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Performance Evaluation of *Mycobacterium bovis* Antibody Test for the Diagnosis of Bovine Tuberculosis in Ethiopia

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Abstract: The intra-dermal tuberculin test is the standard test for the diagnosis of bovine tuberculosis [BTB] although it is not efficient in detecting all infected animals. This necessitates the need for a complementary testing scheme so as to maximize the detection of the BTB infection. The objective of this study was to evaluate *Mycobacterium bovis* [*M. bovis*] antibody test for the diagnosis of BTB. A total of 459 [336 from dairy farms and 123 from abattoir] cattle were used for the evaluation of the test. Comparative intradermal tuberculin [CIDT] test, post mortem examination for TB lesion and mycobacterial culturing were used for the evaluation of the *M. bovis* antibody test. *M. bovis* antibody was measured using enzyme linked immunosorbent assay [ELISA] following the procedure described by the manufacturer. Results revealed that the sensitivity of *M. bovis* antibody ELISA was 50% at the cut-off value of 0.30 considering culture as a gold standard, while its specificity was 99%. On the other hand, receiver operating characteristics [ROC] analysis was performed and a cut-off value of 0.136 was proposed to be used in Ethiopian condition. At cut-off value of 0.136, the sensitivity and specificity of the test were 80% and 96%, respectively. The area under the ROC curve was 0.92. The agreements between *M. bovis* antibody ELISA and CIDT test was fair [k=0.254], while the agreement between *M. bovis* antibody ELISA and post mortem examination was moderate [k=0.437]. In conclusion, the sensitivity and specificity of *M. bovis* antibody ELISA recorded by the present study could suggest the possibility of using *M. bovis* antibody ELISA as ancillary test to CIDT test for the maximal detection of BTB particularly in regions where skin test is not regularly performed for screening of the disease.

Key words: Bovine Tuberculosis · IDEXX *M. bovis* Antibody · Sensitivity · Specificity

INTRODUCTION

Cattle production plays an important role in Ethiopian economy. The country possesses 53.99 million cattle [1] and has great potential for increasing its cattle production, both for local use and for export. However, such potential is constrained by inadequate nutrition, a lack of support services and diseases. BTB is among those diseases that cause economic losses and poses public health risk to the community reviewed by Shitaye *et al.* and Ayele *et al.* [2, 3]. BTB is an infectious and communicable granulomatous disease caused by the acid-fast bacillus *M. bovis*. It is a wide spread zoonosis and affects cattle, other domesticated animals and certain free or captive wildlife species [3]. BTB is endemic in Ethiopia and studies have indicated that the disease is more prevalent in intensive dairy farms than in smallholder farm settings with a prevalence ranging from 3.4% in small holder production systems to 50% in intensive production system [5-9].

The disease control programs carried out in most countries are based on a test and removal strategy utilizing the tuberculin skin test [TST] with purified protein derivative [PPD] [4]. However, the intradermal tuberculin test it is not efficient in detecting the disease at its different stages particularly at its early and advanced stages. Antibody responses to *M. bovis* have been shown to be positively correlated with the mycobacterial-

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elicited pathology and antigen burden [10-12]. Thus, development of serological tests may increase the degree of detection of animals infected with *M. bovis* [13, 14] and could be complementary to tuberculin test. In addition, the use of multiple tests may increase overall diagnostic power by detecting subsets of infected animals missed by skin test [15]. Considering the ease of sample collection and test procedures, serologic tests may be used in a wide range of applications and provide additional testing opportunities not afforded with cell-mediated response-based tests.

IDEXX *M. bovis* antibody test kit is a new commercial ELISA test and manufactured by IDEXX Laboratories LTD for the diagnosis of BTB in cattle. The IDEXX *M. bovis* antibody test kit is designed to detect the presence of antibody *M. bovis* in bovine serum. This test could improve BTB detection and could be easy, cost effective for surveillance. It was validated and certified by the OIE in 2012 with Approval number of 20120107 [16]. Further evaluation of the test under field conditions in Ethiopia is important for future use of this test. The purpose of the present study was to evaluate performance of IDEXX *M. bovis* antibody ELISA.

MATERIALS AND METHODS

Study Animals and Study Design: A cross-sectional study was conducted on 459 cattle, which were sampled purposively from dairy farms and slaughter house. Of the 459 cattle, 336 cattle were selected from six dairy farms, while the remaining 123 cattle were selected randomly from cattle slaughtered at the slaughterhouse. All animals included in this study were above six months of age. Cows in late gestation and those at early parturition were excluded.

Comparative Intradermal Tuberculin Test: The comparative intradermal tuberculin test was conducted following the procedure described by OIE [4]. Briefly, two sites on the right side of the mid-neck, 12 cm apart, were shaved and skin thicknesses were measured with caliper and recorded. Then, 0.1 ml [25,000 IU/ml, 0.5 mg/ml, *M. avium* subspecies *avium*, strain D4ER [Lelystand Biologicals BV, Lelystand, the Netherlands] avian tuberculin was injected into the skin intradermally in the cranial part of the neck while 0.1 ml [20,000 IU/ml, 0.5 mg / ml, *M. bovis*, strain ANS, [Lelystand Biological BV, Lelystand, the Netherlands] bovine tuberculin was injected intradermally in the caudal part of the neck. The results were recorded after 72 hours post-inoculation by measuring the thicknesses of skin folds. The interpretation of the results was made following the recommendation of Office Internationale des Epizooties [4]. A reaction is usually considered to be positive if the increase in skin thickness at the bovine site of injection is more than 4 mm greater than the reaction shown at the site of the avian injection. The reaction is considered to be inconclusive if the increase in skin thickness at the bovine site of injection is from 1 to 4 mm greater than the avian reaction. The reaction is considered to be negative if the increase in skin thickness at the bovine site of injection is less than or equal to the increase in the skin reaction at the avian site of injection.

Blood Collection and Serum Extraction: Blood samples were collected from 336 dairy cattle into plain vacutainers four months after tuberculin testing. Similarly, blood samples were collected from 123 cattle at slaughterhouse just before slaughtering. The samples were kept overnight at room temperature. Thereafter, sera were separated and stored at -20°C until the assay was run.

Post Mortem Examination: Inspection of each of the carcass was undertaken in detail according to Ameni *et al*. [17]. Briefly, lungs and lymph nodes were removed for the investigation of gross lesions of TB. The lobes of the lungs were inspected externally and palpated. Then, each lobe was sectioned into about 2 cm-thick slices to facilitate the detection of lesions. Similarly, lymph nodes namely mandibular, medial retropharyngeal, cranial and caudal mediastinal, left and right bronchial, hepatic and mesenteric lymph nodes were sliced into thin sections and inspected for the presence of visible TB lesions. When gross lesions suggestive of TB were found in any of the tissues examined, the animal was classified as TB lesion positive.

Mycobacterial Culturing: During post-mortem examination TB suspected lesions were collected in individual 50 ml sterile universal tubes [containing sterile saline] and transported at 4°C to the Aklilu Lama Institute of Pathobiology [ALIPB] for culturing. The samples were homogenized, decontaminated with 4% NaOH and neutralized with 1% [0.1N] Hcl with phenol red as indicator. Neutralization was achieved when the color of the solution is changed from purple to yellow. The sediments were then inoculated on two Lowenstein-Jensen media culture, one with supplemented with pyruvet and the other with glycerol. The cultures were incubated aerobically at 37°C in slant position for one week and then in the upright position for about 5-8 weeks with weekly observation for growth of colonies.

IDEXX *M. Bovis* Enzyme-Linked Immunosorbent Assay: Serum samples were tested by IDEXX *M. bovis* antibody ELISA kit according to the manufactures instruction [IDEXX laboratories]. Briefly, serum samples and kit controls were diluted 1:50 in the sample diluents provided with the kit. Thereafter, 100µL were added into the wells and incubated at room temperature for 1 h. This was followed with the removal of the contents of the wells and washing the plates four times after which 100µL of a monoclonal anti-bovine IgG-horseradish peroxidase conjugate was added into each well and incubated for 30 min. Again the plates were washed four times, which was followed by the addition of 100 µL of TMB substrate into each well and incubation for 15-min at room temperature. Further reaction was stopped by addition of 50µL H_2SO_4 and the optical density [OD] values were read using the plate reader [V_{max}, Molecular Devices, Sunnyvale, CA] at 450 nm. Results were presented as sample-to-positive control ratio [S/P] derived by subtracting the mean OD value of kit negative-control from each sample and dividing this value by the corrected positive-control value [mean OD of positive-control minus mean OD of negative-control]. ODs of sample were compared to those of the kit positive control to derive [S/P] ratios. Samples with S/P ratio of >/= 0.30 were considered positive for *M. bovis* antibodies as recommended by the manufacturer.

Data Management and Statistical Analysis: The data were entered into Microsoft Excel 2013 and transferred to SPSS® Version 20 for statistical analysis. Efficiency of IDEXX *M. bovis* antibody ELISA assay to detect antibody against *M. bovis* was validated taking detailed necropsy and culture as gold standards. The sensitivity and specificity of *M. bovis* antibody test was determined at the cut-off values established by the manufacturer [IDEXX Laboratories]. In addition, a receiver operating characteristics [ROC] analysis was made to identify a new cut-off value that can be used under Ethiopian setting. Moreover, the agreement between the *M. bovis* antibody test and the CITD test was evaluated using Kappa statistics.

RESULTS

Evaluation of the Performance of Idexx *M. Bovis* Antibody ELISA: The sensitivity and specificity of the IDEXX *M. bovis* antibody ELISA at the cut-off 0.3 [a cut-off value recommended by the manufacturer] were evaluated using culture as a gold standard. Accordingly, the sensitivity and specificity of *M. bovis* antibody test at the cut-off 0.3 were 50% and 99%, respectively [Table 1]. At the same cut-off value, the positive predictive value and negative predictive value were 83.3% and 95.6%%, respectively.

Table 1: Performance of IDEXX *M. bovis* antibody ELISA using culture as gold standard

		Culture		
		Positive	Negative	Total
M. bovis ELISA [cut-off 0.3]	Positive	5	1	6
	Negative	5	112	117
	Total	10	113	123

Sensitivity=50%, Specificity= 99%

Table 2: Results of IDEXX *M. bovis* ELISA and comparative intradermal tuberculin test

		CIDT test			
		Positive	Doubtful	Negative	Total
M. bovis ELISA [cut-off 0.3]	Positive	46	4	18	68
	Negative	91	12	165	268
	Total	137	16	183	336

The agreement between the two tests was fair [k=0.254]

Table 3: Results of IDEXX *M. bovis* antibody ELISA and post mortem examination

		Gross lesion		
		Positive	Negative	Total
M. bovis ELISA [cut-off 0.3]	Positive	5	1	6
	Negative	10	107	117
	Total	15	108	123

Moderate [k=0.437] agreement

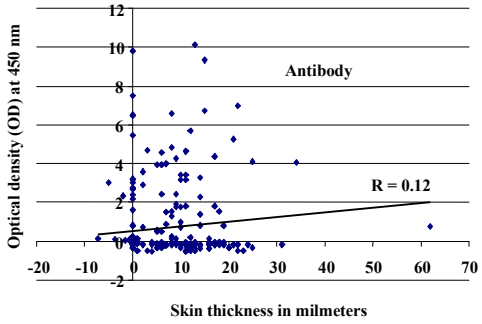

Fig. 1:

The results of IDEXX *M. bovis* antibody ELISA and CIDT test are presented in Table 2. The agreement between *M. bovis* IDEXX antibody test and CIDT test was fair [k=0.254]. Figure [5] shows the antibody titer of IDEXX *M. bovis* antibody ELISA and change in skin thickness [in mm] following CIDT testing. There was a weak positive correlation [r=0.12] between the antibody titer and change in skin thickness.

Moderate [k=0.437] agreement was recorded between *M. bovis* antibody test and post mortem examination. Out of 123 cattle, 15 were positive for TB lesion while the remaining 108 were negative for TB lesion [Table 3].

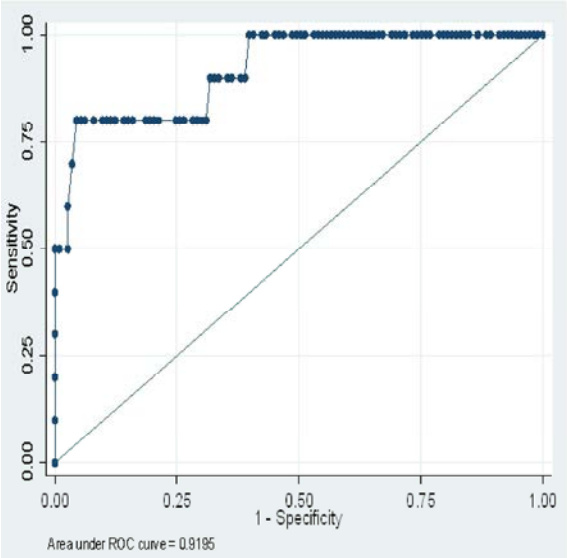

Fig. 2: Receiver operating characteristics [ROC] curve for IDEXX M. bovis antibody ELISA.

Appraisal of a New Cut-off Value for IDEXX *M. Bovis* Test under Ethiopian Condition: The cut-off value recommended by the manufacturer of the kit had lower sensitivity when evaluated under Ethiopian condition. As a result it was found to be important to identify a new cut-off value under Ethiopian condition. Accordingly, receiver operating characteristics [ROC] analysis was performed using culture as a gold standard. ROC curve is depicted in Figure 2 and the area under the ROC curve was 0.92 [95% CI: 0.83-1.00]. Table 4 shows the sensitivities and specificities of IDEXX *M. bovis* antibody ELISA at different cut-off values. As it can be observed from Table 4, sensitivity can be increased without a significant decrease in the specificity of the test. Thus, at a cut-off value of 0.136, the sensitivity could be raised to 80% while the specificity is still maintained high [96%]. Hence, it seems appropriate to use 0.136 as a cut-off value in Ethiopian situation.

Abattoir Survey: The majority of the lesions were detected in the left cardiac lobes [5/15] followed by left diaphragmatic lobes [4/15] and right diaphragmatic lobes [3/15] of the lungs. Among the lymph nodes examined, suspicious lesions were predominantly found in the left bronchial lymph nodes [10/15] followed by retropharyngeal [5/15] and mandibular [3/15] lymph nodes.

Table 4: Sensitivity and specificity IDEXX *M. bovis* antibody test at different cut-off values

Cut Off Value	Sensitivity	Specificity	LH^+	LH^-	
>=.072	90%	67%	2.7486	0.1487	
>=.0721	90%	68%	2.825	0.1468	
>=.0722	80%	69%	2.5829	0.2897	
>=.074	80%	69%	2.6588	0.2861	
>=.0749	80%	71%	2.7394	0.2861	
>=.0756	80%	73%	2.825	0.2825	
>=.0758	80%	74%	3.0133	0.279	
>=.0768	80%	75%	3.1172	0.2723	
>=.077	80%	78%	3.2286	0.2659	
>=.0778	80%	79%	3.7667	0.2559	
>=.0807	80%	80%	3.9304	0.2539	
>=.0817	80%	81%	4.1091	0.2511	
>=.0831	80%	81%	4.3048	0.2484	
>=.0841	80%	84%	5.0222	0.2379	
>=.0849	80%	84%	5.3176	0.2354	
>=.0852	80%	85%	5.65	0.233	
>=.0884	80%	87%	6.45	0.2283	
>=.0893	80%	88%	6.95	0.226	
>=.0896	80%	89%	7.53	0.2238	
>=.094	80%	90%	8.21	0.2216	
>=.0971	80%	92%	10.04	0.2173	
>=.1317	80%	93%	12.914	0.2132	
>=.1329	80%	94%	15.05	0.2112	
>=.1361	80%	96%	18.08	0.2093	new cut off value
>=.1445	70%	96%	19.77	0.311	
>=.1512	60%	97%	22.6	0.4109	
>=.2272	50%	97%	18.83	0.5136	Old cut of value
>=.4261	50%	99%	56.5	0.504	Old cut of value
>=.5454	40.00%	100.00%			0.60
>=.6422	30.00%	100.00%			0.70
>=.6797	20.00%	100.00%			0.80
>=3.8825	10.00%	100.00%			0.90
>3.8825	0.00%	100.00%			0.10

DISCUSSION

In the present study, IDEXX *M. bovis* antibody ELISA was evaluated for its diagnostic performance in Ethiopia. The test was evaluated in dairy farms and in the slaughterhouse on 459 cattle. The sensitivity and specificity of IDEXX *M. bovis* ELISA was determined at the cut-off values established by manufacturer. Mycobacterial culture was considered as a gold standard for the estimation of the sensitivity and specificity of the test. A new cut-off of value was appraised so that the low sensitivity at the present cut-off values is raised without a significant decrease in the specificity of the test. Furthermore, the agreement of this test with the comparative tuberculin test was assessed in dairy farms.

The 50% sensitivity recorded by the present study was lower than the sensitivity [63%] reported by Water *et al.* [14] and the sensitivity [69.4%] reported by Liu *et al*.

[18] and 64.2% sensitivity reported by Lawrence *et al.* [19]. Different factors host related factors and environmental impacts such as parasite burden or exposure to non-tuberculosis mycobacteria could affect the sensitivity of the test in different regions.

Moreover, at the cut-off value recommended by the manufacturer the test had lower sensitivity under Ethiopian condition. Hence, it was found to be necessary to appraise a new cut-off value. The newly appraised cut-off value was 0.136. At this cut-off value the sensitivity was raised from 50% to 80% while the specificity was lowered from 99% to 96%. As one can see, at the new cut-off value the sensitivity was increased significantly while the decrease in specificity was not significant. Therefore, it is of paramount importance to use the new cut-off value under Ethiopian condition. But, before widely using this cut-off value, further evaluation on sufficient number of animals is necessary.

Intradermal tuberculin test has been a standard diagnostic test and has been recommended for the diagnosis of BTB by Office International des Epizooties [4] and the European Union. However, this test cannot detect the disease at all its stages of development. As in many countries, the diagnosis of BTB in Ethiopia has also been based on routine testing with the intradermal tuberculin test which has this constraint. Although, the comparative intradermal tuberculin test is easy to perform and has high specificity, it has low sensitivity [20]. Besides, *M. paratuberculosis* and *M. avium* can cause cross-reaction with intradermal tuberculin test [21]. Under such conditions the use of easier tests such as IDEXX*M. Bovis* antibody ELISA is helpful to supplement the diagnosis of BTB.

There was a fair agreement of IDEXX *M. bovis* antibody ELISA and CIDT test, which suggests that the two tests detect different stages of the disease. As a result, it could be advantageous to use *M. bovis* antibody ELISA as an ancillary test to CIDT test. Earlier studies indicated the usefulness of IDEXX antibody test for the identification of cattle in advanced stages of TB [anergy] [13]. Relatively, the IDEXX *M. bovis* antibody ELISA had lower sensitivity but higher specificity. The lower sensitivity may hamper its utilization in the screening of animals destined for human consumption but the higher specificity makes it a good test to screen animals for eradication programs. On other hand, positive predictive value of IDEXX *M. bovis* antibody ELISA is high. However, negative predictive value of the test was low.

In conclusion, in countries which do not undertake routine screening of their herds using tuberculin test, their herds are expected to consist of animals at the different stages of the disease. Therefore, for surveying their herds of such countries better use a combination of tuberculin test and IDEXX *M. bovis* antibody ELISA. Thus, the IDEXX *M. bovis* antibody test would useful if it is used together with the tuberculin test as it can detect advanced cases which otherwise cannot be detected by tuberculin test.

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