Nontuberculous mycobacteria: their role in human diseases and identification

Dr Rahul Narang[1] and Dr P. Kumar[2]

The laboratories working on isolation of mycobacteria have started reporting isolation of nontuberculous mycobacteria (NTM) in various specimens, but foll short of identifying them up to species level due to lack of facilities and increased work load. National Tuberculosis Institute has the ability to identify NTM up to species level by three confirmatory methods – HPLC, LPA and Gene Sequencing and the Institute is planning to implement these technologies to identify NTM isolates from different IRL s that are under supervision of NTI. This article has tried to discuss the importance of NTM in human disease and their identification.

Soon after Koch's discovery of the human tubercle bacillus in 1882, bacteriologists described many other species of mycobacteria. By 1950, the use of routine culture techniques confirmed the importance of these mycobacteria other than *M. tuberculosis* (MOTT) as agents of human disease. Nontuberculous mycobacteria or NTM is now an accepted name for such organisms which in the past have been given various names like atypical, anonymous, environmental and opportunistic mycobacteria. None of these terms were satisfactory and the name nontuberculous *Mycobacterium* (NTM) seems to have better consensus ever endorsed by American Thoracic Society (ATS) statement. The diseases caused by NTM are known as "other mycobacteriosis". These diseases can involve lung, gastrointestinal tract, skin and subcutaneous tissue, lymph nodes, bones and joints, or surgical wounds and may even lead to systemic infection. Such infections may be indistinguishable from tuberculosis. A number of nosocomial infections are also caused by NTM (Wallace et al 1998).

1. Regional Microbiologist, ICELT (FIND), Bangalore 2.Director, National Tuberculosis Institute, Bangalore

Infections with *Mycobacterium avium* complex (MAC) have gained major prominence particularly after increase of such infections in AIDS patients in the west (Farhni et al 1987, Massenkeil et al 1992, Levy Frebault et al 1987, Meissner and Falkinham 1986, Hampson et al 1989). Even earlier in the Pre AIDS era, these were major cause of pulmonary and other infections (Wallace et al 1990, Good and Snider 1982, O'Brien et al 1987). From AIDS patients, *M. avium* of certain specific serotypes (Portaels et al 1991) and of certain RFLP types have been found to be more commonly isolated so also are *M. avium* strains containing particular plasmid (Hampson et al 1989). As compared to *M. intracellulare*, *M. avium* appears to have greater predilection for causing disease in AIDS cases. Further these may cause mixed infections along with other NTM such as *M. kansasii* and *M. simiae* etc. (Levy et al 1987).

M. simiae was described in 1965 as a novel *Mycobacterium* sp. infecting monkeys (Weiszfeiler 1981). It has been isolated from clinical samples from restricted geographic areas including the southwestern United States (Texas, New Mexico, and Arizona), Israel, India and the Caribbean including Cuba. The organisms have also been recovered from tap water in some of these areas. Clinical disease caused by *M. simiae* is similar to that caused by MAC and includes chronic pulmonary disease, osteomyelitis, and disseminated disease (Falkinham 1996). Pulmonary disease caused by *M. simiae* has been reported in the United States, Israel, Thailand, and France (Lévy-Frébault et al 1987, Lavy 1982, Sriyabhaya 1981). In addition, there have been few reports of disseminated disease caused by *M. simiae* (Rose 1982, Narang 2005a). As is the case for other nontuberculous mycobacteria, AIDS and presumably other immunodeficiencies are also risk factors for *M. simiae* infection (Lévy-Frébault et al 1987). In addition, exposure to possible environmental sources of *M. simiae* or to an animal or another person infected with *M. simiae* may be a risk factor for infection (Weiszfeiler 1981). Geography may be a risk factor for *M. simiae* infection, because isolation appears restricted to a few regions of the world.

M. szulgai has been isolated a number of times in pulmonary disease. It is often confused with some of the scotochromogenic mycobacteria. It has been associated with skin, joint, lymph node and disseminated disease (Maloney et al 1987). *M. szulgai* has been isolated from India also (Chakrabarti et al 1990).

Since long *M. kansasii* has been considered as an important cause of pulmonary diseases (Wolinsky and Rynearson 1968, Wallace et al 1990) and in AIDS era it has become more important (Tortoli et al 1994, Levine and Chaisson 1991, Witzig et al 1995). Although

in vitro susceptibility tests suggest that members of this species are more resistant to anti microbial agents than *M. tuberculosis,* infections with *M. kansasii* frequently respond well to multiple drug therapy. As with MAC infections, these patients may present with advanced AIDS with very low CD4 + counts.

M. terrae complex consists of three species *M. terrae, M. nonchrmogenicum* and *M. triviale*. They appear to be harmless saprophytes but may be associated with disease occasionally (Wolinsky and Rynearson 1968, Wallace et al 1990).

M. marinum has been recognized as causative organism of "swimming pool granuloma" or "fish tank granuloma". It causes common lesions in the extremities which may be confused with sporotrichosis (Collins et al 1985, Chow et al 1987, Lambertus and Mathiesen 1988). It also causes infections of bones, joints, and tendon sheaths especially in AIDS patient.

M. paratuberculosis is closely related to *M. avium* and has characteristic feature of dependence on Mycobactin J. Members of this species have been reported to be causative organisms of enteritis (Johne's disease) in cattle, goats and sheep and can be characterized rapidly with molecular techniques (Vary et al 1990 and McFadden et al 1987). With the help of gene probes, strains belonging to this species have been linked to etiology of Crohn's disease in man (McFadden et al 1987). More recently in situ hybridization has also been used to establish this causative relationship (McFadden et al 1987, Sechi et al 2001).

M. fortuitum and *M. chelonae* (formerly *M. chelonei*), shared a number of characteristics and were associated with similar type of infections; thus they were collectively referred to as the *M. fortuitum* complex. The taxonomic grouping has become satisfactory with the recognition of additional species and taxa within the complex (*M. abscessus* and *M. mucogenicum*) all of whom differ in drug susceptibilities and many differ in the types of clinical disease produced. The *M. fortuitum* group is responsible for a number of different types of sporadic infections including osteomyelitis, cellulitis, surgical and posttraumatic wound infections, otitis media, and chronic pulmonary disease. More than 80% of sporadic cases due to the *M. fortuitum* group are due to *M. fortuitum*. In a large series of infections caused by rapidly growing mycobacteria, the *M. fortuitum* group and *M. chelonae* occurred with equal frequency (Agarwal and Jindal 2001, Sharma et al 1997 Katoch et al 1985, Singh et al 1992, Sack 1990, Kuritsky et al 1983, Safraneck et al 1987, Narang et al 2004). From

the environment, *M. fortuitum* group has been isolated from natural and tap water (Viallier et al 1967), while *M. chelonae* group has been isolated from soil and dust (Wolinsky 1974).

M. abscessus (formerly *M. chelonei* subsp. abscessus) is a nonpigmented, rapidly growing *Mycobacterium* that is most closely related to *M. chelonae*. This species is responsible for almost 90% of the chronic lung diseases due to rapidly growing species. It is a common cause of post-traumatic wound infection viz. post-tympanostomy otitis media, sternal wound infection, mammoplasty wound infections, and bacteremia associated with contaminated haemodialysis equipment (Falkinham 1996). It also produces disseminated cutaneous disease similar to that of *M. chelonae* in patients on chronic corticosteroids or other types of immune suppression. In the environment it has been found in tap water.

M. genavense grows with difficulty and need enrichment with mycobactin J. It grows in liquid media and often prolonged incubation period is required. It has recently been isolated from several countries (Coyle et al 1992, Bottger et al 1992, Bessensen et al 1993). Patients are usually in advanced stage of AIDS and present with weight loss, fever, abdominal pain and diarrhea.

M. haemophilum has been recently recognized as a cause of life threatening infections in individuals with immunocompromised states like AIDS and bone marrow transplant recipients. The organism has been isolated from the skin lesions, lymph nodes, synovial fluid, vitreous fluid, bronchoalveolar lavage (BAL), bone marrow and blood. Recovery of this organism requires cultivation in medium containing haemin or ferric ammonium citrate supplement and incubation at 300C upto 8 weeks. The organism may also be recovered using chocolate agar. Information about its environmental reservoirs is limited (Dawson et al 1981, Dever et al 1992).

Several species of non-tuberculous mycobacteria have been isolated and reported as pathogens for the first time from AIDS and non-AIDS patients (Tortoli et al 1995, Claydon et al 1991). Besides *M. genavense*, other species isolated for the first time from AIDS patients are: *M. celatum* (Tortoli et al 1995) and *M. conspicuum* (Springer et al 1995). *M. malmoense* (Banks et al 1985, Claydon et al 1991) has emerged as another pathogen. Other mycobacteria rarely associated with disease are: *M. smegmatis* (Wallace et al 1988), *M. thermoresistibile* (Weitzman et al 1981), *M. neoaurum* (Davison et al 1988) and *M. vaccae* (Hachem et al 1996).

Brown et al (1999) have reported two rapidly growing species related to *M. smegmatis- M. wolinskyi* and *M. goodie* to be associated with human wound infections. *M. bohemicum* isolates were recovered from Italy in clinical specimens as well as environmental sources (Torkko et al 2001).

M. heckeshornense sp., a new pathogenic slow growing mycobacterial species, has been isolated from lung of immunocompetent patient from Switzerland (Roth et al 2000). *M. triplex* is a slow growing nontuberculous *Mycobacterium* which was isolated from a Finnish patient with pulmonary mycobacteriosis (Suomalainen et al 2001). Schinsky et al (2000) has reported *M. septicum*, a rapidly growing species, to be associated with catheter related bacteremia and Torkko et al (2002) have reported *M. palustre* a potentially pathogenic slow growing *Mycobacterium* isolated from clinical and veterinary specimens and from Finnish stream waters. Turenne et al (2002) have reported *M. elephantis* from human specimens and Turenne et al (2003) have also reported *M. manitobense* a novel pigmented rapidly growing species causing soft tissue infections.

Levi et al (2003) have reported *M. montefiorense* a novel pathogenic *Mycobacterium* related to *M. triplex* from moray eels.

Identification and characterization of mycobacteria:

Studies conducted under the aegis of International Working Group on Mycobacterial Taxonomy (IWGMT) led to identification of biochemical markers for the identification of different mycobacteria including NTM (Wayne et al 1974, 1976). Several biochemical tests (Vestal 1977) have been described for the identification of mycobacterial species. The important among them are the following: - niacin production, nitrate reduction, tween-80 hydrolysis, arylsulphatase, urea hydrolysis, tellurite reduction, TCH sensitivity, catalase (qualitative and quantitative), growth on MacConkey agar, sodium chloride tolerance etc.

According to traditional methods, mycobacteria are usually preliminarily identified by traits such as rate of growth, pigmentation, colony morphology and biochemical profiles. The preliminary grouping may provide presumptive identification of the organism and direct the selection of key biochemical tests to characterize the unknown mycobacteria. The traditional methods are well established, standardized, and relatively inexpensive but are slow in providing clinically relevant information and are limited in scope to the species for which a large number of strains have been studied. In many instances, it is necessary to identify the

organisms based on a best-fit analysis. These traditional methods may result in erroneous identifications due to inter-assay and phenotypic variability; phenotypic homogenicity, with its concomitant lack of differential characteristics; and the bias of traditional identification schemes towards established taxa.

Newer laboratory methods for mycobacterial identification include analysis of mycobacterial fatty acids by chromatography (gas chromatography, high performance liquid chromatography or thin layer chromatography) and genetic investigations through the use of nucleic acid probes (AccuProbeTM, Gen-Probe Inc. San Diego, California; InnoLiPA, Innogenetix N.V. Ghent, Belgium; and GenoType CM/AS, Hain Life science, GmbH, Nehren, Germany), nucleic acid amplification and nucleic acid sequencing,. These new alternatives have limited the role of conventional identification methods.

Chromatographic analysis:

Analysis of mycobacterial cell wall fatty acid composition by GLC and HPLC is recognized as a useful tool for the identification of mycobacteria. Mycolic acids are high-molecular-weight fatty acids that are present in the cell walls of a restricted number of bacterial genera. The number of carbon atoms of mycolic acids is at a minimum (22 to 38) in the genus Corynebacterium and increases in other genera with mycolic acids in the cell wall: Rhodococcus (34 to 52), Nocardia (44 to 60), Gordona (48 to 66), and Tsukamurella (64 to 78). The mycolic acid of the genus *Mycobacterium* contains the maximum number (60 to 90) of carbon atoms.

High performance liquid chromatography (HPLC):

Chromatographic profile analysis can be performed once visible growth is obtained in solid media. After several preparation steps a gradient of methanol and dichloromethane (methylene chloride) is used to separate the mycolic acid esters, which are detected by UV spectrophotometry. HPLC of mycolic acids is a rapid technique; but the basic format requires bacterial colonies grown in culture. HPLC with highly sensitive fluorescent detection system (200-fold more sensitive than UV detection) has been used to detect *M. tuberculosis* and MAC directly in smear-positive sputum specimen (Butler and Kilburn 1988; Butler et al 1991).

HPLC methods are used for the identification of mycobacteria in research laboratories, large public health laboratories including the CDC, supranational (e.g. TRC Chennai) and referral laboratories. Chromatographic analysis of lipids is an attractive alternative to conventional biochemical testing for large reference laboratories, and chromatography is finding increased use as the primary method for identification in those settings. HPLC has been shown to discriminate between species better than biochemical methods and gas chromatography-mass spectrometry (GC-MS) (Vanitha et al 2002).

Molecular Probe Method (GenoType Mycobacterium CM kit):

This assay is a Molecular Genetic Assay for Identification of the clinically most relevant Mycobacterial Species from Cultured Material. The GenoType Mycobacterium CM (Common Mycobacteria) test is based on the DNA STRIP technology and permits the identification of the *M. avium ssp., M. chelonae, M. abscessus, M. fortuitum, M. gordonae, M. intracellulare, M. scrofulaceum, M. interjectum, M. kansasii, M. malmoense, M. peregrinum, M. marinum/M. ulcerans,* the *M. tuberculosis* complex and *M. xenopi* species.

Polymerase chain reaction (PCR):

An ideal diagnostic procedure would detect and identify mycobacteria directly from clinical specimens, thereby avoiding the relatively lengthy time required for culturing. Since specimens usually contain only a small number of tubercle bacilli, direct detection requires either an extremely sensitive and specific assay or a process by which a diagnostically useful component of the target organism can be “amplified” to a detectable level. Nucleic acid amplification (NAA) is one such process being actively developed and evaluated particularly for the diagnosis of tuberculosis and leprosy (Metchock 1999).

PCR assays for the rapid identification of mycobacteria to the species level have been developed targeting hsp65 (Telenti et al., 1993; Taylor et al., 1997; Wong et al., 2001), rpoB (Lee et al., 2000), 16S rDNA (Forthingam and Wilson, 1994; Krischner et al., 1993), and internal transcribed spacer (ITS) 16S-23S rDNA genes (Roth et al., 2000). For species identification by these assays the PCR amplification is either followed by restriction endonuclease assay, sequencing or both.

16 S rRNA sequencing:

The presence of 16S and 23S rRNA genes in all organisms, except for viruses renders it as a suitable target for identifying microorganisms down to the species level. Ribosomal nucleic acids are considered to be genotypically meaningful molecules and have events of evolution imprinted in the sequences of rRNA. The sequence of 16S rRNA genes is specific at the species level and is a stable property of microorganisms. PCR amplification followed by sequencing and BLAST (Basic Local Alignment Search Tool) analysis by comparing the published sequences of 16S rRNA using the BLAST database it is possible to reliably identify a particular bacterium. Sequencing of 16S and 23S rRNA genes (rDNA) has been successfully applied for the identification of Mycobacterial species (Kirschner and Bottger 1998; Springer et al 1996). Turenne et al (2001) created a 16S rRNA sequence database by sequencing 121 American Type Culture Collection (ATCC) strains encompassing 92 species of mycobacteria and have also included chosen unique mycobacterial sequences from public sequence repositories. In addition, the Ribosomal Differentiation of Medical Microorganisms (RIDOM) services have been made freely available on the Internet (http://www.ridom.de/mycobacteria/) for the mycobacterial identification by 16S rRNA analysis. A major limitation of 16S rDNA is lower number of polymorphic sites within the genus *Mycobacterium*. Some species have the same sequence or a very high degree of similarity. *M. simiae* for example is unique among mycobacteria in that part of its 16S rRNA gene sequence is similar to those sequences shared by slowly growing mycobacteria, while another portion resembles sequences shared among rapidly growing mycobacteria. (Rogall et al 1990) This leads to the problem in development of simpler sequence analysis method as restriction length polymorphism analysis or hybridization with probes.