The Burden of Mycobacterial Disease in Ethiopian Cattle: Implications for Public Health

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Abstract

Background: Bovine tuberculosis (bTB), caused by Mycobacterium bovis, is a debilitating disease of cattle. Ethiopia has one of the largest cattle populations in the world, with an economy highly dependent on its livestock. Furthermore, Ethiopia has one of the highest incidence rates of human extrapulmonary TB in the world, a clinical presentation that is often associated with transmission of M. bovis from cattle to humans.

Methodology/Principal Findings: Here we present a comprehensive investigation of the prevalence of bTB in Ethiopia based on cases identified at slaughterhouses. Out of approximately 32,800 inspected cattle, ~4.7% showed suspect tuberculous lesions. Culture of suspect lesions yielded acid-fast bacilli in ~11% of cases, with M. bovis accounting for 58 of 171 acid-fast cultures, while 53 isolates were non-tuberculous mycobacteria. Strikingly, M. tuberculosis was isolated from eight cattle, an unusual finding that suggests human to animal transmission.

Conclusions/Significance: Our analysis has revealed that bTB is widely spread throughout Ethiopia, albeit at a low prevalence, and provides underpinning evidence for public health policy formulation.

Introduction

Bovine tuberculosis (bTB) is an infectious disease of cattle caused by Mycobacterium bovis and characterized by the formation of granulomatous lesions (tubercles) classically seen in the lungs and draining lymph nodes. Infection with M. bovis can be transmitted from cattle to humans, mainly through the consumption of contaminated milk and meat products; because of the route of infection, disease often manifests itself as extrapulmonary TB. Indeed, Kidane and colleagues concluded that among 35 PCR-positive cases of tuberculous lymphadenitis from southern Ethiopia, 29 (82.9%) were caused by M. tuberculosis and six (17.1%) were caused by M. bovis [1]. It is also noteworthy that 36% of incident TB cases in Ethiopia are extrapulmonary [2]. This is exceptionally high, considering that ~15% of incident TB is extrapulmonary in most high burden countries [2].

Ethiopia has ~40 million cattle, the largest cattle population in Africa and the seventh in size in the world (Ethiopian Ministry of Agriculture and Rural Development; http://www.moard.gov.et/statistical.htm). Approximately 80% of the labour-force works in agriculture, with this sector accounting for 47% of GDP and with an export value of agricultural goods of about USD $406 million in the year 2000 [3]. The livestock sector is of national importance and the Ethiopian government has set goals to improve productivity in this sector, such as intensification and the importation of “exotic” high performance cattle breeds such as Holstein.

Previous studies on bTB in Ethiopia have shown that prevalence varies depending on husbandry methods, with rural settings showing a lower prevalence compared to intensive dairy farms [4]. However, there have been no studies to date that have addressed the total burden of mycobacterial infection in cattle, nor do we know the molecular types of M. bovis that are circulating in Ethiopia. Furthermore, given the large cattle population and endemic bTB problem it is possible that some of the incidence of human extrapulmonary TB is caused by infection with M. bovis. While this latter connection has yet to be formally shown, knowledge of mycobacterial strains circulating in Ethiopian cattle is essential baseline data to inform public health policy.

Here we present an extensive analysis of the prevalence study of mycobacterial infection from cattle slaughtered at abattoirs across Ethiopia.
different eco-epidemiological settings of Ethiopia. Suspect tuberculous lesions were processed for culture, with acid-fast isolates typed using molecular methods to provide definitive strain characterisation. The spatial distribution of strains and molecular types could then be mapped across the eco-epidemiological settings. We show that while infection with \( M. \text{bovis} \) is a significant cause of tuberculous lesions in cattle, other acid-fast bacilli (AFB), notably \( M. \text{tuberculosis} \) and non-tuberculous mycobacteria (NTM), are also considerable causes of infection and disease.

**Materials and Methods**

**Study sites**

The investigated cattle specimens in this study were collected from abattoirs located in the rural areas of Gonder (abbrev. Go), Woldiya (Wo), Gimbi (Gi), Butajira (Bu), and Jinka (Ji) in Ethiopia (Figure 1). Each geographical area represents a different eco-epidemiological setting, comprising subsistence farming, agro-pastoral, and pastoral communities. People living in agro-pastoral settings are primarily dependent on cattle for farming their land, while pastoral communities are directly dependent on cattle to provide meat and milk products. To complement this study with samples from an urban setting, specimens from lesioned cattle were also collected at the “Enterprise” abattoir in the capital Addis Ababa (AA).

Identifying a precise geographical origin for each investigated animal was not possible. However, there were no nomads in our study areas, suggesting that any cattle movement was due to local, or possibly national, trading. The five rural abattoirs (Gonder,
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Woldiya, Gimbi, Butajira, and Jinka) documented the markets from where slaughtered animals were purchased by local butchers. Based on this information, and the assumption that no significant cattle movement over large distances was taking place, we determined the approximate area that each abattoir covers (Figure 1). The situation in Addis Ababa is different; the high demand for meat products in the capital necessitates larger supplies. Therefore, merchants buy cattle from a much larger geographical area and transport them to Addis Ababa for slaughter. The area covered by the Addis Ababa abattoir is therefore much greater (Figure 1).

Sample collection, processing, and culturing

The study obtained ethical clearance from institutional (Armauer Hansen Research Institute (AHRI), All Africa Leprosy, Tuberculosis and Rehabilitation Training Centre (ALERT), Ethiopia; VLA, UK) and Ethiopian national ethical review committees. Trained staff at the six abattoirs performed ante-mortem examination (including sex, breed, and body condition etc.) of all or randomly selected cattle that were entering the abattoirs. All ante-mortem examinations were followed up by post-mortem inspections to look for suspect tuberculosis lesions in lungs and a range of lymph nodes (hepatic, mesenteric, bronchial, mediastinal, mandibular, and mediastrophygal LNs). A description of the lesions (purulent, caseous, or calcified) was recorded. All sampled specimens from respective carcasses were pooled together and kept in phosphate buffer saline (pH 7.2) at 4°C in universal containers and transported on ice to the AHRI laboratory in Addis Ababa within one week. Specimens were processed according to standard methods [5]. In brief, the tissue samples were dissected and manually homogenised using a pestle and mortar, followed by decontamination by shaking in an equal volume of 4% NaOH for 15 min and concentrated by centrifugation at 3,000 g for 15 min. The sediment was neutralized with 2 N HCl, using phenol red as an indicator, and then used to inoculate three different media slants: Lowenstein–Jensen (LJ), media with and without pyruvate, and Middlebrook 7H11 medium supplemented as previously described [6]. The slants were incubated at 37°C for six weeks; slants were examined on a weekly basis for the presence of mycobacterial colonies. Cultures were considered negative if no visible growth was detected after six weeks incubation. Microscopic examination of cultures using the Ziehl–Neelsen staining method was performed to select AFB positive isolates. Heat-killed cells of each isolate were prepared by mixing ~2 loopfuls of cells (~20 μl cell pellet) in 200 μl dH2O followed by incubation at 80°C for 1 hour. AFB positive cultures were prepared as 20% glycerol stocks and stored at −80°C.

Molecular typing

Heat-killed AFB positive samples were investigated by multiplex PCR for the presence or absence of RD4, RD9, and TbD1 [7] using the primers listed in Table 1. Oligonucleotide primers used for deletion typing of RD105, RD142, RD150, and RD181 have been described previously [8]. Characterisation of NTM was performed by genus typing [9] and isolates positive for the *Mycobacterium* genus were sequenced at the 16S rDNA locus [10]. 16S rDNA sequences were used in BLAST searches [11] of databases at NCBI and RIDOM [http://rdna.ridom.de][12]; particularly, the sequences of the hypervariable Region A and Region B [13] were considered when determining the *Mycobacterium* species. The PCR amplification mixtures used for RD and genus typing were as follows: reactions were performed in a total volume of 20 μl consisting of 10 μl HotStarTaq Master Mix (Qiagen, United Kingdom), 7.1 μl distilled H2O, 0.3 μl of each oligonucleotide primer (100 μM), and 2 μl DNA template (heat-killed cells, see above). DNA sequencing was performed by The Sequencing Service (College of Life Sciences, University of Dundee, Scotland) using Applied Biosystems Big-Dye Ver 3.1 chemistry on an Applied Biosystems model 3730 automated capillary DNA sequencer. Isolates genetically typed as belonging to the *M. tuberculosis* complex were spoligotyped as previously described [14].

Results

Collection of lesions

In total, about 32,800 cattle were investigated during the years 2006–2008 and the vast majority were of local *Bos indicus* breeds

| Locus | Primer name | Primer sequence | Present | Deleted |
|-------|-------------|-----------------|---------|---------|
| RD4 | RD4_FlankF | CTCGTCGAAGGCCACTAAAG | 335 | 446 |
| RD4 | RD4_FlankR | AAGGGAAAGATCAGCATCAG | |
| RD4 | RD4_InternalF | ACGCTGGCGCAAGATATAG | |
| RD9 | RD9_FlankF | AACAGGGTCAGGTTGCTGTG | 396 | 575 |
| RD9 | RD9_FlankR | CAAGGACGACGTCGTTG | |
| RD9 | RD9_InternalR | TTGCGCTGTCGTTGTTG | |
| TbD1 | TbD1_FlankF | CACACCATCTCGGGTCC | 909 | 486 |
| TbD1 | TbD1_FlankR | CATAGATCCCGGACATGGTG | |
| TbD1 | TbD1_InternalR | AATCGAATCTCGGAAACC | |
| 16S rDNA | MYCGEN-F | AGATTTGATACCTGTCAG | 1030 | NA |
| 16S rDNA | MYCGEN-R | TGCAACCACGCCACAAAGGA | |
| 16S rDNA | 16S_AFB_F | GGCTGCTTTAACACATGCAATC | 660 | NA |
| 16S_AFB_R | TCCCTGATATCTGCGCATC | |

Table 1. Oligonucleotide primers used for molecular typing of *Mycobacterium* isolates and sizes of the expected PCR products.

[^1]: Primer set used for partial sequencing of the 16S rDNA locus [10].

[^2]: NA, Not Applicable.

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patterns are listed in Table 4. Strains of the six study sites were characterised by spoligotyping and their typing was performed to verify the specific deletion of the RD4 region in M. bovis strains (see Materials and Methods; Table 1). Fifty-eight strains were isolated from more than one abattoir.

Culturing and ZN staining results

Approximately 1,500 samples from suspect TB lesions were processed and sown onto the three solid media: 7H11, LJ glycerol, and LJ pyruvate, as described in the Materials and Methods. From the inoculated slants, colonies could be collected at a higher frequency from 7H11 than LJ glycerol or LJ pyruvate (data not shown). In total, 171 AFB were cultured from 1,524 lesioned samples (Table 2), with one animal from Gimbi showing dual infection of M. tuberculosis and M. gordonae.

Characterisation of Mycobacterium isolates by molecular typing

Of the 171 AFB isolated, 135 were subjected to a range of molecular typing techniques to determine their identity. To investigate if these isolates were M. bovis, deletion typing was performed to verify the specific deletion of the RD4 region in M. bovis strains (see Materials and Methods; Table 1). Fifty-eight samples generated a PCR product of 446 bp and were consequently identified as M. bovis (Table 2). In the cases where the RD4 region was shown to be intact, RD9 typing was performed to further identify the species. Eight isolates were shown to have both RD4 and RD9 present, suggesting that they were M. tuberculosis. Of the remaining AFB positive strains, 53 were typed as NTM, and 16 were not from the Mycobacterium genus. The frequency of NTM isolation varied between the collection sites (Table 2). Table 3 shows the NTM strains that were identified by DNA sequencing of the 16S rDNA gene as described in Materials and Methods. Overall, eleven different species were characterised of which M. nonchromogenicum, M. gordonae, M. fortuitum, and M. peregrinum were isolated from more than one abattoir.

Strains of the M. tuberculosis complex and their spoligotypes

Strains that were identified as M. bovis and M. tuberculosis from the six study sites were characterised by spoligotyping and their patterns are listed in Table 4. Strains of M. bovis were represented by seven different spoligotype patterns, of which four had not been registered before in the international M. bovis spoligotype database at www.Mbovis.org. Of the seven patterns SB0133, SB1476, and SB1176 were more prevalent. Interestingly, tendencies toward geographical clustering could be observed for these spoligotypes (Figure 1). First, SB0133 predominates in Jinka, but it is also represented in Addis Ababa, Gimbi, and Woldiya. The second, SB1476, is the most common pattern in both Gimbi and Gonder but it was found in Jinka and Butajira as well. SB1176 is most prevalent among the samples from Addis Ababa but can also be seen in Butajira, Gonder and Woldiya. The remaining four spoligotype patterns, SB0134, SB1488, SB1489, and SB1477, are all highly related to SB0133, and isolates with these patterns were mostly collected from the Addis Ababa or Jinka abattoirs. The largest diversity of M. bovis strains was found in Addis Ababa abattoir with five different spoligotypes, likely reflecting the wide geographical area from which cattle were sourced.

Eight M. tuberculosis strains were isolated from cattle, and they originated from four out of the six abattoirs. The TbD1 region [7] was deleted in all of these eight isolates. The spoligotype patterns of seven of these isolates had been registered previously in the Spoligol-International-Typing (SIT) database [15] and the majority were of the Euro-American lineage (Table 4). Two of the strains were of the Beijing lineage, based on the characteristic spoligotype pattern (SIT1) and by identification of the lineage specific SNPs.

Table 2. Summary of collected lesions and molecular typed AFB isolated from cattle slaughtered at six Ethiopian abattoirs.

| Study Site  | Animals Investigated | Lesioned Animals | Culture and ZN positive | M. bovis | M. tuberculosis | NTM* | Total typed |
|------------|----------------------|-----------------|-------------------------|----------|----------------|------|-------------|
| Butajira   | 4606                 | 281             | 26                      | 4        | 2              | 12   | 18          |
| Jinka      | 3471                 | 278             | 54                      | 13       | 2              | 19   | 34          |
| Gimbi      | 3250                 | 413             | 36                      | 10       | 3              | 13   | 26          |
| Gonder     | 14314                | 261             | 20                      | 11       | 0              | 2    | 13          |
| Woldiya    | 4338                 | 240             | 19                      | 4        | 1              | 7    | 12          |
| Addis Ababa| 2800                 | 51              | 16                      | 16       | 0              | 0    | 16          |
| All abattoirs | 32779               | 1524            | 171                     | 58       | 8              | 53   | 119         |

*NTM = Typed as Mycobacterium species not from the M. tuberculosis complex.

Table 3. Non-tuberculous mycobacteria isolated from cattle in Ethiopia.

| Species             | Woldiya | Gimbi | Butajira | Jinka |
|---------------------|---------|-------|----------|-------|
| M. acapulcensis     | 1       |       |          |       |
| M. colombiense      | 1       |       |          |       |
| M. engbaeki         | 2       | 1     |          |       |
| M. fortuitum        | 2       | 1     |          |       |
| M. gordonae         | 3       | 2     |          |       |
| M. intracellulare   | 2       |       |          |       |
| M. monacense        | 1       |       |          |       |
| M. mucogenicum      | 1       |       |          |       |
| M. nonchromogenicum | 1       | 7     |          |       |
| M. peregrinum       | 1       | 1     |          |       |
| M. vaccae           | 1       |       |          |       |

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Table 4. Spoligotyping patterns of A) *M. bovis* strains and B) *M. tuberculosis* strains isolated from cattle in Ethiopia.

| Spoligotype* | Spacers | Isolates per site | AA | Bu | Gi | Go | Ji | Wo | Total |
|--------------|---------|-------------------|----|----|----|----|----|-----|-------|
| A) SB0134    | 1-2     |                   | 4  | 1  | 5  |     |    |     |       |
| SB0133       | 1-3     |                   | 1  | 2  |    | 2  | 10 | 15   |       |
| SB1488       | 1-5     |                   | 1  | 1  |    |    |    | 1    |       |
| SB1489       | 1-7     |                   | 2  | 1  | 1  |    |    | 4    |       |
| SB1477       | 1-9     |                   | 2  | 8  | 7  | 1  | 18 | 14   |       |
| SB1476       | 1-11    |                   | 8  | 1  | 3  | 2  |    | 14   |       |
| SB1176       | 1-11    |                   | 8  | 1  | 4  |    | 18 | 14   |       |
| B) SIT1688   | 1-11    |                   | 2  | 2  |    |    |    | 2    |       |
| NEW          | 1-11    |                   | 1  | 1  |    |    |    | 1    |       |
| SIT1742      | 1-11    |                   | 1  | 1  |    |    |    | 1    |       |
| SIT59        | 1-11    |                   | 1  | 1  |    |    |    | 1    |       |
| SIT149       | 1-11    |                   | 1  | 1  |    |    |    | 1    |       |
| SIT1         | 1-11    |                   | 2  | 2  |    |    |    | 2    |       |

*SB No = www.mbovis.org; SIT No = Spoligo-International-Typing.
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deletions RD105 and RD181 [16]. However, the RD142 and RD150 regions that are frequently deleted in Beijing strains were present in these two cattle isolates.

Discussion

The aim of this study was to obtain data on the prevalence and epidemiology of bovine TB in cattle in Ethiopia so as to understand the disease burden and to inform public health policies. Ethiopia has the largest cattle population in Africa, and practices such as the consumption of raw milk and meat mean that the opportunity for zoonotic transmission of bTB is high. However, accurate baseline data for the prevalence of bTB in Ethiopia, across a range of eco-epidemiological settings, is needed to ensure that public health policies and disease control strategies are based on sound scientific data. We therefore carried out slaughterhouse surveys across major geographical areas of Ethiopia to determine the burden of mycobacterial infection, the prevalence of bTB, and the molecular types of M. bovis circulating in Ethiopia.

The agricultural settings

The societies in the highlands around the abattoirs of Gonder, Woldeiya, Gimbi, and Butajira, are supported by farming in agro-pastoral settings. This is in contrast to the lowlands in the southern regions of Ethiopia, to the south of Jinka, where farming in pastoral settings is practised. In rural areas the local Zebu cattle are the dominant breeds, while exotic breeds are rare. However, closer to Addis Ababa, in the centre of Ethiopia, farming is more diverse and exotic breeds - such as Holstein-Frisian - are common in dairy farms. The cattle in dairy farms are usually subject to intensive farming methods as compared to husbandry practices among pastoralists who keep their animals grazing outdoors.

Lesion prevalence

Results were based on post-mortem examinations of about 32,000 cattle (Table 2), making this one of the largest bovine TB studies of its kind in Africa. About 1,500 of these animals (~4.7%) carried suspect TB lesions. In 1975 the prevalence of tuberculous lesions at meat inspections in slaughterhouses varied between 0.02–1.83% in different parts of Ethiopia [17], while data from reports in 2003 and 2004 showed an increase to 1.48–5.16%. This is a relatively low number compared to cattle from northern parts of Ethiopia (Gonder and Woldeiya). The latter also raises the question of the risk for cattle-to-human transmission. Future studies are needed to find out if isolation of M. tuberculosis from cattle is a “spill over” effect from humans, or if these M. tuberculosis strains are adapted to cattle. The latter also raises the question of the risk for cattle-to-cattle and cattle-to-human transmission and the impact it may have on public health.

Differentiation of M. bovis strains by spoligotyping

Eighty-five M. bovis strains were spoligotyped and categorised into seven different types (Table 4) with five of these patterns so far apparently unique to Ethiopia. One of these spoligotypes SB1176 was first identified in a government farm in Holeta close to Addis Ababa [32]. Our data from the Addis Ababa abattoir suggests that SB1176 is the most common spoligotype in the central part of Ethiopia. However, it was also identified among strains isolated from the northern part of Ethiopia (Gonder and Woldeiya). The cattle slaughtered in Addis Ababa are traded over a large area of Ethiopia, and as such the mycobacterial strains isolated from these cattle should provide a general picture of strain types in Ethiopia. This is supported by the large diversity of spoligotype patterns that were found at Addis Ababa abattoir.

As well as the five spoligotypes that appear to be unique to Ethiopia, we also identified patterns SB0133 and SB0134. The former is most frequent in Jinka in the southern part of the country. An interesting association is that the SB0133 lineage that has spread over a large geographical area of Eastern Africa. It is therefore possible that SB0133 belongs to an M. bovis lineage that has spread over a large geographical area of Eastern Africa. It is therefore possible that SB0133 belongs to an M. bovis lineage that has spread over a large geographical area of Eastern Africa.
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Belgium [35–37]. It has also been isolated in Africa; a recent publication from Mali suggests that SB0134 is one of the most common patterns in the country [30]. Further chromosomal analysis of strains from Ethiopia and neighbouring countries will help to resolve the population structure of M. bovis in this geographical region, and shed light on how M. bovis has evolved in this location.

**Conclusion**

Our analysis has revealed that bTB is widely spread throughout Ethiopia, although our rates of M. bovis culture-positives from suspect lesions indicate a low prevalence. Indeed, more than 40% of all culture-positives were NTM, underlining the importance of confirming the presence of M. bovis in suspect lesions by culture and molecular methods.

There are reported differences in the susceptibility of Zebu and Holstein cattle breeds to bTB [4]. Similarly, rates of bTB in Ethiopia have been shown to be directly related to husbandry, with intensive farming practices driving increased bTB rates [32]. It is possible therefore that the low prevalence of bTB in our study is at least in part explained by the fact that the vast majority of cattle investigated were of Zebu extraction and from agro-pastoral and pastoral settings (i.e. non-intensive). To gain a more complete picture of the impact of bTB in Ethiopia our current investigations are focussing on intensive farms in periurban areas.

In terms of the impact of bTB on public health, our results suggest that the high rates of extrapulmonary TB seen in humans in Ethiopia are likely due to a combination of factors that include, but are not solely a consequence of, M. bovis transmission from cattle to humans. However, isolation of M. tuberculosis from significant numbers of animals calls for further investigations to elucidate the importance of this finding to public health.

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**Author Contributions**

Conceived and designed the experiments: SB MV HE DBY GH AA SVG. Performed the experiments: SB RF MH EG AM GA. Analyzed the data: SB NHS GH AA SVG. Contributed reagents/materials/analysis tools: LY GA BDR. Wrote the paper: SB SVG.

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