Mycobacterium Bovis Characterization through RD4 Deletion and Spoligotyping Techniques

Yibrah Tekle[1,3]*, Gezahegn Mamo[2] and Gobena Ameni[2]

[1]*Raya University, Maichew, Ethiopia*

[2]*Addis Ababa University, Bishoftu, Ethiopia*

[3]*Harbin Veterinary Research Institute, China*

*Corresponding author: Yibrah Tekle, Harbin Veterinary Research Institute, China

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Abbreviations: NTM: Nontuberculous Mycobacteria; MTBC: M. tuberculosis Complex; BTB: Bovine Tuberculosis; SNNPRs: Southern Nations Nationalities and Peoples Regional state

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 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 [1]. 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 [2-4]. Mycobacteria of the MTBC and NTM have been found to cause infections in immunecompetent and immunocompromised subjects and cause pathology in pulmonary and extrapulmonary sites [4-9]. Bovine Tuberculosis (BTB) is among the principal zoonotic diseases caused by *M. bovis* [10] which affects many vertebrate animals [11] and humans [12,13] and characterized by progressive development of granulomas/ tubercles in tissues and organs [14-16].

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 [17,18] and the most frequent cause TB in man [19]. *M. bovis* is most frequently isolated from domesticated cattle [20-23], 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. Bovine tuberculosis affects broad range of mammalian hosts including humans, cattle, deer, pigs, domestic cats, wild carnivores and omnivores [24]; it rarely affects equids or sheep [25]. Moreover, human TB of animal origin caused by *M. bovis* is becoming increasingly evident in developing countries [26,27]. 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.

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 [28]. 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 [29,30]. 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 [31]. In the areas where the bovine tuberculosis is common and the milk pasteurization is rare, *M. bovis* cases in human estimated 10-15 % [32,33]. Thus, in cattle the main route of infection transmission: aerosol, close contact between animals and ingestion of contaminated products [34-37].

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, 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. Due to inadequate comprehensive abattoir surveillance and lack of diagnostic facilities the BTB has limited information [38-40], particularly on genotypic characteristics of *M. bovis*, a strain affecting the cattle population in Ethiopia [41]. 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 [41-43]. 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

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 Northwest, 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. 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 bout 304,479.
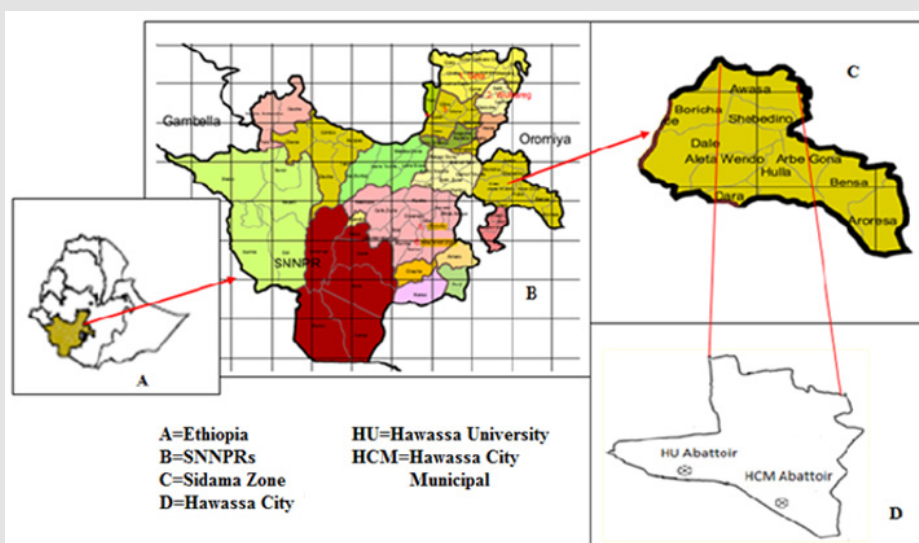

Figure 1: Geographical location map of Hawassa town.

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 [44] 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) (Figure 1).

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 was from Borena and Wolayita markets. All the study animals in this study were cross and local breed.

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.

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 formula:

$$n = \frac{1.96^2 \, x \, p_{exp}\left(1 - p_{exp}\right)}{d^2}$$

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. 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, P_{exp} 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.

Study Methodologies

Postmortem Examination: Postmortem examination had carried us according to the OIE and Meat Inspection and Quarantine Division of the Ministry of Agriculture method [45]. 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 tuberculosis 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 [46]. 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 -200c 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 -200c until bacteriological culture.

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 were 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 [47]. 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 [47]. Whenever, colonies were seen, ZN staining was performed to confirm the presence of Acid-fast bacilli [48-50]. 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 0C heat for 45 minutes, by mixing two loops full of colonies in 200μl distilled water [51-54]. The frozen and heat killed isolates were stored at -20 0C for future Mycobacteriology and further molecular typing analysis was performed at Akililu Lemma Institute of Pathobiology according to the standard developed by [54-59].

Region of Difference (RD)-4 Deletion Typing: For the RD4 deletion typing, the procedure described by Cadmus 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* [55]. 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 1minute 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).

Spoligotyping: Spoligotyping was carried out using the commercially available kit according to the manufacturer's instructions and as previously described by Kamerbeek. 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 and1 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.

Statistical Analysis: Prevalence was calculated as the proportion of suspected lesion positive animals from the total number of animals sampled [60]. 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 Spoligotyping database website http://www.mbovis.org. to retrieved the strain type.

Results

Distribution of Gross Pathological Lesions and Prevalence

The overall prevalence of BTB was 5.84% (95%CI: 4.58 - 8.42), upon detailed postmortem 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 2). 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. 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).
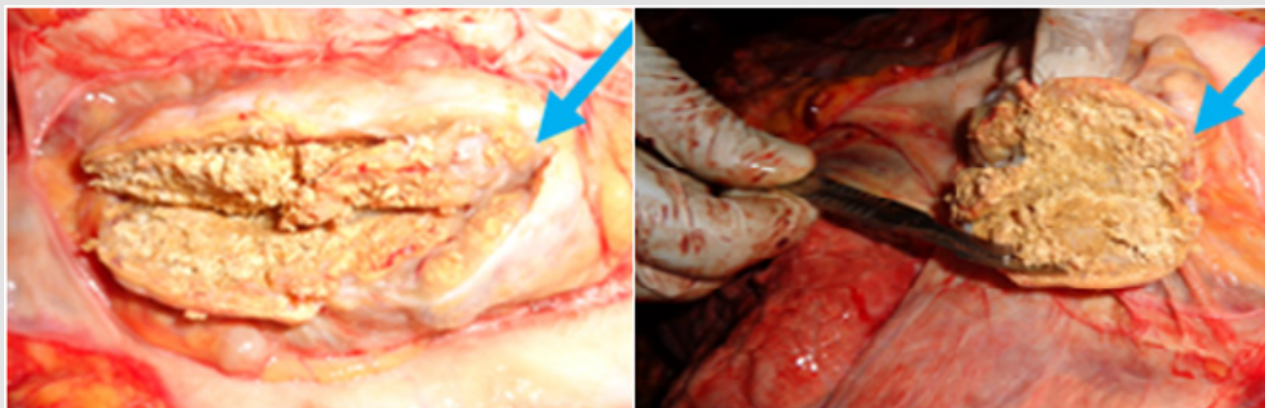
Figure 2: The typical TB lesions of cattle slaughtered in Hawassa abattoirs (Calcified, Caseous and Necrotic Granulomatous lesions in mediastinum lymph nodes, indicated by arrow).

Table 1: Lesion distribution in different organs and anatomic parts.

Anatomical Site	Affected organs	Frequency (%)	Total (%)
Head	Retropharyngeal LN	4 (9.09%)	6(13.64%)
	Mandibular LN	2(4.5%)	
Thorax	Mediastinal LN	31(70.45%)	33(75%)
Abdomen	Mesenteric LN	5(11.36%)	7(15.91%)
	Livers	2(4.55%)	
Total		44(100%)	44(100%)

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 3) 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).

Table 2: Mycobacteriological culture result from suspected TB lesion on LJ medium and ZN staining positivity.

Abattoirs	No. of Sampled	Lesions (%)	Growth on LJ medium			ZN stain positive (%)
			LJP	LJG	Both	
Hawassa University Abattoir	384	31(8.07)	9	6	2	17(70.83)
Hawassa City Municipal Abattoir	369	13(3.51)	2	3	2	7(29.17)
Total	753	44(5.84)	11	9	4	24(100)

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

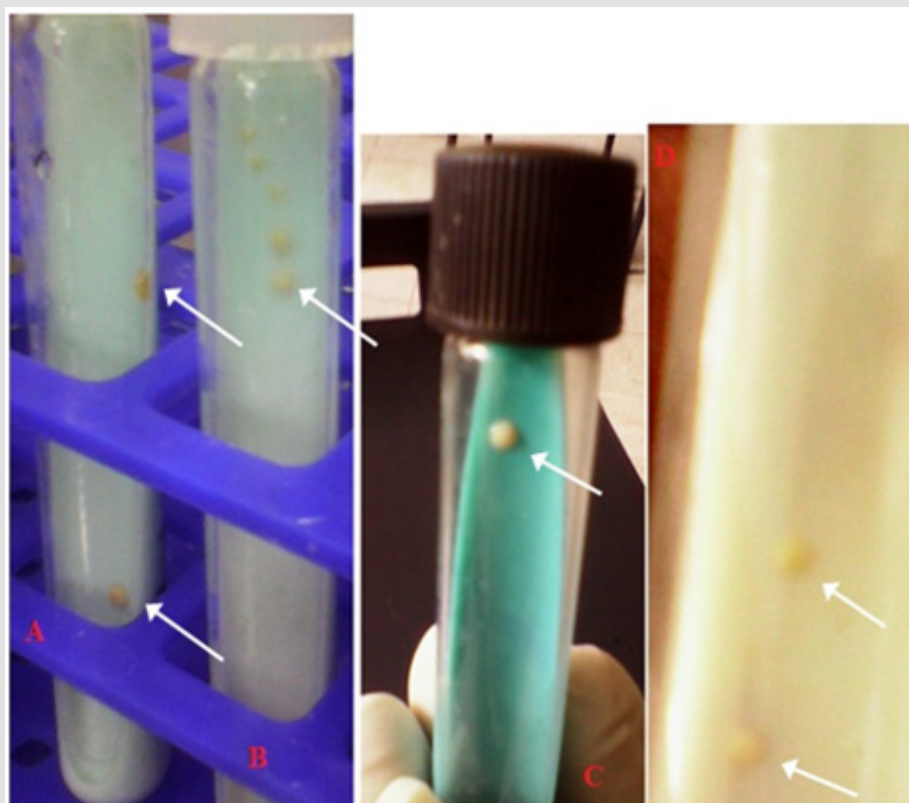| No of Isolates	Source	Time to appear in week		Colony characteristics
		LJP	LJG	
5	Cattle	3		Pinpoint, smooth, whitish, yellowish
13	Cattle	4	5	Pinpoint, smooth, whitish, yellowish
6	Cattle		6	Pinpoint, smooth, whitish, yellowish

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).
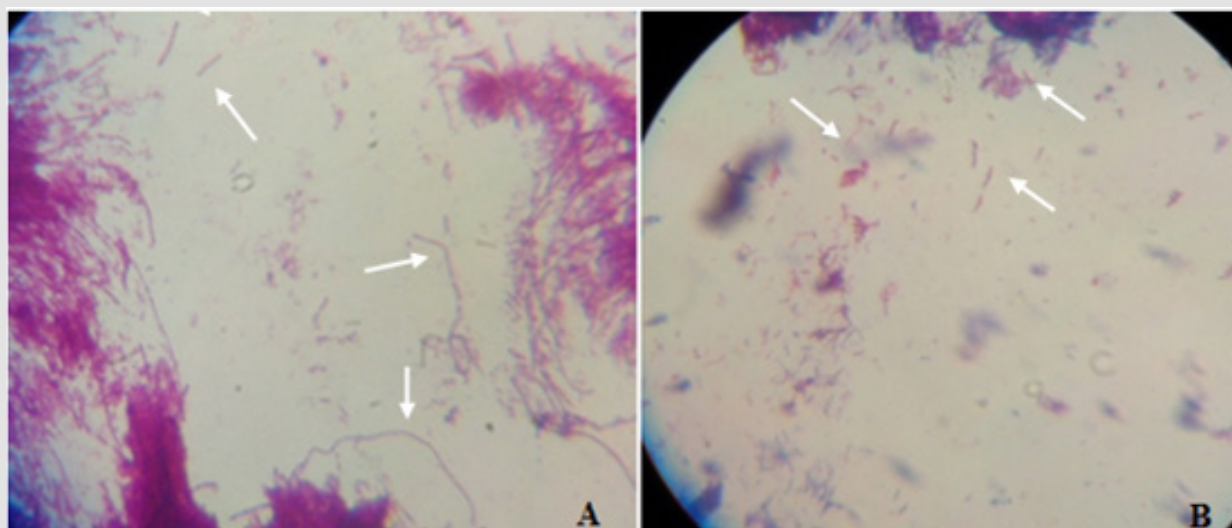

Figure 4: Acid fast bacilli staining positive isolates from mycobacteriological culture colony grows from lymph nodes TB lesion (arrows indicate acid fast bacilli which a short and long, in single or clump).

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 the Spoligotyping of the three *M. bovis* isolates, which were positive in RD4 deletion, showed the typical *M. bovis* Spoligotyping pattern with similar pattern and based on the database website http://www.mbovis.org analysis of the Spoligotyping results isolates were identified as SB1477 (Figure 6).
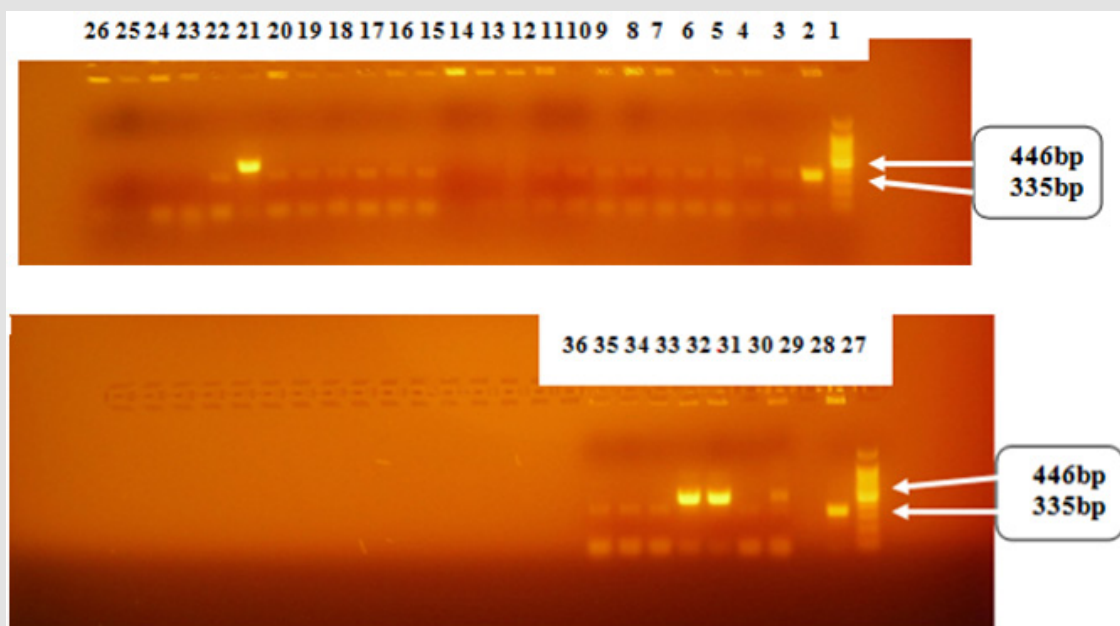
Figure 5: Electrophoretic separation of PCR products by RD4 deletion typing of mycobacteria isolates from 24 isolates sampled culture. Lane 1 and 27- Ladder (100bp), Lane 2 and 28- *M. tuberculosis* H37Rv positive control, Lane 3 and 29- Distiled water negative control, Lane 4 and 30- *M. bovis* SB1176 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*.

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 sample.

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% [61-63]. 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 [64], 4.5% in Hosaana abattoir [65], 5.16% in Adama Municipality abattoir [66,67], 5.1% in Nekemte Municipality abattoir [68], 5% in Kombolch ELFORA abattoir [69], 6.4% in Mekelle town municipal abattoir [70] and 6.79% in Adama municipal abattoir [71] as well as it was the same (5.8%) with research report of Romha 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 in Butajira, 9% [72] in Butajira abattoir, 8.8% by Biffa in Hawassa municipal abattoir, 7.96% by Regassa (1999) in Wolayta, Southern Ethiopia and 24.7% [73] in Adama municipal abattoir.

On the other hand, the finding of this study was higher than the results of Regassa in Hawassa municipal abattoir (1.1%), Gebremedhin in Dilla Municipal Abattoir (2.6%), Asseged in Addis Ababa (1.48%) and Shitaye 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 [74]. 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 [75-78]. Based on the postmortem 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 [79]. 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 [80]. 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 [81-85]; whereas it was higher than the report of Dechassa (67.7%), Firdessa (70%), Miliano-suazo (49.2%) and Regassa (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 [86-90]. 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 [91]. Furthermore, the presence of caseous and/or calcified lesions and miscategorization of tuberculous lesions resembling nontuberculous lesions as tuberculous lesions may not always found to be of mycobacterial origin; viable mycobacteria may not be present in calcified lesions.

However, the result of this study, 54.5%, was higher than the previous report (47%) of Ameni, (23.6%) Araujo, (32%) Shimeles,

(35%) Müller and (31.4%) [51] culture positivity from the lesion positive samples; but this finding was found slightly the same with the report of (56%). 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 Spoligotyping 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 Spoligotyping pattern of the strains has similarities to the strains previously described in Addis Ababa farm, Ethiopia. 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.

Conclusion

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:

a) 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.

b) It's also better genus typing molecular characterization of the pathological lesion /isolates to identifying the presence of nontuberculousis mycobacterium and mycobacterium tuberculosis complex in the study area.

c) BTB control strategies should be designed to reduce the occurrence of BTB in livestock of Southern Region in particular and in Ethiopia in general.

d) 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.

Acknowledgement

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