Conventional and Molecular Epidemiology of Bovine Tuberculosis in Dairy Farms in Addis Ababa City, the Capital of Ethiopia.

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ABSTRACT

A cross-sectional investigation of Bovine Tuberculosis (BTB) was conducted on 1132 dairy cattle from 56 herds using comparative intradermal tuberculin test (CIDT), postmortem examination, bacteriological culturing, and molecular typing of mycobacterium isolates involving spoligotyping and variable number of tandem repeat (VNTR). Herd and individual BTB prevalence rate was found to be 53.6% (95% CI=39.74- 67.00) and 34.1% (95% CI =31.33-36.92) respectively. The individual animal prevalence was significantly affected by both herd size ($\chi 2$ =10.9, $p<0.001$) and age of the animals ($\chi 2$= 56.4, $p<0.001$). Moreover, a strong positive correlation ($r=0.89$, $p<0.001$) was observed between herd size and number of reactor animals. However, there was no statistically significant association ($P>0.05$) between reactor herd and years of establishment of the farm, origin and status of the animal. Typical tuberculous lesions were detected on 72.7% (8/11) of sacrificed tuberculin reactors animals and mycobacterial isolates were recovered by culturing from 100% (11/11) of tissue specimens. Among these, eight isolates were M. bovis while three were M. tuberculosis. All M. bovis isolates were further classified into three different spoligotyping patterns and two different VNTR profiles. In conclusion, the result of the present study showed high prevalence of BTB in Addis Ababa City dairy farms, and the identification of three M. bovis spoligotype pattern from eleven animals only merely suggests a possible presence of more strains in the region.

INTRODUCTION

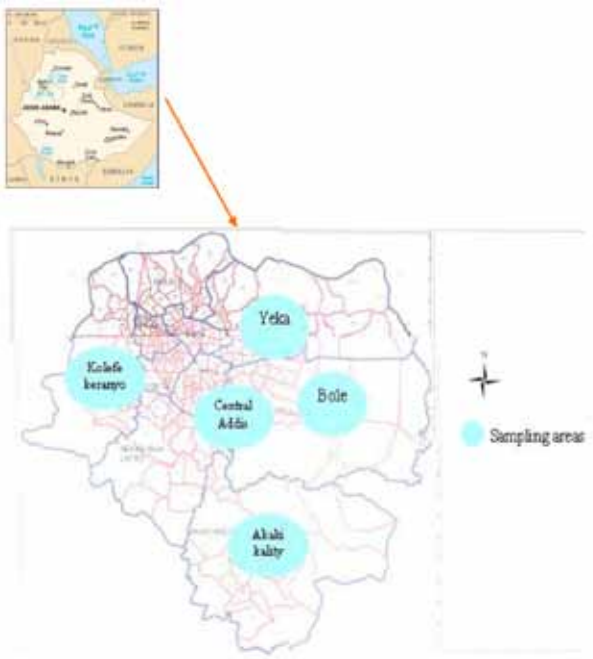
Human and animal tuberculosis are widespread in Africa and are caused with very close genetic and antigenic organisms: M. tuberculosis and M. bovis, respectively both cause identical and clinically indistinguishable disease in humans.[1] The unusually extensive host range of M. bovis, represents a very significant economic problem in numerous countries in both developed and the developing world.[2-4]

Human tuberculosis (TB) of animal origin (zoonotic TB) is an important public health concern in developing countries.[5] More than 94% of the world population lives in countries in which the control of bovine tuberculosis in cattle is limited or absent.[6] In countries where bovine tuberculosis (BTB) is still common and pasteurization of milk is not practiced, an estimated 10–15% of human TB is caused by M. bovis.[7] Rural inhabitants and some urban dwellers in Africa still consume unpasteurized and soured milk potentially infected with M. bovis.[3] Human infection due to M. bovis is thought to be contracted mainly through drinking raw milk[8]; however, cases of pulmonary TB have also been reported particularly in patients from rural areas that live in close contact with cattle.[9,10]

Urban livestock production constitutes an important sub-sector of the agricultural production system in Ethiopia. Specifically in Addis Ababa, with the response to increased demand for milk and milk products linked to growth of human population, relatively large numbers of dairy farms (about 5200) are found in the city and its surrounding with intensive management system. Unfortunately, BTB has shown close links with intensive management system.[11,12] The knowledge of the epidemiology of the disease is a footstep for designing any control strategy to limit the disease spread and to avoid risk for human infection.

Molecular epidemiology has become a very powerful tool in the study of M. tuberculosis and human tuberculosis, where it has been exploited to provide 'added value' to conventional epidemiological approaches (contact tracing) and has often challenged accepted dogmas. For M. bovis and bovine tuberculosis, molecular epidemiology has a key role to play in providing more precise epidemiological data on the issues of inter bovine transmission and the role of wildlife reservoirs in disease maintenance and transmission. M. bovis strains may also differ in key biological properties, such as virulence, transmissibility, stability and antigenic variation, which may help to explain field observations.[13] In this study, conventional and molecular techniques were applied to determine the magnitude of BTB, where dairy farms more intensified.

Figure 1 *Map of Addis Ababa administration showing study area*



MATERIALS AND METHODS

Study area and samples

The study was conducted in Addis Ababa Administrative Region from July 2006 up to June 2007 in five

different part of the city (Figure 1) (north, west, south east and central part of Addis Ababa) where 1132 dairy cattle were sampled from 56 herds. The five sub cities were randomly selected out of the 10 sub-cities of Addis Ababa Cit. Furthermore, random sampling was applied to select the 56 study herds while all animals were sampled within the selected herds.

Tuberculin testing

Comparative intradermal skin test was conducted on a total of 1132 dairy animals (Holstein –Friesians cross breed). Two sites on the skin on the right side of the mid-neck of the animal, 12 cm apart, were shaved, and skin thickness was measured with a caliper. One site was injected with an aliquot of 0.1 ml of 2,500-IU/ml bovine PPD (Veterinary Laboratories Agency, Addlestone, Surrey, United Kingdom), into the dermis, and the other was similarly injected with 0.1 ml of 2,500-IU/ml avian PPD (Veterinary Laboratories Agency, Addlestone, Surrey, United Kingdom). After 72 h, the skin thickness at the injection sites was measured. Results were interpreted according to the recommendations of the Office International des Epizooties.[14] Briefly, when the change in skin thickness was greater at the avian PPD injection site, the animal was considered positive for mycobacterial species other than the mammalian type (M. tuberculosis and M. bovis). When increases were observed at both injection sites, the difference between the two reaction sizes was considered. Thus, if the increase in skin thickness at the injection site for bovine PPD (B) was greater than the increase in skin thickness at the injection site for avian PPD (A); and B minus A was less than 2 mm, between 2 and 4 mm, or 4 mm and above, the animal was classified as negative, inconclusive, or positive for bovine TB, respectively.

Post mortem examination and tissue specimen processing

Eleven skin test reactor cattle were obtained from eight different herds coming from four localities. The animals were sacrificed, and detailed postmortem examinations were performed. The lungs and lymph nodes were removed and checked for tuberculosis lesions. Duplicate tissue specimens were collected from all suspected tuberculosis lesion areas from those animals with visible lesion. Mediastinal, retropharyngeal, bronchial and mesenteric lymph nodes were sampled in case no visible lesions were observed at postmortem examination. Each sample was collected in 5 ml of a PBS solution and then transported to the laboratory. In the laboratory, the samples from a single animal were pooled and sectioned into pieces with sterile blades and then homogenized with a pestle and a mortar. The homogenate was decontaminated by adding an equal volume of 4% NaOH and kept for 15 minute and then neutralized with 1% (0.1 N) HCl with phenol red as an indicator. Neutralization was achieved when the color of the solution changed from purple to yellow.14 Then specimen was centrifuged at 1620g for 15 min and the supernatant discarded. Thereafter, 200 µl of suspension from each sample was spread onto a slant of Löwenstein-Jensen medium. Cultures were incubated aerobically at 37°C for about 5 to 8 weeks with weekly observation for growth of colonies.

Species identification

All isolates grown on Löwenstein-Jensen (LJ) media with pyruvate (0.4%) and/or glycerol were first checked for acid fastness by smear microscopy. Those positive were further characterized. The species identification was performed by PCR using RD10 primers (RD10 FF 5´-CTG-CAA-CCA-TCC-GGT-ACA-C-3´, RD10 Int 5´-GAA-GTC146 GTA-ACT-CAC-CGG-GA-5´, RD10 FR..5´-AAG-CGC-TAC-ATC-GCC-AAG-3´).15 A PCR amplification was performed in a total volume of 25µl (1µl FF, 1 µl FR, 5 µl Int, 12.5 µl Hot start master mix (QIAGEN GmbH Germany), 2 µl heat killed sample and 3.5 µl distilled water were used in all PCR reactions. In all cases, a mix without template DNA was used as PCR internal control. The PCR products were visualized in 1.6% agarose (Sigma) gel with

Table 1: *Herd and individual animal prevalence of tuberculin reactivity among herds in Addis Ababa, 2006-2007*

	Herd Prevalence			Individual animal Prevalence				
	Herds tested	**Positive***	**χ2 P-value**	**Total Tested**	**Positive**	**Negative**	**Inconclusive**	**χ2 (P-value)**
Yeka	16	12 (75%)		294	84 (28.6)	192 (65.7%)	18 (6.1%)	
Akaki kality	18	3 (16.7%)		261	64 (24.52%)	167 (63.9%)	30 (11.4%)	
Bole	4	3 (75%)		112	7 (6.2%)	99 (88.3%)	6 (5.3%)	
Kelefe keranyo	9	6 (66.7%)		343	190 (55.4%)	150 (43.7%)	3 (0.9%)	
Central Addis Ababa	9	6 (66.7%)		122	41 (33.%)	76 (62.5%)	5 (4.0%)	
Total	**56**	**30 (53.6%) CI39.75-67.00**	**16.8 0.032**	**1132**	**386 (34.1%) CI31.34-36.94**	**684 (60.4%)**	**62 (5.5%)**	**38<0.001**

**** At least one reactor animal was found***

Table 2*: Association between positive reaction to the tuberculin test in the farm and selected variables among Addis Ababa dairy animals, 2006-2007.*

			Univariate analysis		Multivariate analysis	
			Odds ratio (95% CI)	**χ2 (p-value)**	**Odds ratio (95% CI)**	**χ2 (p-value)**
Herd size						
<=10	30	30	1		1	
11-<=30	12	66.7	0.15 (0.04 1.63)	18.2 (<0.001)	2.37 (1.12 2.69)	10.9 (0.004)
>30	14	92.8	3.2 (2.04 3.29)		3.30 (2.04 3.43)	
Years of Establishment						
Before 1985	14	78.6	4.58 (0.95 12)	4.7 (0.084)	0.15 (0.07 0.66)	0.27 (0.875)
1985 – 2000	24	45.8	1.06 (0.31 3.61)		0.68 (0.63 0.69)	
After 2000	18	44.4	1		1	
Introduction of New animal*				9.08 (0.003)		3.2 (0.024)
Yes	27	74.0	5.4 (1.72 7.8)		3.15 (3.07 8.68)	
No	29	34.5	1			

**** Since establishment***

0.5µg/ml ethidium bromide.

Spoligotyping

Eight M. bovis and 3 M. tuberculosis isolates from tissue specimens were subjected to spoligotyping. Spoligotyping was performed as previously described.16 The direct repeat (DR) region was amplified by PCR with oligonucleotide primers derived from the DR sequence. Twenty-five microliters of the following reaction mixture was used for the PCR: 12.5 µl of HotStarTaq Master Mix (QIAGEN; this solution provides a final concentration of 1.5 mM MgCl2 and 200 µM each deoxynucleoside triphosphate.), 2 µl of each primer (20 pmol each), 5 µl of the suspension of heat-killed cells (approximately 10 to 50 ng), and 3.5 µl of distilled water. The mixture was heated for 15 min at 96°C and subjected to 30 cycles of 1 min at 96°C, 1 min at 55°C, and 30 s at 72°C. The amplified product was hybridized to a set of 43 immobilized oligonucleotides, each corresponding to one of the unique spacer DNA sequences within the DR locus. After hybridization, the membrane was washed twice for 10 min in 2X SSPE (1X SSPE is 0.18 M NaCl, 10 mM NaH2PO4, and 1 mM EDTA [pH 7.7])–0.5% sodium dodecyl sulfate at 60°C and then incubated in 1:4,000-diluted streptavidin-peroxidase conjugate (Boehringer) for 45 to 60 min at 42°C. The membrane was washed twice for 10 min in 2X SSPE–0.5% sodium dodecyl sulfate at 42°C and rinsed with 2X SSPE for 5 min at room temperature. Hybridized DNA was detected by the enhanced chemiluminescence method (Amersham) and by exposure to X-ray film (Hyper- film ECL; Amersham) as specified by the manufacturer.

Variable number of tandem repeat (VNTR) analysis

VNTR were applied according to 17 for all tissue specimen isolates. PCRs were set up using the QIAGEN HotStarTaq Master Mix with the following mix: 10 µl of Master Mix, 0.5 µl of each primer (at 10 pmol/ml), 7 µl of water, and 2 µl of the suspension from the heat-killed cells. The thermal cycle used for amplification was an initial denaturation at 94°C for 15 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 68°C for 1 min, and extension at 72°C for 2 min; a final extension at 72°C for 10 min was followed by a holding step at 4°C. PCR products were pooled for each strain, such that the six alleles for each strain could be run in a single lane, and products were then electrophoresed through polyacrylamide gels. Tracked gel images were then analyzed using GeneScan and Genotyper to call the alleles at each locus. In the typing nomenclature, the presence of an asterisk over the number of repeats at the D locus denotes the presence of a truncated repeat; the F locus contains two discrete repeats, hence "3.1" means three repeats of the first, followed by a single copy of the second.

RESULTS

Herd and animal prevalence

Out of 56 herds tested, 30 (53.6%) (95% CI =39.74-67.00) contained at least one reactor animal. There was a significance variation in the prevalence of the BTB between the study areas ($\chi 2$ =38, $p<0.001$) (Table 1). The prevalence was significantly higher in those with large size ($\chi 2$ =18.2, $p<0.001$, Table 2). Strong correlation (r=0.89) was observed between herd size and number of reactors. Of 1132 cattle tested 386 were positive giving an animal prevalence of 34.1% (95% CI=31.34- 36.94), considering inconclusive as negative. When inconclusive were considered as positive animal prevalence was 39.6% (95% CI =36.71-42.49). Table 3 shows the multiple logistic regression analysis of association of risk factors with tuberculin test reactivity.

Postmortem findings

Gross lesions of typical bovine tuberculosis was detected in 72.7% (8/11) the reactors. The distribution of lesions in lymph nodes and tissue is presented in Table 4. Presence of lesion was most frequent in mediastinal lymph nodes (72.7%) compared to retropharyngeal (54.5%), mesenteric (54.5%), and bronchial (45.5%). In one animal lesion was

Table 3 *Multivariate analysis of tuberculin reactivity and risk factors among dairy animals in Addis Ababa, 2006-2007.*

Risk Factors		Inconclusive as positive			Inconclusive as negative		
		Odds Ratio	95% CI	χ2 (p-value)	Odds Ratio	95% CI	χ2 (p-value)
Origin	Purchased	1.1	0.93-1.68	2.11 (0.146)	1.2	0.88-1.62	1.30 (0.256)
	Born in the farm	1			1		
Status	Lactating and non-pregnant	1.3	0.88-1.98	3.182 (0.528)	1.1	0.73-1.72	5.50 (0.240)
	Lactating and pregnant	1.1	0.68-1.87		1.1	0.69-1.94	
	non- Lactating and pregnant	1.4	0.87-2.25		1.6	0.94-2.70	
	non-Lactating and pregnant	1.6	0.73-3.60		1.2	0.71-3.53	
	Heifers, male and calves	1			1		
Age group (In years)	< 2	1		32.20 (0.000)	1		21.75 (<0.001)
	2 – < 5	2.4	0.91-2.82		1.2	0.92-2.22	
	5 – < 8	1.4	0.34-2.12		0.9	0.65-1.51	
	>= 8	2.8	1.27-3.42		2.4	2.26-2.86	

also identified in hepatic lymph nodes and in the peritoneum.

Bacteriology

Of 11 isolates from tissue specimen culture, 8 isolates were confirmed as M. bovis (Table4). Other three were identified as M. tuberculosis, one isolate from a tuberculin reactor cow with macroscopic lesion and two isolates from two tuberculin reactor cows that had no evident lesion upon visual postmortem examination.

Strain characterization

The eight M. bovis isolates exhibited three different spoligotype patterns (Table 4). All isolates of M. bovis tested lacked spacer 3, 6, 9, 16 and 39 to 43, a characteristic that distinguishes M. bovis from M. tuberculosis (Table 4). The three M. tuberculosis isolates were identified as two different spologityping patterns. The three M. bovis spoligotype patterns were SB0133, SB0134 and SB1176. VNTR analysis of M. bovis isolates revealed two distinct (5 2 5 4* 3 3.1 and 5 3 5 4* 4 3.1) patterns (Table 4). Two isolates, which had good spoligotype results, failed in the analysis of VNTR. In the case of the M. tuberculosis isolates analyzed with VNTR two types of profiles (3 2 4 3* 3 3.1 and 3 2 3 3* 3 3.2) were evident (Table 4).

DISCUSSION

The presently recorded animal prevalence is much higher than the previously reported (10.5%) in Addis Ababa.[19] Moreover, it is much higher than the prevalence (13.5%) recently reported by 20, and is comparable with the prevalence (46.8%) reported.[20] In addition, there was a rise in herd prevalence was compared to the previous (48%).[19] This

Table 4 *Distribution of tuberculous lesion in Lymph node and tissues of tuberculin reactor animals and molecular characterization of isolates from Addis Ababa dairy farms*

Sample No	Breed	Sampling area *(as shown in Figure 1)*	Herd size	Lesion VL (Visible Lesion) NVL (Non VL)	Infection sites	Isolate *M.tuberculosis / M. bovis*	Spoligotype [b]	VNTR
S-82	Cross	Yeka	62	VL	Mediastinal LN	*M. bovis*	SB0134	5354*43.1
					Retropharyngeal LN			
					Bronchial LN			
2804	Cross	Akakikality	14	NVL		*M .tuberculosis*	SIT149	3243*33.1
651	Cross	Kolefe-keranyo	18	VL	Mediastinal LN	*M. bovis*	SB1176	5254*33.1
					Retropharyngeal LN			
					Bronchial LN			
					Mesenteric LN			
					Lung Tissue			
					Hepatic LN			
652	Cross	Kolefe-keranyo	18	VL	Mediastinal LN	*M. bovis*	SB1176	5254*33.1
					Bronchial LN			
					Mesenteric			
653	Cross	Central Addis Ababa	48	VL	Mediastinal LN	*M. bovis*	SB1176	5254*33.1
					Retropharyngeal LN			
					Mesenteric LN			
					Lung Tissue			
					Peritoneum			
654	Cross	Central Addis Ababa	48	VL	Mediastinal LN	*M. bovis*	SB1176	5254*33.1
					Retropharyngeal LN			
					Bronchial LN			
					Mesenteric LN			
					Lung Tissue			
1817	Cross	Kolefe-keranyo	23	NVL		*M. bovis*	SB1176	5254*33.1
2902	Cross	Akakikality	7	NVL		*M. tuberculosis*	SIT121	3233*33.2
R-12	Cross	Kolefe-keranyo	88	VL	Mediastinal LN	*M .tuberculosis*	IT149	3243*33.1
					Bronchial LN			
3606	Cross	Central Addis Ababa	15	VL	Mediastinal LN	*M. bovis*	SB0133	Failed
					Retropharyngeal LN			
					Mesenteric LN			
3612	Cross	Akakikality	52	VL	Mediastinal LN	*M. bovis*	SB0133	Failed
					Retropharyngeal LN			

rise in prevalence of BTB is due to the absence of control method in Ethiopia.

Typical mycobactrial lesion was detected in 72.7% (8/11) of the animals which is relatively lower than previously reported.[19] The lack of visible lesion in the remaining tuberculin reactor animals can be explained by the fact that animals may be in the early stage of infection when the lesions are too small to be detected grossly with the naked eye or tuberculin sensitivity may be due to infection with Mycobacterium species other than M. bovis.[22] However, those that did not have visible lesions were culture positive and isolates were confirmed as, M. bovis in one and M. tuberculosis in the others two. In several countries, M. tuberculosis has been isolated from the lymph nodes of some positive tuberculin reactors that showed no lesion on postmortem examination[23], which

substantiates the preset result. Infection of cattle by M. tuberculosis from humans leads to transient tuberculin conversion, and occasional small and self-limiting lesions may be established in the lungs and intestines of calves and overt and progressive disease is seldom encountered.[24]

Lesions were predominantly detected in mediastinal lymph nodes and bronchial lymph nodes in the thoracic cavity. Reports from developed countries indicate that tuberculosis lesions are found most frequently in the lymphatic tissues of the thoracic cavity. In intensive husbandry system, 90% of the lesions occur in the respiratory tract.[22,25] Contrary to this finding, in the recent report by [20], 94.5 % of the lesions were detected in mesenteric lymph nodes in animals that kept on pasture.

Three different spoligotype type patterns (SB0133, SB0134 and SB1176) were identified for M. bovis. SB0133 had previously been described[27] from The Netherlands. SB0134 was also described by [26-28] and it was first reported from Belgium. The most commonly found strain was SB1176 and it was first described from Ethiopia.[19] So far, this strain has not been reported either from Africa [29-31]or from other parts of the world.[26,27,32] This pattern is the dominant among the three strains. Two types of VNTR profiles were recorded; though VNTR is a convenient, highly discriminatory, reproducible and high-throughput strain typing technique for M. bovis.[33] The three isolates of M. tuberculosis from the tissue were identified with two spoligotype pattern and two VNTR profiles.

Interestingly, for one isolate the owner of the cow had died with the history of tuberculosis. Even though samples from the owners or herd attendants were not processed for isolation to confirm whether they have similar pattern or not, isolation M. tuberculosis from cattle suggests that cattle owners could be the sources of infection for their cattle. In a study conducted in India from extra pulmonary tissue of tuberculous cattle, 15–28% of the animals were discovered to be infected with M. tuberculosis.[34] Another study from Nigeria also identified one tissue isolate as M. tuberculosis by spoligotyping and VNTR.[32] Even though human-to cattle transmission of M. tuberculosis has been reported (reviewed)3, it is generally held that disease in cattle due to M. tuberculosis is less severe than that caused by M. bovis[23] and the identification of M. tuberculosis in cattle by itself is intriguing.

This study indicated the widespread occurrence of bovine tuberculosis in Addis Ababa City. Unless control measures are designed, the impact on the economy and public health could be enormous. On to this, the identification M. tuberculosis from cattle tissues requires further investigation.

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