MYCOBACTERIUM BOVIS CHARACTERIZATION THROUGH RD4 DELETION AND SPOLIGOTYPING TECHNIQUES

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ABSTRACT

A cross sectional study was conducted on 753 Cattle which selected using systematic random sampling technique from December 2015 to May 2016 to isolate Mycobacterium bovis and its molecular characterization from cattle slaughtered at Hawassa University and Municipal abattoirs. The methods used were postmortem examination, bacteriological culturing, RD4 deletion typing and Spoligotyping. The overall prevalence of the bovine tuberculosis was 5.8% (95%CI: 4.17 - 8.52) on the basis of detailed postmortem examination. Out of 44 tissue samples cultured, 24 (54.5%) were culture positive and all were acid fast staining positive. However, using RD4 deletion only three isolates were confirmed as Mycobacterium bovis. The further Spoligotyping characterizations of these three isolates revealed the strain SB1477. Most of the lesions were found in thoracic cavity lymph nodes (75%), which indicated that respiratory route was the main mode of infection in the study area. Hence, the study confirmed that the low prevalence of BTB and the current Mycobacterium bovis strain circulate in the cattle population being SB1477. Therefore, further investigation needed including large study areas for epidemiological information on circulating strains, ways of transmission and molecular diversity of the M. bovis strains. **Further investigation at large area needed to have the current epidemiological data on the circulating strains, transmission and molecular diversity of the M. bovis.**

**Keywords:** Bacteriological, Culturing, M. bovis, RD4 deletion, Spoligotyping, ZN Staining
INTRODUCTION

Mycobacteria are slow-growing, acid-fast and aerobic organisms whose genus classified in to more than 90 different species (Watterson et al., 1998). A group of Mycobacterium species called M. tuberculosis complex (MTBC) that comprise M. tuberculosis, M. bovis, M. microti and M. africanum, is of utmost clinical importance since it causes tuberculosis in humans worldwide (Kidane et al., 2002; Haddad et al., 2004; Marais, 2008; Rock et al., 2008). Mycobacterium species other than those of the tuberculosis complex, also called nontuberculous mycobacteria (NTM), are widely distributed in the environment and may colonize and occasionally cause infections in humans (Adle-Biassette et al., 2003; Barne et al., 2004; Castro et al., 2007). Mycobacteria of the MTBC and NTM have been found to cause infections in immunocompetent and immunocompromised subjects and cause pathology in pulmonary and extrapulmonary sites (Scarparo et al., 2001; Sachdeva et al., 2002; Stout, 2006; Fanlo and Tiberio, 2007; Glassroth, 2008).

Bovine tuberculosis (BTB) is among the principal zoonotic diseases (Buncic, 2006) caused by M. bovis (Grange et al., 1996) which affects many vertebrate animals (Grange et al., 1996) and humans (Ayele et al., 2004; Thoen et al., 2009) and characterized by progressive development of granulomas/ tubercles in tissues and organs (Amanfu, 2006; OIE, 2010; Hlokwe et al., 2013; Pal et al., 2014). Bovine tuberculosis has been widely distributed throughout the world and has been recognized from 176 countries as one of the important bovine diseases causing great economic loss in animal production (Awah-Ndukum et al., 2013) and the most frequent cause TB in man (Grange et al., 1996). M. bovis is most frequently isolated from domesticated cattle (Grange et al., 1996; Smith et al., 2006), although recent studies indicated that M. tuberculosis has been isolated from cattle and M. bovis from humans infected with BTB and TB respectively. The members of MTBC are characterized by 99.9% or greater similarity at nucleotide level, and are virtually identical at 16s rRNA sequence but they vary in host specificity (Brosch et al., 2002).

Bovine tuberculosis affects broad range of mammalian hosts including humans, cattle, deer, pigs, domestic cats, wild carnivores and omnivores (De Lisle et al., 2002); it rarely affects equids or sheep (Phillips et al., 2003). Moreover, human TB of animal origin caused by M. bovis is becoming increasingly evident in developing countries (Russel, 2003; Mamo et al., 2013). The productivity efficiency of the infected animal reduced from 10-25%; the direct losses due to the infection decreases in milk production 10-18% and meat production 15 %. BTB has both the effect in animal production and public health importance (Radostits et al., 1994; Müller et al., 2013). In developing countries like Ethiopia, the low standard living areas and socio-economic situation for both animals and humans are more contributing in TB transmission between human to
human and human to cattle or vice versa (Ameni et al., 2010b; Ejeh et al., 2013). Organisms are excreted in the exhaled air, in sputum, feces (from both intestinal lesions and swallowed sputum from pulmonary lesions), milk, urine, vaginal and uterine discharges, and discharges from open peripheral lymph nodes of infected animals (Phillips et al., 2003; Radostits et al., 2007). Human infection by M. bovis is thought to be mainly through drinking of contaminated or unpasteurized raw milk. The potential for transmission of M. bovis and other mycobacteria between cattle and humans are the presence of close contact of animal and humans or the rural societies living together with their animals in the same microenvironment and house, raw milk and meat consumption habit, the prevalence of HIV increasing and HIV patient’s susceptibility to TB (Shitaye et al., 2007). In the areas where the bovine tuberculosis is common and the milk pasteurization is rare, M. bovis cases in human estimated 10-15 % (Ashford et al., 2001; Mbugi et al., 2012).

Thus, in cattle the main route of infection transmission: aerosol, close contact between animals (Neill et al., 1991) and ingestion of contaminated products (Menzies and Neill, 2000; Ameni et al., 2007; Cleaveland et al., 2007). Bovine tuberculosis, is an endemic disease of cattle in Ethiopia with prevalence of 1.1%-24.7% in abattoir and 3.5–50% in crossbreed farms (Shitaye et al., 2007; Berg et al., 2009; Biffa et al., 2009; Regassa et al., 2010), like human TB has not received more focus on research and its control strategies plus the test and slaughter control strategies not applied, due to its economic constraints, but its applicable effective method in developed countries (Chukwu et al., 2013). Due to inadequate comprehensive abattoir surveillance and lack of diagnostic facilities the BTB has limited information (Cosivi et al., 1998; Asseged et al., 2000), particularly on genotypic characteristics of M. bovis, a strain affecting the cattle population in Ethiopia (Biffa et al., 2010b). Knowing the M. bovis strains which are circulating in cattle population is using to examining host pathogen relationships to monitor transmission and spread of the disease among cattle (Gagneux and small, 2007; Berg et al., 2011). Therefore, the object of this research paper was to isolated and molecular characterization of isolated Mycobacterium bovis using RD4 deletion typing and Spoligotyping techniques from the cattle slaughtered at Hawassa University and Municipal abattoirs, Southern Ethiopia.
MATERIALS AND METHODS

2.1. Study Area

The study was conducted from December 2015 to May 2016 in Hawassa University and municipal abattoir in Southern Nations Nationalities and Peoples Regional state (SNNPRs), Southern Ethiopia. Hawassa city is located in Southern part of Ethiopia, in Sidama Zone, on the shores of Lake Hawassa in the Great Rift Valley and located 270 km South of Addis Ababa. The city serves as the capital of the SNNPRs, and its total area is 157.21 square kilometers. It bounded by Lake Hawassa on the West and North-west, Chelelaka swampy area on the East and South-East, Tikur Wuha River on the North and Alamura Mountain on the South, It lies on the Trans-African Highway for Cairo-Cape Town, and has a geographic coordinate of 7°3′ N latitude and 38°28′ E longitude and an elevation of 1708 m.a.s.l. (CSA, 2007). The livestock resource of the city is 61,123 cattle, 14,764 sheep, 17,735 goats, 5,544 equines and 56,961 poultry and the total population is estimated about 304,479 (CSA, 2011).

Hawassa city has two abattoirs: one municipality abattoir and second Hawassa University abattoir, in main campus. The Hawassa municipal abattoir supplies the inspected meat to about 304,479 inhabitants (CSA, 2011) and based on the information obtained from personnel working in Hawassa University Registrar Office, the University’s abattoir supplies for about 25,000 students (personal communication).

Even though the abattoirs were fenced, the places used to dispose condemned carcasses were not secured since they were easily accessed by hyenas, dogs and other animals. The overall hygiene and the internal facilities including the drainage were not good in the Municipal abattoir whereas the University’s abattoir was good. Even though the Municipal abattoir has recently built abattoir inside the compound, currently the slaughtering is performing in the old house. The minimum and maximum numbers of cattle slaughtered per day during the study period in Hawassa city Municipal abattoir were about 75 and 175 heads of cattle, respectively and about 8 and 12 heads of cattle were for Hawassa University abattoir, respectively. Few numbers of female cattle were also slaughtered in both abattoirs which have reproductive problems, poor performance and at the end of their reproductive life (aged). The municipal abattoir was operated by one veterinarian and three assistant meat inspectors but the University abattoir was operated only by one assistant meat inspector (Personal communication to the responsible bodies).
2.2. Study Animal

A total of 753 apparently healthy adult cattle slaughtered in the abattoirs regardless of sex, breed and origin were considered in the study. The cattle used for this study were mainly originated from different markets; for the municipal abattoir were from Tula, Arsi Negele, Tukr Wuha, Wolayita, Harrar, Borena, Hawassa and Nazeriat (Adama) Markets and for University abattoir the origin were from Borena and Wolayita markets. All the study animals in this study were cross and local breed.

2.3. Study Design and Sampling Method

A cross sectional study with systematic random sampling was carried out to examine the carcass and sample suspected TB lesions from cattle slaughtered at Hawassa university and municipal abattoirs.

Selection of cattle population to be involved in the study was based on systematic random sampling, from among those cattle slaughtered each day, which was arranged in a systematic manner with each individual animal constituting a sampling unit.

In Hawassa city municipal abattoir on average 20 carcasses were decided to be inspected each day (range from 15 to 25) from among those slaughtered cattle whereas in Hawassa University abattoir the whole cattle slaughtered in each day were considered for postmortem examination.
because the number of slaughter cattle was often fewer (range from 6 to 12) compared the Hawassa city municipal abattoir. The work was carried out in all the weekdays except Wednesday and Friday when animals were not slaughtered due to fasting occasions according to the belief of Ethiopian Orthodox Christianity.

2.4. Sample Size Determination

All animals coming to the abattoirs from different markets during the study period were considered for sampling. The sample size calculation was done by using the Thrusfield (2007) formula:

\[ n = \frac{1.96^2 \times p_{exp} (1-p_{exp})}{d^2} \]

Where

- \( n \) = required sample size
- \( p_{exp} \) = expected prevalence
- \( d \) = desired absolute precision, 5%

The expected prevalence of Hawassa city municipal abattoir was (8.8%) used from the previous works of Biffa et al. (2010a). The calculated sample size for the municipal abattoir was 123 heads of cattle, but to increase the precision of the study this sample size multiplied three times, then \( n = 369 \) heads of cattle. Whereas the calculated sample size of the Hawassa University abattoir, Pexp considered as 50% because there was no previous study on bovine tuberculosis in this abattoir, was 384 heads of cattle. Thereafter, the total animals supervised in both abattoirs were 753 heads of cattle.

2.5. Study Methodologies

2.5.1. Post mortem examination

Post mortem examination was carried out according to the OIE (2009) and Meat Inspection and Quarantine Division of the Ministry of Agriculture method (Hailemariam, 1975). All lymph nodes, livers, kidney and lungs were visualized, palpated, and incised into a size of 2 mm to facilitate the detection of tuberculous lesion from each animal. These include the mandibular, medial retropharyngeal, cranial and caudal mediastinal, left and right bronchial, hepatic, mesenteric lymph nodes as well as the seven lobes of the two lungs, including the left apical, left cardiac, left diaphragmatic, right apical, right cardiac, right diaphragmatic and right accessory lobes were investigated. The animal was classified as suspected tuberculous lesion when tuberculous lesion was found, and if not as non-lesioned.

The cut surfaces were examined under bright light for the presence of abscess, cheesy mass, and tubercles (Corner et al., 1990). In the presence of suspected tuberculous lesion, tissue samples were collected independently in sterile universal bottles containing 0.85% normal saline for culture and kept store froze at -20°C at Hawassa University, School of Animal Science and Rang Management, Dairy section before being transporting to laboratory. The samples were labeled and pooled together and then transported at 4°C under cold chain by ice box with
packed ice to Addis Ababa University, College of Veterinary Medicine and Agricultural (CVMA) and stored at -20°C until bacteriological culture.

2.5.2. Mycobacteria culturing and acid fast staining

For bacteriological culture, tissue samples were macerated in sterile mortal by using surgical blades and forceps to get fine pieces and in order to initiate the release of mycobacteria organisms from body fluid and cells and then homogenized by pestle and mortar. Five milliliters of the homogenized tissue sample was transferred into centrifuge tube and decontaminated with equal volume 4% NaOH followed by centrifugation at 3,000 rpm for 15 minutes. The supernatant was discarded, while the sediment was neutralized with 1 % (0.1N) HCl using phenol as an indicator. Neutralization achieved when the color of the solution changed from purple to yellow (OIE, 2009). Thereafter, 0.1 ml of the suspension from each sample was inoculated on to LJ egg based media. Duplicates of LJ media were used; one enriched with sodium pyruvate, while the other was enriched with glycerol. The Cultures were incubated at 37°C for 1 week and in upright position for the rest 8-12 weeks. The media was in tightly closed tubes to avoid desiccation and slopes were examined for macroscopic growth at intervals during the incubation period for the presence of any mycobacterial colonies (OIE, 2009).

Whenever, colonies were seen, ZN staining was performed to confirm the presence of Acid fast bacilli (Quinn et al., 1999; OIE, 2009). In parallel to the ZN staining, Positive colonies were preserved with freezing media, and some portion of the colonies were heat killed in water bath maintained at 80 °C heat for 45 minutes, by mixing two loops full of colonies in 200μl distilled water (Brosch et al., 2002; Cadmus et al., 2006). The frozen and heat killed isolates were stored at -20 °C for future Mycobacteriology and further molecular typing analysis was performed at Akili Libero Institute of Pathobiology according to the standard developed by Cadmus et al. (2006), Hewinson et al. (2006) and WHO (2012).

2.5.3. Region of difference (RD)-4 deletion typing

For the RD4 deletion typing, the procedure described by Cadmus et al. (2006) was used. Each sample was tested in a separate PCR tube. Primers directed against the RD4 were used to generate a deletion profile that would allow species identification of the isolate. RD4 is 12.7 kb genetic segment that is deleted from M. bovis BCG strain, but present in M. microti, M. africanum and M. tuberculosis (Gordon et al., 1999). RD4 internal F: 5’-ACA CGC TGG CGA AGT ATA GC-3’; RD4 flank R: 5’-AAG GCG AAC AGA TTC AGC AT-3’ and RD4 flank F: 5’-CTC GTC GAA GGC CAC TAA AG-3’ primers were used to check for the presence of RD4 locus.

Each PCR tube consisted of 7μl distilled water (Qiagen), 10μl Hot Star Taq Master Mix, 0.3μl of each of the three primers and 2.1μl of DNA template, thus making the total final volume of 20μl. M. tuberculosis H37Rv and M. bovis SB1176 were used as
positive control, and distilled water was used as a negative control. The mixture was heated in a Thermal Cycler (VWR, International Ltd. Portsmouth, UK ) using an initial hot start at 95°C for 15 minutes and then subjected to 35 cycles of 95°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute and final extension step 72°C for 10 minutes to complete the cycle. The product was electrophoresed in 1.5% agarose gel in 1x TAE running buffer; SYBR Safe/Ethidium bromide at a ratio of 1:10, 100 bp DNA ladder and orange 6 x loading dye were used in gel electrophoresis. The gel was visualized using Syngene Bio Imaging System (Syoptics Group). Interpretation of the result was based on the detection of bands of different sizes. The presence of RD4 (RD4 is intact in M. tuberculosis, M. africanum) gives a product size of 335 bp (RD4 intF + RD4flankR), and its absence (M. bovis) gives a product size of 446 bp (RD4flankF + RD4flankR).

2.5.4. Spoligotyping

Spoligotyping was carried out using the commercially available kit according to the manufacturer’s instructions and as previously described by Kamerbeek et al. (1997). Briefly, the direct repeat (DR) region was amplified with primers DRa (5'-GGT TTT GGG TCT GAC GAC -3' biotinylated at the 5' end) and DRb (5'-CCG AGA GGG GAC GGA AAC -3'). PCR amplification was done for 30 cycles with denaturation and annealing for 1 min at 95°C and 1 min at 55°C, respectively, and extension for 30s at 72 °C in each cycle. The amplified DNA was hybridized to inter-DR spacer oligonucleotides covalently bound to a membrane for 1 hour at 60°C. Reference strains of M. tuberculosis (H37Rv) and M. bovis SB1176 were used as positive control whereas sterile water was used as a negative control. The amplified DNA was subsequently hybridized to a set of 43 oligonucleotide probes by reverse line blotting. The hybridized PCR products were incubated with streptavidin peroxidase conjugate and the signal detection was done with an enhanced chemiluminescence detection system followed by exposure to an X-ray film according to the manufacturer’s instructions. The X-ray film was developed and washed using standard photochemical procedures. The results were entered in a computer-based strain identification system described in international online database website http://www.mbovis.org.

2.6. Statistical Analysis

Prevalence was calculated as the proportion of suspected lesion positive animals from the total number of animals sampled (Thrusfield, 2007). Presence or absence of TB like lesions and affected tissues (suspected lesions) were recorded during postmortem examination. The recorded data was entered and stored using Microsoft Excel computer program. In molecular epidemiology study of isolates from animal tissues, the spoligotype patterns were converted in to binary and entered to the online spoligotype database website http://www.mbovis.org. to retrieved the strain type.
3. RESULTS

3.1. Distribution of Gross Pathological Lesions and Prevalence

The overall prevalence of BTB was 5.84% (95%CI: 4.58 - 8.42), upon detailed post mortem examination of 753 cattle. Gross pathological lesions were observed in lymph nodes and organs of the slaughtered cattle; the majority of the lesions were considered typical of tuberculous lesions which characterized by central round, oval, or irregular, often coalescing areas of caseous necrosis and mineralization (calcification) (Figure 9). Large encapsulated nodules containing thick yellowish cheesy material were mostly observed in the thoracic lymph nodes. Whenever gross lesions of suggestive pathological lesions of TB noticed in any tissue; the tissue was classified as positive for TB.

![Figure 2: The typical TB lesions of cattle slaughtered in Hawassa abattoirs (Calcified, Caseous and Necrotic Granulomatous lesions in mediastinum lymph nodes, indicated by arrow).](image)

According to the anatomical site, 75% (33/44) of the gross lesions were sampled from thoracic cavity followed by abdomen cavity and head region 13.64% (6/44) and 15.91% (5/44) respectively. 70.45% (31/44) of the gross lesions were collected from Mediastinal lymph nodes whereas only 11.36% (5/44) where obtained from mesenteric lymph nodes (Table 1).
Table 1: Lesion distribution in different organs and anatomic parts

<table>
<thead>
<tr>
<th>Anatomical Site</th>
<th>Affected organs</th>
<th>Frequency (%)</th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Head</td>
<td>Retropharyngeal LN</td>
<td>4 (9.09%)</td>
<td>6 (13.64%)</td>
</tr>
<tr>
<td></td>
<td>Mandibular LN</td>
<td>2 (4.5%)</td>
<td></td>
</tr>
<tr>
<td>Thorax</td>
<td>Mediastinal LN</td>
<td>31 (70.45%)</td>
<td>33 (75%)</td>
</tr>
<tr>
<td>Abdomen</td>
<td>Mesenteric LN</td>
<td>5 (11.36%)</td>
<td>7 (15.91%)</td>
</tr>
<tr>
<td></td>
<td>Livers</td>
<td>2 (4.55%)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>44 (100%)</td>
<td>44 (100%)</td>
</tr>
</tbody>
</table>

LN = lymph node

**Mycobacteriological Culture and Acid Fast Stain Results**

Out of the total 44 suspected BTB lesions mycobacteriological cultured; growth was observed in 24 (54.55%) tissue samples on LJ medium culture (Figure 12) and all of them were found to be AFB positive. From this growth, colonies collected at a higher frequency from pyruvate supplemented LJ medium, 12/24 (50%) than glycerol supplemented LJ medium, 8/24 (33.33%) and the remained 16.77% (4/24) from both LJ medium (Table 2). The observed colony morphology was smooth whitish or yellowish color colony (Table 3). These colonies positive for the ZN staining were cocci, short and long rod shape and also found in single and clump (Figure 4).
**Figure 3:** (A & C) Colonies grown on LJ medium glycerol supplemented (B&D) LJ medium pyruvate supplemented (Arrows indicate the colonies growth which are smooth, whitish or yellowish in color)

<table>
<thead>
<tr>
<th>Abattoirs</th>
<th>No. of Sampled</th>
<th>Lesions (%)</th>
<th>Growth on LJ medium</th>
<th>ZN stain positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>LJP</td>
<td>LJG</td>
</tr>
<tr>
<td>Hawassa University Abattoir</td>
<td>384</td>
<td>31(8.07)</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>Hawassa City Municipal Abattoir</td>
<td>369</td>
<td>13(3.51)</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>753</strong></td>
<td><strong>44(5.84)</strong></td>
<td><strong>11</strong></td>
<td><strong>9</strong></td>
</tr>
</tbody>
</table>

**Table 3:** Time appearance of mycobacteria colony growth on the LJ medium and characteristics.

<table>
<thead>
<tr>
<th>No of Isolates</th>
<th>Source</th>
<th>Time to appear in week</th>
<th>Colony characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>LJP</td>
<td>LJG</td>
</tr>
<tr>
<td>5</td>
<td>Cattle</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>13</td>
<td>Cattle</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>Cattle</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 4:** Acid fast bacilli staining positive isolates from mycobacteriological culture colony grows from lymph nodes TB lesion (arrows indicate acid fast bacilli which a short and long, in single or clump).

### 3.2. Molecular Characterization of Mycobacteria Isolates

The RD4 deletion typing of isolates was confirmed only three isolates to be *M. bovis* (Figure 5) that indicated a product size of 446bp; the rest of 21 isolates did not show any signal in RD4 deletion typing.
Figure 5: Electrophoretic separation of PCR products by RD4 deletion typing of mycobacteria isolates from 24 isolates sampled culture. Lane 1 and 27- Ladder (100bp), Lane 2 and 28- M. tuberculosis H37Rv positive control, Lane 3 and 29- Distilled water negative control, Lane 4 and 30- M. bovis SB1176 positive control, Lane 5-26 and 31- 36 isolates from tissue culture positives, Lane 21, 32 and 33 positive for M. bovis and Lane 5-20, 22-26,31,34-36 were negative for M. bovis.

The Spoligotyping of the three M. bovis isolates, which were positive in RD4 deletion, showed the typical M. bovis spoligotype pattern with similar pattern and based on the database website http://www.mbovis.org analysis of the spoligotype results isolates were identified as SB1477 (Figure 6).
<table>
<thead>
<tr>
<th>Code</th>
<th>Strain</th>
<th>Binary Format</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive control</td>
<td>SB1176</td>
<td></td>
</tr>
<tr>
<td>(M. bovis)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative control</td>
<td>SB1477</td>
<td></td>
</tr>
<tr>
<td>(distil water)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HAp 39</td>
<td>SB1477</td>
<td></td>
</tr>
<tr>
<td>H41Med</td>
<td>SB1477</td>
<td></td>
</tr>
<tr>
<td>H39 Med</td>
<td>SB1477</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 6:** Spoligotype patterns of *M. bovis* isolates from TB lesions of cattle from Hawassa abattoirs. The filled boxes (blacks) represent the presence of spacers and the empty boxes (white) represent the absence of spacers. AP39, H41Med and H39Med are isolates from sam
DISCUSSION

Bovine tuberculosis is a chronic infectious disease of animals characterized by the formation of granulomas in tissues and its detection is carried out most commonly on the basis of tuberculin skin testing, abattoir meat inspection and rarely on bacteriological techniques. Bovine tuberculosis has serious economic significance to the livestock sector and public health hazard to human. Tuberculosis caused by M. bovis is clinically indistinguishable from tuberculosis caused by M. tuberculosis and the proportion of human tuberculosis caused by M. bovis is estimated to 10-15% (Ashford et al., 2001; Mbugi et al., 2012).

In the present study, upon detailed post mortem examination of 753 cattle an overall prevalence of BTB was 5.84% (95%CI: 4.58 - 8.42) which is comparably in agreement with the findings of various researchers who reported prevalence of BTB 4.2% in Yabello municipal (Biffa et al., 2010a), 4.5% in Hosaana abattoir (Teklu et al., 2004), 5.16% in Adama Municipality abattoir (Ameni and Wudie, 2003), 5.1% in Nekemte Municipality abattoir (Gudeta, 2008), 5% in Kombolch ELFORA abattoir (Desta, 2008), 6.4% in Mekelle town municipal abattoir (Zeru et al., 2013) and 6.79% in Adama municipal abattoir (Dechassa, 2014) as well as it was the same (5.8%) with research report of Romha et al. (2013) in western Tigray Zone. However, the total prevalence of this study was lower than previous studies carried out by other authors; 11.50% by Abdurohaman (2009) in Butajira, 9% Nemomsa et al. (2014) in Butajira abattoir, 8.8% by Biffa et al. (2009) in Hawassa municipal abattoir, 7.96% by Regassa (1999) in Wolayta, Southern Ethiopia and 24.7% (Biffa et al. 2009) in Adama municipal abattoir. On the other hand, the finding of this study was higher than the results of Regassa et al. (2010) in Hawassa municipal abattoir (1.1%), Gebremedhin et al. (2014) in Dilla Municipal Abattoir (2.6%), Asseged et al. (2004) in Addis Ababa (1.48%) and Shitaye et al. (2006) in Addis Ababa (3.46%). This lower prevalence recorded in the present study could be due to the fact that animals slaughtered in the abattoirs were mainly local breeds (Zebu) (675 out of 753) which are relatively resistant to BTB (Ameni et al., 2007). This the variations in prevalence could be due to the possible difference in the epidemiology of the disease in the animal populations, markets sources of animal (from which they brought to abattoirs either from high BTB prevalent or their local BTB burden), body condition score of the animals and types of production system; The intensive livestock management system could contribute the development of mycobacterial infections than the extensive livestock management system (Radostits et al., 2007; Ameni et al., 2007; Mamo et al., 2013).

Based on the post mortem inspection, the prevalence of TB lesions showed marked variation between the two abattoirs; the cases recorded in cattle slaughtered in Hawassa university abattoir was higher,
8.1% (31/384) than Hawassa municipal abattoir, 3.5% (13/369). Because most of the animal slaughtered at Hawassa University abattoir were from Boren area which might show the high prevalence in the source (Biffa et al., 2010b). The abattoirs have no effects in the development of the TB infection within that short period of time since TB is a chronic disease that needs long period of time.

The best evidence of the transmission route of M. bovis to cattle is the pattern of lesions observed in slaughtered animals (Phillips et al., 2003). In the present study, gross tuberculous lesions were found most frequently in lymph nodes of the thoracic cavity, 75% (33/44); followed by the lymph nodes of the head region, 13.64% (6/44) and the lymph nodes of the abdominal cavity 11.36% (5/44). The occurrence of tuberculous lesions in thoracic cavity was lower than the results of previous studies which reported greater than 84% TB lesions occurrence in the respiratory system (Corner, 1994; Neill et al., 1994; Collins, 1996; Whipple et al., 1996; Teklu et al., 2004); whereas it was higher than the report of Dechassa (2014) (67.7%), Firdessa (2006) (70%), Miliano-suazo et al. (2000) (49.2%) and Regassa et al. (2010) (50%). As a result, this study indicated the main route of transmission and infection being respiratory route and this finding agreed with the previous researchers who reported the same route of transmission and infection, respiratory route (Corner, 1994; Goodchild and Clifton-Hadley, 2001; Phillips et al., 2003; Ameni and Wudie, 2003; Teklu et al., 2004; Regassa et al., 2010; Tigre et al., 2012).

In the current study, the growth rate of mycobacteria on culture media was 54.5% (24/44). M. bovis grows poorly on standard LJ medium (Amanfu, 2006; Cleaveland et al., 2007). Furthermore, the presence of caseous and/or calcified lesions and miscategorizati-on of tuberculous lesions resembling nontuberculous lesions as tuberculous lesions (Teklu et al., 2004) may not always found to be of mycobacterial origin; viable mycobacteria may not be present in calcified lesions (Pritchard, 1988; Diguimbaye-Djaibe, 2006). However, the result of this study, 54.5%, was higher than the previous report (47%) of Ameni et al. (2010b), (23.6%) Araujo et al. (2005), (32%) Shimeles (2008), (35%) Müller et al. (2008) and (31.4%) Woyessa et al. (2014) culture positivity from the lesion positive samples; but this finding was found slightly the same with the report of (56%) Ameni et al. (2007).

RD4 deletion typing of the isolates from 24 animal tissue samples; only three isolates were confirmed to be M. bovis indicating product size of 446bp while the remained isolates were not showed any signal in RD4. Further molecular characterization using spoligotyping on the three M. bovis isolate has designated the isolates as strain SB1477. The outcome was revealed the same spoligotype patterns (clustered stating) were recognized among these M. bovis isolates and defined as of type SB1477 at the international spoligotyping database www.mbovis.org. This spoligotype pattern of the strains has similarities to the strains previously described in Addis Ababa farm, Ethiopia (Firdessa et al., 2012; Mekibeb et al., 2013). This similar spoligopattern show that the
strain has been circulating and transmitting in the cattle population of Borna and wolayta area. Warrants further investigation on their epidemiological and zoontic role in the area.

4. CONCLUSION AND RECOMMENDATIONS

The output of this study has indicated that an overall BTB prevalence of 5.8% of which 8.1% and 3.5% was found at Hawassa University abattoir and Hawassa municipal abattoir respectively. This could be indicating the presence of BTB infection in certain geographical areas. This research also revealed that the respiratory route was the major means of BTB transmission among the cattle population. The isolation and molecular characterization of this study confirmed that the clustered M. bovis strains (SB1477) circulating in the cattle population of the study area. In conclusion, in this study Gross pathological lesions, bacteriological culture, ZN staining and the molecular characterization findings indicated the occurrence of BTB in apparently healthy cattle in the study area and M. bovis has been confirmed as a causative agent of BTB with moderately low prevalence, a threat to livestock production and also for public health.

Basis of findings the present study the following points are recommended:

- Further investigation needed by involving large study areas for epidemiological information on circulating strains, ways of transmission and molecular diversity of the M. bovis strains.
- It's also better genus typing molecular characterization of the pathological lesion /isolates to identifying the presence of nontuberculous mycobacterium and mycobacterium tuberculosis complex in the study area.
- BTB control strategies should be designed to reduce the occurrence of BTB in livestock of Southern Region in particular and in Ethiopia in general.
- A proper postmortem meat inspection should be practiced efficiently in the abattoirs before taking beef to the retail markets to reduce the public health risk.

Authors’ Contributions

YT: collected the data, run the statistical and laboratory works and prepared the manuscript; GM: field supervision, assisted manuscript preparation and critical revision; DZ: Critically and thoroughly review the manuscript; GA: Financial support of the manuscript. All authors have read and approved the final manuscript. All authors read and approved the final manuscript.

ACKNOWLEDGEMENTS

This research was done under the Thematic Research Project of “Mycobacterial Infection in Selected Rural Communities of Ethiopia and their Animals,” a
collaborative research between College of Veterinary Medicine and Agriculture and Aklilu Lemma Institute of Pathobiology and funded by the Addis Ababa University Research and Technology Transfer office and I am thankful for the financial support obtained from the project.

Our Appreciation is run to all staff members of tuberculosis laboratory of the Institute of Aklilu Lemma Pathobiology for their genuine and cooperativeness during the laboratory work, especially Mr. Samuel Tolasa and Mr. Aboma Zewude.

We are grateful to acknowledge Hawassa University, the staff of Faculty Veterinary Medicine, Faculty of Animal and Range Management Science, Dairy Section and Hawassa University Slaughter House staff (particularly Mr. Abera Elias and W/o Emebet Abera) as well as our thank go to the staff of Hawassa city municipal abattoir specially Mr. Tarekegn Birhanu, Mr. Abera Sintayehu, Mr. Betiso Chuko and Dr. Eshetu Belayneh.

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