Molecular typing of *Mycobacterium bovis* isolated from tuberculosis lesions of cattle in north eastern Ethiopia

G. Ameni, F. Desta, R. Firdessa

*Mycobacterium bovis* strains isolated from tuberculosis (TB) lesions from 1138 cattle slaughtered at Kombolcha abattoir in north eastern Ethiopia were characterised. Detailed postmortem examination, bacteriological culturing, regions of difference PCR and spoligotyping were used. At least one TB lesion was observed in 57 (5 per cent) of the cattle, of which 27 (47 per cent) yielded mycobacteria isolates. Of the 27 isolates, 25 were identified as *M bovis* and two as *Mycobacterium tuberculosis*. The *M bovis* isolates were grouped into six clusters of strains, and the *M tuberculosis* isolates were typified to one strain only with the reference SIT262. Three clusters of previously unreported *M bovis* strains were identified and reported to the *Mycobacterium bovis* spoligotype database. They were designated the reference numbers SB1490, SB1491 and SB1492.

The epidemiology and public health significance of bovine TB in Africa remains largely unknown for politicoeconomic reasons, including the high cost of a testing programme, social unrest due to political instability, ethnic wars resulting in displacement of large numbers of people and animals, and a lack of veterinary expertise and communication networks. In addition, there are few or no laboratories that are capable of isolating and characterising mycobacteria from animals and human beings. In Ethiopia, the available reports show that the prevalence of bovine TB ranges from 3.4 per cent in smallholder production systems to 10 per cent in intensive dairy production systems.

BOVINE tuberculosis (TB) is a disease characterised by the progressive development of specific granulomatous lesions or tubercles in the lung tissues, lymph nodes or other organs. Approximately 85 per cent of cattle and 82 per cent of human populations in Africa live in areas where bovine TB is either partly controlled or not controlled at all (Cosivi and others 1995). In such countries, where bovine TB is still common and pasteurisation of milk is not practiced, an estimated 10 to 15 per cent of human TB cases are caused by *Mycobacterium bovis* (Ashford and others 2001). *M bovis*, the causative agent of bovine TB, is a member of the *Mycobacterium tuberculosis* complex (MTBC), a group that includes *M tuberculosis*, *M bovis*, *Mycobacterium africanum*, *Mycobacterium canetti*, *Mycobacterium pinnipedi*, *Mycobacterium caprae* and *Mycobacterium microti* (Smith and others 2006). These species cause similar pathologies in various mammalian hosts. *M tuberculosis* is specifically adapted to human beings, although it is occasionally isolated from other mammals. Similarly, *M bovis* is most frequently isolated from cattle, but it has also been isolated from other bovids and other mammals (Smith and others 2006).

The epidemiology and public health significance of bovine TB in Africa remains largely unknown for politicoeconomic reasons, including the high cost of a testing programme, social unrest due to political instability, ethnic wars resulting in displacement of large numbers of people and animals, and a lack of veterinary expertise and communication networks. In addition, there are few or no laboratories that are capable of isolating and characterising mycobacteria from animals and human beings. In Ethiopia, the available reports show that the prevalence of bovine TB ranges from 3.4 per cent in smallholder production systems to 10 per cent in intensive dairy production systems.

The lack of quarantine and the smuggling of live animals across boundaries in East African countries promote the transmission of mycobacteria from one country to another. In addition, within Ethiopia, cattle move freely from one region to another and from farm to farm, thus increasing the spread of disease. If the Ethiopian government decided to launch a programme to control and neutralise the spread of mycobacteria, then tracing the source of the infection and stopping it from further spread is of paramount importance. Such epidemiological tracing of...
TABLE 1: Proportion of lesioned tissues and severity of lesion of bovine tuberculosis in cattle slaughtered at Kombolcha abattoir, north eastern Ethiopia

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Number examined</th>
<th>Number (%) positive</th>
<th>Mean (±) severity of lesion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung lobes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left apical</td>
<td>57</td>
<td>9 (15.8)</td>
<td>0.47 (0.16)</td>
</tr>
<tr>
<td>Left cardiac</td>
<td>57</td>
<td>11 (19.3)</td>
<td>0.61 (0.17)</td>
</tr>
<tr>
<td>Left diaphragmatic</td>
<td>57</td>
<td>15 (26.3)</td>
<td>0.79 (0.19)</td>
</tr>
<tr>
<td>Right apical</td>
<td>57</td>
<td>10 (17.4)</td>
<td>0.49 (0.16)</td>
</tr>
<tr>
<td>Right cardiac</td>
<td>57</td>
<td>11 (19.3)</td>
<td>0.51 (0.16)</td>
</tr>
<tr>
<td>Right diaphragmatic</td>
<td>57</td>
<td>15 (26.3)</td>
<td>0.74 (0.18)</td>
</tr>
<tr>
<td>Right accessory</td>
<td>57</td>
<td>11 (19.3)</td>
<td>0.54 (0.16)</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mandibular</td>
<td>57</td>
<td>4 (7.0)</td>
<td>0.21 (0.10)</td>
</tr>
<tr>
<td>Retropharyngeal</td>
<td>57</td>
<td>16 (28.1)</td>
<td>0.72 (0.16)</td>
</tr>
<tr>
<td>Mediastinal</td>
<td>57</td>
<td>23 (40.4)</td>
<td>1.04 (0.18)</td>
</tr>
<tr>
<td>Left bronchial</td>
<td>57</td>
<td>18 (31.6)</td>
<td>0.67 (0.15)</td>
</tr>
<tr>
<td>Right bronchial</td>
<td>57</td>
<td>15 (26.3)</td>
<td>0.67 (0.15)</td>
</tr>
<tr>
<td>Hepatic</td>
<td>57</td>
<td>2 (3.5)</td>
<td>0.07 (0.06)</td>
</tr>
<tr>
<td>Mesenteric</td>
<td>57</td>
<td>19 (33.3)</td>
<td>0.79 (0.16)</td>
</tr>
</tbody>
</table>


the source of infection is possible with the use of basic epidemiological tools and molecular epidemiological techniques. Thus, generation of molecular epidemiological data on bovine TB would help in the control of the spread of infection. The objective of this study was to characterise mycobacteria isolated from TB lesions from cattle from north eastern Ethiopia, using molecular tools.

**Materials and methods**

**Study animals**

Cattle slaughtered at Kombolcha abattoir, which is located in north-eastern Ethiopia, were used for the study. A total of 1138 cattle originating from the surrounding zones and districts were randomly selected and investigated postmortem for TB lesions. The cattle were of the zebu breed and were kept by subsistence farmers. Farmers usually keep male cattle for ploughing and female cattle for reproduction and milk production. However, more than 80 per cent of the cattle in the study were old, rendering them, in effect, incapable of these roles. In general, farmers in Ethiopia are reluctant to sell their cattle unless they are certain the animals are past their productive phase and can no longer be used, with younger cattle usually only sold when farmers face immediate financial constraints. All the cattle used for this study were apparently healthy on ante-mortem examination.

**Postmortem examination**

Postmortem examination was performed following the procedure described by Corner (1994). Mandibular, retropharyngeal, left and right bronchial, cranial and caudal mediastinal, and mesenteric lymph nodes, and organs including the lungs, liver and kidneys, were subjected to a detailed postmortem examination. The seven lobes of the two lungs were inspected externally and then palpated. Each lobe was then sectioned into approximately 2 cm-thick slices to facilitate the detection of lesions. Similarly, lymph nodes were sliced into sections of a similar thickness and inspected for the presence of visible lesions. The severity of lesions was scored using a quantitative scoring method of a similar thickness and inspected for the presence of visible lesions. The severity of lesions was scored using a quantitative scoring method developed by Vordermeier and others (2002). Scores ranging from 0 to 3 were assigned to the lymph node lesions on the basis of distribution and size, whereas scores ranging from 0 to 4 were given for the lesions in the lobes of the lungs, again on the basis of distribution and size (Ameni and others 2006).

**Isolation of mycobacteria**

Samples from suspected TB lesions were further processed for isolation of mycobacteria in accordance with World Organisation for Animal Health (OIE) protocols (OIE 2010). Tissue specimens for culture were collected into sterile universal bottles in 5 ml of 0.9 per cent saline solution. They were then transported in an ice box at a latent temperature of 4°C to Aklilu Lemma Institute of Pathobiology. In the laboratory, the specimens were sectioned using sterile blades and were then homogenised with a mortar and pestle. The homogenate was decontaminated by adding an equal volume of 4 per cent NaOH and by centrifugation at 1505 g for 15 minutes. The supernatant was discarded, and the sediment was neutralised by 1 per cent (0.1 N) HCl using phenol red as an indicator. Neutralisation was considered to have been achieved when the colour of the solution changed from purple to yellow. Thereafter, 0.1 ml of suspension from each sample was spread onto a slant of Löwenstein Jensen medium. Duplicate slants were used, one enriched with sodium pyruvate and the other enriched with glyceral. Cultures were incubated aerobically at 57°C for at least eight weeks and with weekly observation of the growth of colonies.

**RD4 deletion typing**

RD4 deletion typing was performed at the Armoured Hansen Research Institute (AHRRI), following the procedure described by Parsons and others (2002). The isolates were harvested for RD4 deletion typing by scraping the growth from the slopes. Next, these shavings were poured into 200 ml of sterile distilled water, the mix was heated to reach a maximum temperature of 80°C, and it was maintained at this temperature for one hour. The primers used were RD4intF, 5’-ACACGGCTGCGGAGTAGTACGC-3’; RD4IntR, 5’-AAGGCGAACAGATTCAGCAT-3’ and RD4IntNF, 5’-CTCGTCGAAAGGCCACTAAAG-3’. The mixture was heated in...
a Thermal Cycler (Applied Biosystems; GeneAMP 9700) for 15 minutes at 95°C and then subjected to 35 cycles of one minute duration at 95°C, one minute at 55°C, one minute at 72°C and 10 minutes at 72°C. Each PCR consisted of 7 µl distilled water (Qiagen), 10 µl HotStarTagMasterMix, 0.3 µl of each of the three primers (1.5µM final concentration) and 2 µl of DNA templates of samples or controls, thus making the total volume of primers 20 µl. M. tuberculosis H37Rv and M. bovis 2122/97 were used as positive controls, and distilled water was used as a negative control. The product was electrophoresed in 1.5 per cent agarose gel in 1 x TAE running buffer. SYBR Safe at a ratio of 1:1000, 100 bp DNA ladder and orange 6 x loading dye were used in gel electrophoresis. The gel was visualised using Syngene Bio Imaging System (Syoptics Group). The presence of RD4 (ie, RD4 is responding to one of the unique spacer DNA sequences within the direct repeat region) was amplified by PCR using oligonucleotide primers derived from the direct repeat sequence. A total volume of 25 µl and a reaction mixture of 12.5 µl and a reaction mixture of 12.5 µl of HotStarTagMasterMix (Qiagen), a final concentration of 1.5mM MgCl2, 200µM of each deoxynucleotide triphosphate, 2 µl of each primer (20 pmol each), 5 µl suspension of heat-killed cells (approximately 10 to 50 ng) and 3.5 µl distilled water was used. The mixture was heated for 15 minutes at 96°C and then subjected to 30 cycles of one minute duration at 96°C, one minute at 55°C, and 30 seconds at 72°C. The amplified product was hybridised to a set of 43 immobilised oligonucleotides, each corresponding to one of the unique spacer DNA sequences within the direct repeat locus. After hybridisation, the membrane was washed twice for 10 minutes in 2 x SSPE (1 x SSPE is 0.18 M NaCl, 10 mM NaH2PO4 and 1 mM EDTA [pH7.7]) and 0.5 per cent sodium dodecyl sulfate at 60°C and then incubated in 1:4000 diluted streptavidin-alkaline phosphatase (Boehringer) for 45 to 60 minutes at 42°C. The membrane was washed twice for 10 minutes in 2 x SSPE and 0.5 per cent sodium dodecyl sulfate at 42°C and rinsed with 2 x SSPE for five minutes at room temperature. Hybridising DNA was detected by the enhanced chemiluminescence method (Amersham) and by exposure to x-ray film (Hyperfilm ECL), as specified by the manufacturer.

Results

Frequency and magnitude of pathology

Gross TB lesions were detected in 57 of 1138 (5 per cent) of the cattle. Characteristic TB lesions were observed in different tissues (Table 1). The lesions were more frequent and severe in the diaphragmatic lobes of both lungs, as well as in the mediastinal, bronchial and retropharyngeal lymph nodes (Table 1). About half (27 of 57) of the suspect lesions were positive for mycobacteria growth.

Molecular characterisation of the isolates

All of the 27 isolates showed evidence for RD4 deletion typing; 25 isolates were M. bovis and two were M. tuberculosis. Fig 1 shows the results of RD4 deletion typing of 24 isolates by the four gels, but the remaining three isolates were not shown. The spoligotype patterns of the 27 isolates are shown in Fig 2. The isolates were grouped into six clusters of M. bovis strains and one cluster of M. tuberculosis. Of the six clusters of M. bovis, three clusters were new and had not been reported to the Mycobacteria bovis spoligotype database previously. They were reported and given the reference numbers SB1490, SB1491 and SB1492. Four, two and one isolate, respectively, belonged to each cluster. The strain with the largest number of isolates (13 isolates) was SB1176, followed by SB0135 (four isolates), and SB0912 (two isolates). SIT262 was the strain of M. tuberculosis isolated from the cattle tissue.

Discussion

The frequency and severity of the lesions were higher in the thoracic lymph nodes than in the mesenteric lymph nodes, which is consistent with the results of previous studies (Lepper and Pearson 1973, Mellroy and others 1986, Corner 1994, Nell and others 1994, Collins 1996, Whipple and others 1996). Such results suggest that respiratory inhalation is the main route by which animal-to-animal transmission occurs (Francis 1972, Smyth and others 2001).

In this study, 27 isolates showed evidence for M. tuberculosis complex species, of which 25 were M. bovis and two were M. tuberculosis. Bovine TB is primarily caused by M. bovis and can occasionally spread

---

**FIG 2:** Spoligotype patterns of mycobacterial isolates recovered from tuberculosis lesions in cattle. Six clusters of spoligotype patterns of Mycobacterium bovis and a cluster of Mycobacterium tuberculosis were detected. Of the six patterns of M. bovis, three (SB1490, SB1491 and SB1492) were identified for the first time. Bold indicates reference strains.
to human beings, whereas \textit{M. tuberculosis} is considered to be a human pathogen. \textit{M. tuberculosis} infection has been reported in a wide range of domestic and wildlife animal species, most frequently in those living in close, prolonged contact with human beings (Steele 1950, Thoen and others 1951, Montal and others 2001, Oh and others 2002, Pavlik and others 2003, Alfonso and others 2004). Other studies conducted in Ethiopia have shown that bovine tuberculosis is the most common zoonotic mycobacterial infection of the milk of skin test-positive cows (Ameni and Aldili 2007, Ragassa and others 2007). Similarly, in Algeria and Sudan, the prevalences of \textit{M. tuberculosis} in cattle were 6.2 and 7.4 per cent, respectively (Boulahbâl and others 1978, Suleiman and Hamid 2002). Animal attendants with active TB in the respiratory tract, urinary tract or gastrointestinal tract also represent an active source of \textit{M. tuberculosis} for animals, spreading the bacillus via the sputum, urine or faeces (Thoen and others 1981).

The characterisation of the strains of \textit{M. bovis} using spoligotyping revealed the presence of 25 isolates grouped into six clusters of spoligotype patterns. The most commonly found strain was SB1176, which was localised in Ethiopia and was also reported earlier by Ameni and others (2007b). SB1035 and SB0912 were also reported previously, however, these were found less frequently than SB1176 (Berg and others 2009, Tsegaye and others 2010). Berg and others (2009) carried out a similar study in Woldiya abattoir, which is located 40 km north of Kombolcha abattoir. The animals slaughtered at Woldiya abattoir are used exclusively for local consumption, whereas those slaughtered at Kombolcha abattoir are used both for export and for local consumption. More cattle are slaughtered at Kombolcha abattoir. From Woldiya abattoir, Berg and others (2009) identified only two strains (SB1035 and SB1176) of \textit{M. bovis}, whereas the present study identified six different strains, of which three were: SB1035 and SB1176 were identified by both Berg and others (2009) and the present study. Furthermore, these strains have been reported by Tsegaye and others (2010) in a study conducted in Addis Ababa. Although other infectious diseases with high mortality and morbidity are increasing in Ethiopia, the incidence and prevalence of bovine TB are on the rise because of the expansion of the dairy farming sector (Ameni and others 2007a, Tsegaye and others 2010, S. Berg, Ameni and Aklilu 2007b) in Ethiopia. The high prevalence and increased severity of pathology of bovine TB in Ethiopia is a predominant factor affecting the pathology of bovine tuberculosis and gamma interferon responses to mycobacterial antigens. In conclusion, there is an urgent need to develop strategies for the effective control of bovine tuberculosis and to implement strategies to control the infection in the animal population.
Molecular typing of *Mycobacterium bovis* isolated from tuberculosis lesions of cattle in north eastern Ethiopia

G. Ameni, F. Desta and R. Firdessa

_Veterinary Record_ 2010 167: 138-141
doi: 10.1136/vr.b4881

Updated information and services can be found at:
[http://veterinaryrecord.bmj.com/content/167/4/138](http://veterinaryrecord.bmj.com/content/167/4/138)

These include:

**References**

This article cites 32 articles, 10 of which you can access for free at:
[http://veterinaryrecord.bmj.com/content/167/4/138#BIBL](http://veterinaryrecord.bmj.com/content/167/4/138#BIBL)

**Open Access**

This is an open-access article distributed under the terms of the Creative Commons Attribution Non-commercial License, which permits use, distribution, and reproduction in any medium, provided the original work is properly cited, the use is non commercial and is otherwise in compliance with the license. See: [http://creativecommons.org/licenses/by-nc/3.0/](http://creativecommons.org/licenses/by-nc/3.0/) and [http://creativecommons.org/licenses/by-nc/3.0/legalcode](http://creativecommons.org/licenses/by-nc/3.0/legalcode)

**Email alerting service**

Receive free email alerts when new articles cite this article. Sign up in the box at the top right corner of the online article.

**Topic Collections**

Articles on similar topics can be found in the following collections

Open access (109)

**Notes**

To request permissions go to:
[http://group.bmj.com/group/rights-licensing/permissions](http://group.bmj.com/group/rights-licensing/permissions)

To order reprints go to:
[http://journals.bmj.com/cgi/reprintform](http://journals.bmj.com/cgi/reprintform)

To subscribe to BMJ go to:
[http://group.bmj.com/subscribe/](http://group.bmj.com/subscribe/)