Magnitude and Geographical Distribution of Nontuberculous Mycobacteria among People Presumed to Have Pulmonary Tuberculosis in Kenya

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Summary

BACKGROUND

Globally, awareness of nontuberculous mycobacteria as causative agents of pulmonary disease is on the rise. In Kenya however, there is very limited information on the burden of nontuberculous mycobacteria, as diagnosis of pulmonary infections is usually by sputum smear microscopy which does not distinguish between *Mycobacterium tuberculosis* complex and nontuberculous mycobacteria, which are both causative agents. This study sought to determine the magnitude, drug susceptibility patterns and geographical distribution of nontuberculous mycobacteria among presumptive tuberculosis patients in selected health facilities including Malindi, Kitale, Busia, Wajir, Nyahururu, Kisii, Machakos and Lamu.

MATERIALS AND METHODS

This was a laboratory-based cross-sectional study in which 124 Ziehl-Neelsen positive isolates from a previous study were further analyzed. The archived isolates were sub-cultured in *Mycobacterium* growth indicator tube (MGIT™; BD Sparks, USA) medium and identification was done using GenoType® *Mycobacterium* assays (HAIN Lifescience, Nehren, Germany). Drug susceptibility against rifampicin, isoniazid and ethambutol was determined by the resistance ratio method and Pearson's Chi-square was used to establish the geographical distribution of species.

RESULTS

The proportion of isolates with nontuberculous mycobacteria was 19.3% and *Mycobacterium intracellulare* was predominant (41.7%). All 24 identified nontuberculous mycobacterium species were resistant to rifampicin, isoniazid and ethambutol. Wajir had the highest number of infections (CI95% 2.46-54.27, p=0.002).

CONCLUSION

There was a high magnitude of nontuberculous mycobacteria among presumptive tuberculosis patients. Inappropriate diagnosis of pulmonary infections caused by nontuberculous mycobacteria may lead to patient mismanagement with routinely prescribed anti-tuberculous drugs.

Keywords: *Nontuberculous Mycobacteria, Lung Diseases, Tuberculosis*

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Introduction

The incidence of pulmonary infections due to nontuberculous mycobacteria (NTM) is continuously increasing globally [1]. However, most reports on incidences of pulmonary infections due to NTM are often from developed countries that are non-endemic to pulmonary tuberculosis (PTB), and few are from sub-Saharan countries, where PTB is endemic [2]. Following the reported increase in pulmonary NTM infections, understanding their geographical distribution is crucial for detection, prevention and control of these infections [3]. These infections vary geographically [4], and are now considered a public health challenge especially in developing countries that have a high burden of pulmonary tuberculosis and NTM infections are often misdiagnosed as they have similar symptoms as tuberculosis caused by *Mycobacterium tuberculosis* complex [5]. Worldwide, the most predominantly isolated NTM is *Mycobacterium avium* [3]. In Japan, *Mycobacterium avium* complex is the most common cause of pulmonary infections due to NTM [6] while *Mycobacterium kansasii* is the major causative species in China [7], the United States and England [8]. In sub-Saharan Africa, the *Mycobacterium avium* complex was found to be the most predominant species isolated from pulmonary samples [9].

In most developing countries where PTB is endemic, Kenya included, diagnosis largely relies on the use of sputum smear microscopy which has several limitations including the inability to differentiate between *Mycobacterium tuberculosis* complex and NTM and cannot diagnose multi-drug resistant tuberculosis [10]. This is further challenged by similarity in clinical presentations of pulmonary infections caused both by *Mycobacterium tuberculosis* complex and NTM [1], leading to underreporting of pulmonary infections caused by NTM and hence the extent of NTM pulmonary diseases is not well known (11]. These challenges [12], coupled with the identification of species involved mostly not being done in cases of *Mycobacterium* pulmonary infections [13] have led to patients being inappropriately managed with commonly used anti-tuberculous drugs that not only lead to treatment failure but are also toxic with long term negative effects and are expensive as well [14].

In western Kenya, a high rate (42.7%) of TB-NTM co-infection was noted [15], with 2.6% of infants developing NTM pulmonary infections 2 years after administration of Bacille Calmette-Guerin vaccine [16]. A high percentage (42.7%) of PTB retreatment patients in Kenya were found infected with various NTM species, with *Mycobacterium intracellulare* (62.7%) being the most predominant species [17]. Since information on the NTM burden in Kenya is still elusive, this study aimed at determining the extent of pulmonary infections caused by NTM in the country.

Materials and methods

Study design

This was a laboratory-based cross-sectional study where isolates of people presumed to have PTB enrolled in a previously conducted study, the East Africa Public Health Laboratory Networking project, in Kenya, were analyzed. In the previous study, sputum specimens were collected from enrolled consenting patients from selected health facilities including Wajir, Machakos, Kitale, Busia, Malindi, Lamu, Narok, Kisii and Nyahururu.

Inclusion criteria

From archived isolates, 124 isolates presumed to be NTM were considered for this study and analyzed at the Kenya Medical Research Institute-Tuberculosis laboratory.

Subculture and isolation of mycobacteria

The isolates were sub-cultured in *Mycobacterium* Growth Indicator Tube (MGIT) media (Becton Dickinson, Sparks, USA) and Ziehl-Neelsen staining was used to confirm the presence of acid-fast bacilli. The absence of *the Mycobacterium tuberculosis* complex was confirmed using the *TBcID* assay (Becton Dickinson, Sparks, USA) test [18].

Mycobacteria characterization using GenoType® Mycobacterium assays:

Bacterial DNA extraction and amplification

Briefly, 1ml of liquid culture was centrifuged at 10000xg for 15 minutes in a 1.5ml Eppendorf tube, the bacterial pellet was re-suspended in 100µl of lysis buffer and heated in a water bath at 95^{o}C for 5 minutes allowing bacterial deoxyribonucleic acid (DNA) extraction. Neutralizing buffer (100µl) was added and centrifuged at full speed (13000xg) for 5 minutes and the supernatant was discarded. The extracted DNA was then stored at -20^{o}C until used.

For amplification, 5µl of the DNA solution was added to a pre-prepared 45µl amplification mix, and amplification was done using GT-Q 96 thermocycler (Hain Lifescience, GmbH, Nehren, Germany) with a set Hot 30 program for culture samples. A Twincubator® with GT 45 automated system (Hain Lifescience, GmbH, Nehren, Germany) was used for reverse hybridization of amplicons to membrane-bound probes and interpretation done following manufacturer's protocol (https://www.hain-lifescience.de/en/downloads/microbiology.html).

Drug susceptibility testing

The resistant ratio (RR) method was used to determine susceptibility patterns to rifampicin, isoniazid and ethambutol. Briefly described, different concentrations of rifampicin (8, 16, 32, and 64µg/ml), isoniazid (0.1, 0.2, 1.0µg/ml) and ethambutol (4, 5.6, 8µg/ml) were incorporated in freshly prepared Lowenstein-Jensen (LJ) media and the media inspissated in a slanting position at 80^{o}C for an hour. The inoculum was prepared from LJ subcultures, using a sterile disposable plastic loop (3mm diameter) to pick colonies and discharged into 7ml screw-capped bijou bottles with 1ml of sterile distilled water. This suspension was vortexed and then standardized by visual comparison with MacFarland tube number 1. One loop full (plastic loop of 3mm diameter) of this suspension was inoculated into the media with drugs and growth compared to that of a known standard mycobacterium laboratory strain ($H_{37}RV$). The resistance ratio of less than 4 and greater than 4 was considered susceptible and resistant respectively, to the drug [19].

Geographical distribution was determined by correlating identified NTM species with information from the facilities where specimens for the larger study were collected from.

Data analysis

Data were analyzed using IBM SPSS (version 24, 2018, USA). Bivariate analysis was done by testing the association between the dependent and single independent variable, using Pearson's Chi-square. The strength of association was estimated using Odds ratio with a corresponding 95% confidence interval. The level of significance was tested at $p<0.05$.

Ethical considerations

Ethical approval to conduct this research was granted by the Ethics and Review committee of Kenyatta University, Kenya.

Results

Magnitude of nontuberculous mycobacteria

From 124 isolates analyzed, 24 (19.3%) were identified as NTM, comprising a total of 6 species, 20 (16.1%) as *Mycobacterium tuberculosis* complex, 56 (45.2%) as other bacteria with high guanine and cytosine content, 21 (16.9%) were negative for any mycobacteria while 3 (2.4%) mycobacteria could not be identified to species level. *Mycobacterium intracellulare* was the most predominant (10/24) (Figure i).

Males were marginally more likely to be infected with NTM compared to females (OR 1.12, 95% CI 0.45-2.81). Identified NTM were distributed across all age groups, with most infected patients being in the age category of 30-39 years (Table i). All 24 NTM species were resistant to rifampicin, isoniazid and ethambutol (Table ii).

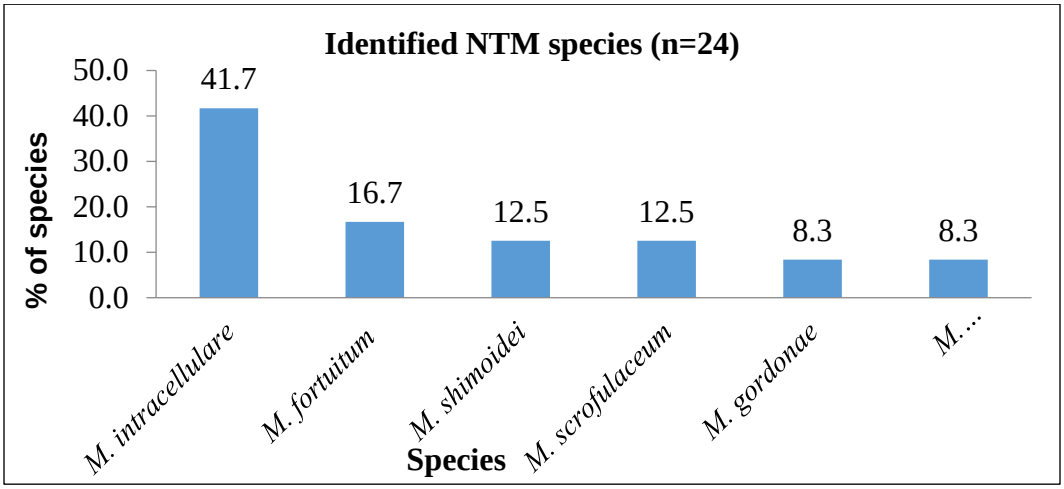

Figure i: Magnitude of Identified NTM Species

Table i: Magnitude of NTM by Demographic Characteristics of Study Participants

	Positive (N=24)		Negative (N=97)		95%CI			
Variables	**N**	**%**	**N**	**%**	**OR**	**Lower**	**Upper**	**P-Value**
Gender								
Male	15	20.5%	58	79.5%	**1.12**	0.45	2.81	0.808
Female	9	18.8%	39	81.3%	Ref			
Age in years								
<20 years	1	10.0%	9	90.0%	0.56	0.05	6.18	0.633
20 - 29 years	5	17.2%	24	82.8%	1.04	0.22	5.01	0.959
30 - 39 years	6	16.7%	30	83.3%	1.00	0.22	4.56	1.000
40 - 49 years	5	26.3%	14	73.7%	1.79	0.36	8.90	0.479
50 - 59 years	4	44.4%	5	55.6%	4.00	0.66	24.37	0.133
>=60 years	3	16.7%	15	83.3%	Ref			

***OR = Odds Ratio; CI =Confidence Interval; P-Value = Probability Value**

Geographical distribution of nontuberculous mycobacteria

The distribution of NTM species varies significantly by region. Among the 8 facilities, NTM were identified in 6 facilities, located in different geographical regions. There was significant variation in the proportions of NTM infections across the facilities, p=0.046, with Wajir having the highest (95%CI: 2.46-54.27, p=0.002) (Table iii). The predominant species, *Mycobacterium intracellulare*, was isolated in five of the eight facilities. *Mycobacterium shimoidei* was only isolated in Wajir and *Mycobacterium mucogenicum* in Kitale (Table iv).

Discussion

The magnitude of NTM

Despite rigorous efforts in sub-Saharan Africa to eliminate TB which have significantly reduced the incidences of *Mycobacterium tuberculosis*, there is a distressing increase in cases of pulmonary NTM infections [17]. Nontuberculous mycobacteria are being recovered in high numbers from people presumed to have PTB [10]. In Kenya, there is scarce information on the burden of pulmonary infections caused by NTM due to the lack of standardized routine surveillance and the use of acid-fast bacilli smear microscopy for the diagnosis of PTB. Our study provides crucial information that will significantly highlight the growing importance of NTM in Kenya.

We found a high magnitude of NTM (19.3%), with a high diversity of 6 species among patients seeking treatment for PTB in various health facilities in Kenya.

Table ii: Susceptibility Patterns of NTM to Three Anti-Tuberculous Drugs

	Resistance		
Species (number)	**Rifampicin**	**Isoniazid**	**Ethambutol**
***M. intracellulare* (10)**	10 (100%)	10 (100%)	10 (100%)
***M. fortuitum* (4)**	4 (100%)	4 (100%)	4 (100%)
***M. shimoidei* (3)**	3 (100%)	3 (100%)	3 (100%)
***M. scrofulaceum* (3)**	3 (100%)	3 (100%)	3 (100%)
***M. gordonae* (2)**	2 (100%)	2 (100%)	2 (100%)
***M. mucogenicum* (2)**	2 (100%)	2 (100%)	2 (100%)
***NTM=Nontuberculous Mycobacteria**			

Table iii: Magnitude of Identified Nontuberculous Mycobacterium Species by Study Site

	Positive (N=24)		Negative (N=97)		95%CI			
Variables	**N**	**%**	**N**	**%**	**OR**	**Lower**	**Upper**	**p-Value**
Study site								
Busia	5	19.2%	21	80.8%	1.96	0.47	8.16	0.353
Kitale	6	20.7%	23	79.3%	2.15	0.55	8.49	0.274
Machakos	1	33.3%	2	66.7%	4.13	0.30	56.39	0.288
Nyahururu	1	12.5%	7	87.5%	1.18	0.11	12.21	0.890
Lamu	0	0.0%	1	100.0%	UD	UD	UD	0.999
Kisii	0	0.0%	5	100.0%	UD	UD	UD	0.999
Wajir	7	58.3%	5	41.7%	11.55	2.46	54.27	**0.002**
Malindi	4	10.8%	33	89.2%	Ref			
***UD=Undefined; OR=Odds Ratio; CI=Confidence Interval; P-Value =Probability Value**								

Elsewhere, a magnitude of 17.4% was identified in Ibadan, Nigeria [2], 15.1% in Iran [20] and 26.2% in Mozambique [21]. Among PTB retreatment patients in Kenya, a high NTM prevalence of 42.4% was identified by Limo and colleagues [17]. Another study identified NTM in a much lower prevalence of 5.9% in China [7] compared to this study. This difference may be due to variations in sample size, with our study having a smaller sample size of 124 compared to that of 24,763 in China.

In our study, males (62.5%) were more likely to be infected with NTM compared to females (37.5%), a similar trend also observed in Nigeria [2] and India [22]. This is however different from a study in Zambia [10], where females were at a higher risk of being infected with NTM as compared to males. In Zambia, the higher prevalence of NTM infections in women was attributed to high female medical seeking behaviour [10]. Similar to findings in Mali [12], those aged 30-39 years in our study were the most affected with pulmonary infections due to NTM and the least affected were aged less than 20 years. In the present study, *Mycobacterium intracellulare* was isolated as the predominant species (41.7%). This was the same case in China 68.33% [23], Japan 44.3% [4] and Nigeria 30.4% [24]. *Mycobacterium fortuitum* was the second most predominant species (16.7%) in our study. Similarly, it was the second predominant species isolated from household members of a camel pastoralist community and camel milk in Samburu county, Kenya [25]. *Mycobacterium fortuitum* is known to cause infections in people with suppressed immunity due to underlying diseases, with such people presenting with symptoms similar to tuberculosis such as a productive cough [26]. This presumably explains its high isolation from people presumed to have PTB in the current study, although the current study did not focus on the presence of underlying diseases in the study population as a possible risk of infection with NTM. *Mycobacterium gordonae* and *Mycobacterium mucogenicum* were the least predominant NTM species (5.3%) in our study. *Mycobacterium gordonae* is commonly encountered in water at very high concentrations of 1,000 colony forming units per litre and its isolation from pulmonary samples may be an indication of contamination [27]. The high magnitude of NTM identified in this study among people presumed to have PTB is an indication that NTM has become a public health problem. This is worrying as identification of species-level of mycobacteria that cause pulmonary infections before treatment initiation is not done in Kenya and there is no standard regimen for treatment of pulmonary infections caused by NTM.

Table IV: Distribution of Nontuberculous Mycobacterial Species by Geographical Site

	M. fortuitum **n=4**	***M. gordonae*** **n=*2***	***M. intracellulare*** **n=10**	***M. mucogenicum*** **n=2**	***M. scrofulaceum*** **n=3**	***M. shimoidei*** **n=3**
Overall	16.7%	8.3%	41.7%	8.3%	12.5%	12.5%
Study site						
Busia(n=5)	0	1	3	0	1	0
Kitale(n=6)	1	0	3	2	0	0
Machakos(n=1)	0	1	0	0	0	0
Nyahururu(n=1)	0	0	1	0	0	0
Wajir(n=7)	1	0	2	0	1	3
Malindi(n=4)	2	0	1	0	1	0
p-value	0.463	0.019	0.560	0.257	0.850	0.139

Drug susceptibility profile

Identification of NTM to species level is important as they have varying drug susceptibility patterns and hence misdiagnosis of pulmonary infections due to NTM may lead to inappropriate patient management [7]. We found all identified NTM species to be resistant to first-line anti-tuberculosis drugs (rifampicin, isoniazid and ethambutol) that are routinely prescribed to patients confirmed to have pulmonary tuberculosis infection in Kenya. Limo *et al.* found almost half (42.4%) of pulmonary tuberculosis retreatment patients in Kenya, who had previously been diagnosed with acid-fast bacilli by direct smear microscopy and put under management with first-line anti-tuberculous drugs were infected with NTM [17]. In China, more than half of *Mycobacterium intracellulare* isolated from patients presumed to have PTB were resistant to rifampicin (88.3%), isoniazid (67.2%) and ethambutol (56.3%). In the same study, nearly all *Mycobacterium fortuitum* isolates were resistant to rifampicin (96.4%), isoniazid (92.9%) and ethambutol (71.4%) [7]. In a recent study conducted in China 27.3% of *Mycobacterium fortuitum* isolates were resistant to rifampicin, 63.9% resistant to isoniazid and 81.8% resistant to ethambutol. In the study, 50% of *Mycobacterium gordonae* isolates were resistant to rifampicin and ethambutol [28]. In Ghana, all isolated *Mycobacterium intracellulare* and *Mycobacterium mucogenicum* were found to be resistant to isoniazid while 88.9% of isolated *Mycobacterium intracellulare* and 66.7% of *Mycobacterium mucogenicum* were resistant to rifampicin [29]. The differences in drug susceptibility patterns in the present study compared to the studies in China may be due to the method and drug concentrations used as we used the resistance ratio method with multiple drug concentrations: rifampicin (8, 16, 32, and 64µg/ml), isoniazid (0.1, 0.2, 1.0µg/ml) and ethambutol (4, 5.6, 8µg/ml) while the first study in China used proportion method with drug concentrations of 40µl/ml, 0.2µl/ml and 2µl/ml for rifampicin, isoniazid and ethambutol respectively. The second study in China used an automated microscopic observation drug susceptibility assay with one concentration of each drug and these were rifampicin 1µg/ml, isoniazid 0.1µl/ml and ethambutol 5µl/ml.

Geographical distribution

The distribution of pulmonary infections due to NTM varies geographically, with the prevalence of particular NTM species also differing by geographic regions [23]. In Kenya however, to the best of our knowledge, the geographical distribution of NTM has never been documented before and hence, our study provides vital information on the distribution of identified NTM.

The highest distribution of NTM in Wajir evident from our study is in line with documented reports on pastoral areas where tuberculosis is considered to be among the most common diseases in both humans and animals [27]. In Kenya, documented articles on NTM are mostly on the prevalence of NTM in selected study populations and not more than one geographical region. The predominant *Mycobacterium intracellulare* and *Mycobacterium fortuitum* were isolated in almost equally distributed in all the study sites. Similar to this current study, they were also isolated in western Kenya [15,16,28]. The extensive environmental distribution of *Mycobacterium intracellulare* [23] may explain its wide distribution in this study. *Mycobacterium shimoidei* was restricted to the northern part of Kenya (Wajir) and has never been isolated in any other study in Kenya.

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