Isolation and molecular characterization of *Mycobacterium tuberculosis* from Humans and Cattle in Namwala district, Zambia

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Abstract

Introduction

Zambia, with a human population of 13 million people, is ranked among the world’s top ten countries with a high burden of human tuberculosis (TB). Although *M. tuberculosis*, the causative agent of TB in humans, is considered primarily a human pathogen it has been reported in a wide range of domestic and wild animals, often living in close prolonged contact with humans.

Methods

Human and cattle samples for isolation of mycobacteria belonging to the *Mycobacterium tuberculosis* complex were collected between April 2011 and July 2012. In humans, sputum samples in which acid fast bacteria were detected in smears were collected from patients at three health facilities in Namwala district, Zambia. Samples from cattle presenting gross lesions compatible with bovine tuberculosis were collected at a local abattoir in the same district. Isolated mycobacteria were identified using classical methods and genotyped using deletion analysis, spoligotyping and mycobacterial interspersed repetitive units-variable number tandem repeats (MIRU-VNTR) with 15 loci to determine the diversity and relatedness of the circulating strains.

Results

A total of 33 isolates of *M. tuberculosis* were detected, 30 from humans and 3 from cattle. Spoligotyping gave a discriminatory power of 0.945. LAM was the largest lineage detected (39.4%). Other lineages were T2 (27.3%), CAS (15%), and X (3.0%). Fifteen percent of the isolates did not correspond to previously reported lineages. MIRU-VNTR typing was highly discriminatory (*h* = 0.98) among the 33 isolates tested. However, the combined spoligotyping and MIRU-VNTR increased the discriminatory power by 0.011, compared with MIRU-VNTR alone.
One cattle isolate shared spoligotype and MIRU-VNTR pattern with two human isolates and one other cattle isolate with another human isolate.

**Conclusion**

This study has for the first time documented the isolation of *M. tuberculosis* from cattle in Zambia and provides molecular evidence of an epidemiological link between *M. tuberculosis* isolates from humans and cattle in Namwala district. It can be hypothesized that animals most likely were infected from human sources given that humans are the natural hosts of *M. tuberculosis* and the high prevalence of tuberculosis in humans in Namwala. A possible spill back of *M. tuberculosis* to humans cannot be excluded and therefore further studies documenting to what extent *M. tuberculosis* is shed in cattle milk are needed.

**Key words:** Mycobacterium tuberculosis, human, cattle, Spoligotyping, MIRU-VNTR, Namwala, Zambia.
Introduction

Zambia, with a human population of 13 million people, is ranked among the world’s top ten countries with a high burden of human tuberculosis (TB) caused by Mycobacterium tuberculosis. The World Health Organization (WHO) estimates the incidence of human TB in Zambia to be 707/100 000 [1,2]. Previous reports indicated that Namwala district has the incidence of human TB to be representative for the situation in all of Zambia (appr. 7 per 1000 individuals). The prevalence of bovine tuberculosis in cattle, caused by Mycobacterium bovis, is estimated to be 17% based on abattoir observations [3,4]. Although M. tuberculosis, the causative agent of TB in humans, is considered primarily a human pathogen it has been reported in a wide range of domestic and wild animals, often living in close prolonged contact with humans [5,6]. M. tuberculosis has previously been isolated from cattle in pastoral regions in Ethiopia, Nigeria and Slovenia [6-8], and in a recent study by Lyashchenko et al (2012), it was isolated from a horse [9]. A study done from India, Srivastava et al (2008) isolated M. tuberculosis from milk samples which could lead to spill back of the pathogen in humans [10]. Due to the increased incidence of human TB worldwide, new strategies to fight human TB are urgently needed. Understanding the epidemiology and identifying the routes of transmission of strains of M. tuberculosis, including from the animal reservoir, are important in the effective control of human TB [11].

The lack of comprehensive molecular epidemiological data in Zambia has limited the understanding of human TB disease dynamics. This underscores the need for isolation, identification and genotyping of mycobacterial isolates circulating in Zambia. Use of molecular tools can contribute to determine the existence, extent, diversity and relationship between human and bovine TB and identify the possible sources of infections [12]. M. tuberculosis has previously never been detected in animals in Zambia, and thus molecular studies comparing isolates of M. tuberculosis in both humans and cattle have never been performed.
This aim of the study was to isolate and describe the molecular diversity and similarity of \textit{M. tuberculosis} from humans and cattle in Namwala District, Zambia.

\textbf{Materials and methods}

\textbf{Study site}

Namwala district is situated in the Southern Province of Zambia. About a quarter of its traditional land is covered by flood plains. It covers an estimated total area of about 10,000 square kilometers and lies between latitudes 15 and 17° S of the equator and longitude 25 and 27° E. It is located in the Kafue Basin area, which is one of the few lacustrine wetlands supporting close to 300,000 cattle [13]. According to the 2010 census of population and housing data, the area has a population of about 102,000 persons [1]. Agriculture is the main economic activity of this rural district and the majority of the people keep livestock. However, humans and animals share the same micro environments and water points, especially during the dry season and in times of drought [14]. This demographic set-up and husbandry practice may increase the risk of transmission of mycobacteria belonging to the \textit{Mycobacterium tuberculosis} complex (MTC, which includes, among others, \textit{M. tuberculosis} and \textit{M. bovis}) between humans and cattle.

Three health facilities were selected for sampling of human sputum namely; Namwala District Hospital, Maala Rural Health center and Chitoongo Rural Health Center.

The district has two commercial abattoirs (Zambeef and Starbeef) from which all cattle meant for public consumption are inspected and slaughtered by well trained and experienced personnel. The Zambeef abattoir was selected for sampling of cattle carcasses.
**Human samples**

During the period April 2011 to July 2012, a total of 100 sputum samples were collected from suspected pulmonary tuberculosis patients in the study area.

After routine microscopy with the Ziehl-Neelsen (ZN) staining technique, all ZN positive smear sputum samples (i.e., samples containing acid fast bacilli) were stored in cetylpyridinium chloride transport medium (Difco, Detroit, MI) and kept at ambient temperature at the District Hospital until they were taken to the Chest Diseases Laboratory (CDL) in Lusaka for further processing.

At CDL, the samples were decontaminated as described by Vestal *et al* (1966) [15] and cultured on duplicate Lowenstein-Jensen media, one containing glycerol and the other pyruvate (BD BBL; Franklin lakes NJ, USA). Culture tubes were incubated at 37°C and read weekly for growth for at least eight weeks. Successfully grown cultures were transported to the University of Zambia, School of Veterinary Medicine for storage and onward transportation of isolates to the Norwegian Veterinary Institute, Oslo, Norway for further analysis. The isolates were further subcultured on Stonebrink and Middlebrook 7H10 (BD Diagnostic, MD) slants at the Norwegian Veterinary Institute.

**Cattle samples**

Between April 2011 and July 2012 slaughtered animals at the local abattoir were examined for gross lesions according to the standard post mortem examination procedures [16]. Organs and tissues with lesions compatible with bovine tuberculosis were collected. Samples were placed in sterile self-zipping histopathological bags and placed in a cooler box with ice packs before transporting them to the University of Zambia, School of Veterinary Medicine, where they were stored at -20°C for 48 hours.
All samples (N=67) were decontaminated in the Biohazard safety cabinet in a Biohazard safety level II laboratory according to standard procedures [17]. The sterile homogenate from the samples were inoculated on Stonebrink and Middlebrook 7H10 slants and incubated aerobically at 37°C for eight weeks with weekly observation. All cultures were confirmed as mycobacteria based on ZN staining. Cultures that stained acid fast were sent to the Norwegian Veterinary Institute, where they further subcultured.

DNA extraction and purification

Mycobacterial colonies on solid media were harvested as a loop full of colony material and suspended in 200 µl of molecular grade water. It was heated at 95°C for 20 m. The crude lysates were purified using the NucliSens easyMAG automated machine (Biomerieux, Netherlands), according to the manufacturer’s protocol, and DNA was stored at -20 °C for further analysis.

IS6110 analysis

All isolates were examined by IS6110 real time PCR as described previously [18]. IS6110 is considered specific for the M. tuberculosis complex (MTC) and the PCR was used as a screening test to identify isolates belonging to the MTC [19].

Deletion analysis

Standard PCR amplification of the genomic regions of difference was performed according to published protocols [20] at the Norwegian Veterinary Institute. The sets of primers targeting the RD1, RD4, RD9 and RD12 regions were employed [20].
Spoligotyping and MIRU-VNTR were performed at Genoscreen, Lille in France. Spoligotyping was conducted using the primers DRa and DRb, which enable amplification of the whole DR region [21]. Chromosomal DNA of *M. tuberculosis* strain H37Rv and *M. bovis* BCG were used as positive controls and water as a negative control. For MIRU-VNTR, the standardized panel of 15-loci were amplified using triplex PCR with fluorescent primers, and the DNA fragments were separated by capillary electrophoresis [22]. Determining the size of the PCR product and assigning the suitable alleles was done using the customized module with GeneMapper® Software (Applied Biosystems, Foster City, CA). The results were reported as the number of repeats per loci in Excel sheet.

### Data assembly and analysis

Molecular results were entered and validated in Excel 2007®. These were then copied into MIRU-VNTR plus (http://www.miru-vntrplus.org/MIRU/index.faces) and the spolDB4.0 database (http://www.pasteurguadeloupe.fr:8081/SITVITDemo) to establish the lineage, sub-lineage and spoligotype international types (SIT) designations and to generate dendograms. Calculation of discriminatory power was done as described by Hunter and Gaston [23]. The allelic diversity (h) for each locus was calculated using described methods [24].

### Ethical approval

The study was approved by ERES converge IRB ethical review Board, Lusaka, Zambia (Ref: 2012-Mar-001) and permission to perform the study in the area was obtained from the District Medical Officer (DMO) and the Provincial Veterinary officer (PVO) through the District Veterinary officer (DVO).
Results

Identification

Out of the sputum samples obtained from 100 humans, acid fast bacilli were detected in direct smear from 65 patients. Mycobacteria were detected by culture in 55 of them. Based on IS6110 real time PCR, 36 isolates were found to belong to the MTC, 34 were further identified as *M. tuberculosis* and two as *M. bovis* by deletion analysis.

And out of the tissue samples obtained from 67 cattle carcasses, acid fast bacilli were detected in direct smear in 55 animals. Mycobacteria were detected by culture in 47 isolates. Based on IS6110 real time PCR, 28 isolates were found to belong to the MTC. Deletion analysis identified 25 of them as *M. bovis* and three as *M. tuberculosis*.

Characterization of *M. tuberculosis* by MIRU-VNTR and spoligotyping

Four human isolates out of 37 showed double alleles at two to five MIRU–VNTR loci and were removed from the analysis. Spoligotyping of the remaining 33 *M. tuberculosis* isolates based on the Spol DB4 (Figure 1) revealed 15 different spoligotypes with a discriminatory power of 0.945. The largest lineage belonged to the LAM (39.4%). Other families included the CAS (15%), T2 (27.3%) and X (3.0%) while 15% of the isolates could not be identified with any lineage in the database. The spoligotype international types (SIT) were generated (Figure 1). Based on the standard 15-locus MIRU-VNTR (Figure 1), the isolates produced 24 different patterns, clustering in eight clusters and 16 singletons with a clustering rate of 0.27. The highest allelic diversity was observed at loci 424 and 4032 (0.70). The lowest allelic diversity was at locus 577 (0.40) while locus 580 gave insignificant allelic diversity in all the isolates (Table 1). The discriminatory
power was 0.98 for MIRU-VNTR alone, while spoligotyping and MIRU-VNTR combined gave a discriminatory power of 0.9811.

**Discussion**

This study reports the analysis of 33 *M. tuberculosis* isolates obtained from human subjects (n=30) and cattle (n=3) in Namwala district, Zambia. This is the first study documenting the isolation and molecular characterization of *M. tuberculosis* from humans in this area, which has a documented incidence of all forms of human TB of 7 per 1000 [3]. Spoligotyping discriminated the *M. tuberculosis* isolates into the LAM, T2, CAS and X lineages. The study showed that the majority of the strains circulating in Namwala district belong to the LAM lineage, followed by the T2 and then CAS lineages. These results corroborate well with findings reported in study conducted in Ndola district, an urban district in the Copperbelt province of Zambia [25]. These genotypes appear to be fairly distributed in the southern African region as they have also been isolated in Zimbabwe, South Africa, Malawi and Zambia [26-28]. They have also been detected elsewhere in Africa, as they have been isolated from Tanzania and Benin [27,29,30]. The other strains circulating in the study area belonged to the X lineage that has been isolated in Tanzania [29]. The other strains circulating in Namwala district are orphan strains as they could not be aligned to any lineage in the database.

The discriminatory power based on MIRU-VNTR was 0.98, while the discriminatory power of the combined methods (spoligotyping and MIRU-VNTR) was 0.9811. This compares well with that obtained from a study done in Ndola, an urban district in the Copperbelt province of Zambia [25]. However, our study showed that the combined discrimination power only was slightly increased compared to MIRU-VNTR alone. Sufficient discrimination can thus be achieved by using MIRU-VNTR alone. Therefore, the use of MIRU-VNTR for typing of *M. tuberculosis* can
be recommended, especially in resource strained studies. High allelic diversity was attained at loci 4032/424, this corroborates with what was found elsewhere [22,31]. However, locus 577 gave low allelic diversity while locus 580 did not provide any discrimination among isolates. These two last loci are thus not suitable for use in Namwala. Four human isolates showed double alleles at two to five MIRU-VNTR loci and were hence removed from the total analysis. Such double alleles might however indicate mixed infections, which has been documented earlier in high prevalence settings [25].

Results reported herein show a high degree of heterogeneity in the circulating strains in the study area. The central location of Zambia, which allows movement of people from the whole region, defines the observed circulating strains which have also been detected in the neighboring countries.

In a study from Ibadan, Nigeria *M. tuberculosis* was isolated and genotyped from humans and cattle living in the same area. The isolates were molecularly dissimilar although possible transmission was suggested [32]. In the present study it has been shown that there is molecular relatedness between *M. tuberculosis* strains isolated from cattle and humans living in the same area. Identical spoligotype and MIRU-VNTR pattern were observed between one human isolate and two cattle isolates. Although we were not able to identify the specific source of *M. tuberculosis* in cattle, the molecular evidence demonstrated that *M. tuberculosis* infection in cattle had an epidemiological link with human tuberculosis in Namwala district. Because the *M. tuberculosis* isolates shared the same or very closely related genotypes, it would be likely that infection in cattle originated from some human patients. However, since this paper was a cross sectional study we could not determine whether cattle to cattle *M. tuberculosis* transmission occurred. Our findings are in line with what was observed in a study done in Croatia [33]. One
isolate from cattle, had a similar spoligotype but a different MIRU-VNTR pattern than two human isolate. The difference was observed at locus 2163b (QUB11).

From the veterinary public health perspective, the isolation of *M. tuberculosis* from cattle could also lead to the spill back of the infection from cattle to human, as observed in a study conducted in India in which *M. tuberculosis* was isolated from cattle milk [10]. Humans could therefore, be infected with *M. tuberculosis* if they consumed unpasteurized milk from infected cattle.

In conclusion, the isolation of *M. tuberculosis* from cattle demonstrates its potential to spill back to humans, which suggests that veterinary public health measures to control human TB, should also take into account the bovine reservoir. This study also showed that MIRU-VNTR based on 15-locus is a useful tool for the epidemiological investigation of *M. tuberculosis* in humans and cattle. However, the isolation and characterization of *M. tuberculosis*, besides *M. bovis* from milk sample is recommended in order to assess the epidemiological importance of cattle as a source of both *M. bovis* and *M. tuberculosis* for humans.

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**Authors’ contribution**

SM, designed the study, carried out sample collection, performed laboratory work, data analysis and drafted the manuscript. JM participated in the design, supervised field and laboratory work
and writing of the manuscript. MM participated in sample collection and drafting of the manuscript, GM participated in the laboratory work and writing of the manuscript, AM helped in data analysis and interpretation of results, ICS helped in study design, interpretation of results and writing the manuscript, BD, JG and TBJ conceived the study, mobilized resources, overall supervision, data interpretation and revised the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Table 1: Showing the allelic diversities of the standard 15-loci used

<table>
<thead>
<tr>
<th>Loci</th>
<th>alias</th>
<th>Allelic diversity</th>
</tr>
</thead>
<tbody>
<tr>
<td>424</td>
<td>Mtub04</td>
<td>0.70</td>
</tr>
<tr>
<td>577</td>
<td>ETR C</td>
<td>0.46</td>
</tr>
<tr>
<td>580</td>
<td>MIRU 4</td>
<td>0.00</td>
</tr>
<tr>
<td>802</td>
<td>MIRU 40</td>
<td>0.55</td>
</tr>
<tr>
<td>960</td>
<td>MIRU 10</td>
<td>0.65</td>
</tr>
<tr>
<td>1644</td>
<td>MIRU 16</td>
<td>0.64</td>
</tr>
<tr>
<td>1955</td>
<td>Mtub21</td>
<td>0.52</td>
</tr>
<tr>
<td>2163b</td>
<td>QUB-11b</td>
<td>0.69</td>
</tr>
<tr>
<td>2165</td>
<td>ETR A</td>
<td>0.47</td>
</tr>
<tr>
<td>2401</td>
<td>Mtub30</td>
<td>0.64</td>
</tr>
<tr>
<td>2996</td>
<td>MIRU 26</td>
<td>0.50</td>
</tr>
<tr>
<td>3192</td>
<td>MIRU 31</td>
<td>0.56</td>
</tr>
<tr>
<td>3690</td>
<td>Mtub39</td>
<td>0.54</td>
</tr>
<tr>
<td>4052</td>
<td>QUB-26</td>
<td>0.70</td>
</tr>
<tr>
<td>4156</td>
<td>QUB-4156</td>
<td>0.51</td>
</tr>
</tbody>
</table>
Figure 1: Spoligotyping and MIRU-VNTR clustering of representative *M. tuberculosis* isolates from Namwala. The dendogram generated using the dice coefficient and the unweighted pair group method with arithmetic averages (UPGMA) using the MIRU-VNTRplus program [34].