Molecular Epidemiology of Mycobacterium Tuberculosis Complex at Nekemte Municipality Abattoir, Western Ethiopia

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Abstract
A cross-sectional study was conducted at Nekemte Municipality Abattoir from September 2009 to May 2010 to estimate the prevalence of bovine tuberculosis (BTB), and characterize its causative agents. Post mortem examination, bacteriological culturing, Ziehl-Neelsen staining, multiplex polymerase chain reaction (PCR), and region of difference-4 (RD4) deletion typing were used for investigation. Cattles (1168) were recruited for the study and the prevalence was found to be 5.9% (70/1186) on the basis of gross lesion. 70% of the gross lesion was detected in the thoracic cavity while 25% of the lesion was found in the abdominal cavity. Only 31.4% (2270) of the suspicious lesions yielded colonies of which 19 were acid-fast positive. Further identification of these 19 isolates using multiplex PCR revealed that 17 isolates belong to the Genus Mycobacterium while the remaining two isolates did not show signal to the Genus. Of the 17 isolates that showed signal to the Genus Mycobacterium, 7 were members of Mycobacterium tuberculosis (M. tuberculosis) complex while the remaining 10 isolates were members of the non-M. tuberculosis complex. Further identification and characterization of the M. tuberculosis complex members using RD4 deletion typing identified four isolates with intact RD4 which could be either M. tuberculosis or M. africanum and three isolates with deleted RD4 thus confirmed to be M. bovis. In conclusion, BTB lesions were caused by both the members of M. tuberculosis complex and the non M. tuberculosis complex Mycobacteria. Hence, as the majority of the isolates was the non M. tuberculosis complex members, the pathogenecity of these members in cattle needs further study.

Keywords
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tuberculosis as well as infection of the cervical and mesenteric lymph nodes (Daborn and Grange, 1993; Edelsten, 1996).

Diagnosis of BTB is helpful to reduce the risk of zoonosis, together with increasing public awareness and proper hygiene in food chain from animal source which may result in eradication (Acha and Szyfres, 2001). Therefore definitive diagnosis can be achieved through isolating and typing the ethological agent through different diagnostic techniques (Acha and Szyfres, 2001).

The advent of genetic engineering has provided alternative DNA based strategies, which have the potential to overcome non-specific reactions. The development of agarose electrophoresis is for the size separation of DNA fragment, followed by the variability of restriction endonucleases which cleave DNA at defined sites lead to the development of restrictionfragment analysis of bacterial DNA; this technique produce a pattern of fragments or finger prints, which uniquely characterizes the strain from which DNA was isolated (Butcher et al., 1996).

Although national data is not available on the prevalence of BTB in Ethiopia, it is assumed that the incidence of the disease is rising because of the present private-oriented economic policy of the government, which thereby promotes the expansion of dairy industry. A few studies have been conducted in central highlands of Ethiopia on the epidemiology of BTB, and the results of such studies have indicated that the disease is prevailing in these areas (Ameni et al., 2003a, and Asseged et al., 2001). Such studies were carried out most commonly using tuberculin skin testing, abattoir meat inspection and rarely on bacteriological techniques. Therefore, expansion of similar studies to the untouched regions of the country will be useful towards made to establish the epidemiology of the disease at the national level. Moreover, complementing such studies with the application of molecular tools so as to identify and characterize the species and strains of Mycobacteria that are pathogenic to cattle is of paramount importance. The current study was formulated to estimate the prevalence, to evaluate the distribution of lesion and to isolate and characterize Mycobacteria from suspicious TB lesions slaughtered at Nekemete Municipality Abattoir, Western Ethiopia.

MATERIALS AND METHODS

Study Area

The study was conducted from September, 2009 to May, 2010 in Western part of Ethiopia at Nekemete municipality abattoir. The town (Nekemte) is located at 331 km West of Addis Ababa. The approximate geographical location of the area is between 9° 4’ 7N to 9° 11’ 02N and 36° 30’E to 36° 43’ 02E. The altitude is from 1500m to 2565m above sea level and the maximum temperature is 27.4°C and the minimum temperature of the area is about 10.2°C. The mean annual rainfall of the area ranges from 1600mm to 2000mm. The area receives long heavy rainy season from June to September and short rainy season from March to May. Among the various soil types in the area, the red brown soil with a PH ranging from 5-7 is the predominant type of soil in the Zone. The area is rich in natural vegetation that comprised of the tropical rain forest tree, all grasses and brushes.

The abattoir is the only source of inspected beef in the town. The overall hygiene of the abattoir, including the drainage, water, lighting and condemned organ and carcass disposal system is medium. Animals from different origin of the surrounding district are the source for slaughtering.

Study Design, Sample Collection and Transportation

Cross-sectional study was followed for the survey to determine the prevalence of BTB in the study area. In a slaughter house about 10 heads of cattle on average was randomly selected from the total of animals slaughtered per day. Tissue lesion samples suspected to be positive for BTB was collected aseptically from the lung lobes, lymph nodes of the head, lung, intestine and other tissue and organs. These samples collected from the abattoir were kept at 4°C in the refrigerator for 5-10 days and transported to Akilu Lemma institute of Pathobiology in ice box packed with ice packs to keep the low temperature during transportation for culture.

The sample size calculation was based on 50% prevalence assumption; 95% CI and P=0.05 (Thrusfeld, 2005). Therefore, the sample size calculated was 384.

\[ n = \frac{Z^2 \times p \times (1 - p)}{d^2} \]

Where \( n \) = required sample size  
\( p \) = expected prevalence  
\( d \) = Desired absolute precision (5 %)  
\( Z \) = Normal distribution constant

Ante Mortem Examination

Physical examination of the animals were carried out before they were slaughtered. Body temperature, pulse rate, respiratory rate, condition of superficial lymph nodes and visible mucus membranes were examined and recorded for individual animals to be slaughtered. Breed and sex was also recorded. Age was estimated as described by Amstutz, 1998 and Body Condition Scoring (BCS) chart was made based on the description by Nicholson and Butterworth, 1986.

Post Mortem Examination in Abattoir

Meat inspection for TB lesion detection was conducted in accordance with the method developed by meat inspection and quarantine division of the ministry of Agriculture (MOA). It involves palpation and incision of the lung, liver and udder, visual inspection of the kidney, palpation and incision of the trachobronchial, mediastinal, prefemoral and prescapular lymph nodes. If lesion is recovered in the above tissue, other lymph nodes and tissues are incised (Teklu et al., 2004). Lymph nodes were sliced in to thin section of 2 mm and other tissues were cut in to slices of 2cm using separate sterile surgical blades. The cut surface was examined under a bright light source for the presence of abscess and tubercules (Patterson and Grooms, 2000; Asseged et al., 2004). In the presence of suspected tuberculous lesions tissue sample were collected in the universal bottles containing normal saline (0.85%) for culture. In the presence of lesions in different tissues of single animal, pooled sample for each animal collected.

Isolation and Identification

Tissue samples were transported in the cold chain using ice box packed with ice packs to keep the low temperature. Then the tissue samples were macerated in sterile mortar and pestle using surgical blades and forceps to get fine pieces and then homogenized for 10 minutes in 5 ml of normal saline. Two ml of homogenate were
transferred to centrifuge tube and decontaminated with equal volume (2ml) of 4% NaOH for 15 minutes; centrifuged at 3000rpm for 15 minutes and neutralized by 1% HCL, employing phenol red as an indicator. Neutertiates M. tuberculosus complex when the suspension M. changed from purple to yellow. After neutralization, 0.1 ml of the suspension from each sample was spread on slants of Lowenstein-Jensen (LJ). Each sample was inoculated on to the set of Lowenstein-Jensen (LJ-tyrpyvate) and glycerol (standard LJ). Cultures were incubated aerobically at 37 °C for up to 12 weeks with weekly observation for growth. When visible colonies were observed, Zeil-Neelesen staining was performed to confirm the presence of acid fast bacilli (WHO, 1998; Quinn et al., 2002).

Microscopic Examination
A direct smear was prepared from pure colony grown on L-J media and stained using the Ziehl-Neelesen acid-fast staining technique; the heat fixed smear were stained with carbol-fuchsin, heated gently and allowed to stand for 10 minutes. The stain was then poured off and the smears washed with tap water and then decolorized with acidic alcohol for 1 minute, each with the slides being washed under tap water between each step. The smears were then counter stained with methylene blue for 3 minutes, dried and examined for the presence of AFB under light microscope employing a 100X oil immersion objective (Quinn et al., 2002).

Molecular Characterization of Mycobacteria Polymerase Chain Reaction (PCR)
Initial identification of Mycobacterial species was based on the rate of growth, pigment production and colony morphology. For further characterization of the species molecular technique was used. The Mycobacterial cell from culture colony was killed in 80°C water bath for 1 hour and the DNA was extracted according to ALIPB-TB-Molecular biology/Immunology laboratory standard operation procedure (SOP) from the Mycobacterial culture isolates (Jordan and Victor, 2002).

Multiplex Polymerase Chain Reaction (m-PCR)
For multiplex PCR, the procedure described by Wilton and Cousins (1992) was followed. This multiplex PCR differentiates M. tuberculosis complex from M. avium species, M. intracellularae and other Mycobacterial species, and either heat-killed bacterial suspensions or extracted DNA was used. The PCR targets the sequence of the Genus Mycobacterium within the 16S rDNA gene (G1, G2), sequences within the hypervariable region of 16S rDNA that is known to be specific to M. intracellularae (MYCINT-F) and M. avium (MYCAY-V), and the MTB70 gene specific for M. tuberculosus complex (TB-1A, TB-1B). The primers used were MYCGEN-F, 5’AGA GTT TCA TCC TGG CTC GA 3’; (35ng/ul), MYCGEN-R, 5’-TGG ACA CAG GCC ACA AGG GA 3’; (35ng/ul); MYCAY-F, 5’-ACC AGA CAT GCC TCT TG 3’; (35ng/ul); MYCINT-F, 5’-CCT TTA GGC GCA TGT CTT TA 3’; (75ng/ul); TB1-F, 5’-GGA CAA TCC GGA GTT GAC AA 3’; (20ng/ul); TB1-R, 5’-AGC ACG CTG CAT ATC AGT AG 3’; (20ng/ul). The reaction was carried out using Thermal Cycler. The mixture was heated for 10 min at 95 °C, further 35 cycles of 1 min at 95 °C, 1 min at 61 °C, and 1.5 min at 72 °C; and 10 min at 72 °C. Each PCR tube consisted of 5.2µl H2O Qiagen, 8µl HotStarTagMasterMix, 0.3µl of each of the six primers (concentration given above), 5 µl of DNA templates of samples or controls making the total volume 20µl. M. avium, M. intracellularae, H37Rv and 212/97 (M. bovis strain) were used as positive controls while H2O Qiagen, was as a negative control. The product was electrophoresed in 2% agarose gel in TAE running buffer. SYBR Safe at a ratio of 1:10 in 2% agarose gel, 100bp DNA ladder, and orange 6x loading dye were used in gel electrophoresis. All members of the Genus Mycobacterium produce a band of 1030bp, M. avium or subspecies such as M. avium subspecies paratuberculosis produces a band of 180bp, M. intracellularae a band of 850bp while members of M. tuberculosus complex produce a band with 372bp.

RD4 Deletion Typing
The RD4 deletion typing was applied to isolates that showed band for M. tuberculosus complex by multiplex PCR. The primers used were RD4infF, 5’-ACA CGC TGG CGA AGT ATA GC-3’; RD4flankR, 5’-AAG GCC AAC AGA TTCAGC AT-3’; and RD4flankK, 5’-CTC GTA GAA GGC CAC TAA AG-3’. The mixture was heated in Thermal Cycler for 15 min at 95 °C, and then subjected to 35 cycles of 1 min at 95 °C, 1 min at 55 °C, and 1 min at 72 °C; and 10 min at 72 °C. Each PCR tube consisted of 7µl H2O Qiagen, 10µl HotStarTagMasterMix, 0.3µl of each of the six primers (concentration), 2µl of DNA templates of samples or controls making the total volume 20µl. H37Rv and 212/97 (M. bovis strain) were used as positive controls while H2O Qiagen, was used a negative control. The product was electrophoresed in 1.5% agarose gel in TAE running buffer 10X. SYBR Safe at a ratio of 1:10 in 2% agarose gel, 100bp DNA ladder, and orange 6x loading dye were used in gel electrophoresis. The gel was read using SYNGENE BIO IMAGING SYSTEM (A Division of Syoptics Group). The presence of RD4 (i.e. M. tuberculosis and M. africanum) gives a product size of 335bp (RD4 intF + RD4flankR) its absence (M. bovis) gives a product size of 446bp (RD4flankF + RD4flankR).

Data Analysis
During the study, individual animal identification number, place of origin, breed, sex, age, organ or tissue affected at abattoir and cultural, staining, PCR and spoligotyping results of laboratory work were entered into MS Excel data sheets. Then, coded and were analyzed using SPSSS version 16 statistical software. The prevalence rate was calculated by dividing the proportion of cattle found infected (either positive reactors or harbouring tuberculous lesions) by the total number of cattle tested or whose carcasses is inspected multiplied by 100%. The risk factors associated with M. bovis infection were calculated by using Chi-square ($\chi^2$) and logistic regression. Odds Ratio (OR) was assessed to investigate the strength of association. A statistically significant association between variables was said to exist if the calculated P<0.05. For the analysis of the effect of different risk factors on bovine tuberculosis status of animals, doubtful reactors were not considered as positive (Thrusfield, 2005).

RESULTS
Results of Abattoir Survey
The prevalence of BTB was investigated and was found to be 5.9 % in abattoir-based surveillance. As shown below, the prevalence of BTB in different district of the study area was different. High prevalence was observed in Guto-gida (11.5%) and low prevalence was recorded in Wayu-Tuka. In the remaining part of animal origin the prevalence is almost similar (Figure 1).
From the risk factors considered (Table 1), only breed was found significantly associated with BTB infection \((P<0.05)\) and the rest were not found significantly associated with the presence of gross tuberculous lesions \((P>0.05)\).

**Table 1:** Level of association of various host and environmental risk factors with *M. bovis* infection

| Variables | Number of animals | Positive | Negative | Total (% of positive) |  \(\chi^2\) | OR | OR 95% CI | P-Value |
|-----------|-------------------|----------|----------|-----------------------|-------------|----|-----------|---------|
| 1. Age    |                   |          |          |                       |             |    |           |         |
| 1-5 yrs   | 2                 | 26       |          | 28 (7.1)              |             |    |           |         |
| 5-10 yrs  | 57                | 960      |          | 1017 (5.6)            | 1.560       | 0.899 | 0.187-4.336 | 0.456   |
| >10 yrs   | 11                | 130      |          | 141 (7.8)             |             |    |           |         |
| 2. BCS    |                   |          |          |                       |             |    |           |         |
| Poor      | 11                | 133      |          | 144 (7.8)             | 2.662       | 0.742 | 0.254-1.993 | 0.262   |
| Medium    | 52                | 909      |          | 961 (5.4)             |             |    |           |         |
| Good      | 7                 | 74       |          | 81 (3.6)              |             |    |           |         |
| 3. Breed  |                   |          |          |                       |             |    |           |         |
| Local     | 68                | 1115     |          | 1183 (5.7)            | 19.995      | 1   | 0.004-0.723 | 0.010   |
| Cross     | 2                 | 1        |          | 3 (6.7)               |             |    |           |         |
| 4. Sex    |                   |          |          |                       |             |    |           |         |
| Female    | 10                | 134      |          | 144 (6.9)             | 0.321       | 1.127 | 0.554-2.292 | 0.571   |
| Male      | 60                | 982      |          | 1042 (5.8)            |             |    |           |         |
| 5. Origin |                   |          |          |                       |             |    |           |         |
| High Land | 15                | 234      |          | 249 (6)               | 0.008       | 1   | 0.482-1.676 | 0.881   |
| Low Land  | 55                | 882      |          | 937 (5.9)             |             |    |           |         |
| Total     | 70                | 1116     |          | 1186 (5.9)            |             |    |           |         |

\(\chi^2\) = chi square; OR= Odd’s ratio; CI= confidence intervals

The distributions of tuberculous lesions in tissues of positive animals were presented in Table 2. The entire lesions observed were localized lesions involving frequently a single organ. A high proportion (70%) of the lesions was located on the thoracic cavity lymph nodes, while 25.7 and 4.3% of the lesions were found in the abdominal cavity and head regions respectively.

**Table 2:** The distribution of tuberculous lesion in the tissues of infected animals

| Region of the body | Anatomic site         | Number of infected tissue | % of positive |
|--------------------|-----------------------|---------------------------|---------------|
| Head               | Mandibular LN         | 2                         | 2.9           |
|                    | Retroparyngeal LN     | 1                         | 1.4           |
|                    | Total                 | 3                         | 4.3           |
| Thoracic cavity    | Cranial mediastinal LN| 21                        | 30.0          |
|                    | Caudal mediastinal LN | 14                        | 20.0          |
|                    | Left bronchial LN     | 11                        | 15.7          |
|                    | Right bronchial LN    | 3                         | 4.3           |
|                    | Total                 | 49                        | 70            |
| Abdominal cavity   | Mesenteric LN         | 17                        | 24.3          |
|                    | Hepatic LN            | 1                         | 1.4           |
|                    | Total                 | 18                        | 25.7          |

LN= Lymph node
Bacteriology

From 70 tuberculous suspected tissue samples on postmortem inspection, that transported ALIPB, at different laboratory entry batch, showed 31.4% (22/70) growth on primary culture media. The outcome of the culturing activity is indicated in Table 3. Among those cultures which showed visible grows, only 31.8% (7/22) were on L-J media enriched with pyruvate and the rest 68.2% (15/22) were on L-J media enriched with glycerol (Table 3).

Table 3: Cultural result of tuberculous tissues from slaughtered cattle

| Sample type   | L-J media with Glycerol | L-J media with Pyruvate |
|---------------|-------------------------|-------------------------|
|               | Total | Positive (%) | Total | Positive (%) |
| Bronchial LN  | 14    | 2(14.3) | 14    | 1(7.1) |
| Mediastinal LN| 35    | 8(22.9) | 35    | 5(14.3) |
| Mesenteric LN | 17    | 5(29.4) | 17    | -     |
| Mandibular LN | 2     | -     | 2     | 1(50) |
| Retropharyngeal LN | 1 | -     | 1     | -     |
| Hepatic LN   | 1     | -     | 1     | -     |
| Total        | 70    | 15(68.2) | 70    | 7(31.8) |

The total of 22 grown cultures were subjected to Ziehl-Neelsen staining technique in the laboratory in order to check for the presence of acid fast bacilli organisms. Out of these, only 13.6% (3/22) were acid fast negative. The remaining 19 grown culture media were confirmed for the presence of acid fast bacilli.

Molecular Analysis

From 19 cultures, positive colonies obtained were from tuberculous tissue samples, up on multiplex PCR, 17 of them showed for the presence of the genus *mycobacteria*. The remaining 2 isolats didn’t show the amplification products characteristic to *mycobacterium* (Figure 2), by using the genus specific primers MYCGEN-F (5’-AGA GTT TGC CCC TGC AG3’) and MYCGEN-R (5’-TGC ACA CAG GCC AGA AGG GA-3’).

RD4 Deletion Typing

The result of m-PCR, which showed band were subjected to RD4 deletion typing. The PCR reaction gave a product size of 335bp for 4 isolates indicating the presence of RD4 and hence the isolates could be either *M. tuberculosis* or *M. africanum*. A product size of 446 bp was produced for 3 isolates indicating the absence of RD4 and therefore, it was confirmed to be *M. bovis* (Figure 3).
The overall prevalence of BTB (5.9%) obtained in the current abattoir survey was high when compared with Regassa et al. 2009 (1.1%) at Hawassa, Asseged et al., 2004 (1.48%) in Addis Ababa, Shitaye et al., 2006 (3.46%) in Addis Ababa and Teklu et al., 2004 (4.53%) at Hossana through similar diagnostic methods but in consistence with previous reports of Ameni and Wudie, 2003b (5.16%) from Adama Municipality abattoir, Gudeta, 2008 from Nekemte Municipality abattoir (5.1%) and Desta, 2008 (5%) at Kombolch meat processing plant, Southern Wallaw, based on postmortem inspection. However, the presently recorded prevalence was low as compared to previous report by Shimels, 2008 at Debre Birhan, Central Ethiopia and Regassa, 1999 (7.96%) at Wolaita Sodo. The infection rate in cattle has been found to differ greatly from place to place (Shitaye et al., 2006) and the difference might be most probably linked to the type of production system (most notably in extensive), which is unlikely to favor the spread of the disease in contrast to the intensive dairy farms as cited by Ameni et al., 2006 and Shitaye et al., 2006.

There was statistical significant ($\chi^2 = 19.9$ and $P = 0.010$) difference between breed of the animal and tuberculous lesion. Regassa et al., 1994 has indicated that Zebu breeds are relatively resistant for BTB than exotic breeds. The remaining assumed risk factors both in abattoir survey and comparative intradermal test revealed no statistical significant difference. The possible reason might be due to in proportionality in the number of the animal compared in specific variable. For example, less number of female animals were come to the study abattoir to be slaughtered and the proportion of very young animal and animal with lean body condition encountered in abattoir was low. However, there is biological association as observed from the Odd’s ratio value.

In the present study, gross tuberculous lesions were found most frequently in lymph nodes of the thoracic cavity (70%); followed by lymph nodes of the abdominal cavity (25.7%) and the lesser frequency was found in the lymph nodes of the head region (4.3%). The occurrence of tuberculous lesions in thoracic cavity was lower than the results of previous studies, when greater than 90% occurrence of TB lesions in the respiratory system was reported in developed countries (Neill et al., 1994; Collins, 1996; Whipple et al., 1996). However it is higher than the report of Regassa et al., 2009 which implies that inhalation is the most important route of infection. Husbandry factors such as enclosures of the animals overnight may facilitate respiratory transmission of the infection (WHO, 2005). The followed high proportion in lymph nodes of the abdominal cavity indicate, the other important route of transmission is ingestion of the agent which may happen during the sucking time from the infected dams or by licking, feeding or drinking contaminated materials (Hardie and Watson, 1992; Morris et al., 1994; Smyth et al., 2001).

In the current study, the chance of growing Mycobacteria was less than 50% which might be due to either loss of the agent during freezing or delayed transportation from the site of collection. WHO (1998) indicated loss of 5-10% due to contamination resulting from prolonged preservation, which in turn resulted into overgrowth of M. bovis with environmental Mycobacteria and a loss of up to 60% due to decontamination procedure. Besides, M. bovis grows poorly on standard Löwenstein-Jensen medium (Cleaveland et al., 2007). Therefore, the use of proper time in culturing and application of standard laboratory technique could increase the chance of recovery of acid fast bacilli. However, the result was higher than the previous report (Araju et al., 2005) were only 17 isolates obtained from 72 (23.6%) lesion positive samples and in agreement with the report (32%) of Shimels, 2008.

Molecular analyses of the isolates were revealed that the lesions were caused not only by the members of M. tuberculosis complex but also by members of the non- M. tuberculosis group. Even majority of the isolates belonged to the later group. Similar nodules (70%) were identified by other workers in Ethiopia (Berg et al., 2009). The techniques employed by the present study could not further identify these isolates to the species level.

CONCLUSIONS

The result of the present study has shown that bovine tuberculosis is prevalent in cattle slaughtered at Nekemet Abattoir. Even though, majority of the isolates were non-M. tuberculosis members, it was observed that the lesions were caused by Mycobacteria that belongs to both M. tuberculosis complex and non-M. tuberculosis complex group. This may be due to contamination of an environment in which they belong (particularly pasture and water body), through faces and air droplet. In addition to this people of the area have the habit of consuming raw meat and milk and share the same microenvironment with their livestock. This further disseminates the causative agent, both through inhalation and ingestion resulting in high economic loss and public health effect. This study reveals a high proportion of tuberculous lesion in the thoracic cavity lymph nodes (70%). It implies that other respiratory rout is the major means of transmission. Similar studies in the slaughterhouse across the country so as to estimate the national prevalence of BTB as well as identification and characterization of the non-M. tuberculosis complex, and evaluation of their pathogenicity in bovine is essential. Detailed abattoir inspection should be implemented by focusing on lymph nodes of the thoracic, mesenteric and head region. Finally Public education to increase the awareness of the community about the potential risk of consumption of raw animal products is necessary.

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