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Comparison of CIDT and INF-γ Release Assay for Detection of BTB in a Dairy Farm Located at Holetta, Central Ethiopia

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Abstract

Bovine Tuberculosis (BTB) is one of the main diseases caused by *Mycobacterium bovis*, a member of the *Mycobacterium tuberculosis* complex. *M. bovis* can infect wide range of domestic and wild animals including human. In Ethiopia, the endemic nature of bovine tuberculosis in domestic animals has long been reported and recent studies also showed that BTB is endemic mainly in cattle, in large parts of the country with considerable magnitude. The disease found to be less prevalent in rural area with characteristics of small holder farms rearing mainly Zebu cattle. However, on urban and semi-urban areas, market oriented production system leads to farming of high grade dairy cattle over a longer period of time exhibiting high prevalence level of the disease.

Keywords: Bovine tuberculosis • *Mycobacterium bovis* • Zebu cattle • Farming

Introduction

Bovine Tuberculosis (BTB) is one of the main diseases caused by *Mycobacterium bovis*, a member of the *Mycobacterium tuberculosis* complex. *M. bovis* can infect wide range of domestic and wild animals including human. Different methods are used for diagnosis of bovine tuberculosis. Tuberculin skin tests are the primary and the most used diagnostic methods of tuberculosis both in human and animals. A group of tests called Interferon Gamma Release Assay (IGRA) are also used for the diagnosis of bovine and human tuberculosis. They detect Latent Tuberculosis Infection (LTBI) by measuring Interferon Gamma (INF-γ) release in response to antigens present in *Mycobacterium tuberculosis*, but not Bacilli Calmette-Guerin (BCG) vaccine and most non-tuberculous mycobacteria. Their high specificity and improved sensitivity in individuals with weakened cellular immunity made them more useful than the older tests [1].

In developing countries like Ethiopia, effective but affordable diagnosis of BTB is crucial to avoid the wide spread of the disease among the less aware community and to put reliable decision for clearance of infected animals. Although tuberculin skin test started long before in Ethiopia, due to some inherent limitation of the test, combined test schemes are recommended. Thus, the objective of this research is to assess the combined or separate use of Comparative Intradermal Tuberculin Test (CIDT) and Interferon Gamma (INF-γ) release assay in different group of animals for diagnosis of bovine tuberculosis [2].

Materials and Methods

Study animals

This study was conducted on 502 heads of cattle in a farm located at Holeta. The cattle were managed under intensive production system. They were well supplemented and graze 5 hours per day except the calves below 1 years old and newly delivered dams. Animals were grouped in to three age categories; ≤ 3 years of age (young), 4-9 years of age (middle aged) and ≥ 10 years (old); two breeds (HF X Boran and Boran) and the two sexes [3].

Comparative intradermal tuberculin test

Two sites on the skin on the right side of the mid-neck of the animal, in average 12 cm apart and at equal distance from cervical lymph-node, were shaved and skin thickness was measured with a caliper and recorded. One site was injected with an aliquot of 0.1 ml of 2,500 IU/ml bovine Purified Protein Derivative (PPD), into the dermis and the other was similarly injected with 0.1 ml of 2,500 IU/ml avian PPD found from similar source. Proper administration of the PPD was appreciated by appearance of pea

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size nodule just after injection on the site. After 72 h, the skin thickness at the injection sites was measured. Results were interpreted according to the Office International des Epizooties recommendations. In brief, increase in skin thickness only at avian PPD injected site indicate the animal was positive for avian tuberculosis but not either of *M. tuberculosis* or *M. bovis* (the mammalian tuberculosis), however, if there is increment of skin thickness for both injections (avian and bovine), the increase for bovine PPD in mm was deducted from the increase of avian PPD to make decision. If the difference is above 4 mm it will be considered as positive for BTB [4].

Whole-blood culture and Bovigam IFN-γ release assay

Blood samples were collected from the jugular vein into heparinized vacutainers and dispensed at 250 µl volume into 96-well flat-bottom culture plates. Antigens (each avian and bovine PPD) were added in 25 µl aliquots to give final assay concentrations of 10 g/ml and 25 µl of saline were used as negative control for each sample. All the samples were duplicated to minimize pipetting error. Cultures were incubated at 37°C, in a humid, 5% CO_2 atmosphere for 48 h and supernatants were harvested and frozen. Levels of IFN-γ in the supernatants were measured by an enzyme-linked immunosorbent assay by using the bovine IFN-γ release assay (Bovigam, Australia) in accordance with the manufacturer's instructions [5].

Statistical analysis

The *chi-square* and fisher exact test was used to analyze the prevalence of BTB among different age groups, breeds and sexes for both diagnostic tests identifying the differences at significant level of $P<0.05$. When three categories are compared in which one of them has minimum sample size, fisher exact test was applied and the indicated significant values were recorded respectively. Categories containing very small number of individual are excluded from computation. Multivariate logistic regression was used to estimate the risk of exposure to the infection quantified by the odds ratio. Cohen's kappa was used to evaluate the level of agreement between the tests for different categories of age, gender and breeds of animals. All the statistics were computed by SPSS statistics v. 17.0 [6].

Results

An overall prevalence of 29.4% (148 out of 502) was found at individual cattle level at cut-off values of >4 mm by CIDT. Whereas, prevalence rate of 34.2% (172 out of 502) was observed by IFN-γ release assay. Out of all animals tested, 19.5% (98 out of 502) of them identified as positive by both tests. Significant difference was observed among the three age groups (≤ 3, 4-9 and ≥ 10 years) of cross breed animals but not borans by CIDT. Again, significant difference observed between the two sexes of cross breed animals by the same test (Table 1). Cross breed animals of ten or more years of age encounter BTB 12 times more likely as compared to young (≤ 3 years) animals and almost eight times as compared to middle aged (4-9) animals after evaluation by CIDT (Table 2) [7].

Variable	Categories	Boran				Cross bred			
		N	No +ve (%)	*Chi-square*/ Fishers' exact	P-value	N	No +ve (%)	*Chi-square*/ Fishers' exact	P-value
CIDT									
Age (Years)	0-3	2	0	4.56	0.101	193	28 (14.5%)	44.1	0.00*
	4-9	46	18 (39.1%)			217	69 (31.8%)		
	≥ 10	13	6 (46.1%)			31	21 (67.7%)		
Gender	M	1	-	N/A	-	46	4 (8.7%)	8.54	0.004*
	F	60	-			395	114 (28.6%)		
INF-γ release assay									
Age (Years)	0-3	2	0	1.55	0.58	193	49 (25.4%)	28.9	0.000*
	4-9	46	18 (39.1%)			217	75 (34.6%)		
	≥ 10	13	6 (46.1%)			31	23 (74.2%)		
Gender	M	1	-	N/A	-	46	11 (23.9%)	2.05	0.187
	F	60	-			395	136 (34.4%)		

Note: N-Total number of animals examined; N/A-not applicable due to small number of individuals; *significant difference ($p<0.005$)

Table 1. Prevalence of bovine tuberculosis and associated risk factors determined by both tests (CIDT and INF-γ release assay).

Variable	Categories	Boran				Cross breed			
		N	No +ve (%)	OR	CI for OR	N	No +ve (%)	OR	CI for OR
CIDT									
Age (Years)	0-3	2	0 (0%)	-	0.72- 2.3	193	28 (14.5%)	1	-
	4-9	46	20 (69%)	1.37		217	69 (31.8%)	4.5	2.0-10.1
	≥ 10	13	9 (31%)	1		31	21 (67.7%)	12.3	5.2-29.4
Sex	M	1	-	N/A	-	46	4 (8.7%)	1	-
	F	60	-			395	114 (28.6%)	2.4	1.9-3.0
INF- γ release assay (Bovigam)									
Age (Years)	0-3	2	0	-	0.86-2.8	193	49 (25.4%)	1	-
	4-9	46	18 (39.1%)	1		217	75 (34.6%)	5.4	2.32-12.8
	≥ 10	13	6 (46.1%)	1.5		31	23 (74.2%)	8.4	3.5-20.0
Gender	M	1	-	N/A	-	46	11 (23.9%)	1	-
	F	60	-			395	136 (34.4%)	1.9	1.5-2.3

Note: N/A-Not applicable due to small number of individuals; CI=Confidence Interval; OR=Odds Ratio

Table 2. Association of host-related risk factors with prevalence of bovine tuberculosis by both tests (CIDT and INF-γ release assay).

Significant difference on proportion of distribution of the diseases observed among the three age groups of cross breed animals but not borans by INF-γ release assay. However, no significant difference observed between male and female cross breed animals by the same test. The INF-γ release assay illustrated that, older (≥ 10) and middle aged (4-9) crossbreed animals were more than eight and five times susceptible to BTB respectively as compared to young (≤ 3) animals [8].

Different agreement levels were scored ranging from disagreement to moderately agree were recorded among the different categories of the tested animals for the two tests (Table 3). The tests were agreed moderately for both boran and cross breed animals as well as all female animals of both breeds (Table 4) [9].

Breed	Variable	Categories	Number of animals examined	K-value	Decision on the agreement
Boran	Age (years)	0-3	2	N/A	-
		4-9	46	0.553	Moderate
		≥ 10	13	-0.46	Disagree
	Gender	M	1	N/A	-
		F	60	0.43	Moderate

Cross	Age (years)	0-3	193	0.347	Fair
		4-9	217	0.398	Moderate
		≥ 10	31	0.221	Fair
	Gender	M	46	0.159	Slight
		F	395	0.429	Moderate

Note: K-Cohen's kappa values; values ≤ 0 as indicating no agreement; 0.01-0.20 as slight; 0.21-0.40 as fair; 0.41-0.60 as moderate; 0.61-0.80 as substantial; 0.81-1.00 as almost perfect agreement

Table 3. Analysis of agreement level between CIDT and INF- γ release assay (Cohen's kappa).

	N	Negative by both tests	Positive by both tests	K-value	Decision
Boran	61 (100%)	26 (42.6%)	18 (29.5%)	0.437	Moderate
Cross	441 (100%)	254 (57.6%)	78 (17.7%)	0.415	Moderate
All females	456 (100%)	247 (54.2%)	94 (20.6%)	0.433	Moderate
All males	47 (100%)	33 (78.6%)	3 (60%)	0.239	Fair

Note: K-Cohen's kappa values; values ≤ 0 as indicating no agreement; 0.01-0.20 as slight; 0.21-0.40 as fair; 0.41-0.60 as moderate; 0.61-0.80 as substantial; 0.81-1.00 as almost perfect agreement

Table 4. Summary of the two tests agreement.

Discussion

In present study, the overall prevalence of BTB both by CIDT and IFN-γ release assay found high. This could be due to the animals were kept indoor which create higher exposure medium to contract the disease *via* infectious aerosol droplets which is supported by earlier study by Ameni et al. who comparing animals kept indoor and in pasture [10].

CIDT

Older cross breed animals (≥ 10 years old) found above four times reactive than middle aged (4-9 years) and 12 times reactive than young animals (0-3 years) of similar breed by CIDT. In borans, although the extent of difference on risk of acquiring BTB among the age groups was little, the highest proportion of reactive animals (69%) found at the middle age group (4-9 years). Similar results were discussed by Ameni and colleagues mentioning that the maximum tubercline reactivity reach between 5-9 years and afterwards declining. Earlier study conducted at Wuchale-Jida district decrease the optimum detection age to 8 years and even lowered to 6 years of age by the same author. In 2013, Brooks-Pollock and colleagues observed that age-specific BTB incidence strictly increase until 12 to 36 months on cattle examined in UK by analyzing through catalytic model. However, there are reports supporting the present finding that, susceptibility of the animals increase as their age increase. Discrepancies among researches on the maximum group at risk could be due genetic difference and difference on the blocking applied on designing of the researches. However, most of the studies agreed that younger animals are less likely reactive to tuberculin test so did to acquire the infection. The relative abundance of γδ T cells in the circulatory system of younger animals rather than the peripheral tissue may contribute to less susceptibility to tuberculosis. Since, the importance of these cells on the immune response against tuberculosis well established. The direct relationship between aging and BTB prevalence frequently explained by exposure time and slow progression of the disease at detectable level [11].

Gender of the animal matters the purpose and management of the animals. In African countries, male cattle used for ploughing of land so that they stayed over longer period of time in the herd in extensive production system. Earlier cross-sectional study conducted in Tanzania, revealed that male cattle were significantly more affected by BTB than female animals. On the reverse, in dairy farms, cows kept overlong period to time for production purpose and frequent stock replacement is very rare due to economical reason while male animals usually culled at younger age and reproduction relay on artificial insemination. In the present study, significant difference on reactivity to tubercline test observed between the two sexes (P=0.004). Female cross breed animals found almost twice as susceptible as male cross breeds. This finding can be related to the hypothesis mentioned earlier since the study population were cattle of a dairy farm. Another possible explanation for susceptibility of female animals than male animals for tuberculosis is occurrence of physiological stresses at different stage of their life. Reports from Eritrea indicate that pregnant lactating cows are risked at most compared to the rest group of the herd. Similarly, Kazwala and colleagues noticed that lactating cows found more reactive than non-lactating cows for SCITT [12].

INF-γ release assay

Interferon-γ release assays are used for the diagnosis of bovine and human tuberculosis. In spite of in vivo stimulation of T-cells by depositing the mycobacterial derived antigen (PPD) under the skin of

the animal by tuberculin skin test, the gamma interferon release assay measure the production of the stable cytokine (INF-γ) *in vitro* by similar group of cells after stimulated by the PPD mostly after incubation for 24 hours. This assay was first reported in 1985 and the main advantage of INF-γ release assay over tuberculin skin test is its ability to detect earlier. As a result, reduce the risk of retaining newly infected animals with such slowly progressing disease. In addition, the INF-γ release assay (*i.e.,* the Bovigam), not affected by poor nutritional condition, mildly by dexamethasone treatment and briefly affected by parturition. However, unlike the well-known tuberculin test, INF-γ release assay has been performed less frequently for diagnosis of tuberculosis in livestock especially in developing countries. This could be due to their high cost, requirement of well-equipped laboratory facilities and trained man-power [13].

Similar to the tuberculin test, no significant difference observed among the three age groups of boran cattle for production of INF-γ (P=0.58). However, the difference was significant for cross breed animals of similar age groups (P=0.00). Older cross breed animals (≥ 10 years) were more than five-fold susceptible to BTB as compared to middle aged (4-9 years) and more than eight-fold when compared to young (0-3 years) cross breed animals by INF-γ release assay.

Both T-lymphocytes (CD4+ and CD8) are responsible for production of INF-γ production during *M. bovis* infection. Mehrzad and Zhao, studied the proliferation capacity and ratio of these cells in cows at different parity level. They have identified greater CD4+/CD8 + ratio in the blood of pluri-parous cows as compared to primi-parous cows. Since, CD4+ cells produce higher amount of INF-γ as compared to CD8+ cells in the presence of *M. bovis* antigens, increase in age indirectly related with increment in parity number for dairy cattle which coincide with current finding on INF-γ level and age. However this explanation does not ignore the concept of aging and longevity for exposure to the bacteria which increase the chance of acquiring the disease in older animals. Although no significant difference observed between the two sexes, female cross bred animals found almost twice likely encounter the disease as compared to male animals [14].

Level of agreement between CIDT and INF γ release assay

In spite of its limitations, kappa is one of the most commonly used statistics to test interrater reliability. In case of diagnosis, the raters can be tests. Moderate agreement level between the two tests was observed in middle aged (4-9 years) and females of boran breed animals. Similarly, the two tests agree moderately for middle aged (4-9 years) and males of cross breed animals. The level of agreement between the two tests was fair for both youngest (0-3 years) and oldest group (≥ 10 years) of cross breed animals. Breed wise comparison for two tests indicate, the tests were agreed moderately for both boran and cross breed animals as well as all female animals of both breeds.

Comparison of CIDT and IFN-γ test by Ameni and colleagues showed slight agreement (k=0.129) between the two tests in study conducted to compare different tests in Ethiopia. In Spain, even lower level of agreement (k=0.036) observed between CIDT and IFN-γ tests in *M. bovis* infected goats. However, higher level of agreement (96.8%) between the two tests was reported by Dondo et al. when the results from cattle from infected herds were compared.

In study conducted to determine the distribution of *M. tuberculosis* on human patients, excellent agreement of these tests observed on BCG non-immunized younger (0-4 years) and older children (5-8 years) at Greece which implies immunization could interfere with diagnostic outcomes. Similarly, Mahomed and colleagues found good agreement (84.8%, kappa 0.70) between QuantiFERON TB gold kit; an IGRA and tuberculin skin test (5 mm cut off) in a study conducted at South Africa involving patient children infected by MTB. In Hong Kong, the test agreement between commercial IFN-γ release assay and tuberculin skin test were found 0.47 and 0.39 respectively at 5 mm and 10 mm cut off values of skin test on human patients. However, in Turkey, low level of test agreement between the two tests observed (Kappa 0.44 to 0.21) on study conducted for the identification of latent TB infection located in different treatment groups. Discrepancies among different studies on the level of agreement between CIDT and IFN-γ release assays could be due to non-specific reaction across the different tests because of infection from environmental bacteria those share protein with bovine PPD.

Studies support combined use of diagnostic tests to make the diseases detection and control strategy sound and economical. Ameni and colleagues highlight the use of a combination of tests. Similarly, a study conducted in Ireland signifies the importance of combined diagnosis since the IFN-γ release assay could identify *M. bovis* infected cattle early before the onset of outbreaks. On the other hand, the conventional apparently low specific IFN-γ release assay could not allow to make decision for disease clearance only by itself [15].

Conclusion

In the present study, high prevalence of BTB was confirmed both by CIDT and INF-γ release assay. This indicate effective diagnosis followed by control of this disease is crucial. The two tests tend to show moderate agreement level. Hence, one less likely replace the other. The combined testing strategy as compared to the sole conventional tuberculin skin test found more effective. Conventionally prepared bovine PPDs share proteins with other environmental mycobacteria thus non-specific reactions complicate skin tests. So, combination of the two tests increase the reliability of the diagnosis.

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