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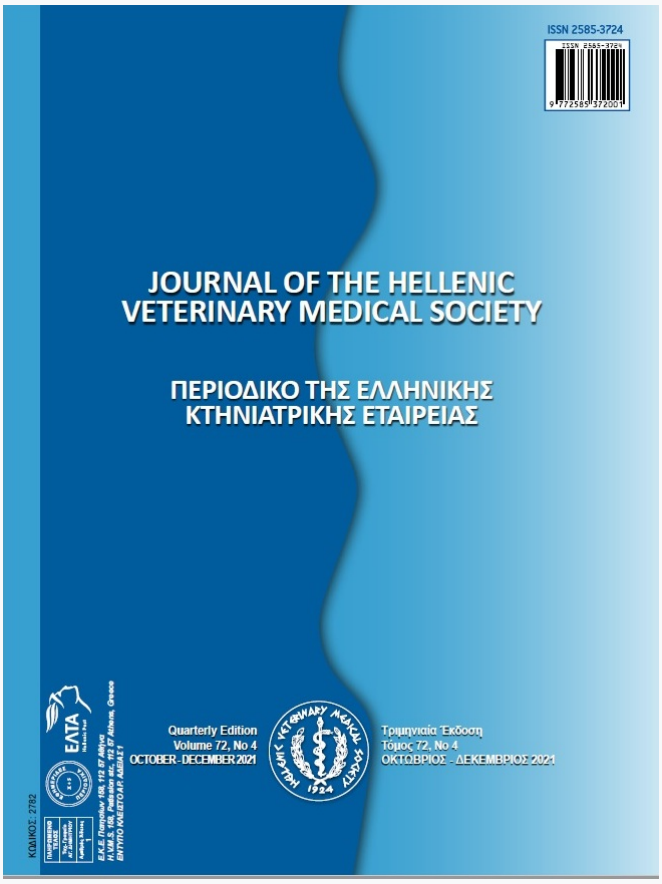

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Research article
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Tuberculin test errors and its effect on detection of bovine tuberculosis

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ABSTRACT: Bovine tuberculosis is an endemic disease in Egypt, a notable gap exists between limited cases identified by single intradermal tuberculin test performed through the national control program and higher detected cases at abattoirs. Therefore, this study aimed to investigate epidemiological situation and causes of the previously mentioned gap in Middle-Delta Region. A total of 25 emergency-slaughtered animals of unknown tuberculosis-status were investigated by cocktail-antigens ELISA and post-mortem examination. Five visible tuberculous-lesion cases were detected and confirmed by PCR, ELISA was sensitive and predictive of the existence of tuberculous-lesions; 4(80%) out of 5 visible-lesion cases were seropositive. True prevalence among the slaughtered animals was 27%. In addition, tuberculin-testing of 400 animals during the national control program was evaluated, many technical and procedural errors were detected, and all animals were negative.Out of them, 55 animals were tested by ELISA before the application of tuberculin test, 30 (54.5%) animals were seropositive. To confirm the effect of the reported errors on reliability of tuberculin test, reference serum of 20 tuberculosis-positive animals that were tested by standard-procedures tuberculin test and their status were confirmed by PCR after slaughtering, were tested by ELISA. A complete matching was evident, the 20 standard-tuberculin positive animals were all seropositive. In conclusion, bovine tuberculosis is endemic at high levels in the study area, reported errors of tuberculin test during national control program may be the cause for missing tuberculosis cases and not tuberculin test itself and finally, a further wide-scale funded study is required to discover the situation throughout Egypt.

Keywords: *Bovine, Post-mortem, Tuberculin, Tuberculosis, Egypt*

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INTRODUCTION

Bovine tuberculosis (TB) is one of the most serious infectious diseases affecting a wide range of domesticated and wild animals. The disease adversely affects animal health, welfare and has implications for the international trade, production of animals and animal products (Fontana et al., 2018).In addition to its negative impact on livestock, TB has a great risk to public health; in 2017 only, 6.4 million new cases of human TB were officially reported to WHO (Global Tuberculosis Report, 2018).

Most countries have official TB control programs that depend mainly on cellular immunity-based tests. The single intradermal tuberculin test (SID) and gamma-interferon assay, which is also used for diagnosis of pseudotuberculosis (Oreiby and Hegazy, 2016), are being used worldwide based on the cellular response to mycobacterial purified protein derivative (PPD).

The SID remains the international field test for TB in bovines. The test has low cost, low logistical demands, a well-documented use, high availability, long history of employing and, for a long time, the lack of alternative field methods to detect bovine TB. Therefore, the test is suitable for routine, systematic surveillance to identify *Mycobacterium* (*M.*) *bovis*-infected animals, slaughter of positive reactor animals, movement tests, epidemiologic trace-back testing, and within TB affected herds to delineate animals going to a slaughter plant versus being condemned for rendering (Bezos et al., 2014; Koni et al., 2016; Waters et al., 2017; Sahli et al., 2018).However, the SID has an imperfect performance caused by nonspecific reactions of the *M.bovis*-crossly reactive bacteria; Mycobacteria, Corynebacteria and *Nocardia* spp. are antigenically related and termed CMN group bacteria (Oreiby, 2015). In addition, the test is complex, require two visits with 72 hrs. in between, variable interpretations between operators in addition to the difficulty to be used in surveillance being requiring professionals in intradermal injection (Brito et al., 2014; Buyuk et al., 2017; Bernttz et al., 2018; Sahli et al., 2018).

Recently, the importance of humoral responses against *M.bovis* infection was highlighted (Infantes-Lorenzo et al., 2019). The pathogen predominantly triggers cell-mediated immunity during its early and intermediate phases. Then after, while the disease progresses, a decrease in the cell-mediated immunity response occurs in parallel with an increase in the production of antibodies resulting in serological responses (Casal et al., 2017; Fontana et al., 2018). The combination of recombinant antigens either as a pool or chimeric antigen (fusion protein) was expected to enable the detection of antibodies from cattle in different stages of *M.bovis* infection (Souza et al., 2012; Infantes-Lorenzo et al., 2017). Thus, great efforts have focused on finding new antigens that would help to improve the performance of the ELISA (Bezos et al.,2014).In addition to the enhanced performance, ELISA is simple and rapid. Therefore, the newly developed commercial ELISA tests, based on a cocktail of carefully selected antigens, have a potential diagnostic value for bovine TB (Riad, 2015).

In Egypt, TB is endemic (Borham et al., 2021), and its control relies on two strategies: test and cull scheme and case detection during meat inspection at slaughterhouses. Test and cull scheme is performed during the national surveillance program against both brucellosis and TB conducted by "General Organization for Veterinary Services", based on annual testing of bovines with the SID. The second strategy is the detection of the affected animals in abattoirs during the regular inspection of meat (Abdellrazeq et al., 2016). A marked unexplained gap exists between the detected TB cases by test and cull scheme, using SID, and that is during meat inspection at abattoirs; the latter is being much higher than the former.

Thus, this work aimed to investigate the prevalence of bovine TB, and to study the causes of the gap between TB-detection of the national control program SID test and that is during routine meat inspection in the study area to be considered in similar endemic countries.

MATERIAL AND METHODS

Study area, animals and sampling

This study was conducted in the Middle-Delta region; Disuk, Baltim, Sidi-Salem, EL-Reyad and Beyala districts of Kafrelsheikh Governorate as well as Tanta abattoir at Gharbia Governorate.

Animals are divided into the followings:

a- Blood samples from 25 animals (19 cows and 6 buffaloes) emergency slaughtered at Tanta abattoir were collected for ELISA examination and these animals were subjected to Post-mortem examination according to (Domingo et al., 2014) for detection of visible TB lesions.

b- A Total of 400 animals (330 cows and 70 buffa-

loes) were tested with SID during the national control program against bovine TB. Of these 400 animals, blood samples for ELISA testing were collected from 55 animals (44 cows and 11 buffaloes) before testing with SID.

c- Reference positive serum samples of 20 (10 cows and 10 buffaloes) TB-affected animals that were tested positive by standard-procedures SID, then subjected to post-mortem examination and their TB-positive status was confirmed by PCR. These serum samples belonged to the TB project (No. 2966, Science & Technology Development Fund, 2017) and were kindly supplied by the tuberculosis unit of the Animal Health Research Institute, Giza, Egypt. The samples were collected 6 months after application of SID.

Evaluation criteria of SID

The SID procedure during the national program was evaluated according to the criteria described in Bovine Tuberculosis Manual(2016). Availability of the test instrument, methods of identification of injection site, measuring the skin thickness, keeping and injection of PPD and final interpretation of the test results were recorded. ThePPD used in the program was *M. bovis* PPD (1mg/mL, Veterinary Serum & Vaccine Research Institute, Egypt). The performance of both the SID conducted during the national control program and that is of the standard SID which was done on the reference positive animals of the TB project (No. 2966, Science & Technology Development Fund, 2017) was judged by a newly developed highly sensitive cocktail-antigens ELISA.

***In Vitro*Testing by cocktail-Antigens ELISA**

The one hundred serum samples were tested using a patent antibody ELISA kit for bovine TB **(**Wuhan UnibiotestCo., Ltd, China).The kit is an indirect ELISA assay for the qualitative detection of *M. bovis* antibody in serum depending on especially selected *M.bovis* antigens that used as detector materials, allowing the detection of the antibodies in specimens with a high degree of accuracy. Briefly, 100 µl of 1:100 diluted serum samples were added into the wells of the pre-coated microplate. Similarly, 100 µl of negative and positive controls were added. The plates were incubated at 37 °C for 30 min. After successive five washing steps, 100 µl of enzyme conjugate (IgG Fc-HRP) were added to each well and incubated at 37 °C for 30 min. The washing was repeated and 50 µl of substrate A and B were added, respectively followed by shaking at 37 °C for 10 min. Finally, 50 µlof the stop solution was added, and the plates were read at 630nm after 10 min using a microplate reader (Model: Clindiag MR-96, Serial No: R2DO003) (Clindiag Systems Co., Ltd, China). The sero-status was calculated according to the following provided equation:

S/P=(Sample OD-Neg.mean)/(Pos.mean-Neg.mean)

If the $S/P \geq 0.17$, the sample was positive while, if it was < 0.17, the sample was negative**.**

Epidemiological investigation

The true prevalence (TP) of bovine TB among emergency slaughtered animals will be calculated as follows:

TP= ((AP+SP-1)/ (SP+Se -1)*100

Where AP is the apparent prevalence of bovine TB among emergency slaughtered animals using post-mortem examination (number of animals with visible lesions and confirmed by PCR/ total examined animals) *100. The Se and Sp are the sensitivity and specificity, respectively, of Post-mortem examination for bovine TB. The Se and specificity were 71% and 99%, respectively, and these estimated were obtained from (Nunez-Garcia et al., 2018).

The apparent of bovine TB among the 55 animals tested by both field SID and ELISA will be estimated as in the previous step as follows:

(The number of seropositive animals to the cocktail-antigens ELISA/55)*100

The statistical difference between apparent prevalence of TB among cows and buffaloes to cocktail-antigens ELISA was examined using Fisher's exact at level of significance $P< 0.05$.

RESULTS

Prevalence of bovine TB among the emergency slaughtered and national control program SID tested animals

Out of the 25 non-tuberculin tested emergency-slaughtered animals, 5 contained visible lesions and TB was confirmed by RTPCR (Data is published in another paper), PM lesions are shown in (Fig. 1 and 2). The true prevalence of the disease among examined animals was 27.14% (95% Confidence interval (CI): 9.74% - 44.5%). The number of true positive animals was estimated at 7 animals.

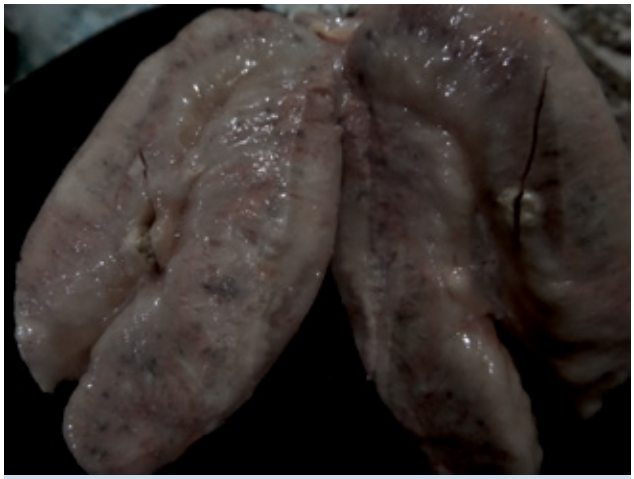

Fig. 1. Incised severely enlarged bronchial lymph node containing tuberculous lesions

Fig. 2. Milliary TB on the peritoneum "Pearls appearance"

Results of ELISA showed that 4 (80%) out of the 5 lesions-contained animals were seropositive, and one (20%) animal was seronegative. On the other hand, out of the 20 non-visible lesion animals, 11 (55%) were seronegative and 9 (45%) were seropositive. These results showed the high sensitivity of cocktail ELISA for detection of infected cases, but on the other hand it showed its low specificity due to the high number of false positive animals. The apparent prevalence among cows and buffaloes are shown (Table 1). There is no significant difference between the prevalence of the disease in cows and buffaloes ($P< 0.644$).

All the 400 SID-tested animals during the national control program were negative. The cocktail-antigens ELISA of the 55 serum-sampled animals showed 30 seropositive animals and an apparent prevalence of 54.5% (95% CI: 41.34% - 67.66%) (Table 1). There is no significant differencein the apparent prevalence among cows and buffaloes ($P< 0.2$).

Evaluation findings of SID performed through the national control program in the study area

Many procedural and technical serious faults were recorded during the official application of the SID. The veterinary units involved in the testing procedures lacked the standard test equipments: syringes, needles, callipers, dark coolers and hair clippers. Moreover, a shortage in animal handling as well as operator safety equipment were evident. The veterinarians involved in the testing process had complained from few numbers of the operators and shortage of transportation vehicles which carry them to the test location. Furthermore, they indicated that sometimes there was a conflict between the scheduled dates of SID and the vaccination campaigns against other diseases such as FMD and LSD. The veterinarians confirmed that there were not any incentives for them in regard of performing the test. They also mentioned that the simultaneous collection of blood samples from the same animals to be examined against brucellosis made much more difficult and slow performance of the SID. The veterinarians thought that the incomplete awareness of farmers about the dangerousness of the disease, lack of their interest in testing against TB and the low compensation value offered by the authority when positive cases exist are seriously affecting the success of the national control program against TB.

Table 1. Results of ELISA in relation to tuberculin test status.

	Seropositve								
	Cows +/t	AP%	95% CI	Buffaloes +/t	AP%	95% CI	Total +/t	AP%	95% CI
A	9/19	47.4	24.95 - 69.85	4/6	66.7	28.98 - 100	13/25	52.0	32.41 - 71.85
B	26/44	59.1	44.75 - 73.45	4/11	36.4	7.97 - 64.83	30/55	54.5	41.34 - 67.66
C	10/10	100	-	10/10	100	-	20/20	100	-

A are the 25 non-tuberculin tested animals.
B are the 55 tuberculin-negative animals of the national control program.
C are the 20 tuberculin-positive reference positive animals.
+/**t** is the number of positive /number of tested animals.

In addition to the previously mentioned procedural errors, several technical mistakes during the application of the test were detected. Most of the SID practitioners didn't shave and clip the hair, none of them disinfected the injection site before the application. Not all the tested animals were numbered and identified. It was noticed that most of the tested animals didn't expose to careful examination for any existing lumps, bruises, adhesions and other skin conditions at the injection site. Also, measuring of the skin thickness was performed using the opened and closed calipers by more than one operator at the same farm during the two visits. During the test, most of the practitioners didn't keep the PPD in dark coolers and it was exposed to direct sunlight. Some practitioners used too long needles not suitable for the intradermal injection. In most instances, disinfection and replacing of needles between animals or herds wasn't performed. During the test interpretation, most operators depended mainly on visual observation and palpation only. To ascertain the effect of these reported errors on the performance of SID, the performance of the cocktail-antigens ELISA was compared to the standard-procedures SID of the reference positive animals.

Comparison between the standard-procedures SID and cocktail-antigens ELISA

A complete matching was existing between the cocktail-antigens ELISA and the standard procedures-SID of the TB project No. 2966. All the 20 animals were SID and ELISA positive. These animals were slaughtered, and their status was confirmed by PCR.

The ELISA Optical density (OD) and sample values (S/P) are illustrated in (Fig. 3 and 4, respectively). In addition, the relationship between the results of the SID and of ELISA is summarized in (Table 1).

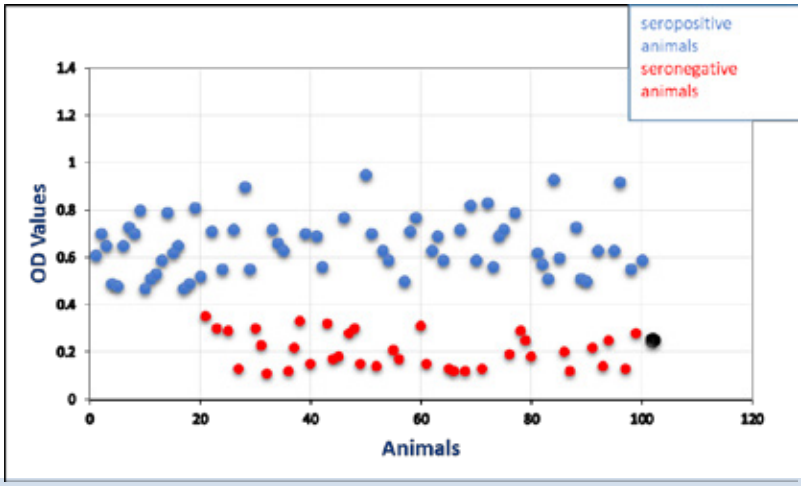

Fig. 3. Optical Density values of ELISA tested animals

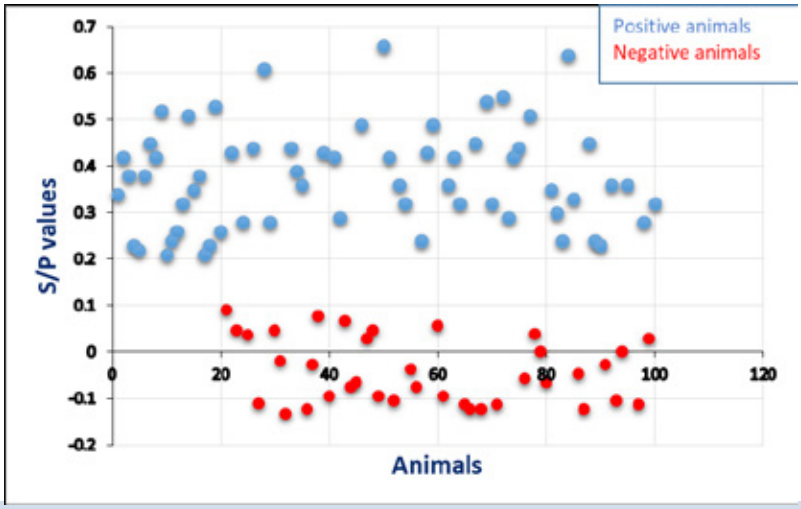

Fig. 4. S/P Values of positive and negative serum samples

DISCUSSION

Based on the official reports recorded by the "General Organization for Veterinary Services"at Kafrelsheikh Governorate in 2005, 2006 and 2007, the prevalence of tuberculin test reactors using SID among cows was 0.014%, 0.17% and 0.088, whilst it was 0.029%, 0.28% and 0.0% among the buffaloes, respectively. These reports indicate the very low percentage of positive reactors detected during the national control program in the study area and comes in harmony with the findings of the current study. In 2007, only 7 positive cases out of 7918 tested cows, and no positive animals out of 3823 tested buffaloes (Khoudair et al., 2009). On the other hand, a higher prevalence rate, 30.11% of the tested animals were positive reactors, was recorded using theSID (EL-Mahrouk and EL-Balawy, 2010). Further study during 2012-2015 in five regions within the Nile Delta had showed a prevalence of 7.3%, whereas it was as high as 45% in some farms (Amin, 2019).Prevalence of the disease in the study areais influenced by increasing animal density, unhygienic practices and stress during inter-current diseases and mass vaccination campaigns (Abou-Eisha et al., 2002). Recent data indicated that bovine TB is increasing sharply in some Egyptian governorates especially that are locatedwithin the Nile Delta and valley compared to the rest of the country being densely populated areas andthe nature of the rural communities where farmers keep the animals inside their houses (Abdellrazeq et al., 2016; Amin, 2019).

Despite of the annual diagnostic efforts for controlling bovine TB in Egypt, the disease still evident. The results obtained in this study confirmed the existence of bovine TB in the study area at a significantly high level; 27.14%. Despite that the examined animals were emergency slaughtered animals, but the findings were relatively like that of animals tested during the national control program. Reasons of such conclusions include the results obtained by ELISA on the SID-negative animals of the national control program which showed that the apparent prevalence among these animals is very high. The reported procedural and technical errors of SID performed during the national program may be responsible for the notable gap between the diagnosed TB cases by SID and those detected during routine meat inspection at abattoirs. It is well known that the SID is of unsatisfactory accuracy, which is considered the major impediment facing the control of bovine TB (Waters et al., 2017). Errors during the application of SID increases the magnitude of poor performance of the test.

In the current study, many procedural and technical errors were detected. In the same context, (Koni et al., 2016) recorded similar errors during the application of the SID which negatively affect the active surveillance against TB in Albania; untrained people, unavailability of test equipment as syringes and needles, lack of the appropriate identification and registration of animals, in addition to the cost of certified tuberculin.

Shortage of animal handling and operator safety equipment in addition to the dangerous temperament of some buffaloes explain, to some extent, the poor performance of the test in these buffaloes. The direct injection of tuberculin by most of the operators without preceding examination of the skin for lesions will affect the test interpretation, especially when the test performed during the outbreaks or even on recovered animals from diseases affecting skin such as oedematous skin disease or lumpy skin disease. Also, the improperly stored PPD, exposed to direct sunlight and the injection of sub-doses might cause false negative results (Humblet et al., 2011; Bezos et al., 2014). Tuberculin should be stored at 4 - 8 ^{0}C, never be shaken and away from sunlight. In addition, the usage of too long needles leads to the deposition of the tuberculin under the skin, especial needles of 3.9mm long must be used to confirm the intradermal injection (Bovine TuberculosisManual, 2016). Moreover, the detection of the formation of a small pea-like swelling at the injection site should be practiced because it is a sign of successful intradermal injection (Bezos et al., 2014). Furthermore, a regular check of the needle for being damaged or bent, and replacing them between animals is very important, otherwise it may act as a vector for the transmission of pathogens (Humblet et al., 2011).The interpretation of the SID without measuring the skin thickness, depending on observation and/or palpation has a negative effect on the test result as it is subjective and may vary between different operators (Bernttz et al., 2018). All these reported errors may explain the negative status of the followed-up SID-tested animals during the national scanning against bovine TB in the study area.

The comparison between the results of cocktail ELISA and that is of both SID performed during the national control program and standard-procedures SID offer a strong evidence about the effect of the reported errors on the performance of SID. The low number of detected bovine TB cases by SID performed by the veterinary authority compared to the

higher number of cases detected at slaughterhouses is mainly because of the errors practiced during the application of SID and not because of the accuracy of the SID itself. A similar study in Brazil reported a significantly greater sensitivity of ELISA than the SID; 46.1% of infected cases were detected by ELISA compared to 5% by SID (Souza et al., 2019).This may reflect the predominance of humoral immune response during the advanced stages of the disease as reported by (Waters et al., 2017) who detected 45% of non-reactor TB cases by ELISA**.** Similarly,(Hassanain et al., 2009) in Egypt, recorded that 43.5% of the tested cattle were positive by ELISA. In addition, the multi-antigen ELISAs, especially that contained a cocktail of MPB70, MPB83, CFP-10, ESAT-6, enhance the overall test sensitivity extremely greater than the single antigen ELISA such as PPD-ELISA, because the latter shares antigens of non-tuberculous mycobacteria resulting in cross reactions (Infantes-Lorenzo et al., 2017; Waters et al., 2017). Other studies reported better detection of bovine TB by the parallel interpretation of SID and serological assays compared with that of each test individually, and the increased sensitivity of ELISA by previous stimulation of the immune system by PPD injection (Koni et al., 2016;Trost et al., 2016;Casal et al., 2017).

Post-mortem examination as a gold standard to judge the performance of the cocktail-antigens ELISA revealed its ability to detect 80 % of visible lesion cases. McCallan et al. (2017) reported one serologically negative animal that was subsequently found to have visible lesions and confirmed for *M.bovis* at post-mortem which may indicate anergy or immunosuppression (Ellner, 1996). On the other hand, 8 non-visible lesion cases were seropositive which may be due to minor lesions that escape the routine PM examination,or even cross-reaction with antigenically related bacteria (Domingo et al., 2014; Picasso-Risso et al., 2019).

In conclusion, bovine TB is endemic at high level and represent a great risk for human and animal population and further wide scale funded studies are required to discover the situation throughout Egypt. On the other hand, the errors performed during the application of SID may be responsible for the gap between the low detection ability of the test and higher detected cases during routine meat inspection in the study area. Finally, cocktail-antigens ELISA is promising as it was able to detect 80% of cases with TB lesions at the slaughterhouse.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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