is the N-acetyl l-cysteine sodium hydroxide technique. NTMs are more vulnerable to decontamination than *M.tuberculosis* and hence the procedure should much more softer than that for *M.tuberculosis*[2].

Smear microscopy

Acid-fast microscopy is the quickest, easiest, and least costly tool for the rapid identification of patients with mycobacterial infections. Though the specificity of acid-fast microscopy is excellent (all mycobacterial species are acid-fast), the sensitivity isn't optimal, and this technique is unable to distinguish among the mycobacterium genus. The sensitivity of microscopy is influenced by various factors like the prevalence and severity of tuberculosis or

NTM disease, the type of specimen, the standard of specimen collection, the number of mycobacteria, present within the specimen, the strategy of processing (direct or concentrated), the method of centrifugation, and most significantly, by the staining technique and also the quality of the examination. Therefore, the overall sensitivity of microscopy is just between 22 – 65%[47].It is recommended that a negative result should be reported solely following the examination of a minimum of three hundred microscopic oil immersion view fields (or equivalent fluorescent view fields). Therefore, when microscopy is performed properly, it is time consuming and laborious. With large specimen loads, fatigue could result in the reporting of a false-negative result, further decreasing the sensitivity of the assay. To facilitate proper patient management, all results should be reported to the physician within twenty four hours of specimen collection or if an offsite laboratory is employed within twenty four hours after receipt of the specimen [47]

Presently, two types of acid-fast stains are used in clinical mycobacteriology laboratories. One is carbolfuchsin [Ziehl-Neelsen (ZN) or Kinyoun staining methods], and the other a fluorochrome (usually auramine or auramine-rhodamine). It is generally accepted that the fluorescent method should be given preference over the ZN and Kinyoun[48].Another study done elsewhere says that the recommended method for staining mycobacteria is the fluorochrome method[2].

Ziehl Neelsen or Kinyoun stain is acceptable but less sensitive. It is more prudent to use a weaker decolorizing agent for staining NTM as they may be more sensitive to the AFB decolorization procedure[2].

Results of a recent survey revealed, however, that the sensitivity and specificity of the ZN and fluorochrome methods are comparable; whereas Kinyoun's cold carbolfuchsin method was found to be inferior to both the ZN and fluorochrome methods [49]. Moreover, fluorochrome stains may stain other bacteria damaged by antituberculous drugs at a higher rate than carbolfuchsin and lead to a false-positive result. This possibility should be considered when the specimen is from a patient on therapy. It is also noteworthy that, because of a tendency toward false-positivity with fluorochrome staining, good laboratory practice requires that any doubtful and smear-positive results on newly diagnosed patients should be confirmed by ZN or by a second examiner

In order to increase the efficacy of acid fast microscopy, a model of a computer-directed automated microscope was constructed [50]. The automation and the time saving provided by this type of microscope can allow the examination of parallel smears from the same specimen, which could well increase the sensitivity. This model equipment still needs further evaluation, however, before it can be put into routine use

Mycobacterial culture:

It is clear that, although acid-fast microscopy and NAA are important adjuncts to the detection of mycobacterial infections, they are not an adequate criterion alone and must be followed by growth detection. Thus, culture for the presence of mycobacteria is still indispensable for the following reasons: (1) culture is more sensitive for the detection of mycobacteria compared to acid-fast microscopy, (2) NTM growth pattern ,not covered by the existing NAA assays is needed for precise identification, (3) viable organisms are needed for the drug susceptibility test and (4) cultured NTM genotyping (ie, *M xenopi, M chelonae*, *M malmoense, M avium, M abscessus,* etc) can be used for epidemiologic purposes and to rule out laboratory errors (cross contamination) [51].

Before culture, clinical specimens from non disinfected body sites should undergo prior treatment involving decontamination, homogenization, and concentration. This procedure will destroy more fastly growing contaminants like normal flora (other fungi and bacteria) but it does not affect the mycobacterial viability [47]. It is noteworthy, however, that the usefulness of the procedures is highly influenced by the exposure time of the reagent used in decontamination, the reagent toxicity, centrifugation effect, and the killing nature of heat during the process of centrifugation [47].There is proof that mildest decontamination methods such as NaOH method/ N-acetyl- L-cysteine kills the mycobacteria by 33% from a

clinical specimen, whereas methods which are more overzealous can destroy up to 70% [47]. In addition, particular patient populations might need special attention regarding the homogenization and decontamination method to be used. This is especially true for respiratory specimens from patients with cystic fibrosis because it has been reported that NTM are being recovered from these patients with increasing frequency. About 80% of these specimens, however, also contain *Pseudomonas aeruginosa,* which grows over the culture medium and thus preventing the NTM isolation. Recently, it was demonstrated that the N-acetyl-L-cysteine/NaOH decontamination procedure, followed by a 5% oxalic acid treatment, can sufficiently reduce the overgrowth by *Pseudomonas aeruginosa*, thus improving the recovery rate of clinically significant NTM [47].

The Centers for Disease Control and Prevention (CDC) recommend the use of both solid and liquid media in sort to decrease the detection time and to raise the yield of growth detection [52]. For many years, the only culture system with the potential to decrease turnaround time was the BACTEC 460TB system (Becton-Dickinson Diagnostic Instrument Systems) [53]. It has been shown, however, that the newly introduced Mycobacteria Growth Indicator Tube (MGIT; Becton-Dickinson Diagnostic Instrument Systems), MB Redox (Biotest AG, Dreieich, Germany), BACTEC 9000 MB (Becton-Dickinson Diagnostic Instrument Systems), MB/BacT (Bio Me´rieux, Inc, Durham, NC) and ESPII (Accumed, Inc, Westlake,

Ohio) systems are suitable nonradiometric and/or fully automated alternatives to the radiometric BACTEC 460 TB system [54]. It is noteworthy that, although all the alternative broth based systems showed a comparable sensitivity to the BACTEC 460 TB system, the MGIT system exhibited a significantly better recovery rate conserning to the *M. avium* complex and other NTM (86% versus 72%, and 69% versus 50%, respectively), and the ESPII showed a higher recovery rate regarding the *M. avium* complex (94.6% versus 75.7%) [54]. However, with the exception of the BACTEC 460 TB and the BACTEC 9000 MB systems, direct inoculation of blood is not done by the novel growth detection systems. Only after lysis and centrifugation steps, blood samples can be inoculated into these systems [54].

Though broth-based systems reduces the detection time to 1–3 weeks, a usage of solid medium must be done for those strains that may not grow well in liquid media [54,55]. It is true in case of *M. haemophilum,* which will grow better on solid media (supplemented with hemin or hemoglobin as an iron source) [56]. Therefore, all acid-fast microscopy positive specimens, specimens from ulcerative skin lesions or septic joints of immune compromised patients, specimens from undiagnosed pulmonary lesions of bone marrow transplant recipients, and specimens from children with adenitis should also be inoculated either onto chocolate plates, or to Middlebrook 7H10 agar with hemolyzed sheep erythrocytes, hemin, or an X-factor strip, or to a Lowenstein-Jensen (LJ) slant with 1% ferric

ammonium citrate [56]. It is noteworthy that *M avium* subspecies *paratuberculosis* also requires additional nutrients (egg yolk and the siderophoremycobactin J) in both liquid and solid media for optimal growth[57]. Other species like *M genavense,* however, show a better recovery rate in liquid media especially at an acidic pH (pH 5.5) [58]. Similar to liquid media, the pH of solid media can also significantly influence the growth of mycobacteria. It has been shown that for slowly growing mycobacteria (>7 days for visible growth in subculture), based on the testing of 16 different species, the optimal pH in LJ medium was between 5.8 and 6.5, with the exception of *M lepraemurium*(pH 5.8–6.1) [59]. As for rapid growers (visible growth of subculture in < 7 days), the optimal pH was between 7 and 7.4, with the exception of *M chelonae*, which preferred an acidic pH [59]. These findings indicate that the routinely applied LJ medium with pH 7 is not optimal for the isolation of all mycobacteria. Therefore, in areas endemic for lung or other diseases caused by NTM, inoculation to an additional LJ slant with an acidic pH, or to Ogawa medium (pH 6), is recommended [59].

Another benefit on solid media for culturing mycobacteria is that colony morphology , growth can be quantified, and pigmentation can be tested , and for performing biochemical tests if needed. Other information also provides clues to find NTM or to guide the selection of other confirmatory tests such as DNA hybridization assays [60].

Use of species-specific probes on AFB-positive sputum smears

Because the stains which are acid-fast cannot distinguish between NTM and M tuberculosis (MTB) complex organisms, Biological approach using fluorescence in situ hybridization (FISH) appears to be beneficial . Probes containing two peptide nucleic acid targets the 16S rRNA and was introduced to identify the NTM in general and MTB complex specifically in positive broth cultures [61].The FISH assay could be an asset in the peripheral laboratory's armamentarium when serving communities with a substantial fraction of NTM diseases since no equipment amplification is needed. Even though the procedure can instantly differentiate between NTM and MTB complex, it is capable to detect the specific NTM present. In addition, due to a single mismatch between the 16S rRNA sequence of this species and that of the MTB-specific probe , it can give a weak false-positive signal with *M marinum* [61]. Another drawback of the method is that the relatively commonly isolated species, *M.fortuitum, M.flavescens, and M xenopi* are not detected by NTM-specific probe [61]. All of these species have more than one mismatch in the capture region of the NTM probe.

Direct nucleic acid amplification assays

Molecular tests such as direct nucleic acid amplification (NAA) assay is directly used on the clinical samples in individuals who are in suspision to have

mycobacterial disease [62]. Nowadays, the only viable test that offer possibility to detect a NTM (*M avium*) along with MTB directly from clinical specimens and from the same amplification reaction are the Amplicor PCR assay (Roche Molecular Systems, Branchburg, NJ) and BDProbeTec (Becton- Dickinson Diagnostic Systems, Sparks, MD) strand displacement amplification technology [63]. But neither of these tests are approved yet for this testing aspect by the Food and Drug Administration in the United States. Recently, to improve the Amplicon polymerase chain reaction (PCR) assay and to detect the presence of a wide range of NTM, a Mycobacterium genus-specific capture probe was developed that can be used in conjunction with the pan-genus primers (targeting the 16S rRNA gene) and the MTB- and *M avium*-specific capture probes of the kit [63]. This genus-specific screening probe was tested with both respiratory and nonrespiratory specimens showing an initial sensitivity of 78.5% and a specificity of 93.5%. One limitation of this genus-specific probe is that it can mount a ‘‘false-positive’’ outcome in the existence of nonsignificant NTM clinically in the specimen. To circumvent this problem, a second screening probe detects only the common potentially pathogenic *(MTB complex, M avium, M intracellulare, M kansasii, M xenopi, M malmoense, M leprae)* mycobacteria but not the clinically less significant ones [64]. The initial sensitivity and specificity of this screening probe were 89% and 93.9%, respectively[64]. Specimen’s positive with either of these screening probes can be

further evaluated by hybridizing the amplicons to species-specific probes. These tests are done in as few as 6– 8 hours on specimens which are processed, would allow same-day reporting of outcome.

Identification of isolates by phenotypic characteristics:

Growth rates of colony, colony pigmentation and tests such as nitrate reduction, urease, arylsulphatase, tellurite reduction, niacin production, catalase (qualitative and quantitative),thiophen-2-carboxylic acid hydrazide (TCH) sensitivity, sodium chloride tolerance,tween-80 hydrolysis, growth on MacConkey agar, *etc*., are enough to make out most of clinically important mycobacteria [65,66]. A study from South India has also given the method to differentiate NTM from *M. tuberculosis* by various biochemical tests as shown in the table[67].

Speciation of NTM by simple biochemical tests

S.NO	BIOCHEMICAL TESTS	*M.tuberculosis*	NTM
1	Niacin test	+	-
2	Growth on LJ at 25^{o}c	-	+
3	68^{o}C catalase	-	+
4	Growth on LJ containing PNB	-	+

This approach is, yet, time consuming and will not be decisive for a lot of isolate with variable characteristics. Thereby, analysing the lipids of mycobacteria by high performance liquid chromatography (HPLC) and thin layer chromatography (TLC) would be considered as an alternating advance. As used by the side of an easier software programmes for quick study, isolates as of solid/ liquid medium could be quickly recognized. Serotyping method via exact serotype for the members of *Mycobacteriumaviumintracellularescrofulaceum(MAIS) complex* been described [68]. Some serotypes of *M. avium* have revealed it to be mostly related to disease in patients with Acquired immunodeficiency syndrome. Proteinelectropherogram and Isoenzyme schemes are for quick characterization and recognition of strains of *M.tuberculosis* and Nontuberculous mycobacteria which may be used even in small lab settings[69]. Based on the dimension of immunological divergence in the enzyme molecule's structure such as superoxide dismutase and catalase, techniques were used for recognition of mycobacteria[70,71]. Most of these techniques are in want of expertise and specific infrastructure but which is not hard to put up.

Immunochromatography test

Routine methods of culture and detection of mycobacteria are very time-consuming, captivating for about 6 to 8 weeks for the growth of organism and yet another a number of weeks to months for identifying biochemically. The beginning of methods based on liquids, on the other hand,

has reduced the times to recognition and detection. In recent times, an add up of assays using MPT64 antigen assays were done to distinguish *Mycobacterium tuberculosis* complex (MTBC) isolates & Nontuberculous mycobacteria by immunochromatography. These kits detects MPT 64 antigen present in the *M.tuberculosis complex* isolates by means of mouse monoclonal MPT 64 antibody MPT64 is nothing but a protein which is produced merely by MTBC and been useful in distinguishing MTBC from that of NTM [72,73]Studies also shown that the *M. tuberculosis* protein 64 is definite for *Mycobacterium tuberculosis* complex, which include *M. tuberculosis*, *M. bovis*, *M. africanum*, & a few, while it is not for all, substrains of *M. bovis* BCG[74,75]. The MPT 64 is an antigen specific to MTBC produced all through the growth of bacteria, and which is an first-rate antigen for recognizing the MTBC[74,75].Various studies have been conducted to find out the validity of this immunochromatography test for identifying NTM. Recent study done in Lucknow showed that the specificity and sensitivity of the

immunochromatography kit was said to be 100 & 99.1% respectively[75].Many other studies conducted elsewhere also concludes the same.

High-performance liquid chromatography

High-performance liquid chromatography (HPLC) done on mycolic acids of *Mycobacterium species* been considered as a fast and reproducible means to make out a wider range of well-known or un-known species of mycobacteria either from culture or from sputum [76]. HPLC Users Group and CDC had given the Standardized procedures and pattern standards[76]. Species identification by mycolic acids is done in comparing with inhouse databases. But a computer- based pattern recognition method and a site on the World Wide Web (http://hplc.cjb.net), maintain the High-performance liquid chromatography Users Group for specific support of the High-performance liquid chromatography detection of mycobacteria, also obtainable[76,77]. The widely used ultraviolet High-performance liquid chromatography is not as sensitive as the fluorescent HPLC, an adaptation that can considerably augment HPLC's sensitivity whereas decreasing the time & the cell mass is essential for mycobacterial detection. Even though it is not as sensitive as NAA assays, fluorescent High-performance liquid chromatography been used limitedly on sputum for fast direct testing. On the whole, High-performance liquid chromatography is hasty (less than 2 hours) and consumables cost are economical; yet, the assay needs a committed and skilled technician

(because of the chromatographic patterns are to be visual interpreted), expensive software & instruments, proficiency on instrumentation preservation, and consistent growth settings together with the want for a great biomass when using UV-HPLC[76].

Nucleic acid hybridization methods

Use of the AccuProbe (Gen-Probe Inc, San Diego, CA) which is a nucleic acid hybridization kits represent a quantum leap forward in the quick detection of the MTB complex, *M avium* complex, *M gordonae*, and *M kansasii*. These assays allowed rapid identification of these mycobacteria (within 2 hours results) as shortly as adequate biomass is got subsequent to growth is in culture[78]. Rarely cross-reaction is recognized in the Accu- Probe for the *Mycobacterium tuberculosis* complex with either *M terrae* or *M celatum* types 1 and 3 isolates as the test is not done accurately as instructed in the insert of the package[79]. Adhering to the appropriate hybridization temp. (between 60˚C and 61˚C quite than 60˚C ± 1˚C) was the mainly the parameter of significance[79]. Although *M gordonae* has not been associated with disease, it is one of the most commonly isolated mycobacterial species. Therefore, rapid identification of this organism using a DNA probe has facilitated patient management by ruling out involvement of mycobacterial pathogens.

A recent study found, however, that not all strains of *M gordonae* were positive with this species-specific probe[80]. As opposed to the nonpathogenic *M gordonae, M.kansasii* is one of the major pulmonary mycobacterial pathogens. In the past, identification of *M kansasii* in the clinical laboratory has relied on growth characteristics, pigmentation, and selected biochemical tests. In recent years, identification of *M kansasii* has been greatly facilitated by use of the commercially available DNA probe (Accu Probe, Gen-Probe, Inc); however, it has been shown that not all subspecies of *M kansasii* are positive with this probe. In order to solve this problem and identify all variants, a new version of the *M kansasii* AccuProbe was developed and made available [82].For the reason that Deoxyribonucleic acid/ Ribonucleic acid probe assays will not embrace an amplification step, these are quiet not receptive enough to be used straight on the specimens; yet, Deoxyribonucleic acid/ Ribonucleic acid probes would frequently able to identify mycobacteria in liquid cultures that are contaminated based on the degree of contamination[83] because they said to have a specificity and sensitivity of about 100% of when 105 organisms are there [84]. Unfortunately, commercial probe assays are not available for the majority of the NTM.

PCR and analysis of restriction fragment length polymorphism

In 1992 developed the concept of differentiating among slowly growing mycobacteria by PCR and restriction fragment length polymorphism analysis

(PRA) using the hsp65 gene was developed [85]. Similar assays targeting the 16S rRNA or the dna J genes were subsequently evaluated, but ultimately a method based on the hsp65 gene was generated for use in routine clinical practice [86]. This procedure can be done on isolates of AFB that develop either on solid or in liquid media. Because of PCR amplification, the assay needs little biomass than either the HPLC or AccuProbe. Originally, 33 PRA patterns of which 19 corresponded to a single species, and 14 were associated with 5 species were also reported [86]. Later, scientists added 5 additional patterns (1 more species and 4 new subtypes), and introduced another 11 patterns (5 additional species and 6 subtypes) into this algorithm [87].

A modified PRA of the hsp65 gene in addition to new algorithm which describes 54 species, including 22 species not described previously was also described[88]. In this assay, 10% polyacrylamide gel electrophoresis (PAGE) seperates the restriction digests instead of agarose gel electrophoresis. They found that 10% PAGE analysis provided a more precise estimate of the size of the restriction fragments and made the detection of mycobacteria whose PRA patterns contained fragments shorter than 60 bp. A drawback of PRA is misidentification because of intraspecies genetic variability (if the PRA pattern is not distinct). Intraspecies variability can be used, however, as molecular epidemiologic markers for particular NTM if the PRA pattern is distinct. In order to overcome this

problem, two new diagnostic algorithms were developed based on the PRA of the 16S-23S DNA spacer region and on the rpoB gene of mycobacteria [89]. The first method shows fourty eight species, fourty subspecies, and four subtypes, whereas the second procedure identified 50 species and 13 subtypes. Both methods were proposed as an alternative to hsp65 PRA.

Line Probe Assay

The commercially available kit-based Inno-LiPA Mycobacteria assay also identifies the mycobacteria by pointing towards the 16S-23S DNA spacer region for [90]. The LiPA method is based on the solid-phase reverse hybridization of biotinylated PCR amplicons of the target region to oligonucleotide probes arranged on a membrane strip. The system as ability for identification of the MTB complex, the*, M avium, M avium complex, , M kansasii, M intracellulare, M xenopi, M gordonae, , M chelonae and M scrofulaceum* from liquid and solid media. A major benefits of the method is the additional ability for the simultaneous indentification of species in mixed cultures[90].

DNA sequencing

DNA sequencing of variable genomic regions offers a more rapid and accurate identification of mycobacteria compared with conventional phenotypic methods. In addition, it is also capable of providing phylogenic information about

the relatedness of strains [91]. DNA sequencing methods are based on the determination of species-specific nucleotide sequences, which for identification are then compared to known sequences of in-house or commercially available databases [92.]The most routinely used and reliable method of this kind is the amplification and sequence analysis of hyper variable regions of the gene encoding 16S rRNA[93.] Using automated sequencers, the assay can be completed and identification results can be reported within 1–3 days. The installation, maintenance and running of automated DNA sequencing on a daily basis are expensive and laborious tasks, however. Nevertheless, DNA sequencing has enabled the discovery of new NTM species that can be non- or poorly cultivable, potentially pathogenic, or not yet characterized [91]. It has also shown that the Mycobacterium genus is more diverse than it was assumed on the basis of the less-accurate conventional identification methods [94].As a note of caution, a recent study demonstrated that clear-cut results with the 16S rRNA gene sequencing are not the rule because public or commercial databases may be inaccurate or may not include all established species [95]. One proposed solution is the Ribosomal Differentiation of Medical Microorganisms system (RIDOM), which is a quality-controlled service, freely available on the Internet for mycobacterial identification by 16S rRNA analysis[95]. Besides sequence data, RIDOM also contains additional information on clinical, phenotypic, and genotypic characteristics of the

established and yet to be established mycobacterial strains. Alternative DNA sequencing methods have been described for the characterization of mycobacteria. These assays are based on the amplification of the rpoB, gyrB, hsp65, and 32-kDa protein genes or the 16S-23S rRNA gene spacer [96,97].

DNA chip technology

A rapidly developing technology that appears promising for clinical mycobacteriology laboratories involves oligonucleotide arrays or ‘‘DNA chips’’ where molecular biology meets computer technology [98]. Reports [99], indicate that using an array designed to determine the specific nucleotide sequence diversity in 10 species of mycobacteria, examined 121 mycobacterial isolates using both DNA sequencing of the rpoB and 16S rRNA genes and analysis of the rpoB oligonucleotide array hybridization patterns. Species identification for each of the isolates was similar, irrespective of the method used. The potential of the DNA chip technology was also demonstrated [100]. As in the previous study, the use of a DNA probe array was based on two sequence databases. The first served species identification (82 unique 16S rRNA sequence patterns from 54 species) and the second, detection of rifampin resistance in MTB (rpoB alleles). Seventy mycobacterial isolates from 27 species and 15 rifampin-resistant MTB strains were tested. The same hybridization conditions could be used for both genes and the

platform that was described can be expanded to analyze other genes simultaneously as well. Epidemiologic markers could also be added to the array for tracing transmission links between strains. With the potential to perform direct testing of clinical specimens for identification and drug susceptibility and genotyping in one step, the DNA chip method hopefully represents the near future of the clinical mycobacteriology laboratory.

Genotyping

Recent technical advances in the field of molecular biology, in conjunction with an increased understanding of the molecular genetics of mycobacteria, have provided the means to type either MTB or NTM reliably at the DNA level [101]. Genotyping or DNA fingerprinting of NTM provides data to assess (1) strain relatedness, (2) the epidemiology of NTM infections and diseases, and (3) whether a positive culture result was caused by cross-contamination in the laboratory [91]. Restriction fragment length polymorphism (RFLP) assays targeting the insertion elements IS1245, IS1311, and IS901 have been developed to type and track specific *M aviumcomplex* strains[102]. A standardized and computer- assisted protocol was also proposed for this species[103]. Molecular characterization methods for other NTM like *M kansasii, M xenopi, M celatum, M chelonae, M abscessus,* and *M malmoense* based on DNA fingerprinting or randomly amplified

polymorphic DNA-PCR genotyping have also been useful tools in the recognition and control of laboratory contamination, nosocomial outbreaks, and pseudo-outbreaks

Determination of sensitivity profiles

The drug susceptibility profile of NTM is usually quite different from *M.tuberculosis.* Firstly, these organisms are usually sensitive at high concentrations of antituberculardrugs[104], so higher cut off values for deciding sensitivity/resistance are recommended. Secondly, rapid growing mycobacteria are usually resistant to rifampicin and isoniazid (INH) whereas these are sensitive to drugs like new generation macrolides, cephalosporins and sulphones. The media usually recommended for the sensitivity screening of *M. tuberculosis* are used for NTM also. Other media like chocolate agar/supplemented with ferric ammonium salts/mycobactins *etc*., will be needed for the sensitivity screening of fastidious species. Newer techniques like BACTEC and E test have been also found to be quite useful for sensitivity determination of rapid as well as slow growing NTM[105]. A new technique using recombinant of*M.avium* expressing beta- galactosidase to evaluate the activities of antimycobacterial agents inside macrophages has been described[106]

Treatment options for NTM

NTM Species: Clinical Aspects and Treatment Guidelines as suggested by American thoracic society are as follows[2]

***General Recommendations*:**

- Treatment recommendations for infrequently encountered NTM are made on the basis of only a few reported cases. With that limitation in mind, unless otherwise stated, the duration of therapy for most pulmonary NTM pathogenesis based on treatment recommendations for more frequently encountered species such as MAC and *M. kansasii*(e.g., 12 months of negative sputum cultures while on therapy). For disseminated disease, treatment duration for most NTM pathogens is the same as for disseminated MAC infection

- The treatment of NTM disease is generally not directly analogous to the treatment of TB. *In vitro* susceptibilities for many NTM do not correlate well with clinical response to antimycobacterial drugs. Recommendations for routine *in vitro* susceptibility testing of NTM isolates are limited. The clinician should use *in vitro* susceptibility data with an appreciation for its limitations.

- Empiric therapy for suspected NTM lung disease is not recommended
- There are no widely accepted criteria for choosing patients with NTM lung disease for resectional surgery. In general, the more difficult an NTM pathogen is to treat medically, the more likely surgery should be considered from a risk/ benefit perspective. Expert consultation is strongly encouraged

MAC Lung Disease

***Recommendations*:**

1. The recommended initial regimen for most patients with nodular/bronchiectatic MAC lung disease is a three-times weekly regimen including clarithromycin 1,000 mg or azithromycin 500 mg, ethambutol 25 mg/kg, and rifampin600 mg administered three times per week
2. The recommended initial regimen for fibrocavitary or severenodular/bronchiectatic MAC lung disease includes clarithromycin 500–1,000 mg/day or azithromycin 250 mg/day, ethambutol 15 mg/kg/day, and rifampin 10 mg/kg/day (maximum, 600 mg). An initial 2 months of ethambutol at 25 mg/kg/day is no longer recommended . Alternative treatment recommendations, including the use of parenteral agents

3. Intermittent drug therapy is not recommended for patients who have cavitary disease, patients who have been previously treated, or for patients who have moderate or severe disease
4. The primary microbiologic goal of therapy is 12 months of negative sputum cultures while on therapy; therefore, sputum must be collected from patients for AFB examination throughout treatment
5. Macrolides should not be used as mono therapy for MAC because of the risk for developing macrolide-resistant MAC isolates .
6. A macrolide with a single companion drug, ethambutol, may be adequate for nodular/bronchiectatic MAC disease but should not be used in patients with fibrocavitary disease because of the risk of emergence of macrolide resistance
7. Patients respond best to MAC treatment regimens the first time they are administered; therefore, it is very important that patients receive recommended multidrug therapy the first time they are treated for MAC lung disease .
8. Expert consultation should be sought for patients who have difficulty tolerating MAC treatment regimens or who do not respond to therapy .

Surgical Treatment For MAC Lung Disease

***Recommendations*:**

1. Surgical resection of limited (focal) disease in a patient with adequate cardiopulmonary reserve to withstand partial or complete lung resection can be successful in combination with multidrug treatment regimens for treating MAC lung disease
2. Surgical resection of a solitary pulmonary nodule due to MAC is considered curative
3. Mycobacterial lung disease surgery should be performed in centres with expertise in both medical and surgical management of mycobacterial diseases .

M. kansasii

***Recommendations*:**

1. Patients should receive a daily regimen including rifampin10 mg/kg/day (maximum, 600 mg), ethambutol 15 mg/kg/day, isoniazid 5 mg/kg/day (maximum, 300 mg), and pyridoxine 50 mg/day . An initial 2 months of ethambutolat 25 mg/kg/day is no longer recommended .
2. Treatment duration for *M. kansasii* lung disease should include 12 months of negative sputum cultures.

3. For patients with rifampin-resistant *M. kansasii* disease, a three-drug regimen is recommended based on *in vitro* susceptibilities including clarithromycin or azithromycin,moxifloxacin, ethambutol, sulfamethoxazole, or streptomycin.
4. Patients undergoing therapy for *M. kansasii* lung disease should have close clinical monitoring with frequent sputum examinations for mycobacterial culture throughout therapy.

M. abscessus

***Recommendations*:**

1. The only predictably curative therapy of limited (focal)*M. abscessus* lung disease is surgical resection of involved lung combined with multidrug chemotherapy
2. Periodic administration of multidrug therapy, including a macrolide and one or more parenteral agents (amikacin,cefoxitin, or imipenem) or a combination of parenteral agents over several months may help control symptoms and progression of *M. abscessus* lung disease .

AIM AND OBJECTIVES

Aim:

To isolate and characterize different species of Non tuberculous Mycobacteria from various clinical specimens received in Mycobacteriology lab in PSG hospitals

Objective

Primary objective:

1. To estimate the incidence of Non tuberculous Mycobacterial (NTM) infections among tuberculosis suspects in PSG hospitals.

Secondary objectives

1. To speciate NTM isolated from tuberculosis suspects in PSG hospitals by phenotypic methods.
2. Comparative evaluation of commercial niacin strip test kit and conventional niacin test for identification of *M.tuberculosis*
3. Evaluation of Immunochromatographic tests to differentiate *M.tuberculosis* from NTM

MATERIALS & METHODS

Study Population:

In patients and out patients of PSG hospital with clinical suspicion of tuberculosis

Study Locale : PSG Hospitals(in and around Coimbatore)

Study period: January 2013 –July 2014

Sample Size Estimation: $n=4pq/d^2$

Where **n** is required sample size; **p** is expected prevalence ;**q** is 100-p and **d** is degree of prevalence

Sampling Method: Convenience sampling

Inclusion Criteria:

All patients with clinical suspicion of pulmonary /extra-pulmonary tuberculosis.

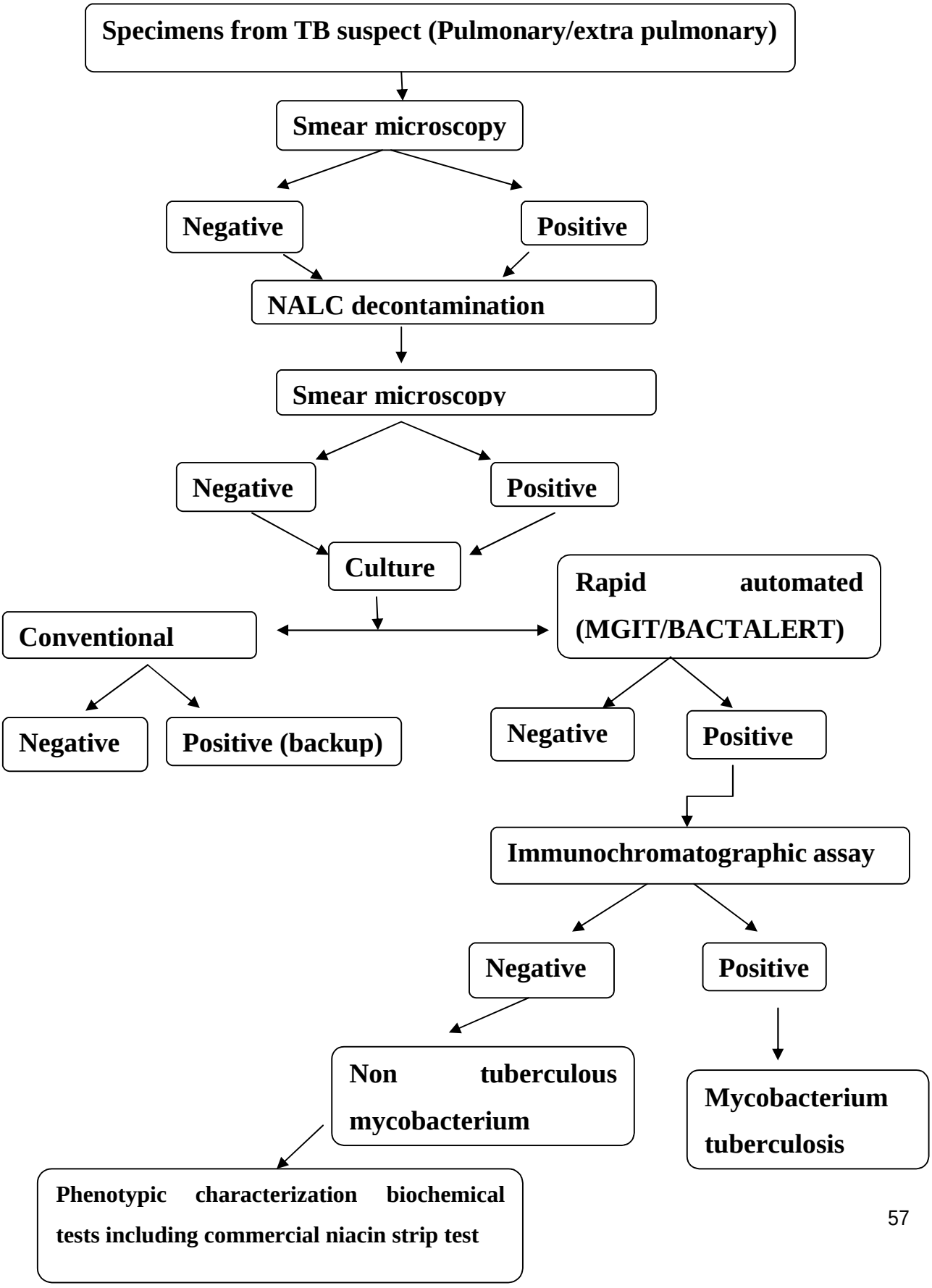
Exclusion Criteria: Nil

Confidentiatility :

Anonymity of patients was done while using their samples.

Type of study - Cross sectional prospective study

FLOW CHART FOR IDENTIFICATION OF NON TUBERCULOUS MYCOBACTERIA



ETHICAL CLEARANCE:

The study was undertaken in the Diagnostic Mycobacteriology Laboratory, Department of Microbiology, PSG Hospitals. Institutional Human ethical clearance was obtained, proof of which has been attached. Confidentiality statement was given to the ethics department stating that patient details disclosing their identity would not be included in the data collection tool.

SAMPLE COLLECTION:

All samples sent for Mycobacteriology culture were collected in sterile leak proof containers. For all samples wide mouth, screw cap containers were used. Collected specimens were transported to the mycobacteriology laboratory and when delayed were refrigerated.

SMEAR MICROSCOPY:

ZIEHL –NEELSEN STAINING PROCEDURE

- Slides were arranged and placed in serial order on the leveled staining bridge.to another.
- Carbolfuchsin stain was added. Entire surface of the slide was covered with filtered Carbolfuchsin solution. If the staining solution drained off, more stain was added to cover the entire slide.

- The slide was heated with the flame of a bunsen burner until steam arises from the stain. The slide was left for ten minutes.
- The slide was tilted to drain off excess stain and then staining solution was rinsed off gentle stream of sterile distilled water. Either a beaker, flask or squeeze bottle was used to pour the water on to the slides. The slide was tilted to drain off excess water.
- The smear was decolorized by covering the whole slide with an acid solution (25% sulphuric acid or 3% hydrochloric acid alcohol solution) and was left for 3 minutes.
- The slide was washed with gentle stream of sterile distilled water and was tilted to drain off excess water.
- The smear was counterstained by covering the entire surface of the slide with methylene blue solution and was left for 30 seconds to 1 minute.
- Slide was rinsed with gentle stream of sterile distilled water to drain off the methylene blue solution under side of the smear was wiped.
- Slide was placed on the slide rack to air-dry. After drying they were screened under microscope.

SMEAR EXAMINATION:

A drop of emulsion oil placed on the dried smear without touching the slide. The smears were examined with the 100 x oil immersion objective.Minimum of 100 fields were examined before the smear was reported as negative. Smears were graded as per RNTCP grading.

RNTCP GRADING OF SPUTUM SMEARS

	Result	Grading	No. of fields to be examined
>10 AFB/OIF	Positive	3+	20
1-10 AFB/OIF	Positive	2+	50
10-99 AFB/100 OIF	Positive	1+	100
1-9 AFB 100 OIF	Positive	Scanty*	100
No AFB in 100 OIF	Negative		100
*Record the actual number of bacilli seen- e.g “scanty 3”			

CULTURE PROCEDURE :

All processing work was done in biosafety cabinet Class II type A2 under strict aseptic precautions. **Illustration 1**

CONVENTIONAL CULTURE

Condensed moisture observed at the of bottom of culture medium slant were removed partly before inoculation. Two slopes of Lowenstein Jensen medium (LJ) were inoculated in case of conventional cultures. In case of MGIT/BactAlert one LG medium was taken as a backup. Each slope was inoculated with 0.2 to 0.4 ml of the centrifuged sediment, distributed over the surface. Inoculated media were incubated in a slanted position for at least 24 hours to ensure even distribution of inoculum. Tops were tightened to minimize evaporation and drying of media.

INCUBATION OF CULTURES:

All cultures were incubated at 35 – 37^0C until growth was observed or discarded as negative after 8 weeks. One slope LJ was incubated at 25-30^0C if the specimens were obtained from skin and superficial lesions.

CULTURE EXAMINATION:

Cultures were examined after 48-72 hours of inoculation to detect gross contaminants. If both LJ slopes were grossly contaminated (those where the surface has been completely contaminated or were medium had been liquefied or

discolored) they were discarded and sample was re-decontaminated and re-inoculated onto two LJ.

Thereafter cultures were examined weekly up to 8 weeks. Slopes were further incubated for 12 weeks, in case of specimens which were smear positive but showed no growth at the end of 8 weeks or in case of suspected M.avium – intracellular complex infection.

MYCOBACTERIA GROWTH INDICATOR TUBE (MGIT) CULTURE

1. The lyophilized PANTA (PolymyxinB, Azlocilin, Nalidixic-acid, Trimethoprim and AmphotericinB) powder was reconstituted with 15 ml of OADC(Oleic acid, Albumin, Dextrose and catalase) supplement solution. Once reconstituted, it was stored at 2-8^{0}C and was used in 6 days.
2. MGIT tube was taken and with the help of marker ,inoculation date and sample number was written on the tube.
3. 0.8ml of PANTA OADC solution was added aseptically to the MGIT tube.
4. Then 0.5 ml of digested and decontaminated concentrated sample was added to MGIT tube.
5. Cap of the MGIT tube was tightened and mixed well.
6. The tubes were incubated at 37^{0}C in the incubator.

7. The tubes were taken out on the 4th day and were read on the BACTEC MICRO MGIT system.
8. First, the calibrator tube was placed in the slot and was adjusted.
9. Then the inoculated MGIT tube was placed in the slot and checked. If the green light was in the black zone it was negative and if it was in the red zone , it was considered as positive.
10. Tubes turning negative were placed back into the incubator and was read until they turn positive or upto 42 days.
11. Tubes turning positive were checked for turbidity or contamination by ZN staining and by streaking on blood agar.

MGIT™TBc IDENTIFICATION TEST:

Principle of the procedure:

The TBc ID test is a chromatographic immunoassay for the qualitative detection of *Mycobacterium tuberculosis* from an AFB smear positive MGIT tube. This product detects MPT64, a mycobacterial protein fraction that is secreted from *M. tuberculosis* cells during culture. When samples are added to the test device, MPT64 antigen binds to anti-MPT64 antibodies conjugated to visualizing particles on the test strip. The antigen –conjugate complex migrates across the test strip to the reaction area and is captured by a second specific MPT64 antibody applied to

the membrane. If the MPT64 antigen is present in the sample, a color reaction is produced by the labeled colloidal gold particles and is visualized as a pink to red line.

Procedure :

1.TBc ID device was removed from the foil pouch immediately before testing and was placed on a flat surface.

2.AFB smear positive MGIT tube was thoroughly mixed by inverting or vortexing. Hundred µl of the sample was pipetted into the sample well of the labeled device.

3.Result was read after 15 minutes.

Interpretation of the test;

- **Positive test-** A pink to red line appears at the Test "T" position and the control "C" position in the read window. This indicates MPT64 antigen was detected in the sample.
- **Negative test-** No pink to red line is visible at the Test "T" position of the read window. This indicates MPT64 antigen was not detected in the sample.

- A line at the control "C" position in the read window indicates proper performance of the test procedure.

This TBcID test helps in identifying NTM from culture positive mycobacteria. Once NTM was identified, duplicate sample was obtained to rule out contamination.After confirmation of NTM infection, further characterization of NTM was done using various biochemical tests.

BIOCHEMICALS FOR IDENTIFICATION :

A.GROWTH ON LJ WITH PARANITRO BENZOIC ACID[107]:

1. A loop full of culture was taken in a McCartney bottle containing 1 ml sterile distilled water (SDW) and 6 glass beads of diameter 3 mm.
2. Vortex was done for 20-30 sec.
3. 4 – 5 ml of sterile distilled water was added slowly with continuous shaking.
4. Coarse particles were allowed to settle down
5. Supernatant was decanted carefully into another sterile bottle
6. McFarland 0.5 standard was matched and a loop full was inoculated on LJ with PNB.

B.TESTING FOR GROWTH RATE AND PIGMENT PRODUCTION[107]:

Principle of photosensitivity testing:

The appearance of yellow pigment in the colonies of photochromogenic mycobacteria is the result of yellowish orange carotene crystals that are produced

by actively metabolizing micro organisms after exposure to bright light. Scotochromogenic species have capability of producing yellow pigment without exposure to light: however the type of pigment is unknown; the pigmentation of young colonies of mycobacteria after growth in the dark or following exposure to light can be an important aid in the identification of certain mycobacterium species

- A primary broth culture of the test organism, diluted sufficiently to produce isolated colonies when was taken
- Materials needed were Biologic safety cabinet, 37^0 C incubator, sterile screw capped test tubes , 20 X 110 or 20 X 125 mm, sterile Pasteur pipette, inoculating wires and loops, three slants of Lowenstein Jensen medium

Procedure:

1. A suspension of growth in sterile distilled water was prepared and matched the turbidity to McFarland 0.5.
2. 0.1 ml of the suspension was inoculated to 3 tubes of LJ agar
3. Two LJ were wrapped with aluminum foil and the third was left exposed
4. One of the wrapped tubes was incubated at 25-30 ^{0} C and the other tubes were incubated at 37^0 C
5. The exposed tube was examined daily for one week and then once weekly for growth. This gave the growth rate of the isolate.

6. If growth was detected in the exposed tube, the wrapped tubes were examined for growth and pigmentation.
7. Anyone one of the wrapped tubes was exposed to a strong light for 5 hours (100W Tungsten bulb). The cap of the culture tube was loosened during this period.
8. Following exposure to light, the tube was returned to the incubator and was inspected after 24-48 hours (72 hrs if at 30^0 C) for the appearance of yellow pigment.

NIACIN TEST[108] (conventional):

Principle

All mycobacteria species produces niacin .*Mycobacterium tuberculosis* gather the maximum quantity of niacin and its recognition is helpful for its ultimate analysis. Niacin negative M. *tuberculosis* strains are rare, while very few other yield positive result.

The following were needed for the procedure:

• Test culture on Lowenstein Jenson medium

(It should be atleast 3-4wks old with 2+ growth).

• Affirmative (+ve) Control: *Mycobacterium tuberculosis* H37Rv

• Non affirmative (-ve) control: Condensed water from uninoculated Lowenstein Jenson medium

• O-toluidine -1.5 percent

• Cyanogen bromide (CNBr) 10 percent

CNBr is highly toxic & hazardous so, a saturated solution of CNBr which is 10%, was used .More distilled water is added and left overnight at four degree Celsius in the refrigerator.

Procedure:

• A four weeks old Lowenstein Jenson culture was selected having atleast 2+ growth & includes both controls

• Laboratory identification number was entered

• Serial number was identified on slopes

• Water of condensation was checked in the culture tube

• 0. 5 or 1 millilitre of sterilized water was mixed if necessary

• Bottles are placed in autoclave at 121 degree Celsius for thirty min & cooled to room temperature

• 0. 25millilitre of the water of condensation was moved to a clean tube

• 0.25 millilitre of 1. 5% O-toluidine was added.

• 0. 25 millilitre of 10 percent CNBr was added & mixed

• Colour formation was observed in five min.

Interpretation:

• If colour is pink- Niacin is affirmative

• If white precipitate is formed-Niacin is non-affirmative

• Test tubes were discarded in 4% sodium hydroxide soln.

Detection of production of Niacin by strip tes using BBL™ Taxo™ TB Niacin Test Strips

Preparation of controls:

An positive(+ve) control with ***BBL Taxo*** TB Niacin Test Control was run along with all series of tests & was equipped as below:

* 0.6 millilitres of sterilized saline or deionized or distilled water is mixed in a 13 x 75 mm tube.

* One ***BBL Taxo***TB Niacin Control disc was supplemented & shuddered slowly 3 times during fifteen minutes in room temperature.

A negative(-ve) control was made ready by mixing 0.6 mL of the same diluent to 13 x 75 mm tube instead of extract.

Test Procedure:

1. Culture being tested for presence of niacin was processed and 0.6 mL of the extract was placed in a 13 x 75 mm test tube .

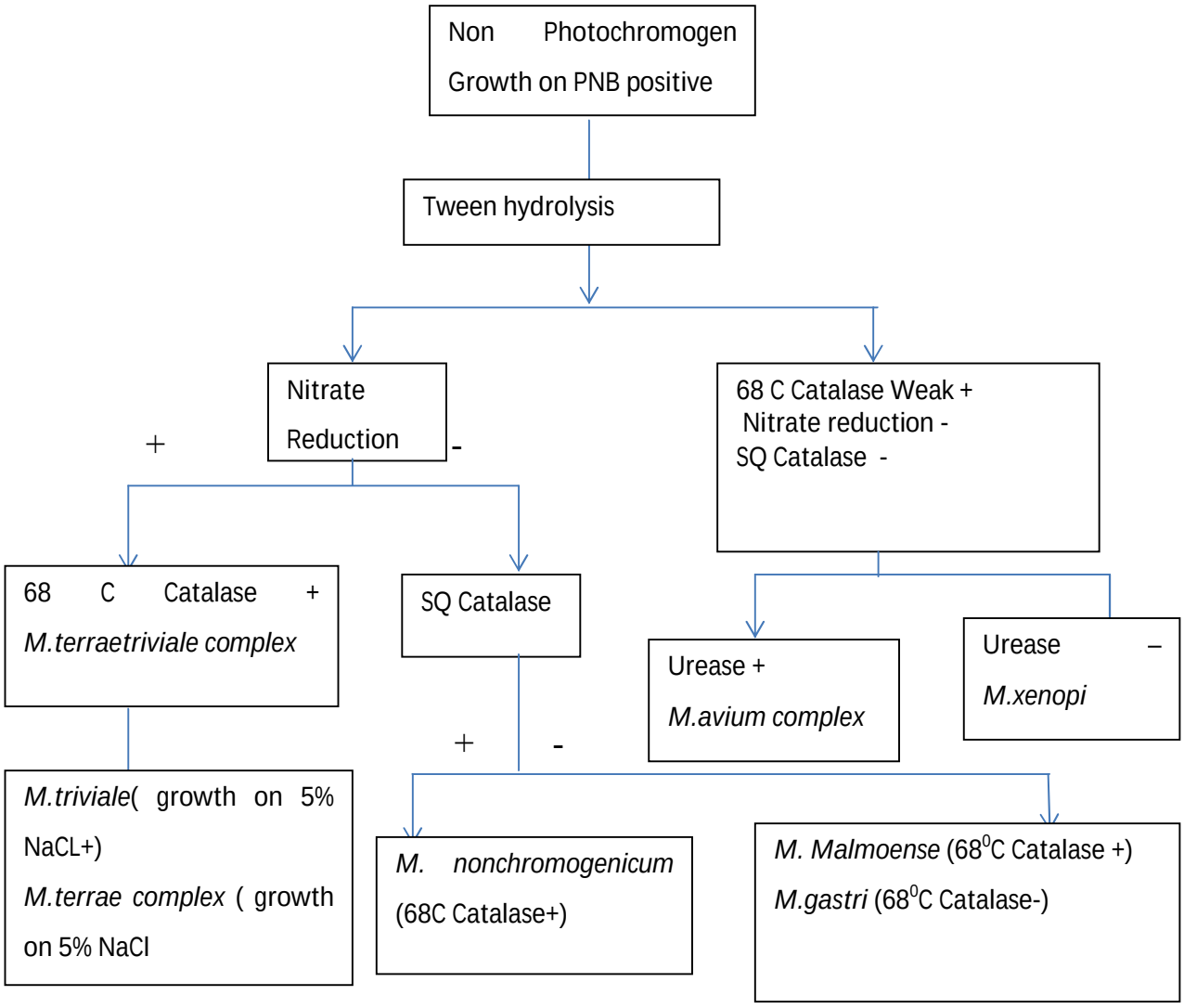
2. Non affirmative (-ve) for this is kept in 13 x 75 millimeter test tube & affirmative (+ve) control were kept ready.
3. Using flamed forceps, *a**BBL TaxoTB** Niacin Test Strip* was dropped arrow pointing downward into each tube & stoppered immediately.
4. Tubes are gently shuddered to mix fluid with reagent on bottom of strip, not tilted. Such smooth shaking was repeated after five to ten min.
5. After twelve to fifteen minutes, but not more than thirty min, color were compared
6. The above tubes were autoclaved after completing the test.

Interpretation:

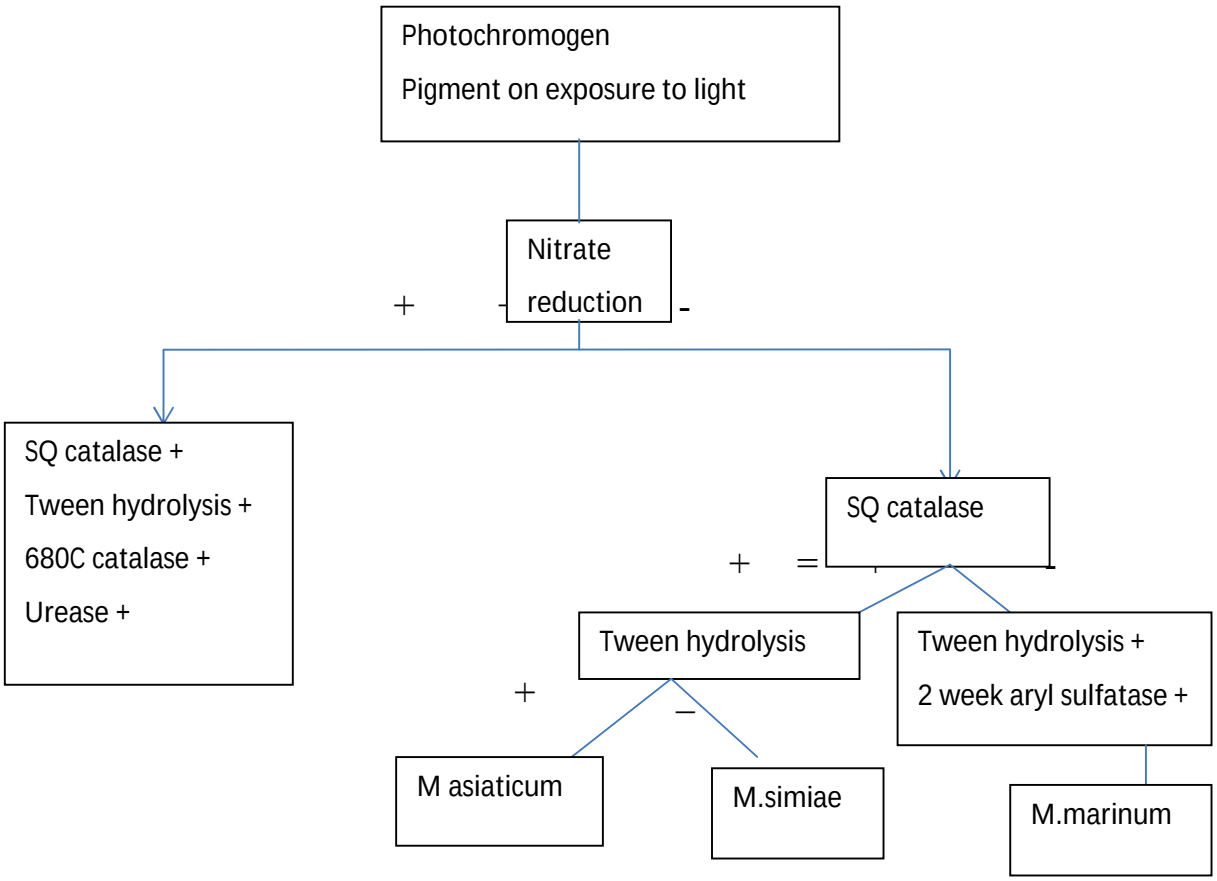
Colour of extract - Yellow - Niacin positive

Colour of extract- Nil - Niacin negative

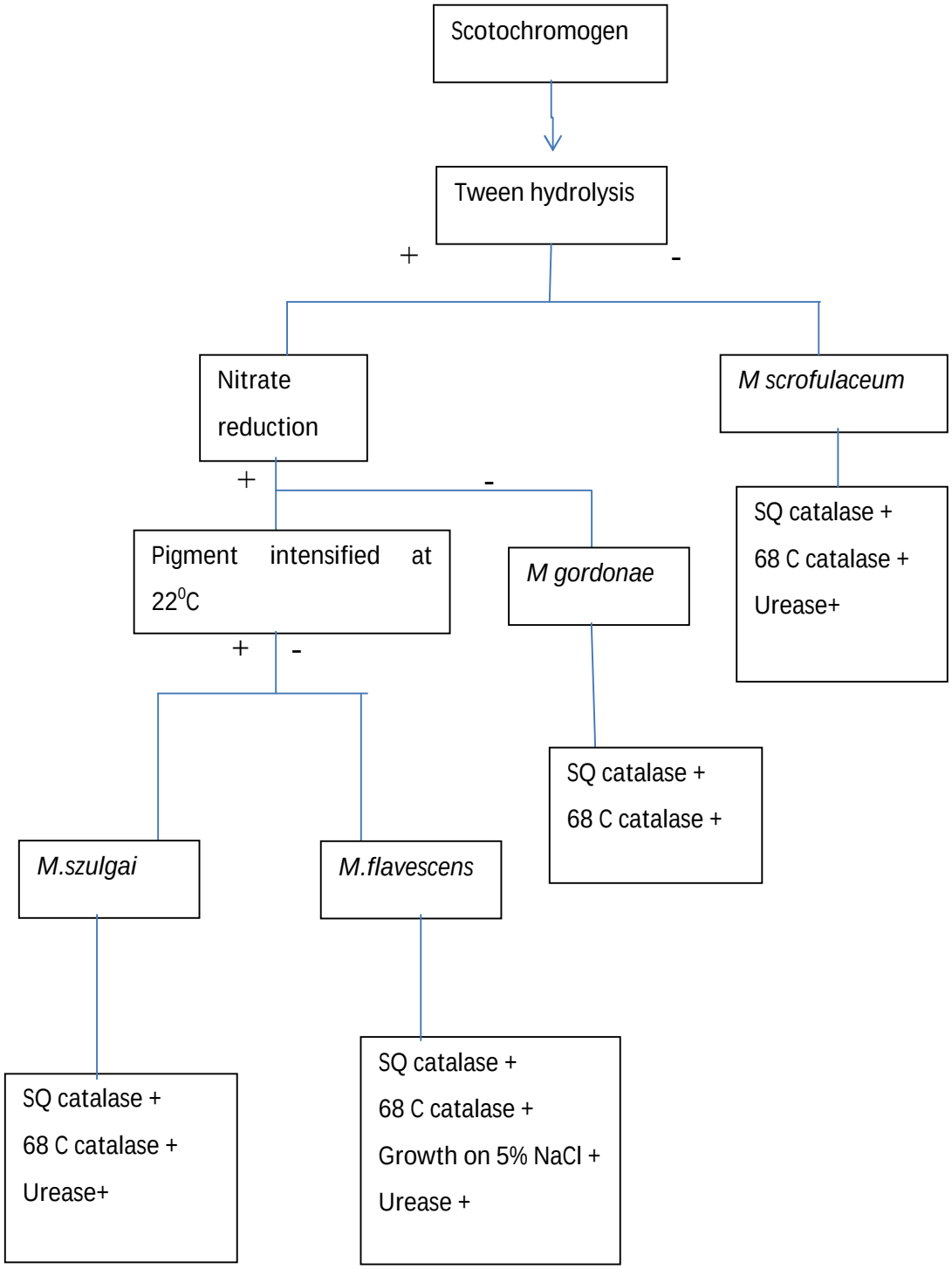
ALGORITHM FOR SPECIATION OF NONPHOTOCHROMOGENS



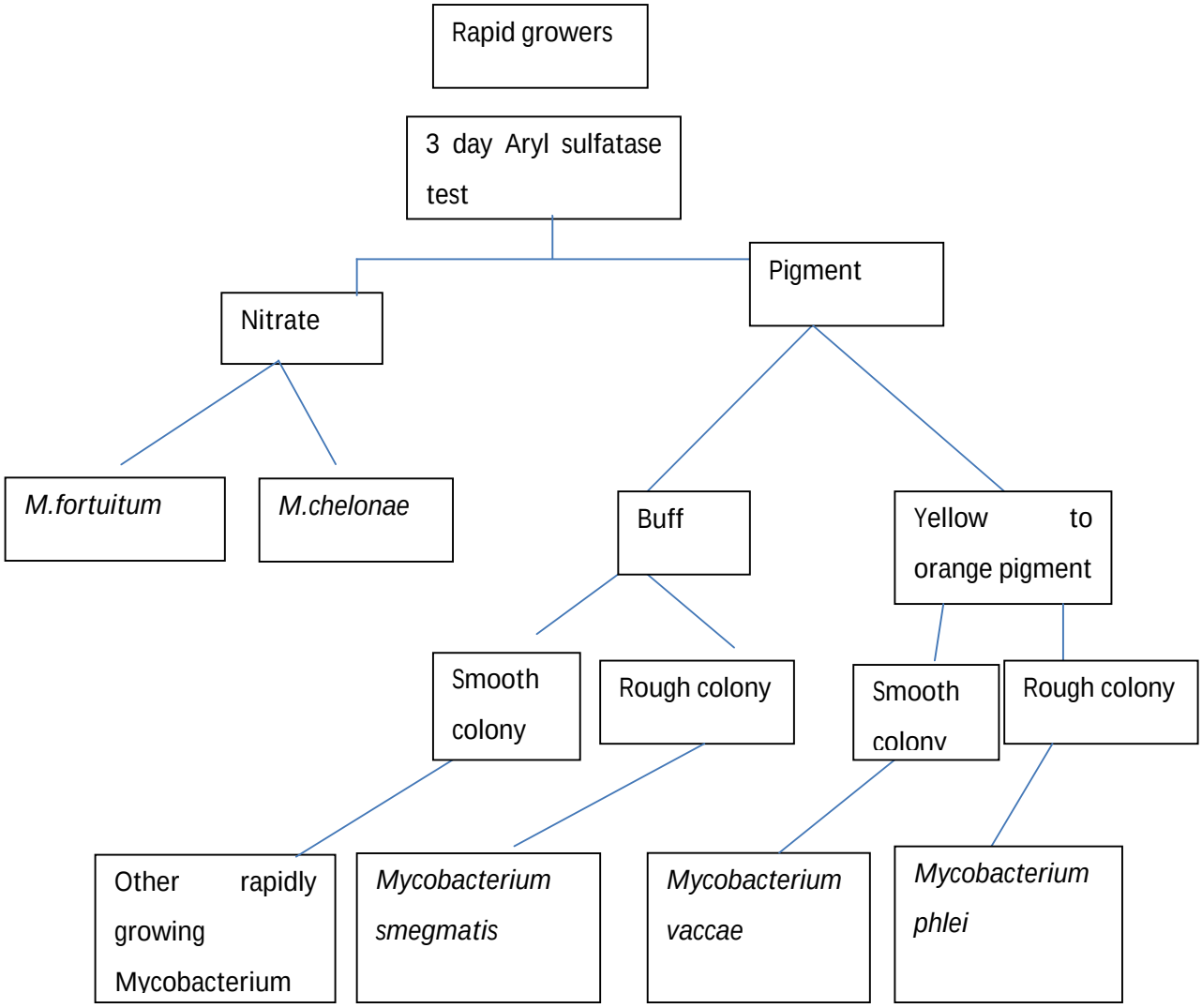
ALGORITHM FOR SPECIATION OF PHOTOCHROMOGENS



ALGORITHM FOR SPECIATION OF SCOTOCHROMOGENS



ALGORITHM FOR SPECIATION OF RAPID GROWERS



CATALASE TEST[107]:

Principle:

Catalase breaks H_2O_2 into H_2O and O_2. The formation of O_2 shows as bubbles, a minimal form of catalase is disactivated by boiling at 60^0 C for twenty min, an important feature for few mycobacterium sp. The H_2O_2 used for the recognition of Mycobacterium sp. varies from H_2O_2 used to find catalase in other types, with use of 30 percent concentration (superoxol) in 10% Tween 80 solution . This helps in dispersing the densely clumped mycobacteria preventing it from aggregating to individual bacilli, increasing the identification of catalase.

- Grown up colonies of the unidentified Mycobacterium sp. Found in from clinical specimen , grown on Lowenstein Jenson slant or middle brook 7H-10 agars were taken
- Materials needed were 68^0 C water bath;Medias – middle brook 7H9 broth, LJ deeps in 25 x 150 mm screw- capped test tubes; Reagents – 30% hydrogen peroxide , 10% Tween 80, M/15 phosphate buffer (0.067 M)

Procedure :

(i) Heat stable catalase test

1. With a sterile pipette 0.5 ml of 0.067M buffer was aseptically added in 16 x 125 millimeter test tubes which are screw capped.
2. Loop full of unidentified culture was suspended in solution containing buffer with sterilized loop.
3. Such tubes which has emulsified culture were placed in a already boiled water bath to 68^0 C for twenty min.
4. The tubes were removed from heat and cooled to room temperature.
5. 0.5 millilitre of newly formed Tween – a mixture with peroxide was added to every tube
6. Development of bubbles on surface of the liquid was observed.
7. Negative tubes were holded for 20 minutes before discarding.

(ii)Semiquantitative catalase test :

1. Liquid medium such as Dubos Tween broth of Middle brook 7H9 broth was inoculated with a loop full of the culture to be tested.
2. Inoculum was incubated for 7 days at 37^0 C
3. After incubation the inoculum was taken and mixed on Vortex mixter for 5-10 seconds

4. 6 drops were transferred to LJ deep
5. LJ deeps were incubated for 14 days at 37^0C .the caps were left loose.
6. I ml of freshly prepared Tween 80 – hydrogen peroxide agent was added to the LJ deep
7. The test tubes were left for five min in room temprature
8. Total column of bubbles was measured

Interpretation :

1.Heat stable catalase test : the formation of bubble shows a affirmative test and absence of bubbles is non affirmative test.

2.Semiquantitative catalase test : high catalase reaction - > 45mm

Low catalase reaction - < 45mm of foam

NITRATE REDUCTION TEST [107]:

Principle :

Those Mycobacteria which produce nitro reductase are able to catalyse reduction of alpha nitrate to nitrite. Here, O2 is extracted from nitrate

$$NO3 + 2e + 2H \longrightarrow NO2 + H2O$$

Nitrate formed is found by the adding αnapthalamine & sulpfanilic acid , which forms the red diazoniumdye,Para sulfobenzene-azo alpha napthalamine.

Specimen:

-A three to four wks old culture of given unidentified organism growing on Lowensten Jenson medium or other coagulated egg medium.

-Materials needed were Biologic safety hood,37^0C water bath or heating block,sterile Pasteur pipettes,sterileforceps;reagents-Nitrate test substrate(0.01 M) in M/45 phosphate buffer(0.02 M,pH 7.0)

Procedure:

One loop of unidentified colonies from solid medium was were emulsified in two ml of substrate

It was mixed well and incubated in thirtyseven ^{0}Celsius for 2 hrs.

Reagents were added in the following method to mixture

1 drop of Conc Hydrochloric acid

2 drops of 0.2 percent sulfanilamide

2 drops of 0.1 percent N-(1-naphthyl) ethylenediamide dihydrochloride

Solution was allowed for 5 min in room temperature.

Formation from pink to red colour was observed.

Results: The appearance of pink or red colour was taken as indicative of a positive test. If no colour change occurred, it was considered negative.lt was confirmed by adding a small amount of zinc dust. A deep red to pink colour after the addition of zinc dust indicated that nitrate was reduced by zinc.

ARYLSULFATASE TEST[107] :

Principle:

Aryl sulphatase is an enzyme that splits free phenolphthalein disulfite.This test is performed for identification of mycobacterium species in a tube having a substrate of phenolphthalein with oleic acid agar. Its incubated for three to fourteen days with subculture of unidentified species, the formation of pink colour after adding Na_2CO_3 proves a affirmative test.

Specimen: Mature colony ,unidentified mycobacterium sp. recovered from clinical material ,developed on Lowenstein Jenson slant .

Procedure :

1.The substrate tube was inoculated with turbid suspension of the test organism and was thoroughly emulsified.

2. It was incubated for 3 days or 14 days(2 week test) at 35^0C in the non CO_2 incubator.

3. After incubation,1 millilitre of 2N Na_2CO_3 reagent was added & mixed. Colour was observed.

Results:

Colour change from pale pink to deep red indicated positive reaction. Lack of colour change indicated negative reaction.

GROWTH ON MAC CONKEY AGAR[107]

Principle

The capacity to develop on special MacConkey agar, without crystal violet formulation, differentiates *Mycobacteria fortuitum, Mycobacteria chelonae & Mycobacteria abscessus*, which are able to grow within Five days, which differs from other specis as they have very slight growth even after 11 days.

Specimen :

A Mature colony of Mycobacterium sp. recovered from specimen , grown on a LJ slant or on middlebrook 7H10 agar is examined

Procedure:

1. A fresh MacConkey was inoculated with 3 drops of organism grown for seven to ten days in 7H9 broth
2. It was incubated at 28 to 30 degrees for eleven days without carbon di oxide

3. At Five and eleven days , absence in growth indicated a negative test and presence of growth indicates positive test.

TOLERANCE TO 5% SODIUM CHLORIDE[107]

Principle : Among slowly growing mycobacteria species, only *Mycobacteria triviale* grows well in media with 5% NaCl ;of the clinically important rapid growing mycobacteria, *M. chelonae subspecies .chelonei* didn't grow in this media.

Specimen :

An three-four week old culture of test species growing on LJ medium or other coagulated egg medium.

Materials needed were biologic safety hood, slant racks, Sterile applicator sticks, sterile 1.0 ml Pipettes, McFarland 1 standard; medias -Lowenstein-Jensen without 5% NaCl in slants, Lowenstein-Jensen with 5% NaCl in slants and Dubos Tween or Middle brook 7H9 broth

Procedure :

1.An actively growing culture (2 to 4 weeks for rapid growers)was used. A suspension of organisms was made in Middlebrook 7H9 broth equal to McFarland 1 standard

2. One Lowenstein-Jensen slant with 5% NaCl was taken and 0.1 millilitres of described suspension was introduced into each tube,.

3. The slants were incubated with the caps loose in a 5 to 10% CO2 incubator. The slants were left flat in a rack during incubation. The slants were incubated for 1 week at 29-30^{0}C

Results :

1. When numerous colonies were seen on the control slant and more than 50 colonies on the medium containing 5% NaCl , test was recorded as positive.

2. When there were colonies on the control slant and no visible growth on the slant containing NaCl, incubation was continued for upto 4 weeks .

TWEEN HYDROLYSIS [107]:

Principle :

Tween is polyethylene derivative of sorbitan mono-oleate. To detect oleic acid is released from Tween 80 due to presence of enzyme.

Colour changes can be observed from orange to pink mainly because of hydrolysis of Tween 80 M/15

Procedure

0.5 Millilitres of Tween-80 was mixed in 100 millilitres of buffer of phosphate .2ml of neutral red solution was added to it. 2 ml of the reagent was poured in test tubes which are screw capped and sterilized at Fifteen lbs pressure for 15 min. The tubes were stored in cool temperature, in containers which had an amber color.

Procedure :

One loopful of culture from L-J slope was added to the substrate tube and was incubated at 37^0C

Result was read at 24 hours, 5 days and at 10-12 days

Change of colour from amber to pink or red was interpretated as positive. The day of observation of the positive reaction was recorded.

TESTING FOR UREASE PRODUCTION BY MYCOBACTERIA[107]

Procedure :

To the urea broth prepared,colony was inoculated heavily from a solid medium. It was incubated at 37^0C for 1-7 days.Development of pink colour(positive test) was observed.

RESULTS

A total of 3050 samples were received and processed in Mycobacteriology laboratory during the study period January 2013 to July 2014.Among them, 363(11.9%) were culture positive and AFB smear positive.

Incidence of NTM during the two year period was found to be 1.3%.

- **Figure 1** shows the distribution of Mycobacterium species among the various samples received. Out of 363 culture positive acid fast bacilli, 40(11.01%) were found to be Non tuberculous Mycobacteria and the others (88.99%) were found to be *Mycobacterium tuberculosis* complex.
- **Figure 2** depicts the distribution of NTM and *Mycobacterium tuberculosis* complex from clinical samples.
- NTMs were most commonly isolated from sputum samples(85%) followed by broncho alveolar lavage(7%),tracheal aspirate (2%) ,pus (2%) and TISSUE(2%)**.Figure 3**shows the pattern of distribution of NTM from various clinical samples
- Growth on Lowenstein Jensen medium with paranitro benzoic acid (PNB) showed 100% positivity. **Illustration 2**
- All isolates of NTM (100%) showed positivity for Immunochromatographic test(MGIT^{TM}TBc Identification test) done with MGIT(Mycobacterium Growth Indicator Tube).Taking culture on LJ with PNB(Para nitro benzoic

acid) as gold standard, the sensitivity, specificity, positive predictive value and negative predictive value were found to be 100% .

- **Illustration 3** shows the results of the Immunochromatography test for detection of MPT 64 antigen which was negative for NTM.
- **Table 1** depicts the results obtained from the Immunochromatographic test kit..

- A representative sample of 50 isolates were tested to compare conventional niacin test with commercial niacin strip(BD BBLTM TaxoTM Niacin strip test) method .Among them,40(80% isolates were niacin test positive and 10 (20%)isolates were negative by both method. Commercial niacin strip test kit was found to be 100% sensitive and specific. Positive predictive value and negative predictive value was also found to be 100%.
- **Table 2** depicts the results of commercial niacin strip test.

- Among the 40 NTM isolated, there were 27(67.5%) rapid growers and 13(32.5%) slow growers. **Figure 4** illustrates the distribution of rapid growers and slow growers among the NTMs isolated.
- **Table 3** and Table **4** show the distribution of rapid growers and slow growers respectively

- **Table 5** shows the speciation of NTM species isolated by various biochemical tests.
- **Illustrations 4-9** show various biochemical tests done to characterize NTM.

Figure 1-Distribution of Mycobacterium among total samples received in Mycobacteriology laboratory

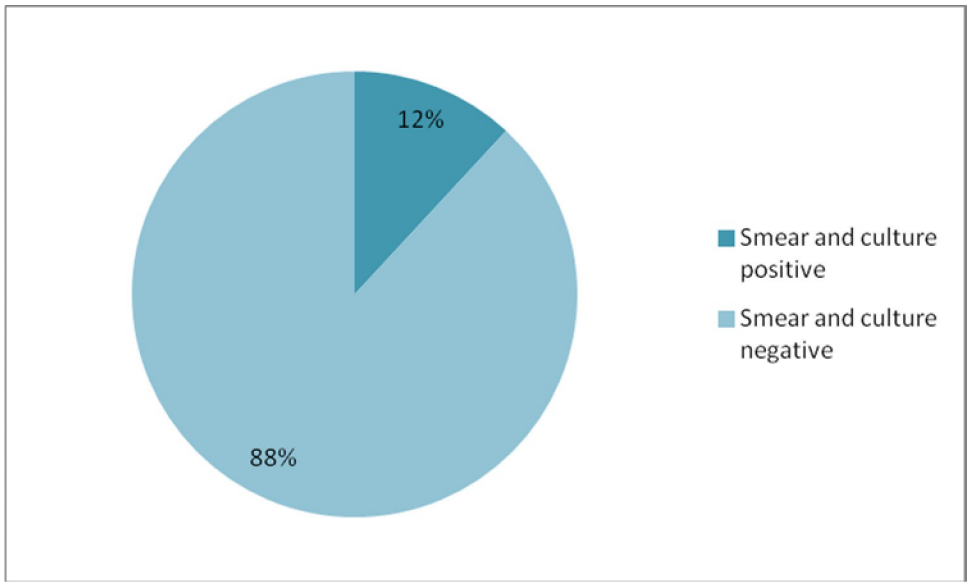


Figure 2- Pattern of NTM among the Mycobacterium species isolated

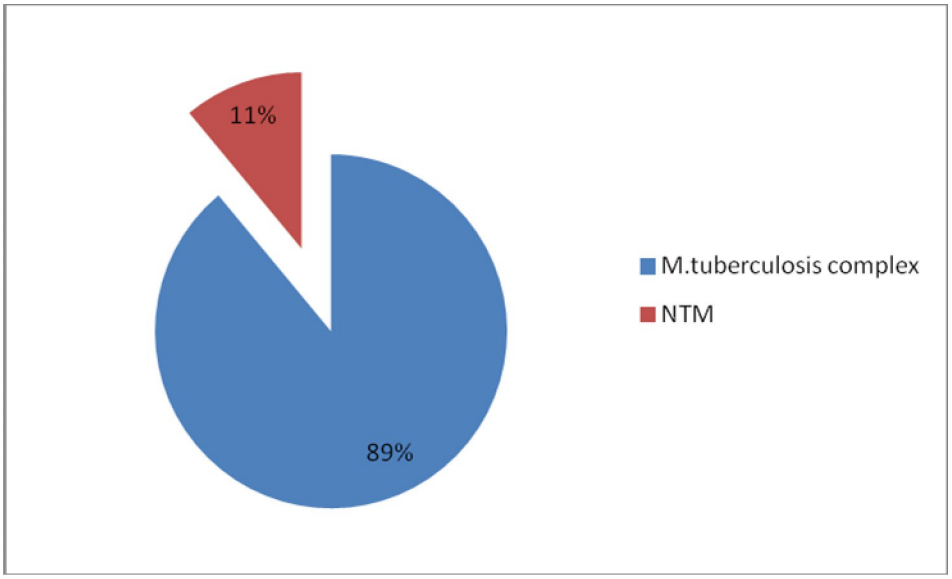


Figure 3-Distribution of NTM among various samples

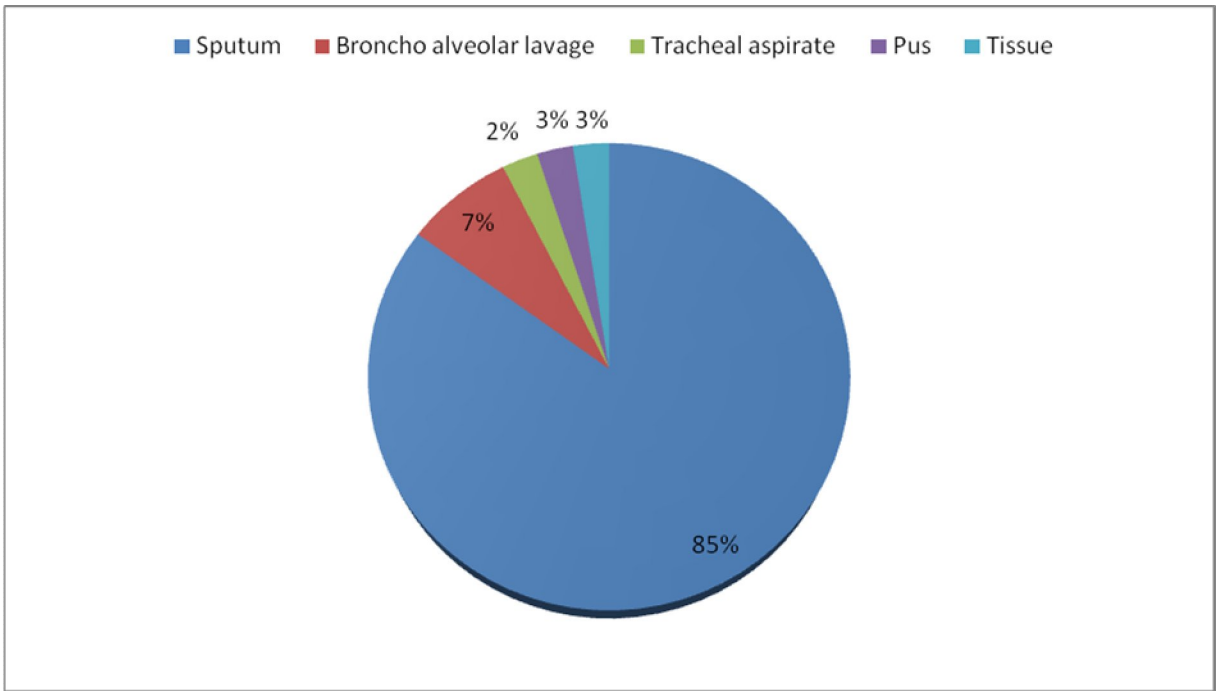


Figure 4 :Distribution of Rapid growers and slow growers

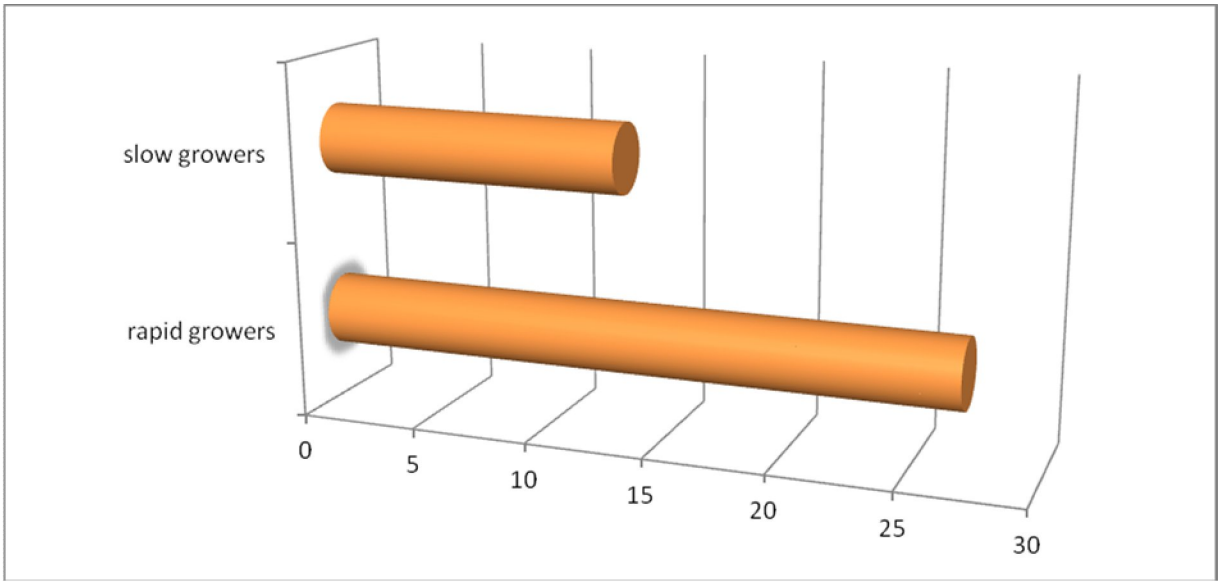


Table1: Results of Immunochromatography test - TBc ID test

		Growth on PNB		
		Positive	**Negative**	**Total**
TBcID test	**Positive**	40	0	40
	Negative	0	0	0
	Total	40	0	40

Sensitivity -100%
Specificity - 100%
Positive PredictiveValue -100%
Negative Predictive Value - 100%

Table 2 : Comparative evaluation of Niacin (conventional) and Commercial Niacin strip test

		Conventional niacin test		
		Positive Total		**Negative**
Commercial niacin strip test	**Positive**	40	0	40
	Negative	0	10	10
	Total	40	10	50

Sensitivity =100%
Specificity = 100%
Positive Predictive Value =100%
Negative Predictive Value= 100%

Table 3 : Distribution of rapid growers

n =27

S.No	Rapid growers	Number
1	*Mycobacterium fortuitum*	11(40.7%)
2	*Mycobacterium smegmatis*	10(37.03%
3	*Mycobacterium chelonae*	4(8.5%)
4	*Mycobacterium vaccae*	2(7.4%)
	Total	27

Table 4: Distribution of slow growers

n = 13

S no	**Slow growers**	Number
	Non Photochromogens	
1.	*Mycobacterium avium complex*	4(30.7%)
2.	*Mycobacterium terrae complex*	4(30.7%)
3.	*Mycobacterium xenopi*	3(23.07)
4.	*Mycobacterium nonchromogenicum*	1(7.6%
	Photochromogen	
5.	*Mycobacterium kansasi*	1(7.6%)
	Total	13

Table 7: Speciation of Non tuberculous Mycobacteria

n=40

Growth characteristics	Rapid growers(27)		Non photochromogen(12)		Photochromogen (1)	
	Positive	Negative	Positive	Negative	Positive	Negative
Nitrate reduction test	17	10	7	5	1	0
68^0C catalase test	27	0	12	0	1	0
3 day aryl sulfatase test	15	12	NA	NA	NA	NA
Tween hydrolysis test	NA	NA	5	7	1	0
Growth on McConkey agar	11	16	NA	NA	NA	NA
Sq catalase	NA	NA	5	7	1	0
Urease test	NA	NA	6	6	1	0

Illustration 1:Biosafety cabinet class II A2

Illustration 2:Growth on LJ with PNB

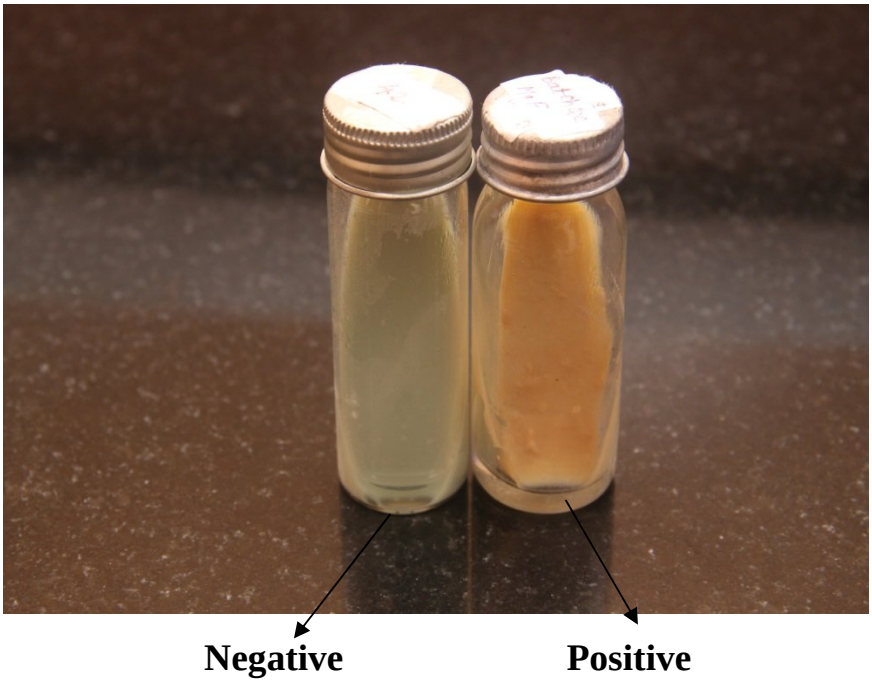
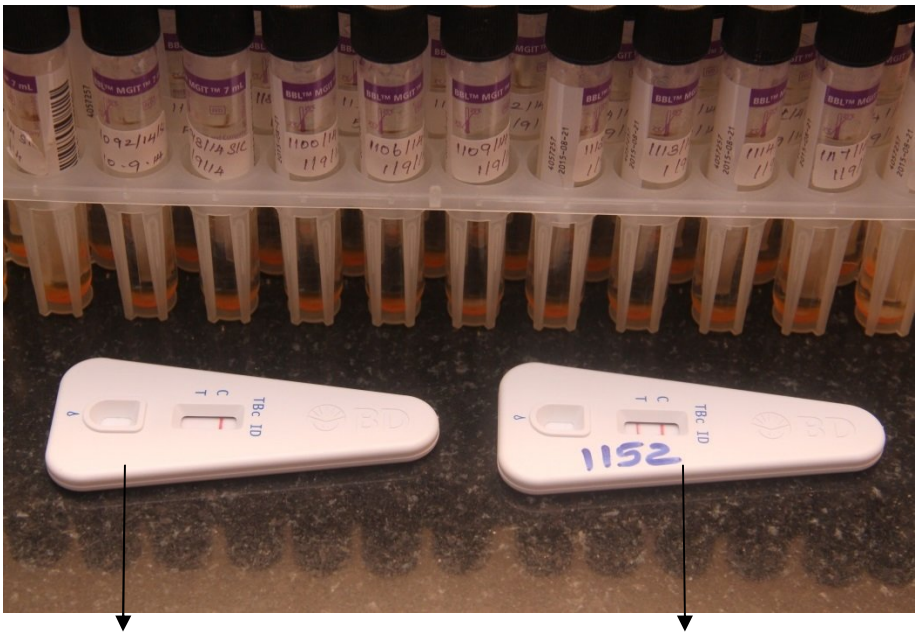


Illustration 3:TBc ID test (MGIT ™TBcID)

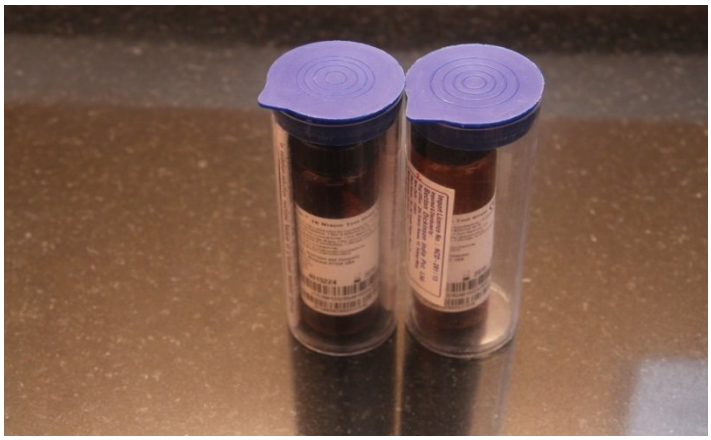


Negative (NTM) **Positive (M.tuberculosis complex)**

Illustration 4:(a)Niacin test-conventional

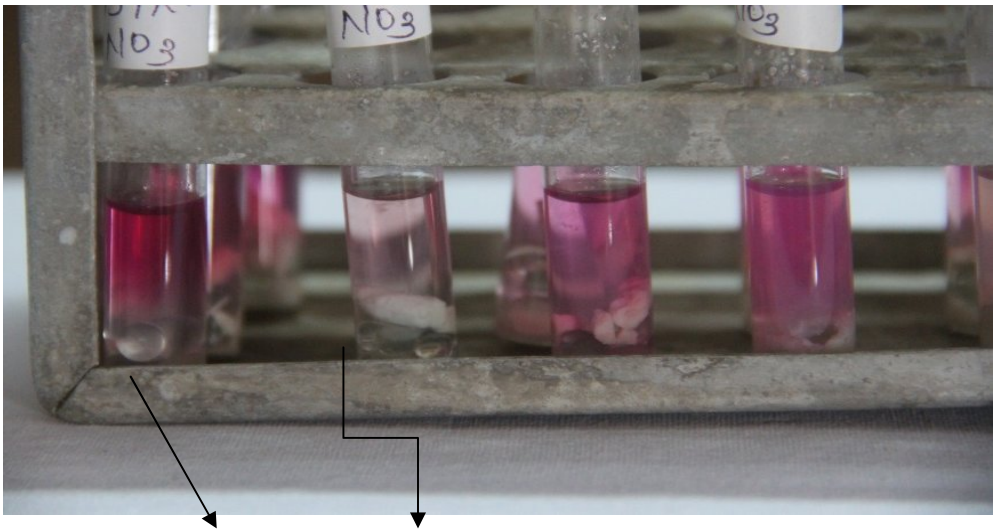
Negative test **Positive test**

(b)Niacin commercial strip test



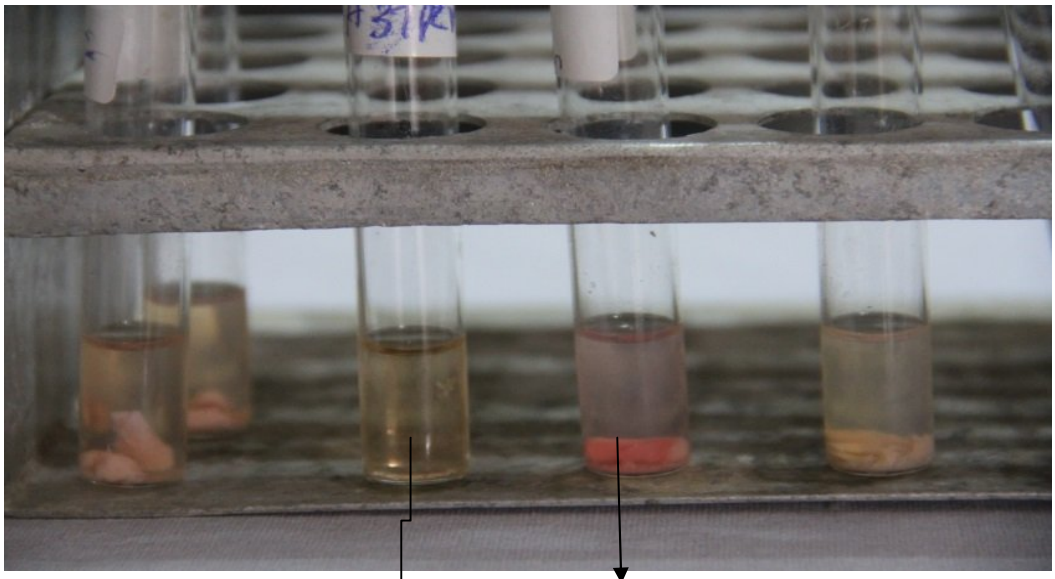
Test positive +ve Control -ve control test negative

Illustration 5:Nitrate reduction test



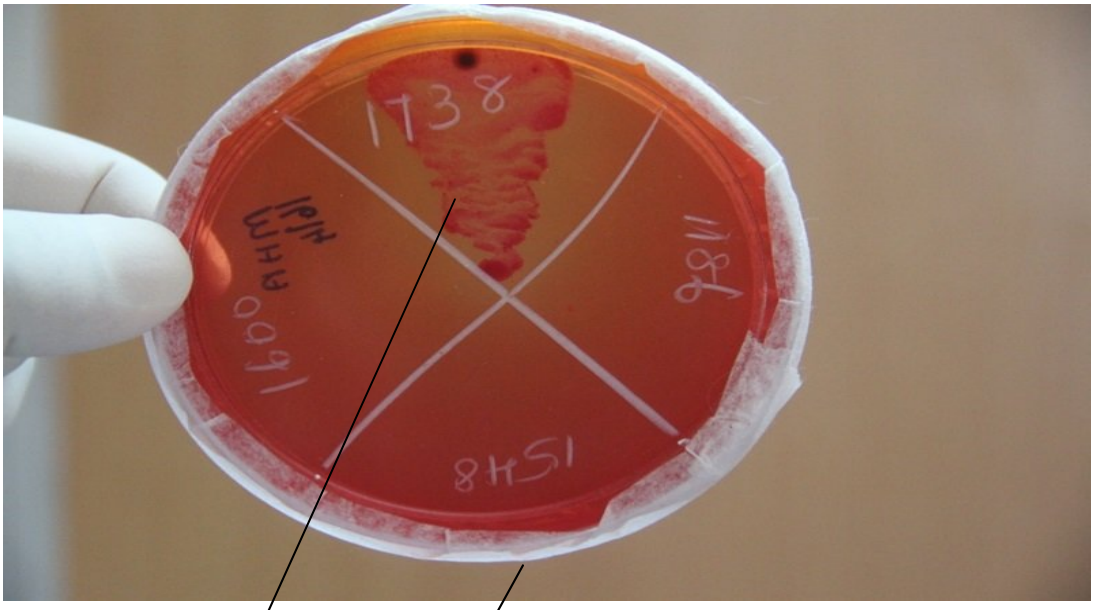
Positive **Negative**

Illustration 6:Tween hydrolysis test



Negative positive

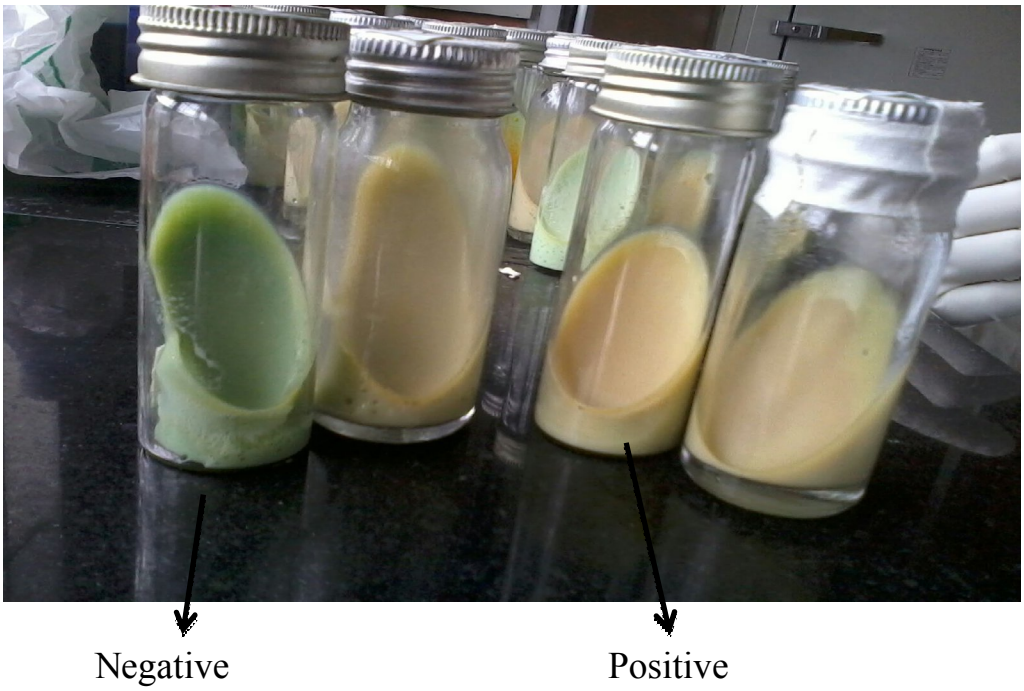
Illustration 7:Growth on McConkey agar



Positive Negative

Illustration 8:Semi Quantitaive Catalase test

Illustration 9:Growth on 5% NaCl



DISCUSSION

Non Tuberculous Mycobacteria has developed into a significant pathogen since AIDS era. Infections are most commonly seen in immune compromised patients and people with respiratory illnesses. In countries like India, where tuberculosis is endemic, there is an under reporting due to various reasons and it continues to be one of the threats for development of multidrug resistance in India. Recent awareness in the spread of infection and the advances in the laboratory diagnosis have made improvement in identification of NTM infections.

In our study, the incidence of NTM was found to be 1.3% among the tuberculosis suspects in our hospital. This is higher when compared with a study conducted in North India which showed prevalence of 0.38%[109].A study from Chennai, South India has reported 8.6% of NTM from sputum specimens of patients in BCG trial area[110].Another study conducted in Canada showed very high prevalence of 41.3% [111].This could be due to actual surge in prevalence of the disease or due to availability of better diagnostic procedures, whereas the lesser prevalence in our country could possibly be due to under diagnosis of NTM infections.

Most of the NTMs were isolated from respiratory samples (95%).A study from North India has reported NTM isolation from respiratory samples to be 45%[109].

Among the isolated NTMs, 67.5% were rapid growers and 32.5% were slow growers .These results correlate well with a study done in South India showed that rapid growers accounted for 67% of the NTM isolated[112]. The common NTM species reported by developed countries were *M.avium* and *M.kansasi*[113].In contrast to them, in the present study *M.fortuitum* (27.5%) was found to be the commonest NTM species. A study conducted elsewhere also has reported *M.fortuitum*(47%) to be the commonest species[114].

PNB positivity was 100% in the present study. Another NTM study conducted in North India also showed 99% PNB positivity[115]. A study from South India has stated that Growth on LJ with PNB, niacin test, nitrate reduction were the tests used to differentiate NTM from *M.tuberculosis* complex[67].

Immunochomatography test with TBcID test kit (BD) revealed 100% sensitivity and specificity. This was comparable with another study done in North India where sensitivity and specificity of Immunochomatography test were 99.1% and 100 % respectively[116].This simple test could be used as an aid to differentiate *M.tuberculosis* from NTM from liquid cultures

Commercial niacin strip test for differentiation of Mycobacteria was found to be 100 % sensitive and specific when compared to conventional method. Similar results were shown in a study conducted in Delhi that the commercial strip test gives comparable results with conventional tests[117].Hence the commercial test

strips could be a good alternative to the laborious conventional method. Moreover, it also has the advantage of avoiding the hazardous exposure of cyanogen bromide which is used in the conventional niacin test.

Treatment options differ once the patient is diagnosed to have NTM infections and species it is recommended that macrolides could be prescribed.

The data collected from the case records of 40 patients, showed that in five cases, the treatment protocol was changed for NTM. All these patients were treated with amikacin, levofloxacin and macrolide (either azithromycin or clarithromycin) combinations. They were found to have responded well to the NTM regimen on follow up. Most of the patients were treated for tuberculosis with first line anti-tubercular drugs and as they were responding well, they were continued on Anti Tuberculous treatment. This could be due to certain drugs like Rifampicin and Ethambutol, which are active against certain NTMs.

The report of acid fast bacilli in smears mandates the start of treatment for tuberculosis by physicians as per the RNTCP. Due to the slow growth of the bacteria culture is delayed and phenotypic methods though available are cumbersome to perform in resource limited settings. Rapid methods like Gene Xpert has revolutionised the detection of MDR tuberculosis. Genotypic methods like Line probe assay, restriction fragment length polymorphism analysis(PRA),gene sequencing etc are available for NTM and are found to be

good alternatives for the early detection and rapid diagnosis which will aid physicians to institute treatment for patients.

CONCLUSION

1. The incidence of Non Tuberculous Mycobacteria was found to be 1.3%
2. The distribution of rapid growers and slow growers were 67.5% and 32.5% respectively.
3. The most common NTM isolated was Mycobacterium Fortuitum(27 %)
4. Growth on LJ with PNB was found to be 100%
5. The TBcID immunochromatography test kit showed 100 % sensitivity and specificity.
6. The commercial niacin strip test kit was found to be 100 % sensitive and specific when compared with conventional niacin test.

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ANNEXURES

Annexure I – Ethics clearance form

PSG Institute of Medical Sciences & Research
Institutional Human Ethics Committee
POST BOX NO. 1674, PEELAMEDU, COIMBATORE 641 004, TAMIL NADU, INDIA
Phone : 91 422 - 2598822, 2570170, Fax : 91 422 - 2594400, Email : psgethics2005@yahoo.co.in

December 17, 2012

To
Dr A Kanimozhi
I Year Postgraduate
Dept. of Microbiology
PSG IMS & R
Coimbatore

The Institutional Human Ethics Committee, PSG IMS & R, Coimbatore -4, has reviewed your proposal on 13th December, 2012 in its expedited review meeting held at College Council Room, PSG IMS&R, between 2.30 pm and 4.30 pm, and discussed your application to conduct the study entitled:

"Isolation and characterization of non tuberculous mycobacteria from clinical specimens"

The following documents were received for review:

1. Duly filled application form
2. Proposal
3. Confidentiality Statement
4. Budget
5. Data Collection Tool
6. CV

After due consideration, the Committee has decided to approve the above study.

The members who attended the meeting held on 13.12.2012, at which your proposal was discussed, are listed below:

Name	Qualification	Responsibility in IHEC	Gender	Affiliation to the Institution Yes/No	Present at the meeting Yes/No
Dr P Sathyan	DO, DNB	Clinician, Chairperson	Male	No	Yes
Dr S Bhuvaneshwari	M.D	Clinical Pharmacologist Member - Secretary	Female	Yes	Yes
Dr Sudha Ramalingam	M.D	Epidemiologist Alt. Member - Secretary	Female	Yes	Yes
Dr Y S Sivan	Ph D	Member – Social Scientist	Male	Yes	Yes
Dr D Vijaya	Ph D	Member – Basic Scientist	Female	Yes	Yes

The approval is valid for one year.

PSG Institute of Medical Sciences & Research
Institutional Human Ethics Committee

POST BOX NO. 1674, PEELAMEDU, COIMBATORE 641 004, TAMIL NADU, INDIA
Phone : 91 422 - 2598822, 2570170, Fax : 91 422 - 2594400, Email : psgethics2005@yahoo.co.in

We request you to intimate the date of initiation of the study to IHEC, PSG IMS&R and also, after completion of the project, please submit completion report to IHEC.

This Ethics Committee is organized and operates according to Good Clinical Practice and Schedule Y requirements.

Non-adherence to the Standard Operating Procedures (SOP) of the Institutional Human Ethics Committee (IHEC) and national and international ethical guidelines shall result in withdrawal of approval (suspension or termination of the study). SOP will be revised from time to time and revisions are applicable prospectively to ongoing studies approved prior to such revisions.

Kindly note this approval is subject to ratification in the full board review meeting scheduled on 31.12.2012.

Yours truly,

17.12.12

Dr S Bhuvaneshwari
Member - Secretary
Institutional Human Ethics Committee

SECRETARY
PSG IMS & R
COIMBATORE-641 004
INSTITUTIONAL HUMAN ETHICS COMMITTEE

Annexure II – Data Collection Tool

Sample no: Age: Sex: M/F

Ward /OP:

Brief clinical history:

New case/Old case:

Date of collection:

Specimen: _____ If sputum: Spot/morning

Smear report:

Date of reporting	Report	RNTCP grading
	25% ZiehlNeelsen Direct smear : Concentrated smear:	
	Any other specify:	

Primary culture:

Date of inoculation	Method	Expected date of final report
	Conventional Automated	

Annexure III – Media and Reagents

1. LOWENSTEIN AND JENSEN MEDIUM:

Mineral salt solution	
Potassium dihydrogen phosphate	2.4grams
Magnesium sulphate	0.24grams
Asparagine	3.6grams
Magnesium citrate	0.6grams
Glycerol	12ml
Distilled water	600ml
Malachite green solution (2%)	1000.0ml

The ingredients of mineral salt solution were dissolved by heating.It was sterilized by autoclave at 121℃ for 20 minutes and then stored at 4℃

A 2% solution of malachite green was prepared in sterile water by dissolving the dye in the incubator for 1-2 hours

Eggs were freshly washed with soap water and rinsed for 30 minutes and were placed in sterile cover. Alternatively the eggs may be dried by cleaning shell with methylated spirit and burning it.

The eggs were beaten with sterile egg beater and there is no need to filter the beaten egg.

For complete medium the following were mixed

Mineral salt solution 600ml

Malachite green solution 20ml

Beaten egg 1000ml

The prepared media was distributed in 5 ml amount in sterile screwed tube or McCartney bottles and screw caps were tightly closed.

2. TWEEN HYDROLYSIS

Ingredients

- 80 M/15 phosphate buffer, Ph 7.
- Tween -80
- Neutral red solution , 0.1% gm of neutral red powder in 100 ml of distilled water.

0.5 ml of Tween-80 was mixed with 100 ml of phosphate buffer . 2ml neutral red solution was added to it. 2 ml amounts were distributed in screw-capped test tubes and sterilize at 15 lbs pressure for 15 minutes. The tubes should be amber coloured and were stored in the cold.

3. **UREA BROTH**

Ingredients

Peptone	1.0 gm
Dextrose	1.0 gm
Sodium chloride	5.0 gm
Potassium phosphate,	
Monobasic	0.4 gm
Urea	20 gm
Phenol red 1% solution	1ml
Tween 80	0.1ml
Distilled water	1000ml

The ingredients were dissolved in distilled water and the pH was adjusted to 5.8+/-0.1.It was then sterilized by filtration. 1.5 ml of the broth was distributed in screw capped tubes. It was stored at 4^0C up to 2 months.

4. CATALASE REAGENTS

1.30% Hydrogen peroxide(commercially available as superoxol)

2.10% tween 80

3.M/15 phosphate buffer(0.067M)

5. NITRATE TEST SUBSTRATE

$NaNO_3$ - 0.8 gm

KH_2PO_4 - 1.17 gm

Na_2HPO_4 - 1.94 gm

H_2O - 999 ml

The chemicals were dissolved and mixed.pH was adjusted to 7.It was then autoclaved and 2 ml of it was distributed in sterile tubes.

6. NIACIN TEST REAGENTS

1) 4% aniline solution

Aniline 4 gm

Ethanol 100 ml

Both were mixed in amber bottle and were stored in refrigerator in dark

2) Cyanogen bromide solution , approx. 10%

A saturated aqueous solution of cyanogen bromide of approx. 10% was prepared.It was then stored at 4^0C in the refrigerator.

Both the reagents must be freshly prepared.

7. ARYL SULFATASE TEST

Stock substrate;

- 2.6 gm of phenolphthalein disulphate tripotassium salt was dissolved in 50 ml of sterile deionized water
- It was sterilized by membrane filtration (0.22um pore size)
- It was stored in the refrigerator at 2-8^{0}C

Annexure IV- Plagiarism check

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