Sero-diagnosis of Bovine tuberculosis using a rapid lateral flow test and ELISA in dairy cattle in Egypt

By

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Abstract:

Mycobacterium bovis (M. bovis), the cause of bovine tuberculosis (BTB) in cattle, remains a major zoonotic and economic problem in many countries. This study aimed to apply different serological tests for detection of bovine tuberculosis as a complementary for intradermal tuberculin test. The tested 4250 cattle in different Egyptian governorates by single intradermal cervical comparative tuberculin test (SICCT), 90 (2.1%) cattle reacted positively. The postmortem (PM) examination of the positive reactors revealed 65 (72.2%) out of 90 slaughtered cattle with visible lesions (VL) while the other 25 (27.8%) reactors showed no visible lesions (NVL). The visible lesions detected in head were 10 (15.4%) out of the 65 VL, in respiratory system were 32 (49.2%), in the digestive system were 5 (7.7%), in

mixed infection were 11 (16.9%), and in generalized infection were 7(10.8%). The bacteriological examination of processed samples from the 90 slaughtered cattle revealed 55 M. bovis (61.1%); 51 (78.5%) were from 65 VL and 4 (16.0%) were from 25 NVL. Enzyme linked immune sorbent assay (ELISA) was used for the tuberculin positive animals' sera and gave 36 (55.4%) out of 65 cattle with and 2 (8%) out of 25 cattle with NVL by a total 38 (42.2%) out of the 90 tuberculin positive animals. The results of lateral flow test gave 32 (49.2%) out of 65 cattle with VL and 1 (4%) out of 25 cattle with NVL by a total 33 (36.7%) out of the 90 tuberculin positive animals.

Keywords: Bovine tuberculosis (BTB), SICCT, VL, NVL, ELISA, lateral flow assay.

Introduction

Globally, BTB is a considerable animal health problem, a chronic disease that impacting both public health and livestock industry (**Jang, *et al.*, 2020**), and endemic primarily in low-income and middle income countries, caused by ***M. bovis***, a member of the Mycobacterium tuberculosis complex (MTBC) **(Shannon, *et al.*, 2020)**.

M. bovis is a highly pathogenic organism, characterized by progressive development of tuberculous-like lesions in many tissues especially in lungs and lymph nodes. In terms of virulence, transmissibility and antigenicity, ***M. bovis*** possesses great potentials for infecting man, numerous other domestic and wild animals **.**Worldwide, eradication programs for ***M. bovis*** are based on detection of infected animals and their removal from the herd (test and slaughter) to limit both zoonotic transmission and economic losses, that requires an accurate diagnosis of infected animals (**Sven, *et al.*, 2016**).

Recently, several diagnostic methods have helped more effective programs of prevention, control and eradication of the disease, include direct detection of the etiologic agent in

biological material and indirect detection through the identification of a host immune response to the etiologic agent (**Collins, 2006**). Among which we can mention the tuberculin test, culture, PM examination, ELISA (**Schiller, *et al.*, 2010**) and lateral flow rapid test.

Disease control programs implemented in most countries are based on the testing of cattle with PPD-tuberculin and slaughter of reactors (**Monaghan, *et al.*, 1994**). Detection of ***M. bovis*** in most African countries is based on the PM findings of tuberculous lesions like an abscess with yellowish pus and tubercles which may be caseous or gritty calcification in the carcasses (**Grist, 2008)**, but not all infected animals express lesions at carcass inspection, so presence of visible lesions suggests that the disease is at an advanced or late stage (**Shitaye, *et al*., 2006)**.

Anergic animals may be found at advanced stage of disease and per parturient cows had been reported to be poor responders to tuberculin test (**Good, M., and Duignan, A., 2011)**, so it is preferable to use more than one technique for detection of ***M. bovis***.

Serological assays generally are simple, rapid and inexpensive, but the development of an improved sero-diagnostic assays also require understanding the bovine tuberculosis humeral immune mechanisms which is characterized by heterogeneous antigen recognition (**Whelan, *et al.*, 2011**).

ELISA has been described based on using ***M. bovis*** antigens, such as bovine-PPD antigen. That assay was successful in detecting circulating antibodies to ***Mycobacteria*** (**Mcnair, *et al.*, 2001**).

Advances in humeral based responses tests have led to the recently development lateral flow test. Ab Test Kit is a solid phase chromatography immunoassay for a qualitative detection

of M. bovis antibody in serum or plasma **(Wernery, *et al.*, 2007; Nasr, *et al.*, 2016)**. The lateral flow test has become an extremely popular tool for the rapid diagnosis of bovine tuberculosis **(chambers, 2013)**. When tuberculin skin test and lateral flow used in parallel offered improved detection of BTB compared to individual tests (**Danbirni, *et al.*, 2013)**.

The purpose for conducting this study was to detect prevalence of bovine TB in dairy farms at different Governorates of Egypt in recent years, with an evaluation of ELISA and lateral flow as complementary tests to the tuberculin skin test for detection of bovine TB.

Material and Methods

1- Tuberculin Skin Testing (Brahma, *et al.*, 2019):

-Tested Animals:

A total of 4250 cattle from different Governorates in Egypt were scanned for TB in this study. All animals were tested with SICCT using bovine tuberculin and avian tuberculin as performed by **OIE, 2018**, gratefully supplied by in the bacterial diagnostic products, Veterinary Serum Vaccine Research Institute (VSVRI), Abbasia; Cairo, Egypt.

SICCT was conducted at two sites on the side of mid neck, 10 - 12 cm apart, injection sites were shaved and the thickness of skin was measured in millimeters with a caliper before the injection of tuberculin and recorded as the first reading, 0.1ml of bovine tuberculin and 0.1ml of avian tuberculin were injected intradermally into the separate clipped sites. After 72 hours the thickness of the skin at the both sites were measured again and recorded as the second reading, then the difference between first and second readings was calculated.

-**Serum of Reactor Animals**:

Blood samples were drawn from jugular vein of the tuberculin reactor cattle, then the sera were separated and stored at- 20°C till be used in serological tests.

3) Post mortem examination:

After slaughtering of tuberculin reacted animals, inspected carefully to detect any pathological changes including the presence of congestion, or any suspected tuberculous lesions such as caseation, calcification and nodular lesions, that might be present in the internal organs and lymph node (**Gobena, *et al.*, 2008**). Lesions in the animals were classified as visible lesions (VL) when detected by the nacked eyes, while in which lesions were not detected were classified as non-visible lesions (NVL). Suspected tuberculous lesions were collected, using aseptic techniques, then placed in an ice box and submitted as soon as possible to the laboratory where they were processed for isolation and identification of the organism microscopically and bacteriologically.

2-Sampling according to (OIE, 2018)

A) Organs:

The suspected internal organs as, lungs; livers; spleens; intestine; head; tongue; diaphragm and peritoneum and its associated lymph nodes.

B) Lymph Nodes

- Lymph nodes of head; sub maxillary, retropharyngeal and parotid lymph nodes.
- Bronchial lymph nodes; the left bronchial and mediastinal lymph nodes.
- Hepatic, mesenteric and renal lymph nodes.
- Prescapular, precrural, popliteal, supra-mammary and internal iliac lymph nodes of both sides were also collected from generalized infected carcasses.

3- Bacteriological isolation and identification of the mycobacterial isolates (OIE, 2018):

Suspicious tissues from slaughtered animals were processed for isolation of Mycobacteria on Lowenstein-Jensen medium. Prepared sections were stained with Ziehl – Nielsen's stain. Samples were cultured on tubes of Lowenstein-Jensen slants after being decontaminated with 4% H_2SO_4. Obtained isolates were identified by conventional methods (rate of growth, colonial morphology, pigmentation, and biochemical properties).

4- ELISA (Collee, *et al.*, 1996 and Nasr, *et al.*, 2018):

ELISA was applied on sera of tuberculin positive cattle using PPD-B. ELISA plates (Immulon II- Nunc) were coated with 5µg/ml PPD-B and incubated overnight at 4°C. Blocking of free binding sites was done by 3% bovine serum albumin PBST. Individual cattle serum samples were analyzed in two fold dilutions starting with 1/50. Anti-bovine horseradish peroxidase labeled secondary antibodies was diluted 1/15000 and Ortho-phenylene diamine (OPD) was used as a substrate. The optical density (OD) was measured at 450, using spectra III ELISA reader. The cut-off value was then calculated according to **Nassau, *et al.*, (1976)**, which is equal to the mean OD of negative control plus 2 standards deviation.

- Interpretation of the test:

The serum dilution was considered positive if it yielded a mean OD of each group equal to or greater than the cut-off value.

5- Lateral Flow Rapid Test according to Nasr, *et al.*, (2018):

For rapid detection of antibodies, TB lateral flow rapid test uses selected mycobacterial antigens immobilized on a nitrocellulose strip and a blue latex signal detection system for rapid detection of antibodies, each animal tested by a strip (**Abdullah Kaya, *et al.*, 2015**).

The testing of sera was carried out according to the manufacturer's instructions using the Anigen Rapid Bovine TB antibody test kit, (Anigen, Korea) as described by **Nasr, *et al.*, (2016**):

-Interpretation of the test:

- Negative result: when there is only one color at control (C) band within the result window.
- Positive result: when there is tow color bands at test (T) band and C band within the result window. (Even if the intensity of the band color is faint it is considered as positive).
- Invalid: if there is no color band within the result window, or a color at T band while no color appears at the C band, the result is considered invalid and the specimen is retested.

Results

1- Tuberculin skin test and PM examinations:

About 90 (2.1%) positive tuberculin of total 4250 tuberculin tested cattle by SICCT from different farms in different Egyptian Governorates were recorded. The 90 (2.1%) positive reactors when examined after slaughtering revealed that the number of reactors with NVL were 25 (27.8%) animals, while the number of reactors with VL found to be 65 (72.2%) animals as shown in table (1). On other hand, about 10 (11.1%) of lesions were localized in the head, 32 (35.6%) in the respiratory system, 5 (5.6%) in the digestive, while 11 (12.2%) of reactors had mixed lesions (respiratory and digestive rotes) and 7 (7.8%) of reactors had generalized lesions according to site of the lesion as illustrated in table (2).

Table (1) Results of Tuberculin skin test and PM finding of slaughtered positive cattle.

No. of tested cattle	Reactor cattle		PM findings			
			Visible Lesions		Non-Visible lesions	
	No.	percentage	No.	percentage	No.	percentage
4250	90	2.1	65	72.2	25	27.8

Table (2) Results of Tuberculin skin test and PM findings according to site of lesion of tuberculin reactor cattle.

No. of examined animals	No. of tuberculin reactors		P M Findings											
			VL										NVL	
			Head		Respiratory		Digestive		Mixed		Generalized			
	No	%	No	%	No	%	No	%	No	%	No	%	No	%
4250	90	2.1	10	11.1	32	35.6	5	5.6	11	12.2	7	7.8	25	27.8

2- Bacteriological examination:

Bacteriological examination of suspected tuberculous and caseated tissue samples that were processed from the tuberculin reactors revealed ***M. bovis*** isolation, about 7 (7.8%) cattle from head lesions, 26 (28.9%) respiratory lesions, 2 (2.2%) digestive lesions, 9 (10%) mixed lesions, 7 (7.8%) generalized lesions, totally about 51 (56.7%) cattle showed VL and 4 (4.44%) cattle from those with NVL, by a total 55 (61.1%) cattle of the 90 reactors, as illustrated in tables (3).

Table (3) Results of bacteriological isolation of *M. bovis* related to site of lesion in PM.

PM Findings				*M. bovis* isolation	
VL	Site of Lesion	No.	Percentage	No	Percentage
	Head	10	11.1	7	7.8

	Respiratory	**32**	**35.6**	**26**	**28.9**
	Digestive	**5**	**5.6**	**2**	**2.2**
	Mixed	**11**	**12.2**	**9**	**10.0**
	Generalized	**7**	**7.8**	**7**	**7.8**
	Total	**65**	**72.2**	**51**	**56.7**
NVL		**25**	**27.8**	**4**	**4.4**
Total		**90/4250**	**2.1**	**55**	**61.1**

3- ELISA results:

ELISA was performed on sera of the 90 tuberculin reactors using PPD-B antigen. A pilot test was conducted at first to determine the optimum serum dilution which was used in the subsequent analysis. It was found that serum dilution 1/100 gave the best results with the antigen used in this study. PPD-B gave superior results with the tuberculin reactors either with VL or NVL. From 90 tuberculin reactor cattle, 38 (42.2%) reactors were positive for ELISA using PPD-B, 36 (40%) reactors that showed VL and 2 (2.22%) reactors with NVL compared to ***M. bovis*** isolation rate of 55 (61.1%), 51 (56.7%) for reactors with VL and 4 (4.44%) for reactors with NVL, as shown in table (4).

Table (4) Results of ELISA in comparison with bacteriological isolation and PM findings.

Test	**PM Findings**		**Bacteriological Examination**				**ELISA**			
	Positive		**Positive**		**Negative**		**Negative**		**Negative**	
	No.	**percentage**	**No**	**percentage**	**No**	**percentage**	**No**	**percentage**	**No**	**percentage**
VL	**65**	**72.2**	**51**	**56.7**			**36**	**40**		
NVL	**25**	**27.8**	**4**	**4.44**			**2**	**2.22**		
Total	**90**	**2.1**	**55**	**61.1**	**35**	**38.9**	**38**	**42.2**	**52**	**57.8**

4- Lateral flow results:

Results of Rapid Bovine TB Ab test kit for tuberculin reactor cattle in comparison to the type of lesions VL or NVL and results by ELISA. It is cleared from table (5) that 33 (36.7%) of tuberculin reactor cattle were positive with Anigen Rapid

Bovine TB kit, while 57 (63.3%) were negative, also it was noticed that 32 (35.6%) of the reactors with VL are positive by lateral flow rapid bovine TB kit while only 1 (1.1%) of the reactors with NVL is positive by lateral flow. Table (6) and figure (1), illustrated a comparison between results of PM findings, bacteriological isolation of ***M. bovis***, ELISA using PPD-B and lateral flow technique for tuberculin reactor cattle.

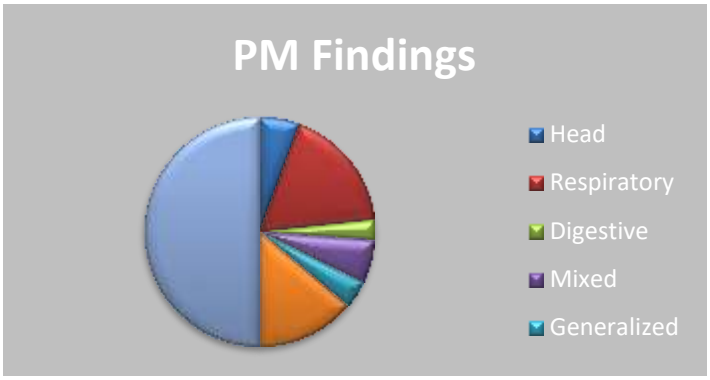
Table (5): Comparison between results of PM findings, ELISA and lateral flow technique for tuberculin reactor cattle.

Test	PM Findings		ELISA				Lateral Flow			
	Positive		Positive		Negative		Positive		Negative	
	No	%	No	%	No	%	No	%	No	%
VL	65	72.2	36	40	29	32.2	32	35.6	33	36.6
NVL	25	27.8	2	2.2	23	25.6	1	1.1	24	26.7
Total	90	2.1	38	42.2	52	57.8	33	36.7	57	63.3

Table (6) Comparison between results of PM findings, bacteriological isolation of *M. bovis*, ELISA using PPD-B and lateral flow technique in tuberculin reactor cattle according to site of lesions.

PM Findings				*M. bovis* isolation		ELISA		Lateral Flow	
	Site of Lesion	No	%	No	%	No	%	No	%
VL	Head	10	11.1	7	7.8	4	4.4	3	3.33
	Respiratory	32	35.6	26	28.9	20	22.2	18	20.0
	Digestive	5	5.6	2	2.2	0	0	0	0
	Mixed	11	12.2	9	10.0	5	5.6	4	4.44
	Generalized	7	7.8	7	7.8	7	7.8	7	7.8
	Total	65	72.2	51	56.7	36	40.0	32	35.6
NVL		25	27.8	4	4.4	2	2.2	1	1.1
Total		90/4250	2.1	55	61.1	38	42.2	33	36.7

Figure (1):- The correlation between results of PM findings, bacteriological isolation of *M. bovis*, ELISA using PPD-B and lateral flow technique in tuberculin reactor cattle according to site of lesions.



Discussion

Bovine tuberculosis is an important zoonotic disease transmitted by direct contact, respiratory pathway, ingestion of unpasteurized milk and milk product, raw or uncooked meet. Tuberculosis couldn't be diagnosed basically on the clinical signs (**Abdullah Kaya, *et al.*, 2015**).

The objective of this study was to field evaluate the two serological tests; ELISA and Lateral Flow, for detection of BTB in dairy farms in parallel to the traditional diagnostic tools.

The results in table (1) illustrated the prevalence of tuberculin reactors in dairy cattle farms in different Egyptian Governorates and PM findings of slaughtered tuberculin reactor cattle. From table (1), a total of 4250 tuberculin tested cattle, 90 tuberculin reactor cattle with a prevalence rate of 2.1%. The prevalence rate recorded in the present study is relatively similar to that given by (**Mohamed, *et al.*, 2011**, 2.46%) and (**Nasr, *et al.*, 2016,** 2.6%) but less than that given by other investigators in

Egypt as (**Lotfy, *et al.*, 1960**, 6.9%; **Guindi, *et al.*, 1965**, 26.5%; **El- Sabban, *et al.*, 1992**, 24% and **El battawy, 2008**, 4.6 %) and in other countries of the world 11.6% (**Ameni and Erkihun, 2007**) in Ethiopia; 8% (**Borna, *et al.*, 2009**), in Chad; 5.6% (**Pereira, *et al.*, 2009**) in Brazil and 7.3% **(Durnez, *et al.*, 2011**) in Tanzania**.**

In the present study, the low incidence of infection could be attributed to many factors such as herd size, density of animals, breeding and management system (**Abuo–Eisha, *et al.*, 1995**). At the same time, the prevalence recorded in the present study is comparatively higher than that given by other investigators 1.3 % (**Shirma, *et al.*, 2003**) and 0.9% (**Cleaveland, *et al.*, 2007**) in Tanzania.

On other hand, the number of tuberculin reactors with NVL as shown in table (1) were 25 (27.8%) reactors, a finding which may be attributed to the non-specific reaction to the tuberculin test which may be due to sensitization by other mycobacteria rather than ***M. bovis*** or even closely related microorganisms especially of the genus Nocardia or a combination of liver fluke infestation with saprophytic mycobacteria (**Cortina and Vera, 1986**). Moreover, (**O'Reilly, 1992**) and (**Huitema, 1994**) described the cause of non-specific reaction to the assumption that reactors may be slaughtered at stage of the disease where the tuberculous lesions are invisible or the lesions may be found in parts of carcass such as bone or skin. As shown in table (1) the VL found with an overall percentage of 72.2% which are less than that reported by **Zivkovic, *et al.*, (1984**), 75.2% and **Nasr, (1997**), 73.4%. On the other hand, other authors claimed a much higher percentage (**Kilian, 1982)**, 96.3% in Germany and (**El- Sabban, 1992**), 100% in Egypt.

Table (2) showed that the higher lesion severity was observed in the respiratory system; lungs, trachea and its lymph nodes 35.6%, 28.9%, 22.2%, 20%, by PM examinations. This

may be due to the intensive husbandry systems which make the respiratory excretion the main route by which animal– to –animal transmission occurs (**Smyth, *et al.*, 2001**).

Culture is considered to be the "gold standard," but this is a very slow and labor-intensive procedure and may become positive only several weeks after inoculation, especially for samples containing low numbers of mycobacteria (**Fawzy, *et al.*, 2013)**. The total isolation rate of ***M. bovis*** form carcasses of tuberculin reactors with VL and with NVL lesions was demonstrated in tables (3). From a total of 90 carcasses, 55 positive cultures were recovered with an isolation rate of 61.1 %. The obtained results were less than that mentioned by (**Tammemagi, *et al.*, 1973**), 89.1 % and (**Naglaa, 2008**), 70.59 %. Other investigators reported lower ***M. bovis*** recovery rate, by (**Adawy, 1986**), 17.5 % and (**Zschoc, *et al.*, 1990**), 1.6 %.

ELISA could detect the immune status of the herd and could be used as a complementary to the skin test to determine the status of disease and reduce the frequency of misdiagnosis as it could detect the early infection and also the advanced stages of infection (generalized form) and localized forms of tuberculosis (**Fawzy, *et al.*, 2013)**.

It is cleared from table (4) that 38 (42.2%) of tuberculin reactor cattle were positive with ELISA using B-PPD, by a compare with 55 (61.1%) in bacteriological isolation. While, 52 (57.8%) were negative by ELISA by a compare with 35 (38.9%) in bacteriological isolation. The obtained results were less than that of (**Sayin, and Erganis, 2013**) 45.9% in Turkey and (**Asiak, *et al.*, 2007**), who recorded 45.7% but higher than that of (**Gutierrez*, et al.*, 1998**) 40.7%.

Table (5) showed the difference between results of ELISA and the lateral flow as serological tests. The Anigen Rapid Bovine TB AB Test Kit has detected 33 (36.7%) of tuberculin

reactor cattle, while the ELISA with B-PPD antigen has detected 38 (42.2%) of tuberculin reactor cattle. The results agreed with that of (**Danbirni*, et al.*, 2013**), who mentioned that the Anigen rapid bovine TB Ab test alone is not efficient in detection of BTB and the serological tests like ELISA must be used to validate results, but disagreed with (**Danbirni, *et al.*, 2009**) who found that 62% of cows were positive by Anigen rapid bovine test TB Ab test, and (**Kalaf, *et al.*, 2014**) who showed that out of 28 cows examined with comparative tuberculin test, 21 (75%) cows showed positive tuberculin reactions and 22 (78.57%) cows showed positive results for Anigen rapid bovine TB (**Awah-Ndukum, 2010**).

Table (6) and figure (1) illustrated the difference between the SICCT, PM examinations, bacteriological isolation, ELISA using B-PPD and lateral flow for detection of BTB. The Negative ELISA explained that the use of ELISA was not effective for detection of BTB. These results were in agreement with the reports of (**Auer, 1987**), (**Placket, *et al.*, 1989**), (**Ritacco*, et al.*, 1990**), (**Casillas, *et al.*, 1995**), (**Keskin, 2001**), (**Keskin, and İzgür, 1996**), and (**Akçay, 2000**), who reported that ELISA was unsuitable as an alternative to tuberculin testing for the detection of tuberculous cattle. However, anergic tuberculous cattle, which are false-negative to the tuberculin test, can be detected by ELISA (**Placket, *et al.*, 1989**, **Neill, *et al.*, 2001**). The immune response to bovine tuberculosis is multifaceted and diagnostic parameters are likely to evolve differently as the disease progresses (**Pollock, *et al.*, 2005**). Antibody titers in tuberculous animals generally depend on the stage of the disease. High positive titers reflect the active stage of the disease (**Vordermeier, *et al.*, 2004**).

The Negative Anigen Rapid Bovine TB kit is explained by the fact that low titer of antibodies to mycobacterial antigens which may be associated with heavy infection and that antigens

may be released into the blood circulation and cause temporary suppression to antibody formation (**Krambovitis, 1986**) and that agree with **Thorns and Morris, (1983)** who cleared the level of specific antibodies in many ***M. bovis*** infected cattle may be low or undetectable, but this is supported by (**Amadori, *et al.*, 1998**) who pointed that the antibodies to mycobacterial antigens were investigated with various rates of success since the humeral immune response to ***M. bovis*** is late and irregular during the course of the disease.

Conclusions

The humeral immune test using ELISA and lateral flow as the Rapid Bovine TB Ab kit test for detection of BTB was not efficient, but it may be useful as complementary tests for skin test especially in bovine tuberculosis eradication programme.

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