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## Isolation and Characterization of Nontuberculous Mycobacteria from Patients with Pulmonary Tuberculosis in Ghana

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### Abstract

**Background**—Nontuberculous mycobacterial (NTM) species are assuming public health importance in pulmonary diseases; they are increasingly being isolated, and importantly, most NTMs do not respond to routine tuberculosis (TB) drugs. This study aimed to identify NTMs isolated from pulmonary TB cases and also determine their susceptibility to streptomycin (STR), isoniazid (INH), and rifampicin (RIF).

**Methods**—A total of 1755 mycobacterial isolates, obtained between August 2012 and July 2014, from 2036 smear-positive pulmonary cases were identified using polymerase chain reaction amplification of IS6110, and *hsp65* gene sequencing analysis. Drug susceptibility testing (DST) was then performed for the identified NTMs against STR, INH, and RIF using microplate Alamar blue assay. The results were analyzed against patients' biodata for statistical associations.

**Results**—Of the 1755 analyzed isolates, we identified 43 (2.5%) NTMs, which included 18 (41.9%) *Mycobacterium intracellulare*, 13 (30.2%) *Mycobacterium avium* subs. *paratuberculosis*, 5 (11.3%) *Mycobacterium abscessus*, 3 (7.0%) each of *Mycobacterium mucogenicum* and *Mycobacterium colombiense*, and 1 (2.3%) *Mycobacterium simiae*. Patients infected with NTMs (52.0%) were more likely to be human immunodeficiency virus-positive ( $P = 0.001$ , odds ratio = 6.6, 95% confidence interval = 2.7–16.2) than those infected with *M. tuberculosis* complex (5.8%). All the 43 (100%) NTMs were resistant to INH, whereas 32 (74%) and 19 (44%) were resistant to RIF and STR, respectively. Furthermore, 16 (37.2%) NTMs were resistant to all three drugs, 20 were resistant to INH and RIF, and 3 were resistant to STR and INH. All the *M. abscessus* isolates were resistant to all the three drugs, whereas all the *M. avium* isolates were resistant to INH and RIF, but only three were resistant to STR. Among the *M. intracellulare* isolates, 8, 18, and 15 isolates were resistant to STR, INH, and RIF, respectively.

**Conclusion**—The observed high-resistance level to INH and RIF supports the need for rapid species identification and DST of nonresponding TB cases before retreatment.

### 1 Background

Nontuberculous mycobacteria (NTMs) species are mycobacteria other than members of the *Mycobacterium tuberculosis* complex (MTBC) and *Mycobacterium leprae*. There are over 170 species identified to date, and unlike *M. tuberculosis*, NTMs are generally free-living,

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ubiquitous organisms in the environment.[1] The ecology of NTMs makes it easier for human exposure.[2] Until recently, NTMs were not considered clinically important because they were not found to cause diseases.[2] However, lately, the prevalence of clinical NTMs, especially pulmonary NTMs, has been on the increase worldwide.[1,3] The prevalence rates have been shown to vary among countries globally,[1] and in regions of high tuberculosis (TB) endemicity, NTMs are often unrecognized, misdiagnosed, and/or under diagnosed due to overreliance on smear microscopy.[4–6] Immune suppression (due to factors, such as human immunodeficiency virus [HIV] infection, and chemotherapy) and underlying lung diseases, such as cystic fibrosis, have been identified as contributing factors for the increasing global incidence of NTMs-associated infections.[1,7] Although Africa is a TB/HIV-endemic region,[8] information concerning isolation and characterization of pulmonary NTMs are scanty. This lack of knowledge regarding the contribution of NTMs inevitably may lead to an overly high rate of misdiagnosis of NTMs as MTBC since smear microscopy, the most widely used diagnostic tool, cannot differentiate NTMs from MTBC, which could have dire consequence on the control of TB. [4] Ghana has TB and HIV coinfection rate of approximately 21%;[8] however, there is not a single report regarding isolation and characterization of pulmonary NTMs.

Most NTMs are innately resistant to the conventional anti-TB drugs, [9] leading to treatment failure of the standard TB regimen. Therefore, NTMs infections are likely to be misdiagnosed as multidrug-resistant TB; therefore, patients are put on second-line anti-TB drugs,[6,9] which may also fail. Unlike MTBC infections, there is no single treatment regimen for NTMs because of the diverse spectrum of drug resistance influenced by the genetic background of the infecting species/ strain.[10–12] Therefore, it is imperative that proper identification of NTMs is performed to advice an appropriate treatment regimen.[10–12]

Although smear microscopy is limited by its low sensitivity and discriminatory power, it is the most widely used diagnostic tool for mycobacterial infections, especially in resource-limited countries.[13] Classical identification of NTMs by culture-based biochemical tests has an improved sensitivity and discrimination; however, it is tedious, requires expertise, and the results are sometimes not reproducible.[14] The use of nucleic acid-based typing tools, such as 16S rRNA and *hsp65* encoding a 65 kDa protein conserved in all *Mycobacteria species* with species-specific polymorphic loci have revolutionized the diagnosis of NTM. [15,16] The DNA-based assays are rapid, very reproducible, and highly discriminatory for differential diagnosis to advice on species specific therapy.[14]

We set out to use *hsp65* gene sequencing to identify NTMs isolated from pulmonary TB cases from Ghana and to phenotypically test the identified NTMs against isoniazid (INH), rifampicin (RIF), and streptomycin (STR). Our findings suggest that NTMs infections may play a vital role in causing lung diseases, thereby impacting the management of TB in endemic settings such as Ghana.

## 2 Methodology

### 2.1 Recruitment of study participants

Sputum smear-positive patients reporting to selected health facilities in the Accra Metropolitan Authority of the Greater-Accra Region and in the northern region (Mamprusi East District and Tamale Metropolis) of Ghana between August 2012 and July 2014 were recruited for the study. The procedures used for sample collection were as routinely employed by the National Tuberculosis Program. The structured standard questionnaire was employed to gather patients' demographic and clinical data. The questionnaire and protocols for this work were reviewed and approved by the Institutional Review Board of the Noguchi Memorial Institute for Medical Research with federal assurance number FWA00001824.

### 2.2 Cultivation of mycobacteria from sputa

All specimens were decontaminated using the 5% oxalic acid decontamination method,[13] inoculated on Lowenstein–Jensen medium supplemented with glycerol, incubated at 37°C, and observed for macroscopic growth until 12 weeks. The isolates obtained were suspended in nuclease-free water, deactivated at 98°C for 60 min and spun at 14,000 rpm to pellet cells for DNA extraction.

### 2.3 Isolation of genomic DNA

The protocol used for DNA extraction was a hybrid of the protocols as outlined by van Soolingen *et al.*[17] and Käser *et al.*[18] Briefly, the mycobacterial cell wall was disrupted by the addition of lysozyme solution (50 µL lysozyme of 10 mg/mL), vortexed, and incubated overnight. Next, 75 µL of 10% SDS and 10 µL of proteinase K (20 mg/mL) was added to the suspension, vortexed, and incubated at 65°C for 15 min. After incubation, 100 µL of 5M NaCl was added to the suspension, followed by CTAB/NaCl which was prewarmed at 65°C, and incubated at 65°C for 10 min. After vortexing, the extracted DNA was purified using the chloroform/isoamyl alcohol extraction method. The DNA contained in the upper phase was precipitated with isopropanol, washed with ethanol, dried, resuspended in 100 µL of Tris-EDTA buffer, and stored at 4°C until use.

### 4.2 Polymerase chain reaction detection of nontuberculous mycobacteria

All isolates obtained were typed as MTBC or NTM by polymerase chain reaction (PCR) amplification of the insertion sequence *6110* (IS*6110*) with MTBC-specific primers as previously described.[19] All IS*6110*-negative strains were suspected as NTMs and were subsequently used for further analysis.

### 2.5 Hsp65 gene amplification, DNA sequencing, and blast analysis

Oligonucleotide primers used for the mycobacterial *hsp65* gene were Tb11 (5'-CAACGATGGTGTGTCCCAT-3') and Tb12 (5'-CTTGTCGAACCGCATACCCT-3'). Briefly, the PCR reactions contained 3 µL of 10X buffer, 1.8 µL of 15 mM MgCl<sub>2</sub>, 3 µL of Q solution, 0.6 µL of 10 mM dNTP mix, 1.8 µL of each primer, 0.2 µL of hot-start Taq polymerase from Qiagen, 14.8 µL of nuclease-free water, and 3 µL of template DNA. Cycling conditions were (a) initial denaturation at 95°C for 15 min and 35 cycles of

denaturation at 96°C for 1 min, (b) annealing at 60°C for 1 min, (c) extension at 68°C for 1 min, and (d) final extension at 72°C for 10 min. Amplification of the 441-bp product was confirmed by 1.5% agarose gel electrophoresis and UV transillumination. PCR products were purified and sequenced by outsourcing (Macrogen Europe). Vector sequences in the sequenced *hsp65* genes were removed, the sequences were processed into gap experimental files with pregap4, edited with gap4 of the Staden package,[20] and the corresponding consensus sequence was saved in FASTA format for further analysis. The consensus *hsp65* gene sequence of each isolate was analyzed by NCBI nucleotide blast against the microbial database of representative genomes only and selecting highly similar sequences (megablast), whereas leaving all other settings to default.

## 2.6 Drug susceptibility testing

Microplate Alamar blue assay (MABA) for STR, INH, and RIF drug susceptibility testing was performed in clear-bottomed, 96-well microplates (Nunc International, Rochester, NY, US). Outer perimeter wells were filled with sterile water to prevent dehydration in experimental wells. The drug concentrations used were prepared, and the assay was set up by following the standard method,[21,22] with few modifications as described by Otchere *et al.*[23] and reduced incubation time from 7 to 4 days. All manipulations were conducted in an appropriate, safe working area in the P3 laboratory of the Noguchi Memorial Institute for Medical Research.

## 2.7 Statistical analysis

The Fisher's exact test was used to assess the associations between patient characteristics and species identified. All statistical analyses were performed in STATA 10.1 (Stata Corp., College Station, TX, USA)[24] with significant threshold set to  $P < 0.05$  at 95% confidence interval (CI).

## 3.0 Results

### 3.1 Patients with nontuberculous mycobacteria infection

In total, 2036 smear-positive patients were recruited in the study. We excluded 278 from further analysis because no macroscopic cultures were obtained from their sputa after 12 weeks of observation; the remaining 1755 (86.2%) unique isolates were used for further analyses. The characteristics of the study population are summarized in Table 1. Out of 43 patients, comprising 27 (62.8%) male and 16 (37.2%) female patients of average age 39.4 (range, 10–75) years, 43 (2.5%) isolates were identified as NTMs after unsuccessful PCR amplification of *IS6110*, but positive *hsp65* gene amplification [Figure 1]. Furthermore, 25 patients consented to HIV testing, and 13 (52.0%) of them were seropositive. Univariate analysis comparing patients with HIV and NTMs coinfection (52%) and those with HIV and MTBC coinfection (5.8%) showed a statistical association of NTMs infection with HIV ( $P = 0.001$ , odd ratio [OR] = 15.6, 95% CI = 7.7–32.4). Among 10 patients who consented to diabetic testing, only 1 (10%) was diabetic. Out of the 25 patients with information on Bacillus Calmette–Guerin, 16 (64%) had the scar.

### 3.2 Species identity of the isolated nontuberculous mycobacteria

The DNA sequencing *hsp65* gene identified 13 (30.2%) *Mycobacterium avium* subs. *paratuberculosis*, 3 (7.0%) each of *Mycobacterium colombiense* and *Mycobacterium mucogenicum*, 18 (41.9%) *Mycobacterium intracellulare*, 5 (11.3%) *Mycobacterium abscessus*, and 1 (2.3%) *Mycobacterium simiae* [Figure 2]. No species-specific statistical association was found between the identified NTMs and any of the patient's clinical data.

### 3.3 Drug susceptibility profiling of nontuberculous mycobacteria against streptomycin, isoniazid, and rifampicin

All the 43 NTMs identified were screened for their susceptibility profiles against INH, STR, and RIF using MABA. All 43 (100%) identified NTMs were resistant to INH, 26 (60.5%) were resistant to all the 3 drugs tested, 11 (25.6%) were resistant to both INH and RIF, and 3 (6.9%) were resistant to RIF and STR in addition to INH resistance. Moreover, 3 (6.9%) of the isolates were susceptible to STR and RIF, 6 (13.9%) were susceptible to RIF irrespective of INH and STR resistance, and none was susceptible to INH [Table 2].

Stratifying drug profile by species, all the 13 *M. avium* subs. *paratuberculosis* strains were resistant to INH and RIF irrespective of INH resistance, whereas 7 (53.8%) were additionally resistant to STR. All the 5 *M. abscessus* (100%) strains were resistant to all drugs, whereas 12 (66.7%), 18 (100%), and 16 (88.9%) of the *M. intracellulare* strains were resistant to STR, INH, and RIF, respectively. All the 3 *M. colombiense* strains were resistant to INH, whereas 2 (66.7%) and 1 (33.3%) were resistant to STR and RIF, respectively. While all the 3 *M. mucogenicum* strains were additionally resistant to STR, 2 (66.7%) out of the 3 were additionally resistant to RIF. Last, the single *M. simiae* strain was susceptible to STR and RIF, but resistant to INH [Table 3].

## 4.0 Discussion

Pulmonary infections caused by NTMs are often misdiagnosed as conventional TB caused by members of the MTBC because of the nonspecificity of sputum smear microscopy.[4–6] In addition, because of their intrinsic resistance to most first-line anti-TB drugs,[9,10] NTMs-associated TB may be normally diagnosed as TB and managed as MDR with its associated public health implications.[25–28] In Ghana, the prevalence and epidemiology of NTMs are largely unknown.[25] We sought to (1) determine the proportion and identity of NTMs among our prospective collection of mycobacteria isolates from smear-positive pulmonary TB cases reporting to selected health facilities in Ghana; (2) establish their susceptibility to the anti-TB drugs INH, RIF, and STR; and (3) analyzed for possible association of NTMs infection with patient characteristics. We found that (1) 2.5% of our mycobacterial isolates were NTMs and were dominated by *M. intracellulare*; (2) most of the identified NTMs were resistant to the two backbone drugs (INH and RIF) of the Directly Observed Treatment Short-course (DOTS) regimen; and (3) there was a significant association between NTMs-infected patients and HIV. Compared with the global incidence and a recent report on NTMs from Ghana, which identified 8% of NTMs among clinical mycobacteria from presumptive TB cases, our report of 2.5% is relatively small.[29–32] However, the previous study in Ghana involved only HIV-positive presumptive TB patients,

which may explain the high rate of NTMs from that study. Nevertheless, for individual patient care and public health purposes, 2.5% is still high enough to reiterate the importance of rapid identification of the infecting species to facilitate effective treatment of pulmonary TB cases.

Our data set was dominated by *M. intracellulare* and *M. avium* subs. *paratuberculosis* [Figure 2], which constitute the *M. avium* complex (MAC). This finding compares with earlier reports of the dominance of MAC among pulmonary NTM infections.[1,29,32] This result is suggestive of the possible dominance of the MAC species in the environmental settings of the country. In addition, our results give more credit to the use of *hsp65* gene sequence analysis for rapid identification of NTMs [Figure 2]. Compared with the earlier report from Ghana that could identify 17/38 (44.7%) of the NTMs using genotyping tools other than *hsp65* gene sequencing,[32] we were able to identify all the isolated NTMs using the *hsp65* gene sequencing. Our study, therefore, highlights the discriminatory power of the use of *hsp65* gene sequencing for mycobacterial speciation.

Individuals that are immunocompromised due to HIV infection are at a risk of NTMs infection as evidenced by our study. We found a significant association between NTMs-infected patients and HIV ( $P=0.001$ , OR = 15.6, 95% CI = 7.7–32.4). This finding supports earlier reports implicating the global HIV pandemic on the increasing global cases of NTMs-associated infections[33,34] and also compares with earlier works done in Tanzania and South Africa,[29,35] where HIV coinfection was implicated as a predictor of pulmonary NTMs infections. It is, therefore, imperative that TB treatment failures on the standard DOTS, especially among HIV-coinfected patients, are properly investigated to exclude the possibility of treating NTMs infection as an MDR-TB case.

NTMs pose a major challenge for TB treatment programs since such patients are managed mainly on the basis of smear microscopy,[9] which cannot determine their drug susceptibility profiles leading to their misdiagnoses as MDR-TB cases. Most of the NTMs (60.5%) identified were resistant to all the three drugs (INH, STR, and RIF), whereas 11 (25.6%) were resistant to the two most important drugs of the DOTS regimen (INH and RIF). None of the 43 isolates was sensitive to INH. This result is in agreement with the already established high resistance of NTMs to the drugs commonly used in the management of pulmonary TB cases[36,37] and reinforces the need to correctly identify the causative agent (to the species level) of such patients before they are put on an appropriate treatment regimen. Comparing independent resistance to the three drugs (43 INH, 29 STR, and 37 RIF) suggests that STR was the most active drug against our identified NTMs [Table 3] even though the proportion of identified NTMs resistant to STR (67.4%) was still high. Among the identified species, *M. abscessus* was the only species that had all strains resistant to all the three drugs [Table 3] and had the highest minimal inhibitory concentrations (S1). This finding supports earlier reports of resistance of *M. abscessus* to various antimicrobials, hence, making the treatment of *M. abscessus* infections very difficult.[9,38,39] This extreme resistance of *M. abscessus* has been partly attributed to the unique genetic constitution, probably due to horizontal gene transfer, a trait that is largely missing in the MTBC.[39–42]

## 5.0 Conclusion

Our results emphasize the importance of NTMs in pulmonary infections, which is dominated by the *M. intracellulare* complex. The high level of resistance of the identified species to INH and RIF calls for rapid identification and susceptibility testing of the causative agents of nonconverting TB patients or treatment failures for proper management.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

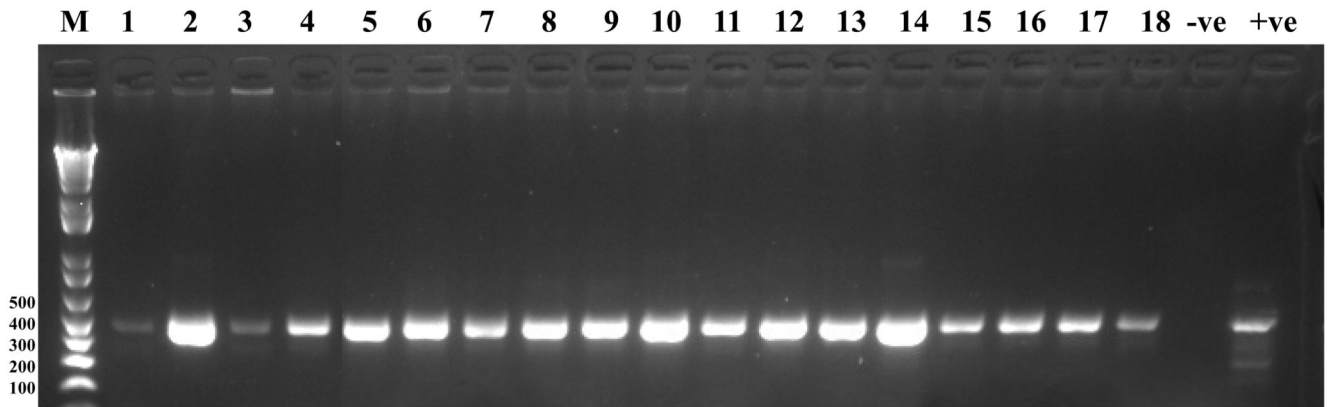
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**Figure 1. Agarose Gel Picture of PCR Amplified hsp65 Gene of Suspected NTM Strains**

Lane M: DNA size marker, lane 1 to 18 are the 441 bp hsp65 gene amplified from candidate NTM strains. Lane -ve is the negative pcr control, Lane +ve is the positive control amplified from *Mycobacterium tuberculosis* H37Rv

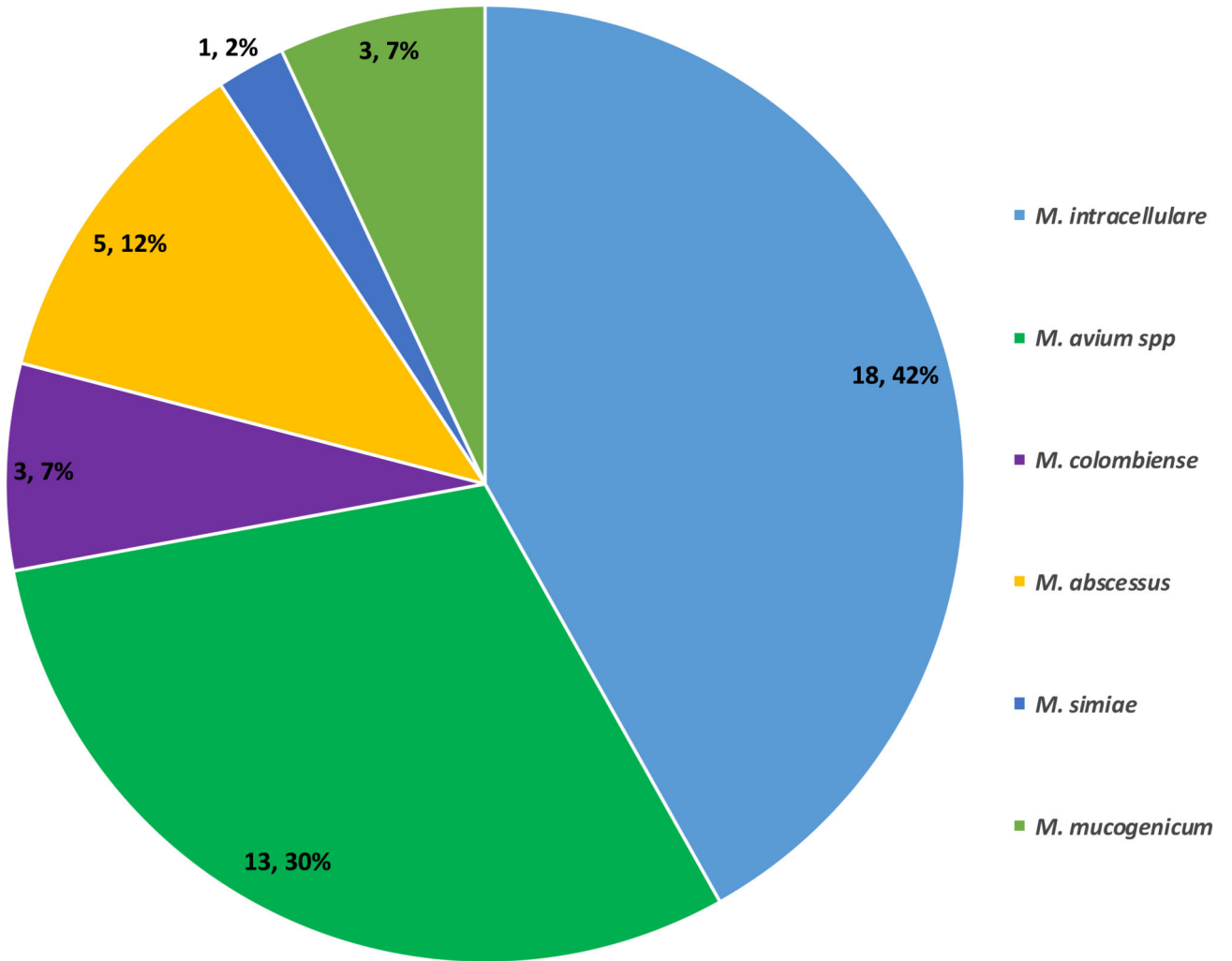


Figure 2. Distribution of Identified NTM

**Table 1**  
**Biodata of Patients from Whom NTM were Isolated**

<b>Feature (Number of Patients)</b>	<b>Number (%)</b>
<b>Average Age (43)</b>	39.4 years
<b>Gender (43)</b>	
Male	27(62.8)
Female	16(37.2)
<b>HIV available (25)</b>	
Positive	13(52.0)
Negative	12(48.0)
<b>HIV Not Available (18)</b>	
<b>Diabetes Available (10)</b>	
Positive	1(10.0)
Negative	9(90.0)
<b>Diabetes Not Available (33)</b>	
<b>BCG Scar Available (25)</b>	
Present	16(64.0)
Not Present	9(36.0)
<b>BCG Scar Not Available (18)</b>	

**Table 2**  
**Distribution of Drug Resistance Among the NTMs**

<b>Resistance</b>	<b>Number (Percentage)</b>
INH + RIF + STR	26 (60.5%)
INH + STR	3 (6.9%)
RIF + INH	11 (25.6%)

**Table 3**  
**Drug Susceptibility Profile of Identified NTM**

Species (Number)	Resistance		
	STR	INH	RIF
<i>M. avium</i> paratuberculosis (13)	7 (53.8%)	13 (100%)	13 (100%)
<i>M. colombiense</i> (3)	2 (66.7%)	3 (100%)	1(33.3%)
<i>M. intracellulare</i> (18)	12 (66.7%)	18 (100%)	16 (88.9%)
<i>M. abscessus</i> (3)	5 (100%)	5 (100%)	5 (100%)
<i>M. mucogenicum</i> (3)	3 (100%)	3 (100%)	2 (66.7%)
<i>M. simiae</i> (1)	-	1 (100%)	-
Total Number of isolates	29 (67.4%)	43 (100%)	37 (86.0%)