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Isolation and Identification Non-Tuberculous Mycobacteria from Presumptive Tuberculosis Patients

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A B S T R A C T

Silent presence of non- tuberculous mycobacterium (NTM) has been observed since last 100 years, but now the increasing incidence of NTM is threatening the successful implementation of ongoing Tuberculosis control programme. While for identification and control of Mycobacterium tuberculosis many advanced efforts are being made, at the same time the silently growing menace of non-tuberculous mycobacterium is receiving little attention. This study is aimed at providing early and accurate detection of NTM from isolated mycobacterium by using routine biochemical tests. In the laboratory, specimen were processed by modified Petroff''s and cultured in a pair of Lowenstein Jensen (LJ) medium and MGIT culture, samples were processed by NALC - NaOH method, and inoculated in MGIT culture tube. The LJ medium were incubated at 37°C for 8 weeks and LJ slopes were examined daily for one weak and then every week for colonies of acid fast bacilli (AFB). Once the growth appeared, it was confirmed by Ziehl-Neelsen staining. Mycobacterium isolates were identified by batteries of biochemical tests. All the identification tests were standardized and monitored by including standard mycobacterium cultures as positive and negative controls. During the study period, a total of 4104 culture positive for Mycobacterium were found which included LJ positive (3060) and MGIT positive (1044) cultures. The total 60 mycobacterium growths were identified as NTM which included 41 NTM from LJ positive culture and 19 from MGIT culture. NTM isolated using LJ culture from the male was 29 and in female 12. In MGIT culture system NTM isolated were 13 from male and 06 from female Tb suspects. The mycobacterium species identification results showed that NTM isolated in our laboratory belong to all the 4 groups of runyon classification. Total number of NTM found was as follows: in Gr1 (5,) Gr 11 (08), Gr111 (31) and Gr1V (16). The most common species identified in this study was *M. simae* (12%) followed by *M. avium* (10), *M. gordonae* (08%) and *M. kansasi* (08%) etc. The study showed that most of the NTMs were isolated from sputum (37%) followed by pleural pus (21.66), Lymph node aspirate (20%), pleural fluid (7%), bronchial wash (8%), pus (3%), CSF (1.66%) and ascitic fluid (1.66%). The isolation of NTMs from all types of samples indicated that they not only cause pulmonary but are also responsible for extra pulmonary diseases. This study is giving a clear message to clinical microbiologists that any positive growth of Mycobacterium cannot be left for discard till the whole process of identification and sensitivity of the organism is complete.

Introduction

The reports of non tuberculous mycobacterium (NTM) associated with pulmonary and extrapulmonary diseases are increasing every day. More than 125 species of NTM have been cataloged and available online out of which at least 42 species associate with disease in human (Tortoli, 2003). NTM was initially recognized as important only in 1982, when *Mycobacterium avium* complex (MAC) was isolated and considered as most common opportunistic bacterial infection in AIDS patients. Thereon, NTM has been identified in many immunocompromised and immunocompetent patients with significant pulmonary and extrapulmonary diseases (Huang *et al.,* 1999; Mc Garvey and Bermudez, 2002; O'Brien *et al.,* 1987). In India NTM isolation and identification rate in pulmonary diseases varied from 0.7% to 34% and the species reported were *M. avium, M. fortuitum, M. scrofulaceum*, etc. (Paramasivan *et al.,* 1981; Das *et al.,* 1982; Choudri *et al.,* 1979)

The various species of the NTM are continuously being reported from western countries also. In USA, every year around 300 cases of Mycobacterium avium complex (MAC) are reported from lymphadenitis cases and other diseases (skin, soft tissue, tendons and joints). Since 1980, in US the association of MAC in AIDS patients is well known which has gone up to 37,000 cases in 1994 and *M. kansasii* reported as the second most common NTM that produces diseases in immunocompromised and immunocompetent patients (O'Brien *et al.,* 1987).

Till recently, NTMs were construed as laboratory or environmental contaminants. Thus, not getting due attention as a pathogenic organism. In most of the Indian studies M. tuberculosis has been found as major cause of mycobacterial infection and the portion of NTM has been considered low (Katoch, 2004).

Globally, medical practitioners and clinical microbiologists are facing the threat of increasing NTM associated pulmonary and extrapulmonary diseases. In India the comprehensive data depicting occurrence of NTM in pulmonary and extrapulmonary diseases is scanty. It is now clear that prompt and accurate identification of NTM for appropriate patient treatment and management is a pressing need. The precise magnitude of the problem is not well understood. Despite many limitations, increasing prevalence and geographical variability in nature of NTM prompted us to carry the research on fact finding of NTM diseases at LRSI, Delhi. The aim of the study was to determine the disease burden caused by NTM by culturing the mycobacteria using LJ media and MGIT separately and identify them upto species level.

Materials and Methods

The study was conducted in the Department of Microbiology at LRS Institute of TB & RD during 2009-2010. The Institute has a National Reference Microbiology Laboratory engaged in smear microscopy, culture, drug sensitivity testing (DST) and implementing the DOTS & DOTS plus programmes catering a population of around 1.3 million with help of chest clinics at peripheral level. This study was undertaken as institutional project in 2009 and had approval of Ethical Committee of the Institute. In this study sputum samples were collected as per Revised National Tuberculosis Control Program (RNTCP) (Revised National Tuberculosis Control Programme Central Tuberculosis Division, 1999). In the laboratory specimen were processed by modified Petroffs and cultured in a pair of Lowenstein Jensen (LJ) medium and for MGIT culture samples were processed by

NALC - NaOH method, and inoculated in Mycobacterium growth indicator tube (MGIT) culture (Revised National Tuberculosis Control Programme Central Tuberculosis Division, 1999; Kent and Kubica, 1985). The LJ medium were incubated at 37°C for 8 weeks and LJ slopes were examined for colonies of acid fast bacilli (AFB) daily for one weak and then every week.

Once the growth appeared, it was confirmed by Ziehl-Neelsen staining. In MGIT culture the positive mycobacterium were identified by growth value and positive culture tubes were identified to species level after sub culturing on solid LJ media. For the identification of NTMs various biochemical tests were used as per ATS guidelines.

The ATS diagnostic criteria of nontuberculous mycobacterial lung disease include the following (American Thoracic Society, 1997):

Clinical criteria; chest radiograph showing cavitation or high resolution computed tomography (HRCT) scan that shows multifocal bronchiectasis with multiple small nodules.

Microbiological criteria; positive culture report from 2 separate sputum or positive culture report from at least one bronchial wash or bronchial lavage or transbronchial / lung biopsy with mycobacterial histopathologic feature and positive culture for NTM mycobacterium.

A total of 13889 samples were processed during the study period for isolation of mycobacterium in L.J media (11921) and MGIT tube (1968) culture. In the LJ media, 10229 pulmonary and 1692 extrapulmonary samples were processed. The extrapulmonary samples were from pleural fluid (420), pus (456), pleural pus (300), fine needle aspiration (312), bronchial wash, (96), lymph node aspirate (84) and ascitic fluid (24). In the MGIT system 1716 pulmonary and 252 extrapulmonary samples were processed. Extrapulmonary samples for MGIT culture were from lymph node aspirate(84), fine needle aspiration (84), pus (24), urine (24), cerebrospinal fluid (12), tissue biopsy (12) and ascitic fluid (12) (table 1). These Mycobacterium isolates were identified by battery of biochemical tests. In the beginning, all the mycobacterium isolates were subcultured in LJ media containing 500 ug/ml of para-aminobenzoic acid (PNB). PNB positive clinical isolates were subjected to further identification upto species level on the basis of morphology, growth rate, growth at 25°, 37°C and 44°C, pigment production in dark, pigment production on exposure of light, no pigment production, Niacin test, Nitrate Reduction test, heat resistance catalase test at 68°C for 20 minutes, semi-quantitative Catalase test (SQCT), -2-Carboxylic Acid Hydrazide (TCH) Susceptibility Test, Tween hydrolysis, Aryl sulphatase test (3 days and 14 days), Sodium chloride tolerance test, pyrazinamide test (4 & 7 days), iron uptake and growth on MacConkey agar (Pfyffer, 2007; Witebsky and Kruczak-Filipov, 1996).

All the identification tests were standarised and monitored by including standard mycobacterial cultures as appropriative positive and negative controls.

Results and Discussion

The total culture positivity was found to be 4104 during the study period, which included 3060 positive growth in LJ media and 1044 mycobacterium grown in MGIT culture media. In the LJ media 2988 pulmonary and 72 extrapulmonary specimens were positive for mycobacterium growth. In the MGIT culture media, 1008 sputum specimen and 36 extrapulmonary specimens were positive for mycobacterium.

The elaborative biochemical test identification process of total 4104 mycobacterium strain showed 60 non tuberculous mycobacterium strains.

In the LJ media, 41 mycobacterium were identified as NTM including NTM from sputum (32) and extra pulmonary (09) samples.

In the MGIT positive culture total 19 mycobacterium were identified as NTM comprising of 12 NTM from pulmonary samples and 07 from extra pulmonary specimens.

The 60 biochemically identified NTM were from 42 males and 18 females (Table 4).

The mycobacterium species identification results showed that NTM isolated in our laboratory belonged to all the 4 groups of runyon classification. Total number of NTM found was in Gr1 (5), Gr 11(08), Gr111(31) and Gr(16) (Table 5). The most common species identified were *M.simae* (12%), *M.avium* (10), *M.gordonae* (08%), & *M.kansasi* (08%) in this study (Table 5).

The study showed that most of the NTM were isolated from sputum (37%) followed by pleural pus (21.66), Lymph node aspirate (20%), pleural fluid (7%), bronchial wash (8%), pus (3%), CSF (1.66%) and ascitic fluid (1.66%).

This is the comprehensive study from the northern India where clinically significant and microbiologically proven NTM were identified & described at a tertiary care institute having laboratory of International repute. The meticulously taken study showed that 1.36% of the total positive mycobacterium strains were NTM representing a population of 1.3 million approximately.

Karak *et al.,* from Kolkata, have reported an NTM prevalence of 17.4% from sputum specimens. This was comparatively higher than the reports of the other workers. Chakrabarthi *et al.,* from Chandigarh documented NTM isolation rate of 7.4% from various clinical specimens and M.fortuitum was the commonest isolate.

Paramasivam *et al.,* from Chennai, South India has reported 8.6% of NTM from sputum specimens of patients in BCG trial area. *M. avium* intracellulare was the species most frequently isolated in their study. Das *et al.,* reported isolation of 8.3% NTM from various clinical specimens from Delhi and Kasauli. (Paramasivan *et al.,* 1981; Karak *et al.,* 1996; Chakrabarti *et al.,* 1990; Das *et al.,* 1982).

The study from the other nations showed that the number of NTM identified were 8.3/100000 in Europe, 6.2/100000 in North America, 7.2/100000 in Australia and 12.6/100000 in Ontario, Canada & 15/100000 in Asia (Marras *et al.,* 1997-2003).

In this study, the isolation of NTM is higher in MGIT culture as compared to LJ culture (1.81 vs 1.33). The higher yield of NTM from MGIT culture has been described by Maras *et al.,* also in his research paper (Marras *et al.,* 1997-2003). Upto 30% increase in sensitivity in isolation of NTM has been observed by using the MGIT system as described by (Hanna *et al.,* 1999).

Harsh decontamination procedure could be the reason for lesser isolation rate in LJ culture media. Another reason for higher isolation of NTM in MGIT system may be due to selected specimen usually resistant form of disease not responding to treatment when clinician want result in earliest possible time for giving accurate treatment to patients. Those not responding to ATT treatment found infected with NTM species.

Table.1 Distribution of pulmonary and extrapulmonary specimens

Culture	Samples										
	Pulmonary	Extrapulmonary									
	Sputum	PL fluid	Pl Pus (empyema)	FNA	Bronch wash	LN	Asc fluid	Pus	Urine	CSF	Tissue biopsy
LJ Culture N=11921	10229	420	300	312	96	84	24	456	-	-	-
MGIT Culture N=1968	1716	-	-	84	-	84	12	24	24	12	12
Total N=13889	11945	428	300	396	96	168	36	480	24	12	12

Pl.flu; pleural fluid, Pl. pus; pleural pus, FNA; Fine needle aspiration, Bronch wash; bronchial wash, Asc.fluid; ascetic fluid; CSF; cerebero spinal fluid.

Table.2 Mycobacterium positivity in LJ / MGIT Culture

Samples	LJ culture (n=10229/1692)		MGIT CULTURE(n=1716/252)	
	+VE	-VE	+VE	-VE
Sputum	2988(29.21%)	7241(70.78%)	1008(58.74%)	708(41.25)
Extrapulmonary	72(4.25%)	1620(95.74%)	36(14.28)	216(85.71)
Total (11921/1968)	3060(25.66%)	8861(74.33%)	1044(53.04)	924(46.95)

Table.3 Non-tuberculous Mycobacterium in clinical samples

Specimen	M.Tb positive (N=4104)			NTM positive (N=4104)		
	LJ Medium	**MGIT**	Total	LJ medium	MGIT	Total
Pulmonary	**2956/2988 (98.92%)**	**996/1008 (98.80%)**	3952/4294 (0.92%)	32/2988 (1.07%)	12/1008 (1.19%)	44/4294 (1.02%)
Extrapulmonary	**63/72 (87.50%)**	**29/36 (80.55%)**	92/108 (85.18%)	09/72 (12.50%)	07/36 (19.44%)	16/108 (14.81%)
Total	**3019/3060 (98.66%)**	**1025/1044 (98.18%)**	4044/4402 (91.86%)	41/3060(1.33%)	19/1044(1.81%)	60/4402(1.36%)

M.Tb=Mycobacterium tuberculosis, NTM= Non-tuberculous mycobacterium

Table.4 Age/sex distribution of patients positive for NTM isolation

Age distribution (years)	*Total Number of Pulmonary NTM*				*Extra pulmonary NTM*			
	LJ medium		*MGIT*		*LJ medium*		*MGIT*	
	Male	*Female*	*Male*	*Female*	*Male*	*Female*	*Male*	*Female*
1-10	*2*	*0*	*1*	*1*	-	-	-	-
11-20	*1*	*2*	-	-	*2*	*1*	*1*	*0*
21-30	*4*	*1*	*3*	*1*	*3*	*1*	*2*	*1*
31-40	*5*	*3*	*3*	*2*	-	-	*1*	*1*
41-50	*4*	*2*	-	-	*1*	-	-	-
51-60	*3*	*1*	-	-	-	-	*1*	-
61-70	*1*	*1*	*1*	-	*1*	-	-	-
>70	*2*	*0*	-	-	-	-	-	-
Total	*22*	*10*	*08*	*4*	*7*	*2*	*5*	*2*

Table.5 Distribution of NTM isolates according to runyon group n=60

Runyon Group	Species	No of isolates	%age of NTM
Group1	*M.kansasi*	05	8.33
Group 11	*M.gordonae*	05	8.33
	M.szulgai	02	3.33
	M.scrofulaceum	01	1.66
Total		08	13.33
Group 111	*M.avium*	06	10.00
	M.intracellulare	01	1.66
	M.xenopi	01	1.66
	M.ulcernans	01	1.66
	M.malmoense	01	1.66
	M.terrae	04	6.66
	M.trivale	02	3.33
	M.simiae	07	11.66
	M.vaccae	02	3.33
	M.tusciae	01	1.66
	M.triplex	01	1.66
	M.malmoensae	01	1.66
	M.septicum	01	1.66
	M.flaviscens	02	1.66
Total		31	51.66
Group 1V	*M.fortuitum*	05	8.33
	M.chelonei	05	8.33
	M.pheli	05	8.33
	M.mucogenicum	01	1.33
Total		16	30.00

Table.6 Number of nontuberculous mycobacterial species in clinical samples

Specimen	Bron-washing	Lymph node	Empyema	Pl-fluid	CSF	Pus	Ascitic fluid	Sputum	Total
M. pheli		1	1		1			2	5
M.simiae	**1**	1		2				3	7
M.avium	**1**	1	*1*	-	-			3	6
M.fortuitum		1	1	1				2	5
M.chelonae	**1**	1	1					2	5
M. kansaci		1	1	1				2	5
M.vaccae			1					1	2
M.godonae		2	1					2	5
M.trivale		1	1						2
M.flavacenc			1					1	2
M.terrae	**1**	1	1					1	4
M.mucogenicom			1						1
M.triplex			1					1	2
M.szulgae	**1**		1						2
M.tuscae		1							1
M.septicum		1							1
M.scrofullacaeum								1	1
M.intracellulare							1		1
M.xenopi						1			1
M.ulceransm						1			1
Total	**5**	**12**	**13**	**4**	**1**	**2**	**1**	**22**	**60**

The developed nations have reported isolation of common NTM species such as *M. avium*, *M.kansasi*, *M.gordonae* (Cook, 2010). In this study too except *M.simiae* (11.66%) the other isolates were common as identified and reported by developed nation, *i.e., M. avium* (10.00%), *M. kansasi* (8.33%), *M. gordonae* (8.33%) and *M. terrae* (6.66%) NTM species. Jesudason *et al.,* from south India observed that *M. chelonae* and *M. fortuitum* accounted for 67% of the total NTM isolates along with others *i.e., M. szulgai M. terrae M. scrofulaceum M. flavescens M. gordonae M. simiae* and *M. smegmatis* (Jesudason and Gladstone, 2005). This is in contrast to our report where the 56% NTM belong to the slow grower group of class 111 as per runyon classification. In common with our study Meena *et al.,* from Amritsar reported 54% (approx) were slow grower mycobacterium strains which includes M. interacellulare (15.4%), *M.kansasi* (7.7%), *M. gordonae* (7.7%), *M. terrae* (15.4%), *M. fortuitum* (7.7%) (Cook, 2010). They identified 46% of total NTM isolates belongs to the runyon group 111 and this trend has been observed in other Indian studies also (Aggarwal *et al.,* 1993; Vanitha *et al.,* 2002; Chakrabarti *et al.,* 1990; Das *et al.,* 1982).

M.simae has been identified as the most common NTM species in this study. Report published by Cook JL in British medical bulletin 2010 described that *Mycobacterium simiae* is more common in arid region and is a common NTM species found southwest USA, Cuba and Israel (Cook, 2010). The temperature of Delhi is also warm, airy and

dry supporting the growth of *M.simiae* (ref). Rapidly growing mycobacterium is also the major component of NTM species. Reports from the Asian region (Taiwan, China Singapore etc) showed that16% of the total NTM are rapid grower *i.e., M.fortuitum, M.abscesus* and *M.chelonae* (Simon *et al.*, 2011). In this study also almost 30% of the NTM were identified as rapid grower mycobacterium consisting of *M.fortuitum* (8.33%), *M.chelonae* (8.33%), *M.pheli* (8.33%) and *M.mucogenicum* (1.33%). Jesudason *et al.*, described 54% of rapid grower which includes *M.fortuitum* (41%) and *M.chelonae* (13%). Marras *et al.*, described 13% of rapid grower which includes *M.abscessus, M.fortuitum* and *M.chelonae* (21).

Simon *et al.*, and other researchers showed higher infectivity rate of 79% (543/689) mycobacterium isolates from male patients. Other reports from India and our study also confirm NTM isolation is more common in young and adult and with male preponderance (Simon *et al.*, 2011). NTM isolated in this study were from 70% males and 30% females.

The changing pattern of age group being infected by NTM species i.e from older generation to young adult is well observed worldwide. Now more number of young adults are infected by NTM species than the older ones.

In this study 45% of NTM were from sputum and bronchial wash samples. The rest of the 55% of the NTM were isolated from lympnode aspirates, empyema, pleural fluid, cerebro spinal fluid, pus and ascitic fluid. Li *et al.*, showed that significant number of NTMs were isolated from sterile sites, i.e. surgical tissues, bronchial washing fluid, bronchial alveolar lavage fluid and others (Li *et al.*, 2009). Other researchers also showed that different NTMs may cause localized pulmonary diseases, lymphadenitis, soft tissue infection, infection of joints/bones, bursae, skin ulcers and generalized diseases in lukaemia and transplant patients (Pinner, 1935; Wolinksky, 1979). This study reflects more detailed research are required for identification and DST of NTM so that early diagnosis and treatment can be started in pulmonary and extrapulmonary NTM associated diseases.

The isolation of NTM at LRSI is the reflection of growing burden of NTM associated diseases in India. The isolation of NTM from all types of samples indicated that it is causing pulmonary diseases as well as extra pulmonary diseases. This study is giving a clear message to clinical microbiologists that any positive growth of Mycobacterium cannot be left for discard till the whole identification and sensitivity processing of the organism is complete. This study is an eye opener for clinician who is treating the Tb patients without having proper culture and sensitivity report of the isolated organisms. More planned studies are required to see the impact of NTM in many diseases and follow up of patients where to conclude the outcome of the disease.

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