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RESEARCH ARTICLE

IDENTIFICATION OF NON-TUBERCULOUS MYCOBACTERIA FROM CLINICAL SPECIMENS REFERRED TO ADAMA TB REFERENCE LABORATORY, ETHIOPIA

Mulualem Agonafir[1] , Adey Feleke[1] and Gurja Belay[1,*]

ABSTRACT: Non-tuberculous mycobacteria (NTM) are opportunistic pathogens that can be acquired from the environment like water and soil. They can cause pulmonary and extrapulmonary diseases in both HIV-positive and HIV-negative individuals. There is no information in Ethiopia on the identity and diversity of NTM in clinical specimens. The aim of this study was to identify and characterize NTM from clinical samples stored between November 2016 to May 2017 at Adama TB reference laboratory. A total of 47 NTM isolates were grouped using Runyon system and speciation was determined using molecular method of GenoType Mycobacterium CM/AS assays. Of the 47 isolates, 24 (51%) were found to be slow growers while 23 (49%) were rapid growers (Group IV). Of the slow growers, 4(16.7%) were photochromogen (Group I), 7(29.2%) scotochromogen (Group II) and 13 (54.2%) non-photochromogen (Group III). NTM species distribution among identified isolates revealed the following groups: *M. intracellulare* (n=6), *M. abcessus*/*M. immunogenum* (n=4), *M. gordonae* (n=4), *M. simiae* (n=3), *M. fortuitum* (type 1) (n=3), *M. chelonae*/*M. immunogenum* (n=1), *M. scrofulaceum*/*M. paraaffinicum* (n=1), *M. avium* (n=1) and *M. mucogenicum* (n=1). The study showed that there are different species of NTM that were known to cause NTM infection as well as disease. *Mycobacterium avium* complex (*Mycobacterium avium* and *intracellulare* 29.2%) was found to be the most common species out of the identified stains. Based on our findings, we suggest that due emphasis should be given to NTM while diagnosing TB and laboratories should be capacitated to properly identify NTM.

Key words/phrases: CM/AS assays, GenoType Mycobacterium, Non-tuberculous mycobacteria, Runyon grouping.

INTRODUCTION

Non-tuberculous mycobacteria (NTM), also known as atypical mycobacteria, has been recognized since Koch's time. Many of them are free living saprophytes detected and isolated from a wide variety of

[1] Department of Microbial, Cellular and Molecular Biology, College of Natural and Computational Sciences, Addis Ababa University, P.O. Box 1176, Addis Ababa, Ethiopia. E-mail: gurja.belay @aau.edu.et
*Author to whom all correspondence should be addressed

environment, including water, soil, dust, unpasteurized milk, and fish (Falkinham, 1996). Several NTM species are recognized as causative agents of human pathology, with variable severity and prognosis (Tortoli, 2003). Most have been shown in human infection with a variety of clinical presentations that involve the lung, skin, lymph nodes, or other parts of the body (Runyon, 1959b). Detection and identification of NTM to the species level is essential because patients with NTM infections show clinical findings that are similar to those of patients with tuberculosis, despite the different chemotherapeutic regimens (Raju *et al*., 2016; Riello *et al*., 2016).

Since the advent of effective treatment for tuberculosis (TB) in the 1950s, the isolation of *Mycobacterium tuberculosis* (MTB) has become a routine task in diagnosis. Some of the cases initially described as TB infection were subsequently recognized as cases of infection with non-tuberculous mycobacterium infection (NTM) (Runyon, 1959a). The frequency of pulmonary disease from NTM is reportedly on the rise in different parts of the world (Prevots and Marras, 2015; Falkinham, 2016; Ringshausen *et al*., 2016; Winthrop *et al*., 2017). The increasing disease prevalence due to NTM is caused by interactions with environmental conditions as well as a growing number of immunosuppressed population due to human immunodeficiency virus/Acquired Immunodeficiency Syndrome (HIV/AIDS), malignancy or medical intervention. Many of the recognized NTMs are significant opportunistic pathogens in patients with severe immunosuppression, while others have emerged as causes of disease in immunocompetent individuals (Brown-Elliott *et al*., 2002). The improvement of public health services for TB and technological advances in NTM recovery and identification has increased interest and knowledge of NTM identification for the proper control of the diseases (Runyon, 1959b; Morris and Harrison, 2003).

Despite control efforts since the 1960s, tuberculosis remains one of the major health problems in Ethiopia. The country is among the top 30 high TB/ Multidrug resistant TB (MDR-TB) burden countries (WHO, 2018). One of the factors contributing for the rise of multidrug resistance is the limitation of smear examination in differentiating tuberculosis Mycobacterium from non-tuberculous mycobacterium (NTM), thus missing the real causative agent (Gopinath *et al*., 2007; Gopinath and Singh, 2010). It is further important to speciate NTM because treatment among the NTM species is also different (NCCLS, 2007; Duan *et al*., 2016). Although the epidemiology of TB is well described, the epidemiology of NTM in Ethiopia is not well documented. Therefore, the objective of this study was

to identify and characterize NTM species from stored samples at Adama TB reference laboratory.

MATERIALS AND METHODS

Study design

A descriptive study was conducted on 45 stored isolates identified phenotypically and stored as NTM in the years of 2016 to 2017. Information about the samples was collected from the hospital log book after permission was granted.

Microscopic examination and sub-culturing

Stored isolates were sub-cultured and microscopic examination was performed from the primary sub-cultured isolates before starting any laboratory. Smear from culture isolates were prepared on clean new glass slides and stained with Ziehl Neelson (ZN) staining technique. ZN stained slides were examined under oil immersion lens of light microscope according to national AFB smear microscopy guideline (AFB, 2009). This was used to confirm AFB and to see if there is any cross contamination; thereby confirming purity (WHO, 1998).

Using a loop of 3 mm, colonies from Lowenstein-Jensen (LJ) stored isolates were scraped from all over the culture and a loopful of culture was picked up and added to a screw capped glass tube that contains 6−8 glass beads. This was then vortexed for few seconds and 5 ml of distilled water was added to make a suspension. Then the tube was let stand for 15−30 minutes to allow larger aggregates of bacteria settle. From the supernatant, a McFarland 1 bacterial suspension was prepared using a standard control (Kent and Kubica, 1985).

To check viability, contamination and presence of mixed colonies of the isolates, 100 µl of bacterial suspension of LJ isolates was inoculated into LJ medium from McFarland standard 1 suspension and incubated at 35−37°C. Cultures were read every day for a week and weekly after for 8 weeks. Isolates that were positive on culture were checked again for AFB; those did not grow and were contaminated were excluded from the study. Mixed colonies were further purified by sub-culturing and were included in the study.

Phenotypic grouping of mycobacterial isolates

Runyon grouping was carried out using growth rate and pigmentation tests on LJ medium according to Kent and Kubica (1985). For primary isolation

harsh digestion-decontamination procedures were used. As a result some rapid growers might take 3 weeks to appear in primary culture medium. Therefore, growth speed was determined by using subcultures diluted sufficiently in order to yield isolated colonies on solid medium (Kent and Kubica, 1985).

From McFarland 1, 10^{-2} and 10^{-4} dilutions were made and 100 µl of bacterial suspension was inoculated into two LJ medium tubes. Cultures were incubated at 35−37°C and reading was done daily for one week and weekly thereafter for six weeks. *Mycobacterium* that formed clearly visible colonies within 7 days on subculture were termed as rapid growers, while those requiring greater than 7 days were termed as slow growers (Kent and Kubica, 1985). Control strains H37Rv (ATCC 27294) and *M. fortuitum* (ATCC 6841) for slow and rapid growers were used, respectively.

Light reaction

Bacterial suspension was prepared by taking a loopful of culture from LJ stored culture and suspending into 5 ml of distilled water as described above. Then, 100 µl of 10^{-2} and 10^{-4} diluted bacterial suspensions were inoculated in to two LJ medium tubes. One tube was wrapped with aluminum foil to protect exposure to light. Both tubes were then incubated at 35−37°C. Reading of uncovered cultures was done every day for a week and then after every week for the remaining five weeks.

When growth was seen in the uncovered tube, the wrapped aluminum foil was removed and pigmentation was recorded. If growth on the slopes were pigmented in the dark, the actual colour of the pigment was recorded and the isolates were termed as a scotochromogen. If the examined tubes were not pigmented, they were exposed to light by placing a 60 watt bulb 20 to 25 cm from cultures for 3 to 5 hours. After the light exposure, the cultures were re-incubated and examined at 24, 48 and 72 hours and recorded as photochromogen if pigmented. If not pigmented, the culture results were recorded as non-photochromogens.

For the above procedure, control strains of H37Rv (ATCC 27294), *M. kansasii* (ATCC 12478) and *M. gordonae* (ATCC 14470) were used for non-photochromogen, photochromogen and scotochromogen, respectively.

Capilia TB test

Even though isolates were stored as NTM, those slow growers and non-pigmented (group III) isolates might have similar growth as *M. tuberculosis* complex, Capilla TB Neo (TAUNS Laboratories Inc, Numazu, Japan),

identification test device was used to distinguish group III NTM isolates from *M. tuberculosis* complex. The control strains H37Rv (ATCC 27294) and *M. kansasii* (ATCC 12478) for positive and negative specimens were used, respectively. Culture isolates that were positive for capilia test were excluded from the study.

Molecular identification

GenoType Mycobacterim CM/AS (Hain Lifesciences) assay was used to identify NTM species from culture isolates. The tests are based on DNA strip technology that includes amplification and subsequent reverse hybridization line probe assay (Rong *et al*., 2011; Lee *et al*., 2009).

DNA extraction

The DNA extraction of mycobacteria was performed according to extraction protocol put by the manufacturer. Briefly, 1−2 colonies were suspended in 300 µl distilled water and heat inactivated at 95°C for 20 minutes using beat block (Digial Block Heater HX-l, HAIN Life science Germany). Then it was centrifuged (MIKRO 200, HAIN Lifescience, Germany) for 15 minutes at 10,000 x g. After centrifugation, the supernatant was removed and pellets were resuspended by vortexing in 100 µl molecular grade water. The processed specimen was incubated in an ultrasonic bath for 15 minutes and again centrifuged for 5 minutes at 13,000x g. Finally, the supernatant containing DNA was transferred into sterile tube and stored at -20°C until further use.

Amplification

A premix was prepared for each PCR samples to a final volume of 50 µl. Each reaction mixture contained 5 µl of template DNA, Primer Nucleotide Mix (35µl), 10X buffer (5 µl), $MgC1_2$ (2 µl), Water (2.8 µl) and HotStar Taq (0.2 µl). The amplification was performed using thermocycler (Applied Biosystems Model 2720, USA) involving 95°C pre-denaturation for 15 minutes followed by 10 cycles of 95°C for 30 sec, 58°C for 2 min, 20 cycles of 95°C for 40 sec and 70°C for 40 sec, followed by final extension of 72°C for 8 minutes.

Hybridization and detection

Hybridization and detection were carried out in an automated system (Twincubator, HAIN Life science Germany) according to the manufacturer's instructions. Briefly 20 µl of amplification product was incubated for 5 minutes at room temperature with 20 µl of denaturation reagent. Crossing

over troughs was avoided to minimize cross contamination of samples. To each trough, 1 ml of hybridization buffer at 45°C was added carefully. Then, nitrocellulose strips with the probes were placed and hybridization was carried out for 30 minutes at 45°C. The strips were washed in 1 ml of Stringent Wash Solution at 45°C for 15 minutes. The strips were then rinsed by 1 ml of Rinse Solution (RIN) at room temperature for 1 minute followed by incubation for 30 minutes at room temperature with 1 ml of alkaline phosphates conjugated with streptavidin, diluted with an appropriate buffer. The strip was washed three times at room temperature: twice in 1 ml of RIN for 1 minute and once in 1 ml of water for 1 minute. The washed strip was incubated in 1 ml of the substrate at room temperature, in the darkness, for between 3 and 20 minutes, until the Conjugate Control (CC) band was stained. The colour reaction was inhibited by washing the strip twice with water. The strips were allowed to dry and then pasted on the evaluation sheet provided. The pattern of stained bands consistent with specific DNA probes was assessed by comparing it with the pattern provided by the manufacturer. Assessment was only carried out with respect to strips in which the control bands had stained.

For the above molecular procedure, control strains of H37Rv (ATCC 27294), *M. kansasii* (ATCC 12478), *M. fortuitum* (ATCC 6841), *M. gordonae* (ATCC 14470) and *M. marinum* (ATCC 927) were used as positive controls. Molecular grade water (Ambion, the RNA company) was used as negative control.

Data management and statistical analysis

Data were entered and analyzed using Microsoft Office Excel 2010. The socio-demographic variables and proportion of the species identified were described using descriptive analysis.

RESULTS

From the 45 stored isolates, 20 (44.4%) and 22 (48.9%) were from female and male patients, respectively. Three isolates were without proper recordings and hence, not known. The patients were from 20 to 72 years old: 62.2 % of the specimens were from the age group 20 to 39 (Table 1).

Table 1. Proportion of patients by age and sex.

Sex	**Proportion of patients in different age groups**						
	20–29	**30–39**	**40–49**	**50–59**	**>59**	**Unknown**	**Total**
Female	8	6	4	0	2	0	20
Male	5	6	5	4	2	0	22
Unknown	0	1	0	0	0	2	3
Total	13	13	9	4	4	2	45

Phenotypic grouping of isolates

In the phenotypic Runyon grouping method, 24 (51%) isolates were found to be slow growers of which 4 (16.7%) were photochromogen (Group I), 7 (29.2%) scotochromogen (Group II) and 13 (54.2%) non-photochromogen (Group III). The remaining 23 (49%) isolates were rapid growers (Group IV) (Table 2).

Table 2. Grouping of culture isolates based on growth speed and colony pigment using Runyon grouping method.

No of isolates	Growth speed	Pigment	Runyon group
14	Rapid	Nonchromogen	IV
13	Slows	Nonchromogen	III
9	Rapid	Scotochromogen	IV
6	Slow	Scotochromogen	II
5	Slow	Photochromogen	I

Source and species typing

The sources of clinical specimens were as follows: 34 (75.6%) pulmonary (sputum), 8 (17.8%) extrapulmonary samples that constituted urine (n=4), pleural fluid (n=3), pus (n=1) and source-undetermined (3) (Table 3). While trying to get pure cultures, two mixed colonies were obtained from two sputum cultures which increased the total number of isolates to 47 for further investigation.

Of the 34 sputum-sourced isolates, 19 (55.9%) were identified as non-tuberculosis mycobacterial (NTM) species. Thirteen (38.2%) isolates were identified as other *Mycobacterium* species which did not belong to the *Mycobacterium* species included in the kits. The remaining two (5.9%) isolates were identified as high G+C Gram-positive bacteria. Mixed colonies were identified as *M. mucogenicum* and *M. tuberculosis* complex species in one stored sample and *M. scrofulaceum*/*M. paraffinicum* and *Mycobacterium* species in the other sample. From the extrapulmonary culture isolates, three (37.5%) NTM species and five (62.5%) genus *Mycobacterium* species were identified (Table 3).

Table 3. Specimen type and GenoType CM/AS result.

Sample type (No of samples)	Genotype CM/AS result (No of isolates)
Sputum (34)	*M. intracellulare* (4)
	M. fortuitum type 1 (2)
	M. abcessus/ *M.* immunogenum (4)
	M. gordonae (4)
	M. chelonae/ *M. immunogenum* (1)
	M. scrofulaceum/ *M. paraffinicum* (1)
	M. simiae (2)
	M. mucogenicum (1)
	Mycobacterium spp. (13)
	M. tuberculosis complex (1)
	High GC Gram-positive bacterium (2)
	Indeterminate (1)
Pleural fluid (3)	*M. simiae* (1)
	Mycobacterium spp. (2)
Urine (4)	*M. intracellulare* (2)
	Mycobacterium spp. (2)
Pus (1)	*Mycobacterium* spp. (1)
Unknown (3)	*M. avium* (1)
	Mycobacterium spp. (1)
	M. fortuitum type 1 (1)

Molecular species identification

Out of the 47 clinical isolates, the GenoType Mycobacterium CM/AS assays identified 24 samples as non-tuberculous mycobacterium (NTM). From these, the GenoType Mycobacterium CM was able to identify NTM species in 21 (87.5%) clinical isolates and GenoType Mycobacterium AS system detected the remaining 3 (12.5%).

GenoType Mycobacterium CM and GenoType Mycobacterium AS assays together identified 19 (40.2%) isolates as genus *Mycobacterium* species. Two isolates were identified as high G-C content Gram-positive bacteria and one isolate was unidentified.

Mycobacterium avium complex [*M. avium* (n=1) and *M. intracellulare* (n=6)] species were the most frequent isolates identified accounting for seven (29.7%) of the isolates followed by *M. abcessus*/*M. immunogenum* (n=4), *M. gordonae* (n=4), *M. simiae* (N=3), *M. fortuitum* (type 1) (n=3), *M. chelonae*/*M. immunogenum* (n=1), *M. scrofulaceum*/*M. paraffinicum* (n=1), and *M. mucogenicum* (n=1) (Table 4). One isolate was found to belong to *M. tuberculosis* complex (n=1) that was grouped as nonphotochromogen (group 3) in Runyon grouping (Table 4).

Table 4. GenoType Mycobacterium CM and AS assay's result pattern.

No of isolate	CM pattern	Geno Type CM result	AS pattern	Geno Type AS result
1	1,2,3	*Mycobacteium* spp.	1,2	High GC Gram-positive
6	1,2,3,9	*M. intracellulare*	NA	
16	1,2,3,10	*Mycobacterium* spp.	1,2,3,12	*Mycobacterium* spp.
3	1,2,3,7,14	*M. fortuium* type I	NA	
4	1,2,3,5,6,10	*M. abcessus*/ *M. immunogenum*	NA	
3	1,2,3	*Mycobacterium* spp.	1,2,3,4,6	*M. simiae*
2	1,2	High GC Gram-positive	1,2,	High GC Gram-positive
3	1,2,3,8,10	*M. gordonae*	NA	
1	1,2,3,5,10	*M. chelonae*	NA	
1	1,2,3,9,10	*M. scrofulaceum/ M. paraffinucum*	NA	
1	1,2,3,4	*M. avium*	NA	
2	1,2,3	*Mycobacterium* spp.	1,2,3,12	*Mycobacterium* spp.
1	1,2,3,10,16	*M. tuberculosis* complex		
1	1,2,3,10	*Mycobacterium* spp.	1,2,3,5,12	*M. mucogenicum*

NOTE: NA = not applicable

DISCUSSION

In the current study, species which are known to cause NTM diseases have been classified into groups which were identified by CM/AS assays to include several *Mycobacterium* species, suggesting the concordance between the Runyon system of classification and the CM/AS assays. For instance, species which were found to be slow growers by conventional method were confirmed to be slow growers by the CM/AS assays. Seventy-two percent of the isolated NTM were pulmonary. The most frequently identified species known to cause NTM disease in the present study were also reported to be responsible for most of *Mycobacterium avium* complex (MAC) associated lung disease in different part of the world (Koh *et al*., 2005; Cook, 2010). The remaining species that were identified in the study were reportedly associated with pulmonary infections in both immunocompetent and immunocompromised patients. A study conducted in India had shown that *M. intracellulare*, *M. simiae*, *M. abscessus* and *M. fortuitum* were NTM most commonly isolated from pulmonary specimens (Shenai *et al*., 2010). Another study in Zambia reported that *M. intracellulare* was the second most species isolated from pulmonary specimens (Buijtels *et al*., 2010). Moreover, *M. abcessus*, *M. scrofulaceum*, *M. goradonae*, *M. fortuitum* and *M. simiae* have also been described as a cause of pulmonary NTM disease in AIDS patients (Wayne and Cubica, 1986).

The isolation of *M. gordonae* in the absence of invasive disease known was reported to create diagnostic misinformation with implications in therapy; and also causing unnecessary wastage of time and expense in the laboratory. However, there are also studies showing that *M. gordanae* cause lung and skin infection, principally in immunocompromised patients (Weinberger *et al*., 1992; Pedro *et al*., 2008).

When isolated from respiratory specimens, *M. mucogenicum* is most often a contaminant. In one study that included 54 respiratory *M. mucogenicum* isolates, only two (4%) were found to be clinically significant (Wallace *et al*., 1993). Because we do not have the detailed clinical, radiological and socio-demographic information, we were not able to substantiate this claim in our report. Over the last few years, a growing number of NTM isolates, often of newly described species, are being submitted to laboratories for identification. The clinical relevance of this Mycobacterium group is under constant evaluation (Gitti *et al*., 2006). An early identification may lead to the initiation of faster treatment and a better prognosis. Although we did not perform comparative study evaluating the CM/AS assays, DNA line probe assays such as GenoType Mycobacterium CM/AS are reported to be sensitive (100%) and specific (94.4–100%) for the identification of most common mycobacteria (Mäkinen *et al*., 2006).

Of the NTM species identified in the clinical specimens, *Mycobacterium avium complex* constituted the majority (35%) of the strains identified from both pulmonary and extrapulmonary specimens. Similar trend was reported by different scholars (Massenkeil *et al*., 1992; Griffith *et al*., 2007). Drinking water distribution systems were identified as source of infections in the United States of America (Wu *et al*., 2017; Falkinham, 1996). We were not able to relate this evidence with our findings because of lack of data in this study. Nevertheless, it is important to study the source of infection by extrapulmonary MTB in drinking water distribution systems in Ethiopia.

There are studies conducted by different scholars to identify NTM from various samples using CM/AS kits. Researchers from Turkey were able to identify 10 different *Mycobacterium* species from 44 respiratory samples (sputum, bronchial aspiration fluid and bronchial lavage) belonging to 30 patients. *M. fortuitum*/*M. pereginum* complex (n=5), *M. intracellulare* (n=4), *M. avium* complex (n=4), *M. gordonae* (n=4). *M. simiae* (n=1) and *M. scrofulaceum* (n=1) were some of the species that were identified. They have also found two isolates with unidentified atypical mycobacteria

(Mycobacterium spp.) (Biçmen *et al*., 2007).

Another study done in Portugal reported that *Mycobacterium avium* complex was the most frequent NTM from specimens received from 12 Lisbon hospitals over a three year period using GenoType Mycobacterium (CM/AS) assays (Couto *et al*., 2010). In a study done in Taiwan on urine specimen, *Mycobacterium avium* complex was the most common species, which is in agreement with the results of our study (Huang *et al*., 2010).

Using the GenoType Mycobacterium CM/AS assays, we were not able to identify 19 (40.4%) of the mycobacterial species below the genus *Mycobacterium* level, with one isolate exhibiting discrepancy. Clarification of such ambiguities requires the need to use complimentary tools such as sequencing technologies.

CONCLUSION

The study clearly showed the presence of different species of NTM that were known to cause NTM infection as well as disease. Among the different NTM species, *Mycobacterim avium* complex was found to be the most common identified species. The use of molecular methods such as GenoType Mycobacterium CM/AS kit in our study has helped in the identification of isolates precisely and this will greatly contribute for the proper diagnosis and treatment of NTM infections.

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