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Sensitive and Specific Diagnostic Assay for Detection of Tuberculosis in Cattle

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Abstract: The present study was planned to estimate the sensitivity of the single intra dermal tuberculin test, dipstick assay, ELISA (using Bovine PPD, Cured culture filtrate, ESAT-6, MBP70 and CFP10) and histopathlogical examination of the positive slaughtered animals for the detection of bovine tuberculosis (BTB). A total number of 1850 of cattle from different farms in Egypt were examined for BTB by tuberculin intradermal test using mammalian purified protein derivative (MPPD). A total of 36/1850 (1.90%) were positive reactors by single cervical test. Histopathological examination of 288 lymph nodes and 72 organ tissues (Liver and Lung) revealed that 75 lymph nodes and 25 organ tissue samples showed typical granuloma for tuberculosis. The percentage of the collected samples from those showing positivity by Ziehl Neelsen stain was 52.43 % (151/288) for the lymph nodes and 56.94 % (41/72) for liver and lung tissues. Positive reactors of ELISA assay using different antigens for skin tuberculin tested cattle were increased to 61.67 and 63.33% by using ESAT-6 and MPB70, respectively. Generally, the sensitivity and specificity of ELISA and dipstick assays were recorded 100% by using crude culture filtrate and CFP10 as capture antigens. The range of sensitivity (89 - 97 %) and specificity (88 - 96) was slightly decreased by using Bovine PPD, ESAT-6 and MPB70 for serodiagnosis of BTB. The study has provided a clearer understanding of the kinetics of antibody responses to defined mycobacterial antigens at the subclass level in bovine tuberculosis and has made it possible to develop a novel dipstick system which may be useful in disease diagnosis. In conclusion, the dipstick of the present study is inexpensive (relative to ELISA), sensitive and specific diagnostic assay for detection of tuberculosis in cattle and could be particularly useful in developing countries or remote areas that may lack access to expensive testing equipment for diagnosis of tuberculosis.

Key Words: Bovine Tuberculosis · Tuberculin Test · ELISA · Dipstick Assay

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INTRODUCTION

Bovine tuberculosis caused by *Mycobacterium bovis* is an animal health problem throughout the world and also constitutes a major threat to human public health. *Mycobacterium bovis* is a zoonotic organism and, during diagnostic examination, should be treated as a risk/hazard with appropriate precautions to prevent human infection occurring. Although cattle are considered to be the true hosts of *M. bovis*, the disease has been reported in many domesticated and non-domesticated animals [1-3].

Eradication of this zoonotic disease remains an important goal in several countries. The strategy of test and slaughter has been used widely in an attempt to control dissemination of the disease and is based on the tuberculin skin test as a means for bovine tuberculosis diagnosis. However, implementation of tuberculin tests is cumbersome, requiring a second visit by the veterinarian three days after the tuberculin injection. Some bovine tuberculosis eradication programs have incorporated variants of this test but suboptimal sensitivity and specificity have frequently been reported [4]. Therefore, more sensitive and specific tests, probably incorporating better defined antigens, are required for efficient detection of this disease.

Mycobacterium bovis can cause severe economic losses due to its effects on domesticated livestock and zoonotic infections. *M. bovis* infected cattle that have been skin tested prior to subsequent slaughter and postmortem can be subdivided into several groups depending on their tuberculin skin test results and pathological findings. In regard to the single intradermal cervical tuberculin test (SICTT), they can be designated either skin test positive, negative, or inconclusive. Herd removal operations were carried out on farms with persistent and severe BTB herd breakdowns. In these cases, the whole herd is depopulated and all cattle are slaughtered irrespective of their SICTT responses. The poorly characterized antigens used are mycobacterial extracts containing components that are not species specific [5].

The present study was planned to estimate the sensitivity and specificity of the single intra dermal tuberculin test, dipstick assay and ELISA [using bovine PPD, crude culture filtrate and recombinant peptides (ESAT-6, MBP70 and CFP10)] with histopathological examination of the positive slaughtered animals for the detection of bovine tuberculosis.

MATERIALS AND METHODS

Tuberculin Testing of Animals: The single intradermal tuberculin test (SITT) was carried out on the neck of the animals. A fold of skin was pinched with tips of fingers; its thickness was measured and 0.1 ml (0.5 mg/ml) of the mammalian tuberculin (Veterinary Serum & Vaccine Research Institute, Cairo, Abbassia, Egypt) was injected intradermally. The injection site was encircled with indelible ink. The injection sites were examined for swelling and thickness after 72 hours post injection [5]. The interpretation of the results to the test was done according to O.I.E. [6] and Nasr *et al.* [7]. For delayed hypersensitivity and maximum specificity the negative tested animals were re-examined after 96h, 3weeks and 6 weeks following inoculation in order to reduce the probability of false-negative reactors.

Animals: Thirty herds from different governorates were subjected to tuberculin testing of total number of 1850 cattle. The animals which showed positive tuberculin test were slaughtered approximately two to three weeks after being tested and the post slaughter examinations for any tuberculous lesions were recorded.

Blood Samples: Blood samples were collected by using sterile plane venipuncture tubes. The serum samples were separated and stored at -20°C till analysis.

Post-Mortem Examination: A total of 36 positive reactor animals were thoroughly examined postmortem at the abattoir. The prescapular, axillary, supra mammary, prefemoral, suprarenal, mesenteric, ileocaecal, popliteal, retropharyngeal, hepatic and pulmonary lymph nodes (288 samples) were examined. The liver and lungs (72 organ samples) were inspected visually and by palpation. All macroscopic lesions and any congestion detected at the abattoir were removed for laboratory examination.

Bacteriological Examination and Preparation of Crude Culture Filtrate: The samples were processed for isolation of mycobacteria following standard procedures for homogenization, suspension, centrifugation and decontamination [8]. The processed samples were inoculated on Lowenstein-Jensen (L-J) media with and without pyruvate and incubated at 37°C for a maximum period up to 8weeks. The prepared sections were stained

with Ziehel-Nelson stain and the isolates were typed as described by Collee *et al.* [9,10] and Vestal [11]. Bacteria were removed by filtration and the culture filtrate was concentrated by 80% ammonium sulphate precipitation. Precipitated proteins were dissolved in buffer and dialyzed against distilled water and lyophilized [12].

Histopathological Examinations: Detection of acid-fast bacilli was done by histopathological examination of collected specimens (stained with Ziel Nielsen stain) from tuberculin tested positive slaughtered animals. Autopsy specimens were taken from the lymph nodes and organs and fixed in 10% formol saline. Paraffin sections of 5ì thickness were prepared and microscopically examined after staining with hematoxylin and eosin [13].

Enzyme-linked Immunosorbent Assay (ELISA): Different antigens [Bovine PPD, crude culture filtrate and recombinant proteins (ESAT-6, MBP70 and CFP10- Genosphere Biotech. Co. France)] were used separately as capture antigens in ELISA assay as previously described by Lightbody *et al.* [14] and McNair *et al.* [15]. The optimal antigen concentration, antibody and conjugate dilutions were chosen after preliminary checker board titration. All reaction mixture was set in duplicate, with the mean value being used for recording and calculations. Results were read on SOFTmax PRO ELISA reader (Molecular Device Corporation, California) at a wave length of 405°A. The diagnostic value of the ELISA was evaluated in terms cut off value. The cut off value in the current study was calculated as mean O.D. value of the healthy control animals [7].

Dipstick Assay: Dipstick strips were prepared according to the method described from our laboratory by Al-Sherbiny [16]. Briefly, the PPD, Crude culture filtrate, ESAt-6, MpB70 and CFp10 were dotted in 5.2 cm long slots onto nitrocellulose sheets (*Cat. No.* CNPC-SS12, Arista Biologicals Inc. USA) using the Bio-Rad mini-protein II multiscreen apparatus (BioRad). Dipstick assay was developed following the method described earlier by Van Etten *et al.* [17] with slight modifications according to Al-sherbiny [16]. After preparation of sticks they were placed in Sarsdt tubes containing the primary antibody, the strips were incubated with peroxidase-labeled affinity purified antibovine IgG, IgM or IgA (H+L) (kPL, Germany). The reactions were developed with DAB/H_2O_2 substrate solution and stopped by several dH_2O washing.

Specificity and Sensitivity Analysis: Specificity and sensitivity analysis of ELISA and dipstick assays were recorded according to Rajul Parikh *et al.* [18].

RESULTS

Age and Tuberculin Tested Cattle from Different Dairy Farms in Egypt: Results of tuberculin reactions, age and PM findings in slaughtered cattle are demonstrated in tables 1 & 2. Thirty six (1.95%) positive tuberculin reactors were recoded from the total tested animals (1850) in different dairy farms in Egypt. The age of the tested animals (1200) was between 3 and 5 years, while 650 animals were above 5 years. The positive tuberculin test was 1.8% of the first category (3-5 years) and 2.15% of the second category (above 5 years- Table 1). The post mortem examination of the slaughtered tuberculin positive animals showed that, 32 animals (88.9%) had visible lesions (VL), while 4 animals (11.1%) had non-visible lesions (NVL- Table 2).

The locations of the lesions were confined to 288 lymph nodes (prescapular, axillary, supra mammary, prefemoral, suprarenal, mesenteric, ileocaecal, popliteal, retropharyngeal, hepatic and pulmonary) and 72 specimens of 2 organs (liver and lungs- Tables 3 & 4). The degree of lesion varied from caseated lymph node to severe and mild congestion.

Macroscopic Examination of Lesions of Tuberculin Positive Animals: The percentages of the collected samples from those showing visible lesions were 11.8 % (34/288- Table 3) for t he lymph nodes and 12.50 % (9/72- Table 4) for liver and lung tissues, while the rest of samples showed non-visible lesions.

Microscopic Examination of Lesions Using Ziehl Neelsen Stain of Tuberculin Positive Animals: The percentages of the collected samples from those showing positivity

Table 1: Age and tuberculin tested cattle from different dairy farms in Egypt

		Tuberculin test			
Total No. of examined animals	Age (years) of animal (No.).	Positive		Negative	
		No	%	No	%
1850	3-5 (1200)	22	1.83	1178	98.17
	>5 (650)	14	2.15	636	97.85
Total	1850	36	1.95	1814	98.05

Table 2: Postmortem lesions (location and type) of tuberculin positive cattle from different dairy farms in Egypt (n=36).

Macroscopic examination			
No. of samples showing visible lesion (VL)		No. of samples showing non-visible lesion (NVL)	
No.	%	No.	%
32.00	88.89	4.00	11.11
lesions type	Location		
Varied from caseation to severe & mild congestion	Prescapular, axillary, supra mammary, prefemoral, suprarenal, mesenteric, ileocaecal, popliteal, hepatic retropharyngeal and pulmonary lymph nodes.		

Table 3: Macroscopic and microscopic examination of lymph nodes of tuberculin positive animals (n=36)

		Macroscopic examination		Microscopic examination		
		No. of samples showing	No. of samples showing	Using Z.N. stain		
Type of lymph node	Total No. of examined L.N.	visible lesion	non-visible lesion	Positive	Negative	No. of samples showing granuloma (H&E)
Prescapular	36	4	32	20	16	14
	(100%)	11.11	88.89	55.56	44.44	38.89
Axillary	18	2	16	10	8	7
	(100%)	11.11	88.89	55.56	44.44	38.89
Supra mammary	36	2	34	21	15	9
	(100%)	5.56	94.44	58.33	41.67	25
Prefemoral	36	4	32	13	23	13
	(100%)	11.11	88.89	36.11	63.89	36.11
Suprarenal	21	4	17	9	12	4
	(100%)	19.05	80.95	42.86	57.14	19.05
Mesenteric	36	7	29	15	21	3
	(100%)	19.44	80.56	41.67	58.33	8.33
Ileocaecal	6	0.00	6	6	0.00	0
	(100%)	0.00	100	100	0.00	0.00
Poploteal	6	0.00	6	6	0.00	0
	(100%)	0.00	100	100	0.00	0.00
Retropharyngeal	21	2	19	10	11	0
	(100%)	9.52	90.48	47.62	52.38	0.00
Hepatic	36	3	33	17	19	7
	(100%)	8.33	91.67	47.22	52.78	19.44
Pulmonary	36	6	30	24	12	18
	(100%)	16.67	83.33	66.67	333.33	50.00
Total	288	34	254	151	137	75
	(100%)	11.81	88.19	52.43	47.57	26.04

Between practice = Percentage relative to total number of the lesions.

Table 4: Macroscopic and microscopic examination of body tissues in tuberculin positive cattle

		Macroscopic examination		Microscopic examination		
		No. of samples showing	No. of samples showing	Using Z.N. stain		
	Total No. of	visible lesion	non-visible lesion	Positive	Negative	
Type of tissues	examined tissues	16.76	83.33	66.67	33.33	Showing granuloma (H&E)
liver	36	3	33	17	19	7
	(100%)	8.33	91.67	47.22	52.78	19.44
Lung	36	6	30	24	12	18
	(100%)	16.67	83.33	66.67	333.33	50.00
Total	72	9/72	63/72	41/72	31/72	25/72
	(100 %)	(12.50%)	(87.50%)	(56.94 %)	(43.06%)	(34.72%)

Between practice = Percentage relative to total number of the lesions

Table 5: ELISA assay using different antigens for tuberculin tested cattle (n=60)

Tuberculin reactor cattle		ELISA using different antigens									
		Bovine PPD		*CCF		ESAT-6		MPB70		CFP10	
	No.	No.	%	No.	%	No.	%	No.	%	No.	%
-VE RC	24	0/60	0.00	0/60	0.00	1/60	1.67	2/60	3.33	0/60	0.00
+ve RC	36	36/60	60.00	36/60	60.00	36/60	60.00	36/60	60.00	36/60	60.00
+ve R-ELISA		36/60	60.00	36/60	60.00	37/60	61.67	38/60	63.33	36/60	60.00

*CCF= Crud culture filtrate.
-ve RC= Negative tuberculin reactor cattle.
+ve RC=Positive tuberculin reactor cattle.
+ve R-ELISA= Total number of positive reactor ELISA sera.

Table 6: Sensitivity and specificity of ELISA for diagnosis of bovine tuberculosis (n=60)

Tuberculin reactor cattle		ELISA using different antigens									
		Bovine PPD		*CCF		ESAT-6		MPB70		CFP10	
	No.	No.	%	No.	%	No.	%	No.	%	No.	%
-VE RC	24	0/60	0.00	0/60	0.00	1/60	1.67	2/60	3.33	0/60	0.00
+ve RC	36	36/60	60.00	36/60	60.00	36/60	60.00	36/60	60.00	36/60	60.00
Sensitivity		100.00% (36/36+0)		100.00% (36/36+0)		100.00% (36/36+1)		94.73% (36/36+2)		100.00% (36/36+0)	
Specificity		100.00% (24/24+0)		100.00% (24/24+0)		95.83% (23/23+1)		91.67% (22/22+2)		100.00% (24/24+0)	

*CCF= Crud culture filtrate
-ve RC= Negative tuberculin reactor cattle
+ve RC=Positive tuberculin reactor cattle

Table 7: Sensitivity and specificity of dipstick assay using different antigens for tuberculin tested cattle (n=60)

Antigen	Bovine PPD	*CCF	ESAT-6	MPB70	CFP10
Dipstick +ve	35/60 (58.33)	36/60 (60.00%)	32/60 (53.33%)	34/60 (56.67%)	36/60 (60.00%)
Dipstick -ve	25/60 (41.67%)	24/60 (40.00%)	28/60 (46.67%)	26/60 (43.33%)	24/60 (40.00%)
Sensitivity (%)	35/35+1=97.22	36/36+0=100	32/32+4=88.88	34/34+2=94.44	36/36+0=100
Specificity (%)	25/25+1=96.15	24/24+0=100.00	28/28+4=87.50	26/26+2=92.86	24/24+0=100.00

*CCF= Crud culture filtrate
Dipstick +ve = Number of TB dipstick Positive Sera
Dipstick -ve = Number of TB dipstick Negative Sera
Between practice = Percentage relative to total number of the lesions

with Ziehl Neelsen stain were 52.43 % (151/288- Table 3) for the lymph nodes and 56.94 % (41/72- Table 4) for liver and lung tissues.

Microscopic Examination of Lesions Using H&e Stain of Tuberculin Positive Animals: Histopathological examination of 288 lymph nodes and 72 organ tissues revealed that 26% (75/288) lymph nodes and 34.72% (25/72) organ tissues showed typical granuloma for tuberculosis (Tables 3&4).

Diagnosis of Bovine Tuberculosis by Elisa Assay: Results of ELISA by using different capture antigens showed that 60% of the animals (36/60) were positively reacted by using bovine PPD, crude culture filtrate and CFP10. The percentage of positive reactor ELISA sera was increased to 61.67% (37/60) and 63.33% (38/60) by using ESAT-6 and MPB70, respectively (Table 5).

Sensitivity and Specificity of ELISA for Diagnosis of Bovine Tuberculosis: The sensitivity and specificity of ELISA for diagnosis of bovine tuberculosis were recorded 100% by using bovine PPD, crude culture filtrate and CFP10 as capture antigens (Table 6). Whereas, the specificity (95.83% & 91.67%) and sensitivity (97.30% & 94.73%) were to some extent decreased by using ESAT-6 and MPB70, respectively (Table 6).

Diagnosis of Bovine Tuberculosis by Dipstick Assay: The sensitivity and specificity of dipstick assay were recorded 100% by using crude culture filtrate and CFP10 as capture antigens (Table 7). The range of sensitivity (88.88-97.22%) and specificity (87.50-96.15) were decreased by using bovine PPD, ESAT-6 and MPB70 (Table 7- Fig 1, 2 & 3).

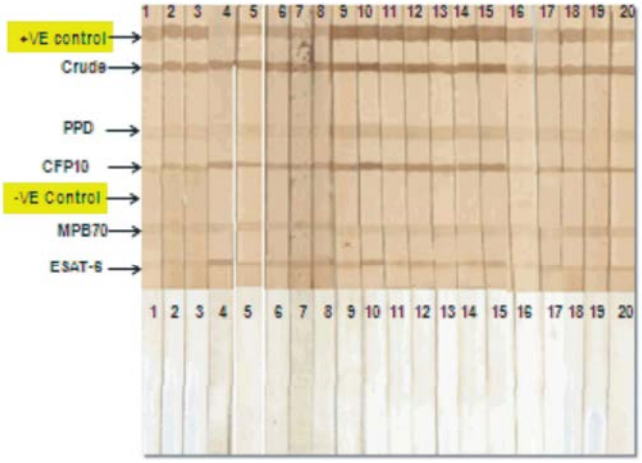

Fig. 1: Dipstick positive sera using different antigens [Bovine PPD, Crude culture filtrate, ESAT-6, MPB70 and CFP10] for samples 1 to 20 of tuberculin positive animals.

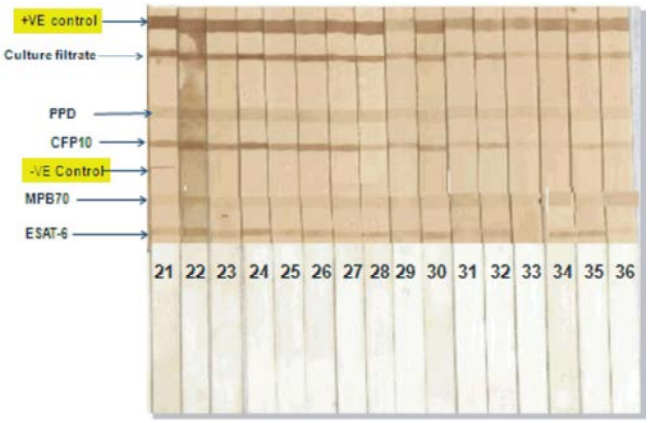

Fig. 2: Dipstick positive sera using different antigens [Bovine PPD, Crude culture filtrate, ESAT-6, MPB70 and CFP10] for samples 21 to 36 of tuberculin positive animals.

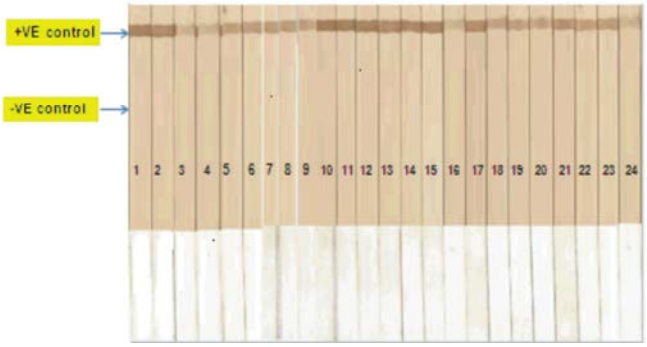

Fig. 3: Dipstick negative sera using different antigens [Bovine PPD, Crude culture filtrate, ESAT-6, MPB70 and CFP10] for samples 1 to 24 of tuberculin negative animals.

Generally, the sensitivity and specificity of ELISA and dipstick assays were recorded 100% by using crude culture filtrate and CFP10 as capture antigens (Table 8). The range of sensitivity (89 - 97 %) and specificity (88 - 96) were slightly decreased by using bovine PPD, ESAT-6 and MPB70 for serodiagnosis of bovine tuberculosis (Table 7).

DISCUSSION

Detection of bovine tuberculosis in cattle is often made on history, clinical and necropsy findings, tuberculin skin tests and abattoir meat inspection. Research on improved diagnostic assays of tuberculosis in animals has resulted in a change in the interpretation of tuberculin skin testing results in cattle by veterinarians. The interpretation of skin test in cattle may result in decreased numbers of false positive results, decreased destruction of non-infected animals and decreased cost to General Organization of Veterinary Egyptian Services (GOVES). Bovine tuberculosis remains a costly disease in many countries despite extensive eradication and control efforts. An accurate diagnostic test for bovine tuberculosis is urgently needed to improve control strategies. *Mycobacterium bovis* infection is certainly an occupational hazard to agricultural workers who may acquire it by inhaling cough spray from infected cattle [19].

Tuberculin test was applied on 1850 cattle in dairy farms. The age groups of cattle were divided into 3-5 years (1200) and > 5 years (650) and 1.95% (36/1850) of the cattle gave a positive tuberculin results (Tables 1& 2).

On the other hand, animals producing false positive tuberculin may be associated with non-visible lesion reactors (NVL reactors) to the tuberculin test which may be due to sensitization by other mycobacteria rather than *M. bovis* or even closely related microorganisms specially of the genus *Norcardia* or a combination of liver fluke infestation with saprophytic mycobacteria [20,21]. Furthermore, there were some of the examined animals that gave a false negative tuberculin but can't be detected due to early stage of infection and technical errors (including skin handling, reagent dose and needle type and size). The sensitivity of the tuberculin test is affected by the potency and dose of tuberculin administered the interval post-infection, desensitization, deliberate interference, post-partum immunosuppression and observer variations. Specificity was influenced by sensitization as a result of exposure to *M. avium*, *M. paratuberculosis* and environmental mycobacteria and by skin tuberculosis [5, 22]. Also, the current study noted that the disease affects all age groups of susceptible hosts and is accountable for more deaths throughout the world than any other bacterial disease every day as recorded by Omer *et al*. [23] and Ghazy *et al*. [24]. These facts has led several research groups to the identification of antigens recognized by immune cells and tuberculin test must be carried out on the side of the neck, with hair clipping at the site of testing, accurate intradermal injection and careful pre- and post-inoculation skin thickness measurement using caliper to obtain results that are valid [24- 27].

The relationship between the reactivity of tested cattle to tuberculin test and post slaughter (PM) findings was investigated in the current study (Tables 3& 4). The macroscopic examination of the collected lymph nodes and organ tissues (liver and lung) reported high incidence in mesenteric, suprarenal and pulmonary ones (16-19%) and in lung tissues (16%) of the tuberculin positive animals, respectively. At the same time the percent of tuberculin reactors with NVL was 88.19% (254 out of 288) for lymph nodes and 87.50% (63/72) for liver and lung tissues. These results are in agreement with those reported by De Oliveira *et al.* [28] and Zivkovic *et al.* [29]. Post mortem examination and bacteriological examination of suspected lesions in cattle are important tools to confirm their presence [30, 31].

The histopathological examination of the collected lymph nodes, using Z.N stain, revealed that 52.43 % (151/288) were positive for acid fast bacilli and a large group of tissues stained from those collected specimens came from non-visible lesion reactors (NVL-88.19%) of the positive tuberculin tested animals (Table 3). Therefore, the opportunity to animal's samples that gave a response to a spectrum of SICTT was pathological descriptions using Z.N stain. Isolation of *M. bovis* from NVL (non-visible lesion reactors) may be described to the fact that in early stage of infection, no or only microscopic lesions are found in the lymph nodes of the reactors and in turn *M. bovis* may be recovered on culturing [30, 32]. However, similar results were obtained by Adu-Bobi *et al.* [33] who stated that the ZN microscopy is effective in detecting the presence of mycobacteria, as 73.1% of the suspected samples (64 lung tissues and 95 lymph nodes) were acid fast bacilli positive. Histopathological examination by H&E of lymph nodes and organ tissues revealed that lymph nodes (26.04%) and organ tissue (34.72%) samples showed typical granuloma for tuberculosis (Table 3 & 4). Histopathological examination may increase the specificity of the diagnosis where the prevalence of bovine tuberculosis is high [34].

The current ELISA results showed that 60-63% (Table 5) of the animals positively reacted by using different capture antigens (Bovine PPD, Crude culture filtrate, ESAT-6, MPB70 and CFP10) in serodiagnosis of bovine tuberculosis in dairy cattle. While, the results of

Hassanain *et al*. [35] revealed that 43.5% (20/46) of the examined animals were positive for ELISA using mammalian PPD (MPPD). Also, lower results were reported by many investigators [36-38]. Ritacco *et al.* [36] stated that the low sensitivity of ELISA limits its usefulness as a diagnostic tool for BTB eradication campaigns. However, it could be helpful in epidemiological surveillance if its efficiency to identify infected herds is demonstrated. The lower results may be due to using non specific antigen of MPPD and the present work aimed to improve the tuberculin reagents which have all the specific antigens. Our data demonstrated that the main advantage of using different capture antigens (cocktail antigens) as a diagnostic reagents would be its capability to detect infected animals escaping skin testing (false negatives) at a level exceeding those of tuberculin and PPD, Crude culture filtrate, ESAT-6, MpB70 and CFP-10. It is likely that a proportion of skin test negative tuberculous cattle are at an earlier time point post infection than skin test positive animals because cellular immune responses dominate early after infection. During early infection stages, it is likely that only a limited number of antigens will be recognized [7, 39].

In the current study, the degree of sensitivity and specificity of ELISA and dipstick assays were nearly similar by using different capture antigens (Table 8). Consequently, the dipstick strips, which produced results in less than 30 minutes, could be particularly useful in developing countries or remote areas that may lack access to expensive testing equipment for diagnosis of tuberculosis.

From the previously mentioned and recorded results about animal tuberculosis we noted that there is a great agreement between serodiagnosis of active tuberculosis in cattle using a specific cocktail antigen (by dipstick assay) and with macroscopic lesion (VL). On the basis of these findings, we suggest that a specific cocktail antigen will be tested in the near future with large numbers of field animals after completing the validation of newly developed diagnostic assay.

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