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Molecular Isolation of Pathogenic Non-tuberculous Mycobacteria in Free Ranging Migratory Wildebeests (Connochaetes taurinus) and Cattle of Masai Mara, Kenya

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Abstract The genus Mycobacterium consists of the members of the Mycobacterium (M.) tuberculosis complex (MTC), non-tuberculous mycobacteria (NTM) and M. leprae. MTC and some NTM organisms cause tuberculosis or tuberculosis-like diseases in humans and other animal species respectively. In Masai Mara conservation area in Kenya, a potential interface between wildlife, livestock and humans is existing due to frequent wildlife and livestock movements and interactions. This study proved the presence of various NTM species in free ranging migratory wildebeests wildebeests (Connochaetes taurinus) and cattle (Bos indicus) through culture, isolation, polymerase chain reaction (PCR) and DNA sequencing techniques using 16S rRNA genes followed by hsp65 gene to confirm the results. The study revealed the presence of M. kansasii sub-species VI in 4/60 (6.7%) wildebeests and in 10/89 (11.2%) cattle. One of the wildebeests and 3/89 (3.4%) cattle had M. lentiflavum. M. gordonae was isolated from only one wildebeest while M. intracellulare was detected in 1/89 (1.1%) cattle. No MTC organisms were isolated. The DNA extracts of the isolated mycobacteria were sequence-analysed and published in Genbank with accession numbers assigned. The occurrence of NTM organisms in free ranging migratory wildebeests and cattle may pose a great risk of human infection through consumption of cattle and other livestock or bush-meat products.

Keywords Wildebeests; M. Kansasi; M. lentiflavum; M. intracellulare; hsp65 gene; 16S rRNA gene

Background Mycobacterial organisms are the causative agents of tuberculosis and other tuberculosis-like diseases in humans and animals. Mycobacterial infection has a negative impact on livestock production, wildlife conservation and poses an increased risk to humans who may get infected through consumption of uncooked meat or untreated milk from infected animals (Minja et al., 1998). Vice versa, animals may acquire the infection from tuberculosis-infected humans (Alexander et al., 2002). Consumption of infected prey is considered to be the predominant route by which lions in the Kruger National park became infected with M. bovis (Keet, et. al., 2000b).

Mycobacterial organisms belonging to the Mycobacterium (M.) tuberculosis complex (MTC) are the causative agents of tuberculosis disease in humans and a broad range of mammalian species. Mycobacterium tuberculosis is the major cause of tuberculosis in humans causing mainly lung disease, while all mammals are susceptible to M. bovis infections,
including humans causing mainly extra-pulmonary pathology.

Although the causative agent of tuberculosis in birds is normally *M. avium*, under certain conditions even birds may acquire mammalian tuberculosis. *M. avium* belongs to the numerous groups of the non-tuberculous mycobacteria (NTM) which are of minor pathogenicity for humans and mammals. Besides these, there are many other emerging NTM species which have been reported to cause disease especially in immunocompromised humans and animals alike. Currently there are over 125 mycobacterial species already identified; both pathogenic and non-pathogenic (Shirin et al., 2010).

The Masai Mara ecosystem is characterized by intensive wildlife-livestock-human interactions favourable for disease transmission among different species. The prevalence and distribution of pathogenic and non-pathogenic mycobacterial organisms in wildlife and livestock has not been documented in the ecosystem. This study investigated the presence of both pathogenic and non-pathogenic mycobacteria organisms in wildebeests (*Connochaetes taurinus*) and cattle (*Bos indicus*) in this area. The role of NTM in wildebeests and cattle in Masai Mara need to be investigated if they have any impact on public health.

Masai Mara ecosystem is situated in Narok County on the south-western part of Kenya. The ecosystem comprises the Masai Mara National Reserve (MMNR) surrounded by several adjacent community-owned wildlife conservancies (Figure 1).

MMNR is a government protected area for wildlife conservation which covers an area of approximately 1510 square kilometers and is located on the South-Western part of Kenya along the Kenyan-Tanzanian border between 1°13’ and 1°45’ South and 34°45’ and 35°25’ East. The reserve extends to Serengeti National Park (SNP) in Tanzania where tuberculosis has been reported in lions (Figure 1).

The ecosystem has dense populations of wildlife including large mammals such as African elephants, lions, leopards, African buffaloes, black rhinoceros, wildebeests and several antelope species (Mijele et al., 2013). The area is inhabited mainly by Masai pastoral communities keeping large herds of cattle, sheep and goats.

Figure 1 Map of Masai Mara National Reserve in Narok county and wildebeests sampling sites along the Mara river

There is regular interaction between wildlife, human and livestock within and outside the reserve (Mijele et al., 2013) and frequent cross-border movements of wildlife and livestock between Kenya and Tanzania. MMNR is also known for spectacular annual wildebeests migration between SNP and MMNR in search of adequate pastures (Ogutu et al., 2011). Wildebeests usually drown and die in large numbers while crossing the Mara River during the annual migrations. Frequent animal movements in the area have the potential of enhancing disease transmission among the wildlife-livestock-human populations across the border of Kenya and Tanzania.

1 Results

There were no lesions or pathology related to tuberculosis infection in all the 60 wildebeests and 89 cattle examined. Twenty PCR-positive mycobacterial isolates from 6 wildebeests and 14 cattle were
sequence-analyzed. Sequencing the 16S rRNA gene yielded DNA stretches of 749 to 923 bp in all but one strain where only a length of 311 bp was achieved. Sequencing of the hsp65 gene yielded DNA stretches of 297 to 421 bp. The maximal identity to the data base entries was 99% for the 16S rRNA gene and 99%–100% for the hsp65 gene (Table 1).

### Table 1 Percentage of similarities between mycobacterial species isolated from wildebeests and cattle of Masai Mara and sequences already published in the Genbank

<table>
<thead>
<tr>
<th>No</th>
<th>Animal species</th>
<th>FLI number</th>
<th>Mycobacterial species (16S rRNA gene)</th>
<th>Mycobacterial species (hsp65 gene)</th>
<th>New Genbank Accession numbers</th>
<th>Closest existing accession numbers in Genbank</th>
<th>Percentage similarity</th>
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<td>*W/beest</td>
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<td>M. kansasii/gastri</td>
<td>M. kansasii ssp VI</td>
<td>KF687951</td>
<td>HE575963.1</td>
<td>99%</td>
</tr>
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<td>M. lentiflavum</td>
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<tr>
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<tr>
<td>8</td>
<td>Cattle</td>
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<td>99%</td>
</tr>
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<td>99%</td>
</tr>
<tr>
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<td>Cattle</td>
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<td>M. kansasii/gastri</td>
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<td>100%</td>
</tr>
<tr>
<td>18</td>
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<tr>
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<td>99%</td>
</tr>
</tbody>
</table>

### 1.1 Wildebeests results

From the 60 wildebeest lymph node samples examined from the Mara River, members of the MTC or M. avium were not isolated. Six out of 60 (10%) wildebeests were found positive for non-tuberculous mycobacteria. Sequences for 16S rRNA gene revealed *Mycobacterium kansasii/gastri* in retropharyngeal lymph nodes of three (5%) wildebeests and in mediastinal lymph node of one wildebeest (1.7%). One wildebeest (1.6%) had *M. lentiflavum* in mesenteric lymph node and one (1.6%) had *M. gordonae* in mediastinal lymph node (Table 2).

Further sequencing of the same samples using hsp65 gene confirmed that the four samples which could not be differentiated using 16S rRNA, were all *M. kansasii* sub-species VI. The results for the other two wildebeests samples remained the same showing *M. lentiflavum* and *M. gordonae* respectively (Table 2).

### 1.2 Cattle results

A variety of non-tuberculous mycobacteria species were isolated from 14 out of 89 (15.7%) cattle lymph node samples examined at the Ewaso Nyiro slaughterhouse. Sequencing of the 16S rRNA gene revealed *Mycobacterium kansasii/gastri* in ten (11.2%) cattle. *Mycobacterium lentiflavum* was detected in three (3.4%) cattle and *M. intracellulare* in one cattle (1.1%) as indicated in (Table 2). Members of the MTC or M. avium were not isolated.
Table 2: Mycobacterial species isolated from lymph node tissue samples of wildebeests and cattle of Masai Mara

<table>
<thead>
<tr>
<th>No.</th>
<th>Sample number</th>
<th>Animal species</th>
<th>FLI number</th>
<th>Lymph node</th>
<th>Mycobacterial species (16S rRNA gene)</th>
<th>Mycobacterial species (hsp65 gene)</th>
<th>Accession numbers</th>
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<tr>
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<td>WB46MES</td>
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<tr>
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</tr>
<tr>
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<td>KF687952</td>
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<tr>
<td>5</td>
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<td>Mediastinal</td>
<td>M. gordonae</td>
<td>M. gordonae</td>
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</tr>
<tr>
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<td>W/beest</td>
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<td>M. kansasii/gastri</td>
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</table>

Sequencing of the hsp65 gene confirmed that all the M. kansasii/gastri in 10 cattle were M. kansasii sub-species VI. The results for the other four cattle samples remained the same showing M. lentiflavum and M. intracellulare, respectively (Table 2). There was a mixed infection in one cattle Talek01 in which M. lentiflavum was isolated from the mediastinum lymph node and M. kansasii sub-species VI isolated from the retropharyngeal lymph node.

2 Discussion

The presence of NTM organisms in free-ranging wildebeests and cattle generates a risk for human infection and may be responsible for the presence of human disease compatible with tuberculosis in Masai Mara ecosystem of Kenya.

NTM organisms are ubiquitous and are usually recovered from the environment. They can cause disease to humans or animals through respiratory, cutaneous, parenteral and gastrointestinal exposure. The most pathogenic NTM species, M. avium causing tuberculosis in birds and tuberculosis-like disease in humans, especially in immunocompromised persons (e.g. HIV, elderly people) has not been detected. However, other NTM species which have been isolated in the course of this study are also known to cause disease in humans and animals alike.

Mycobacterium kansasii is a slow-growing acid-fast bacillus (AFB) and belongs to the NTM group of mycobacteria (Sang et al., 2010) also known as environmental mycobacteria or even called “atypical mycobacteria”. Local water supplies are considered as the major reservoir for M. kansasii, evidence of person-to-person transmission has not been reported. The most common presentation of M. kansasii infection in humans is a chronic bronchopulmonary disease, which manifests typically in adult patients with chronic obstructive pulmonary disease or cystic fibrosis (Sang et al., 2010). In addition, M. kansasii...
can cause skeletal infections, skin and soft tissue infection, cervical or other lymphadenitis, and disseminated infection (Brown-Elliot et al., 2004). *M. kansasii* is pathogenic for humans and may cause severe tuberculosis-like disease (Sang et al., 2010). *M. kansasii* isolation is more common in HIV-positive persons, patients with hematologic malignancy, or patients receiving long-term steroid regimens (Wallace et al., 1997). Infection with *M. kansasii* requires the same treatment as *M. tuberculosis* infection (Evans et al., 1996).

In wildlife, cattle and other livestock *M. kansasii* is not pathogenic but it may cause immune reactions and thus interfere with tuberculosis skin test or gamma interferon release assay tests. There is a considerable risk of transmission from cattle or wildebeests to humans through consumption of meat or even contact. This is the first time *M. kansasii* has been isolated from wildebeests of Masai Mara ecosystem.

Even though *M. kansasii* infection has not been reported in Kenya or elsewhere in Africa, it is the second most frequently recognized NTM pathogen and second most frequent cause of disseminated NTM disease, after *M. avium* complex (MAC), in the United States and Japan (Tsukamura et al., 1988; Lillo et al., 1990). In southeast England, *M. kansasii* is more common than *M. avium* Complex (Yattes et al., 1997).

In South Korea, *M. kansasii* is the fourth most commonly isolated NTM pathogen, after MAC, *M. abscessus-chelonae* complex, and *M. fortuitum*, but its incidence has increased, especially in highly industrialized areas (Yim et al., 2005). Most patients with *M. kansasii* infection show clinical and radiologic evidence of infection regardless of HIV status.

*Mycobacterium lentiflavum* was recently described as non-tuberculous mycobacterial species (Springer et al., 1996). It is described as a causative agent of cervical lymphadenitis in immuno-compromised human patients and children. *M. lentiflavum* is resistant to most first line antituberculous drugs. *M. lentiflavum* is a slow growing acid-fast bacillus (AFB) that has biochemical characteristics identical to those of organisms belonging to the *Mycobacterium avium* complex (MAC) and mycolic acid and fatty acid chromatography patterns very similar to those of *Mycobacterium simiae*, so genetic analysis is necessary for conclusive identification (Springer et al., 1996). This organism has been isolated from clinical samples in Italy, Switzerland, Germany, France and Spain (Springer et al., 1996; Tortoli et al., 1997; Niobe et al., 2001; Ibanez et al., 2002) and from sputum samples in Brazil (da Silva Rocha et al., 1999) and Italy (Molteni et al., 2005). Recently, cases of human disease have been reported, including chronic pulmonary disease (Molteni et al., 2005), cervical lymphadenitis (Cabria et al., 2002), liver abscess (Tortoli et al., 2002) and fatal disseminated infection (Ibanez et al., 2002). Our findings are the first evidence of *M. lentiflavum* infection reported in Kenya. The main reservoir in the environment has not been firmly established, but organisms with *M. lentiflavum*-like 16S rRNA gene sequences were detected in soil samples from the UK and from France (Mendum et al., 2000) and the species seem to be frequently present in drinking water distribution systems in Finland (Torvinen et al., 2004).

*Mycobacterium gordonae* is the least pathogenic and its isolation is typically regarded as a contaminant (Lessnau et al., 1993). The organism is ubiquitous and it is most commonly isolated from soil and water (Caterina et al., 2009). Clinically significant disease caused by *M. gordonae* has been reported (Weinberger et al., 1992). Its presence in wildebeest samples could have been due to environmental contamination of the carcasses.

*Mycobacterium intracellulare* is closely related to *Mycobacterium avium* and similarly causes pulmonary disease in humans. Environmental sources, especially water are the main reservoir for most human infections. Wildebeests and cattle could have acquired *M. intracellulare* from the environment.

The high similarities between mycobacterial organisms in wildebeests and cattle is an indication that there could be cross-transmission of mycobacterial organisms between cattle and wildebeests and possibly to humans or other animals thereby putting human beings at a higher risk of
infection. There is a possibility that the isolated mycobacteria exists in the environment from where wildebeests and cattle acquired infection from water or pasture.

3 Methods

3.1 Wildebeests sampling

Postmortem examination was done on 60 wildebeests (Conchaeta taurinus) that drowned in three sites along the Mara River in August 2010. The three sites included Mara bridge (30 wildebeests), Serena (20 wildebeests) and Little Governors (10 wildebeests) (Figure 1). The sampling of wildebeests was opportunistic and any wildebeest carcass irrespective of age or sex was retrieved from the river and a detailed postmortem examination conducted to detect granulomas or any other lesions compatible with tuberculosis. Lymph node tissue samples were then collected from retropharyngeal, mediastinal and mesenteric lymph nodes. The samples were labeled and immediately preserved in liquid nitrogen before being transferred to 80°C freezer at the Kenya Wildlife Service laboratory in Nairobi.

3.2 Cattle sampling

Detailed postmortem examination was performed on 89 cattle at Ewaso-Ngiro slaughterhouse within the Masai Mara ecosystem. The selection of cattle for post-mortem was simple random and animals above 2 years of age were targeted. Each carcass was examined for existence of any lesions or pathology related to tuberculosis infection. Tissue samples were collected from retropharyngeal, mediastinal and mesenteric lymph nodes irrespective of whether the animal had lesions or not. The samples were immediately preserved in liquid nitrogen and later transferred to a -80°C freezer at the Kenya Wildlife Service laboratory in Nairobi. All the samples collection was done at the same time in August, 2010.

3.3 Laboratory analysis

Frozen lymph node tissue samples from wildebeest and cattle were transferred to the tuberculosis laboratory at Sokoine University of Agriculture (SUA), Tanzania, where they were processed according to standard methods for isolation of mycobacteria from tissue (Hosek et al., 2006) and cultured on Loewenstein-Jensen (LJ) media for 8 weeks. Colonies that grew on LJ media were stained according to Ziehl-Neelsen and examined for the presence of acid fast bacilli (AFB) under light microscope. From positive cultures, material was taken for polymerase chain reaction (PCR) analysis at SUA and DNA sequence analysis was performed at Friedrich-Loeffler-Institute in Jena, Germany.

Colony material was suspended in sterile water, boiled for ten minutes to inactivate the mycobacteria and centrifuged. The supernatant was taken as source of DNA and introduced into mycogenous standard PCR (Kirschner and Boettger 1998). The PCR product was run on an agarose gel with Ethidium Bromide and visualized under UV light. The PCR product band was cut out of the gel and DNA was extracted from agarose gel using QIAquick Gel Extraction kit (Qiagen, Hilden, Germany). The resultant DNA together with sequencing primers was submitted to GATC Biotech Company (Konstanz, Germany) for sequencing.

3.4 DNA-Sequencing

Sequencing of the 16S rRNA gene to identify mycobacterial species was performed according to Kirschner et al. (1998) using primer 271 as sequencing primer. The primers were obtained from Jena Bioscience, Jena, Germany. The sequence data were analyzed at FLI, Jena, Germany, using NCBI Blast software to identify the mycobacteria with the highest match to the sequences.

Sequencing of hsp65 gene was performed to differentiate M. kansasii and M. gastri using the primers TB11 (Devallois et al., 1997). Subspecies identity of M. kansasii was determined according to Richter et al., 1999. All the sequence data was published in Gen-bank and provided with accession numbers (Table 2). BioEdit software was used to assess the similarities between the new sequences and the existing sequences in the database.

Authors Contributions

DM IL FG RK IM MT MO conceived and designed the experiments for the paper. DM FG IL IM MT performed the experiments. DM IM RK IL analyzed
the data. DM RK IL IM contributed reagents, materials and analysis tools. DM IL FG RK IM MT MO wrote the paper. All authors read and approved the final manuscript.

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