Prevalent non-tuberculous Mycobacteria (NTM) species among presumptive MTBDR patients from peripheral health facilities referred for testing at KEMRI TB Laboratory-Kisumu, Kenya

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Abstract
Objectives: The aim of this study was to establish prevalent Nontuberculosis Mycobacteria among presumptive multidrug resistant TB (MDRTB) patients in western Kenya. Sputum samples obtained from laboratory confirmed MDRTB patients were processed using standard culture methods, fluorescence microscopy (FM), MGIT culture, Xpert MTB/RIF. Mycobacteria tuberculosis complex (MTBc) and NTM were identified using line probe assays techniques.

Results: Between January 2012 to December 2015, a total of 287 samples from presumptive MDRTB individuals were flagged positive with liquid culture (MGIT), subsequently had line probe assay (LPA) performed for potential nontuberculous mycobacteria species. Of these, 155 (54%) had NTMs and 14 (4.87%) MTBc by LPA technique, respectively. Males constituted 140 (49%) of which 64 (22.3%) were HIV infected, while females were a total of 160 (55.7%), with 96 (33.4%) HIV infected. 41 (14.3%) individuals with unknown HIV status. M. intracellulare 62 (21.6%), and M. scrofulaceum 13 (4.5%) were most prevalent NTMs species identified.

Keywords: Nontuberculous mycobacteria, multidrug resistance tuberculosis, pulmonary, diagnosis, line probe assay, microscopy.

Introduction
In 2019, there were an estimated 10 million new (incident) Tuberculosis (TB) cases worldwide, of which 5.6 million (56%) were among men, 3.2 million (32%) among women and 1.2 million (12%) among children[1]. Globally, an estimated 1.4 million TB deaths were reported in 2019; 0.2 million of these deaths occurred in people living with HIV (PLWH)[1]. In addition, 8.2% of new TB cases reported globally occurred in PLWH in 2019[1]. Simultaneous infection with HIV and Mycobacterium tuberculosis (MTB) is a deadly combination affecting large populations in Africa, Asia, Latin America and Eastern Europe. Progressive immune dysfunction caused by HIV infection increases susceptibility to MTB infection as well as progression from latent infection to active tuberculosis (TB) disease[2].

More recently, other species of mycobacterium causing clinical disease have been identified in many geographical regions where they cause significant disease burden than MTB[3]. Mycobacteria other than tuberculous (MOTT), Mycobacteria species and M. leprae are known as nontuberculous mycobacteria (NTM), previously, they were referred to as “atypical” mycobacterial...
species and they share many common properties with MTB, such as acid fastness, ability to cause pulmonary and extra-pulmonary granulomatous disorders and pathogenicity. These properties vary among different species and may cause opportunistic infections, however, the lung seems to be their most common site of involvement, same as MTB.

Although MTB and NTM cause chronic lung infections, only MTB spreads from person to person by inhalation of organisms expectorated into the air. NTM infections are acquired directly from environment, where they are often present in soil and various water sources, thus considered opportunistic pathogens. Since pathologic findings of NTM and MTB regarding granulomatous inflammation and even cavity are very similar, it is difficult to distinguish them from pulmonary TB.

*Mycobacterium tuberculosis* diagnosis is an important disease control tool, consisting of both conventional methods (acid-fast microscopy, a primary procedure in most health facilities, culture, biochemical identification, anti-tuberculosis drug-susceptibility testing (AST) and modern molecular techniques, hence laboratory plays an important role in diagnosing tuberculosis (TB) and the identification and drug sensitivity testing (DST) of *Mycobacterium tuberculosis*. Kenya is considered a high TB-burden country, with a TB prevalence of 553/100,000 (KTBPS, 2016) and with limited access to mycobacterial culture. 95% of pulmonary bacterial infections are due to Mtb while 5% by NTM. Western region has a TB prevalence of 14%, twice the national rates making TB/HIV co-infection a serious health concern.

The incidence of disease caused by NTM is somewhat independent of that of TB, but is determined by the number, distribution, and species of NTM in the environment and susceptibility of human population. Little is known about the relative burden of NTMs, including Kenya, where public laboratories performing bacteriological cultures, primarily test for MTBc but seldom test for NTMs.

The reports of nontuberculous mycobacteria (NTM) associated with extrapulmonary diseases are increasing in tertiary care hospitals. Despite a significant increase in knowledge about NTM infections, they still represent a diagnostic and therapeutic challenge.

The high costs to the patient and society should lead health care providers to consider NTM in all patients suspected of having TB. Even in low resource countries where mycobacterial culture and molecular identification procedures are not routinely practiced, there could be great saving of country resources associated with correctly diagnosing and treating patients. It is another reason for National TB Programs to integrate mycobacterial culture and molecular identification procedures into routine practice.

Identifying NTM frequency and alerting physicians to this problem may increase awareness, help better understand the epidemiology, get patients on appropriate therapy sooner, and decrease the stigma and cost of this care.

In our demonstration of NTMs, we used line probe assay kits from HAIN LIFESCIENCE, which provides for both the common mycobacteria (CM) and atypical species (AS) of mycobacteria species. These kits have the ability to distinguish the various types of species of mycobacteria of interests. The molecular based technologies that provide the genomic identification and demonstrates the presence of the corresponding DNA for mycobacteria species. In 2012, the Kenya’s MOH TB program scaled up MDR surveillance across the country, by authorizing KEMRI CGHR TB laboratory to support with culture and resistance testing for presumptive multidrug resistant patients in Western and north rift region of Kenya. The study aimed to show the prevalent NTMs in these populations through the various techniques employed.
Main Text
NTM presence shows positivity with common routine Ziehl Nelsen (ZN) microscopy methods available hence patients may routinely be initiated on wrong medications due to MTBc. Hence parallel diagnosis for tuberculosis diagnosis is required to classify MTBc to inform patient management.

Methods
The Study Design
This was a cross sectional study, where samples were collected at one-time point and sent for culture. Stored sputum samples collected from presumptive MDRTB patients western Kenya, were processed for and determined for MTBc and NTM presence.

These are patients who had been initiated into the TB programs, primary regimen but did not show signs of improvement.

Laboratory Procedures
Sputum samples were collected from January 2012 through December 2015 as part of Multidrug resistance surveillance, an activity by the MoH through the peripheral facilities. Patients were asked to produce at least one sputum sample during their hospital visits. These samples were then packaged and transported via courier delivery or hand delivered to the TB laboratory for processing.

In the laboratory, samples were processed based on the national TB program testing algorithm. All presumptive MDRTB cases, had Xpert Mtb tests, smear microscopy (ZN/FM), and all positive smears had Line probe assays (LPA) tests as well as MGIT cultures of which positives had MTBc identification carried thereafter drug susceptibility tests for first line drugs.

During processing, samples were digested and decontaminated using N-acetyl-L-cysteine/ 4% sodium hydroxide-sodium citrate (NALC/NaOH Na-citrate, [final concentration of NaOH 1%]), neutralized with pH 6.8 phosphate buffer solution (PBS), concentrated through centrifugation @ 3,000 x g for 15 minutes, decanted, and resuspended in 2.0mL of fresh PBS. Thereafter inoculated into MGIT tubes, a drop (0.03ul) for smear preparations, 0.5ul aliquoted for Xpert Mtb Rif PCR and also LPA (HAIN CM& AS) kits. The tubes were incubated at 37°C per culture SOP, in the BACTEC 960 and observed for any growths per, subsequent positive flags were processed for identification using Brain heart infusion (BHI) and Ziehl Nelsen smear.

Microscopic evaluations were performed on all tubes showing positive flags, as well as on BHI culture plates for confirmation of AFB; growth consistent with mycobacteria was identified using an immunochromatographic assay (MGIT TBc ID, BD Sparksville Maryland, MD). All positive smears, were processed using Line probe assays, the Genotype Mycobacterium Common mycobacteria or Atypical species (CM/AS) line probe assay (Hain LifeScience, Nehren, Germany). Quality control procedures included inoculation of MGIT with processed artificial sputum (AS) spiked with an American Type Culture Collection (ATCC) - H37rv control strain for positive control and non-spiked AS for negative control.

Classification of Culture Results
The MTBC yield was compared to the prevalent NTMs identified through the performance of Line probe assay (LPA) with respect to contamination, NTMS and MTBC yield. The final outcome was categorized as: MTBC positive, negative, contaminated, or NTM positive. Cultures were considered MTBC positive if growth obtained from the cultures was identified as such using the immunochromatographic assay, negative if no growth was observed after the standard 6 weeks (42 days) of incubation, contaminated if only non-acid-fast organisms grew, and NTM if growth was negative for MTBC by the immunochromatographic assay and identified as NTM using the line probe assay. The NTMs were identified and classified into the various species using HAIN (CM&AS) kits.

Statistical Analysis
Participant data retrieved from source documents were keyed in and stored in a password-protected
Laboratory Information System (LIMS). Data was analysed using STATA version 14 for Windows. Frequencies and percentages were used to present the participant characteristics, HIV results and the MTB results for HAIN CM/AS, Ziehl Neelsen (ZN)/ Fluorescent (FM) microscopy and Xpert Mt rif PCR.

Ethical Review
We sought ethical approval from KEMRI’s IRB (SERU) to conduct this study. As part of collaboration with MOH, we also had a MoU with National TB Leprosy and lung diseases program, which permitted use of left over samples, these presented No or minimal risk to participants. Patients’ confidentiality was adhered to maintained and protected, during the study.

Results
Table 1. A total of 287 samples flagged positive on liquid culture (MGIT), WHO gold standard procedures, were processed for presumptive MDRTB patients using a molecular technique (line probe assay), to determine potential nontuberculous mycobacteria species. The demographic characteristics of these participants with NTMs, 147(51.2%) were females and 140(48.8%) males. Mean age was 38.1 (14.8%), and a prevalence of MTBe of 62 (21.6%). HIV positive individuals with NTMs were 156 (54.4%), 92(32.1%) and 39 (13.6%) with no HIV status indicated.

Table 2 shows risk factors associated with NTM infection, where gender showed a significance of p-v 0.036, with an OR (0.77) while HIV status was insignificant, with an OR (0.89) and a p-v 0.225. 167 (58%) individuals had comorbidity of NTM to either Mtb and/or HIV or both. 26 (0.6%) had NTM only, 84 (2.1%) had both NTM and Mtb co-infections.

Table 4 shows the prevalent mycobacteria species in relation to HIV status and gender. Of the species identified, Mycobacterium intracellulare were 62(22.1%), of which 37(66%) among HIV positive, and 33(53.3%) in males. Mycobacteria scrofulaceum were 13(4.5%), of 9(69%) from HIV infected, 7(30%) and 6 (30%) among females and males respectively. Of note, other high grade cocci (HGC) organisms were prevalent, 45(16%) were identified of which 29(70.7%) were HIV infected, 25(55.6%) and 20(44.4%) were females and males respectively. Other NTMs identified were Mycobacterium fortuitum 11(3.8%), Mycobacteria celatum 10(3.5%), Mycobacteria abscessus 7(2.5%), Mycobacterium gordonae 7(2.5%), Mycobacterium malmoense 6(2.14%), Mycobacterium avium 3(1.1%), and Mycobacterium interjectum and Mycobacterium kansasii each having 1(0.3%) respectively.

Table 5 shows the participants having both NTM and Mtb based on their HIV status. Of the 122 (42.5%%) with NTM only, 84(45.6) of them were HIV positive while 26(14.1%) HIV non-infected. From among 62 (21.6%) individuals with both Mtb and NTM comorbidity, 32(17.3) HIV infected and 25(13.5%) HIV non-infected.

Table 6 shows the prevalence of NTM species in relation to HIV status and gender. Out of the 287 individuals presumed to have NTMs, 155(55.6%) had various types of NTMs, with the most prevalent NTMs were 62(21.6%) M. intracellulare, 23(8%) Mycobacteria species, 13(4.5%) M. scrofulaceum, 14(4.9%) MTBC, 10(3.9%) M. celatum, 7(2.4%) M. abscessus, 11(3.8%) M. fortuitum, among others as shown in Table.
Appendix: Tables

**Table 1: Participant Characteristics (demographics and HIV status)**

| Demographic and risk factors | Overall (n = 287) |
|------------------------------|------------------|
| Gender                       | N                |
| Female                       | 147              |
| Male                         | 140              |
| Age: Mean (SD)               | 38.1 (14.8)      |
| Age groups (years)           |                  |
| <20                          | 27               |
| 20-40                        | 128              |
| 40-60                        | 99               |
| >60                          | 22               |
| Prevalence of MTBC           | 62               |
| Concurrent conditions        |                  |
| HIV +ve                      | 156              |
| HIV –ve                      | 92               |
| Unknown                      | 39               |

**Table 2: Risk factors associated with NTM infection**

| Gender        | OR   | 95 % CI     | p-value |
|---------------|------|-------------|---------|
| Male          | 0.77 | 0.61 - 0.98 | 0.036   |
| Female        | 0.89 | 0.73 - 1.08 | 0.225   |

**Table 3: Comorbidity of NTM with either *M. tb* and or HIV among individuals with NTMs**

| Type of infection  | N   | %   |
|--------------------|-----|-----|
| NTM alone          | 26  | 0.6 |
| HIV +NTM           | 84  | 2.1 |
| NTM + M.tb         | 25  | 0.6 |
| HIV + NTM +M.tb    | 32  | 0.8 |
| Total              | 167 |     |

**Table 4: Comorbidity of NTM with either *M. tb* and or HIV among individuals with NTMs by Gender**

| Type of infection  | n   | Male (%) | Female (%) |
|--------------------|-----|----------|------------|
| NTM alone          | 26  | 15 (0.68) | 11 (0.60)  |
| HIV +NTM           | 84  | 38 (1.72) | 46 (2.52)  |
| NTM + M.tb         | 25  | 19 (0.86) | 6 (0.33)   |
| HIV + NTM +M.tb    | 32  | 13 (0.59) | 19 (1.04)  |

**Table 5: Comorbidity of NTM with *M. tb* by HIV**

| Prevalence of NTM and NTM infection | n(%) | Positive | Negative | Others |
|------------------------------------|------|----------|----------|--------|
| NTM Only                           | 122(42.5) | 84 (45.6) | 26 (14.1) | 12 (6.5) |
| M.tb + NTM                         | 62(21.6)  | 32 (17.3) | 25 (13.5) | 5 (2.7)  |
Table 6: Species distribution of the NTM isolated from PDCs/hospitals by HIV Status and Gender

| Prevalence of NTM infection | HIV Status n (%) | Gender n(%) |
|----------------------------|-----------------|-------------|
|                           | Positive | Negative | Others | Male | Female |
| M. gordonae                | 5        | 4 (2.50) | 1 (1.14) | 0    | 4 (2.86) | 1 (0.68) |
| M. Abscessus               | 7        | 1 (0.63) | 3 (3.41) | 3 (7.69) | 1 (0.71) | 6 (4.08) |
| M. Asiaticum               | 4        | 1 (0.63) | 2 (2.71) | 1 (2.56) | 1 (0.71) | 3 (2.04) |
| M. Genavense               | 2        | 1 (0.63) | 1 (1.14) | 5 (12.82) | 1 (0.71) | 1 (0.68) |
| M. interjectum             | 1        | 0        | 1 (1.14) | 0    | 0        | 1 (0.68) |
| M. intracellularae          | 62       | 37 (23.1) | 18 (20.45) | 7 (17.95) | 31 (22.14) | 31 (21.09) |
| M. scrofulaceum            | 13       | 9 (5.63) | 4 (4.55) | 0    | 6 (4.29) | 7 (4.76) |
| M. Mucogenum               | 2        | 0        | 2 (2.27) | 0    | 2 (1.43) | 0 |
| M. Smegmatis               | 2        | 1 (0.63) | 0        | 1 (2.56) | 0        | 2 (1.36) |
| MTBC                       | 14       | 7 (4.38) | 3 (3.41) | 4 (10.26) | 7 (5.00) | 7 (4.76) |
| Myco SPP                   | 23       | 15 (9.38) | 6 (6.82) | 2 (5.13) | 12 (8.57) | 11 (7.48) |
| M. avium spp               | 3        | 3 (1.88) | 0        | 0    | 0        | 2 (3.04) |
| M. fortuitum               | 11       | 6 (3.75) | 3 (3.41) | 2 (5.13) | 8 (5.71) | 3 (2.04) |
| M. kansasii                | 1        | 0        | 1 (1.14) | 0    | 1 (0.71) | 0 |
| M. lentiflavum             | 2        | 1 (0.63) | 1 (1.14) | 0    | 0        | 2 (1.36) |
| High GC                    | 49       | 28 (17.5) | 15 (17.05) | 6 (15.38) | 24 (17.14) | 25 (17.01) |
| M. celatum                 | 10       | 4 (2.50) | 1 (1.14) | 5 (12.82) | 5 (3.57) | 5 (3.40) |
| M. malmoense               | 5        | 4 (2.50) | 0        | 1 (2.56) | 4 (2.86) | 1 (0.68) |
| NTM not identified          | 58       | 30 (18.75) | 23 (26.14) | 5 (12.82) | 29 (20.71) | 29 (19.73) |
| Negative                   | 13       | 8 (5.00) | 3 (3.41) | 2 (5.13) | 4 (2.86) | 9 (6.12) |

Discussions

NTM have been increasingly recognized as an important cause of morbidity in the developing countries\(^{(14)}\). The identification of NTM is important because positive microscopy cannot differentiate *M. tuberculosis* complex from NTM infection, causing diagnostic and clinical dilemmas. Management of patients with MTBC and NTM is entirely different; therefore, prompt isolation, detection, and differentiation are necessary for suitable management\(^{(6, 14, 19)}\).

In this study, the prevalence of NTMs among presumptive MDR patients was 3.7% lower than a study done in Iran\(^{(15)}\) which found 30% of NTMs among MDR TB patients. Similarly, it was also lower than a study conducted earlier to show prevalence of NTMs among MDR patients, such as; Tabarsi *et al.* studied 105 suspected MDR-TB subjects in the center of Iran and found 12 (11%) cases of NTM. Aliyu *et al.* found 69 (15%) NTM out of 444-mycobacterial positive cultures from consecutive new cases of suspected TB in Nigeria\(^{(20)}\). The present study presents similar outcomes on gender as shown in a study done in Korea previously showing higher rates in females than males\(^{(21)}\) also showing increased prevalence among older people, while our study, we had mid aged population\(^{(21)}\).

Among the HIV infected, individuals with NTM were lower than the study conducted in USA\(^{(22)}\). While the participants in this study were of middle ages, this was not consistent with other studies showing people of older ages having NTMS, from USA\(^{(23)}\). This study showed risk factors associated with NTM infections to be gender and HIV status, while gender proved significant, HIV infection showed no significance risk, in this study contrary to a study conducted in Spain (2015), which found out that HIV was associated with NTM infections\(^{(24)}\). The study was similar to one conducted in Dhaka, Bangladesh which showed almost similar representation among the gender, while the MTBC infection was observed in all gender in equal numbers, contrary to the global situation as shared that in 2017 close to 6 million adult men contracted TB and around 840,000 died from it. This compares with an estimated 3.2 million adult women who fell ill and almost half a million who died from TB\(^{(25)}\).
In this study, among these participants with presumed multidrug resistance tuberculosis, the most common NTMs species identified were *M. Intracellulare*, *M. Scrofulaceum*, among other species. Notably MTBC, was found present at 4.86%, other non-identified mycobacteria species and NTM were also found in distribution.

This study showed *Mycobacteria intracellulare* (22.1%) to be prevalent NTM, it was also significant among immunocompromised individuals, especially in HIV people, it is readily available in the soil, water and environment as well as airborne(5) followed by *M. scrofulaceum* among others, contrary to a study of a similar population conducted in Northern India, which found that M. fortuitum was more prevalent(14).

There was co-infection of both TB and NTMs as 14 (4.87%) cases of MTBC were found. Other studies have also showed co-infection rates, a similar in Canada, had NTM/Mtb co-infection of 11%,(26) and another in USA at 14%(27). The study showed presence of species of high grade cocci (High GC) at 16% (microorganisms), these organisms resemble Mycobacteria but are from a different genera (Rhodoccus), these organisms mimic those properties, as acid fastness, thus readily being confused as MTB using the old ZN technology, this shows that if the smear tests were done at the peripheral facilities (16%) of these would have been captured as TB positive. The high rate of NTM in patients with suspected MDR-TB due to TB treatment failure has important implications for healthcare economics, epidemiology, antimicrobial stewardship, and especially the care and quality of life of the individual patient(15).

Conclusions
The prevalence of NTMs 287 (12.5%) which includes *M. intracellulare* 62 (21.6%) and *M. scrofulaceum* 13 (4.5%) were the most frequently isolated rapid growing mycobacteria among the presumed multidrug resistant tuberculosis participants in Western Kenya. Overall rapid identification and differentiation to species level by molecular assay may help in targeted therapy and management of infections caused by different mycobacterial species and indirectly, it will also help in reducing the developing of antimicrobial drug resistance among NTM isolates in the community.

Limitations
A limitation to this study, was the inability to test all the possible species of other mycobacteria and other possible NTMs due to the unavailability of a kit, nor using advanced molecular technique.

Declaration

**Ethics approval and consent to participate**
This study was reviewed and approved by the Kenya Medical Research Institute’s Scientific and Ethics Review Unit. Protocol Number; KEMRI/SERU/CGHR/084/3410.

**Consent for publication**
Not applicable

**Availability of data and material**
All data generated or analyzed during this study are included in this published article.

**Competing interest**
The authors declare that they have no competing interests.

**Funding information**
Funding for this study was provided by the U.S. President’s Emergency Plan for AIDS Relief (PEPFAR) through Cooperative Agreement 5U19GH000041 from the U.S. Centers for Disease Control and Prevention (CDC), Division of Global HIV/AIDS and the United States Agency for International Development (USAID). Funding for the sub-study was provided by KEMRI.

**Disclaimer**
The findings and conclusions in this reports are those of the authors and do not necessarily represent the official position of the U.S. Centers for Disease Control and Prevention or the Government of Kenya.
Author’s contribution
Albert Okumu and Steve Wandiga designed the study and drafted the manuscript. Stephen Asito and Isaiah Omondi contributed to the data collection. Christine Ogolla, Clement Shiluli and Wilfred Murithi performed literature search, review and data analysis. Ronald Odero and Timothy Malika carried out sample collection, isolation, culture and identification of isolates. All authors agree to be accountable for all aspects of the work and approved the final version of the manuscript.

Acknowledgments
We thank the Kenya Ministry of Health (Division of Leprosy, Tuberculosis and Lung Disease and the National AIDS and STI Control Program) and the Kenya Medical Research Institute- Centres for global health research (KEMRI-CGHR), Centers for Disease Control (CDC) in addition to Joseph Orure, Ruth Sitati, Ronald Odero, Christine Agollah and Wilfred Murithi for high quality laboratory testing and data collection.

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